ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

DEPARTMENT OF BIOCHEMISTRY

Effect of resveratrol, metformin and *Eucalyptus* oil on visceral fat deposition, serum glucose, lipid profiles and liver function tests of Swiss albino mice fed a high-fat diet.

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A thesis submitted to Addis Ababa University School of Graduate Studies, Department of Biochemistry in partial fulfillment of the requirements for the Degree of Master of Science in Medical Biochemistry.

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This is to certify that the thesis prepared by Tigist Matheos entitled: *Effect of resveratrol, metformin and Eucalyptus oil on visceral fat deposition, serum glucose, lipid profiles and liver function tests of Swiss albino mice fed a high-fat diet,* and submitted in partial fulfillment of the requirements for the Degree of Master of Science in Biochemistry, complies with the regulations of the University with respect to originality and quality.

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Examiner

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Abstract

Introduction: Obesity results from environmental and genetic factors and is rapidly growing public health problem with its leading co-morbidity type 2 diabetes. Great emphasis has been put on establishing treatments for obesity, including pharmacological intervention using animal models of obesity.

Objective: To develop an obesity model in Swiss albino mice using a high fat diet, that is feasible and affordable in Ethiopia, and to assess the effect of resveratrol, metformin and *Eucalyptus* oil on body weights, visceral fat deposition liver histology, serum glucose, lipid profiles and liver function tests in these mice.

Methods: Male Swiss albino mice were fed a normal chow diet, high fat diet (52% fat, w/w) containing lard made from choma (crude beef fat), a high fat diet with resveratrol (75 μg/ml) in the drinking water for 12 weeks, a high fat diet with intraperitoneal metformin injections (250 mg/kg/day) and a high fat diet with *Eucalyptus* oil (10μL/mouse/day) administered intraperitoneally for 8 weeks. Body weight, visceral fat mass, blood glucose, serum lipid profiles, serum liver enzymes, and liver histopathology were assessed.

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Results: The high fat diet did not cause significant changes in total body weight of mice, but there was significantly more visceral fat deposition as large fat pads in mice fed a high fat diet for 12 weeks (1140.0 ± 248.8 mg, as compared with 66.5 ± 27.6 mg in normal diet fed mice) and also in mice fed a high fat diet for 8 weeks (862.4±148.7 mg, as compared with 611.7 ±178.3 mg for mice fed a normal diet). The high fat diet did not induce any obvious prediabetic or diabetic state and did not raise fasting blood glucose level above the normal range. Resveratrol administration to Swiss albino mice fed a high fat diet did not cause any significant effect on body weight, visceral fat mass, fasting blood glucose, liver enzymes, or serum lipid profile. Treatment with metformin did not have any significant effect on body weight, fasting blood glucose level, visceral fat mass, serum liver enzymes, serum total cholesterol level or serum triglyceride level, but metformin significantly increased serum HDL level, and decreased serum LDL level, and raised serum AST level. *Eucalyptus* oil did not have any significant effect on body weight and visceral fat mass, serum ALT level, serum ALP level, serum total cholesterol and on serum triglyceride level, but it significantly lowered serum LDL levels and raised serum HDL level, as well as weakly increasing serum AST. *Eucalyptus* oil also lowered blood glucose levels even though they were within the normal range to begin with.

Conclusion: The high-fat lard diet developed in a Swiss albino mouse model may be a useful model for visceral obesity, but, without modification, was not a model for prediabetes or diabetes. Resveratrol had no effect on body weight, fat deposition and metabolic profiles. Metformin caused elevation of HDL and lowering of serum LDL. *Eucalyptus* oil lowered fasting blood glucose and serum LDL levels and it elevated serum HDL levels. The significance of these findings are discussed.

Keywords: Obesity, High fat diet, AMPK, Resveratrol, Metformin, *Eucalyptus* oil.

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Abbreviations

AAP	Aminoantipyrine
ADP	Adenosine diphosphate
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AMP	Adenosine monophosphate
AMPK	AMP-activated Protein Kinase
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
BMI	Body Mass Index
CaMKKβ	Calcium/Calmodulin-Dependent Protein Kinase β
cAMP	Cyclic AMP
DAP	Dihydroxyacetone phosphate
DHBS	Dichlorohydroxybenzene
GLUT 4	Glucose transporter type 4
HDL	High Density Lipoprotein
LDL	Low Density Lipoprotein
NAD+	Nicontinamide Adenine Dinucleotide
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-α
pNP	p-nitrophenol
pNPP	p-nitrophenylphosphate
WHO	World Health Organization

Chapter 1

Introduction

1.1. General features of obesity

Obesity is a complex disorder which results from changes in living standards and the environment in which humans live, superimposed on a genetic susceptibility (Pereira *et al*., 2014; Haththotuwa *et al*., 2012). However, genes have not changed significantly over the past 50 years, which implies that the main contributors for the explosion of the global obesity epidemic are high calorie intake and physical inactivity. Obesity is now so common within the world's population, therefore, it should no longer be considered as a cosmetic problem affecting certain individuals, but an epidemic that threatens global public health (Kopelman, 2000).

BMI (body mass index), calculated as weight in kg/ (height in m) ^{2}, is classified according to the criteria of the National Institutes of Health in the United States and is recommended for defining obesity by the WHO (world health organization). In general, BMI less than 18.5 kg/m^2 is considered underweight, a BMI from 18.5 to 24.9 kg/m^2 is normal, a BMI from 25 to 29.9 kg/m^2 is overweight, and a BMI of 30 kg/m^2 or greater is considered as obese (Haththotuwa *et al* ., 2012).

New obesity classification criteria based on body fat composition, rather than on body weight and BMI alone, have also been proposed. According to this classification, at least four phenotypes of individuals have been described as: (1) People with normal BMI and no excess fat, (2) People with normal BMI, but excess percentage of fat, (3) People with an overweight/obese BMI who appear to be metabolically normal and not prone to

complications of obesity, and (4) People with an overweight/obese BMI who are metabolically abnormal and prone to metabolic complications of obesity (Castro *et al* ., 2014 ; De Lorenzo *et al*., 2016). These new classifications are based on the fact that there are individuals with normal BMI who have excess visceral fat that produces adipokines and predisposes to complications of obesity including diabetes. Likewise, there are individuals with high BMI (over 25) who have very little visceral fat but large amounts of subcutaneous fat and appear to be relatively resistant to developing diabetes and other metabolic complications of obesity.

Obesity involves deposition and expansion of excess adipose tissue or increased percentage of body fat, which have a role in lipid and glucose metabolism, (Siriwardhana *et al*., 2013) and leads to an extent of impairment of health and well-being (World Health Organization, 2000). Generally, adipose tissue can be classified mainly as white adipose tissue and brown adipose tissue.

White adipose tissue is an important energy storage and endocrine organ with active metabolism (Kalupahana *et al*., 2012) which contain fewer mitochondria and have one large lipid droplet, which is able to store triglycerides at a high energy density (Nishino *et al*., 2008). Energy storage in this form is efficient because of higher caloric value of lipids than carbohydrates and, in contrast to carbohydrates (glycogen), triglycerides can be stored with little associated water (Rezaee and Dashty, 2013). In addition, white adipocytes, are used as endocrine cells that secrete abnormal or high levels of adverse, and low levels of beneficial adipocytokines (Maury and Brichard, 2010), which leads to activation of proinflammatory signaling pathways and result in the induction of several biological markers of inflammation (Fuentes *et al*., 2013; Zagotta *et al*., 2015).

White adipose tissue can be further classified as visceral/ central fat and peripheral fat based on anatomic location in the body, histology, cellular and biochemical characteristics. Visceral/ central fat accumulates mostly around organs in the abdominal and pelvic regions, in the central region of the body, in the liver, omentum and around the heart, and peripheral fat is stored subcutaneously especially around hips, thighs and buttocks and has possible immunological benefits and protective effects against obesity associated metabolic disorders and provides insulation from heat and cold (Wajchenberg, 2000).

Brown adipose tissue cells have numerous small lipid droplets in their cytoplasm and are abundant in mitochondria. Therefore, the energy in the form of triglycerides in lipid droplets is accessible for rapid hydrolysis and oxidation of fatty acids, with production of heat by brown adipose tissue. Brown adipose cells are well adapted to converting the energy of fatty acids to heat, rather than fat storage, in particularly because their mitochondria contain uncoupling protein 1, which uncouples electron transport from ATP (adenosine triphosphate) production (Cinti, 2002; Tharp and Stahl, 2015).

Obese individuals differ not only in the amount of excess fat mass, but also in the regional distribution of the fat within the body. Central obesity, which is common and associated with excess abdominal visceral fat deposition, is a public health problem. It is becoming clear that many people may have significant visceral fat deposition yet still having normal BMI range (Janssen *et al*., 2011) and excess abdominal visceral fat has recently been considered as a prerequisite for diagnosis of metabolic syndrome (Pereira *et al*., 2014).

1.2. Epidemiology of obesity

Obesity has reached epidemic proportions globally and its prevalence has more than doubled worldwide since 1980. WHO in 1997 formally recognized obesity as a global epidemic by designating obesity as a major public health problem. According to WHO, in 2008, more than 1.5 billion adults were overweight, and of those, over 200 million men and nearly 300 million women were obese (World Health Organization, 2010; (Haththotuwa *et al*., 2012). According to the global database on BMI compiled by WHO, the prevalence of obesity varies widely in adults across countries as; 33.9% in United States, 23.1% in Canada, 22.7% in the United Kingdom, 16.9% in France, and 16.4% in Australia (World Health Organization, 2012).

The worldwide prevalence of overweight and obesity increased by 27.5% for adults and 47.1% for children between 1980 and 2013. The number of overweight and obese individuals increased from 857 million in 1980, to 2.1 billion in 2013. Since 1980, the prevalence of overweight and obesity has increased remarkably in high income countries; from 16.9 % to 23.8% for boys and from 16.2% to 22.6% for girls in 2013. The prevalence is also rising in children and adolescents in developing countries, from 8.1% to 12.9% for boys and from 8.4% to 13.4% in girls between 1980 and 2013 (Ng *et al*., 2014).

Overweight and obesity are now highly prevalent also in many low and middle-income countries including Ethiopia. It is currently estimated that as many as 20% to 50% of people who live in urban populations in Africa are classified as either overweight or obese and it is estimated that by 2025, three quarters of the obese population worldwide will be in low-to-middle income countries (Teshome *et al* ., 2013).

In Ethiopia, 4.6 % of boys, 6.3 % of girls (under 20 years old) and 4.0 % of men and 8.0 % of women (over 20 years old) are overweight or obese (Ng *et al*., 2014). In a cross-sectional survey conducted among a total of 463 children from elementary school in Addis Ababa, the overall prevalence of being overweight was 46/463 (9.9%) and of obesity was 13/463 (2.8%) (Gebremichael and Chere, 2015). In another institution-based cross-sectional study conducted on 456 randomly selected adolescents from 20 high schools in Addis Ababa city, the prevalence of overweight in the study participants was 9.7 % and the prevalence of obesity was 4.2%. The combined prevalence of obesity and overweight was 13.9 %. Prevalence of overweight and obesity was higher in adolescents studying in private high schools than governmental high schools and the reason might be that adolescents in private schools come from families with higher socioeconomic status, and with an increased obesogenic lifestyle, than that of low income families (Shegaze *et al*., 2015).

1.3. Complications and comorbidities of obesity

Obesity is closely associated with an increased risk of various metabolic diseases such as cardiovascular disease, dyslipidemia, insulin resistance, nonalcoholic fatty liver disease, osteoarthritis, some obesity-associated cancers and with type 2 diabetes. Enlarged adipose tissue results in the infiltration of macrophages and imbalance of pro-inflammatory and anti-inflammatory factors secreted by adipose tissue (Jung and Choi, 2014).

Obesity increases cardiovascular risk through increased plasma triglycerides, elevated LDL (low density lipoprotein) cholesterol, reduced HDL (high density lipoprotein) cholesterol, elevated blood glucose, elevated insulin levels and high blood pressure.

An important link between obesity, the metabolic syndrome and dyslipidemia seems to be the development of insulin resistance in peripheral tissues (Klop *et al*., 2013). In insulin resistance, adipocytes and muscle cells have reduced responsiveness to insulin binding and signaling and recruitment of GLUT 4 (glucose transporter type 4) to the cell surface. Thus, their uptake of glucose is reduced, hepatic cells also fail to convert glucose into glycogen. As a result, blood glucose levels are increased, and insulin secretion is also increased, causing hyperinsulemia (Chen *et al*., 2011) which is one of the earliest hallmarks of the prediabetic state (Ghoshal and Bhattacharyya, 2015). Insulin resistance also occurs in pancreatic beta cells and this, coupled with lipotoxicity and glucotoxicity, contributes to the worsening of beta-cell function in conjunction with regulation of insulin secretion (Ogihara and Mirmira, 2010).

Obesity can also result in nonalcoholic fatty liver disease (Fabbrini *et al.*, 2010). High fat diet induced fatty liver is characterized by excessive accumulation of triacylglycerol in the liver, impaired fatty acid oxidation, and increased hepatic *de novo* lipogenesis. Long-term high-fat diet induced dysregulation of hepatic lipid metabolism increases lipolysis, causing an increase in free fatty acid levels. These metabolic changes trigger fatty liver and lead to systemic aggravation of lipid metabolic dysfunction (Li *et al.*, 2014).

Obesity also has a role in osteoarthritis which is a painful degenerative condition that can affect one or more of the joints and weight-bearing joints (spine, hip, knee, and ankle). Increased weight translates to increased force on the weight-bearing joints and mechanical forces exerted on the joints are a significant cause of osteoarthiritis, often requiring surgical joint replacement, and one of the most modifiable risk factors with respect to weight loss and activity modification (Lementowski and Zelicof, 2008).

Obesity is also associated with an increased risk of numerous cancers, in particular uterine adenocarcinoma, postmenopausal breast cancer and colon cancer. The association between obesity and cancer is rooted in a fundamental and intimate link between the processes that regulate cellular energy homeostasis and those that regulate cell survival, proliferation, and apoptosis. Obesity leads to dysregulation of inflammatory, endocrine, and metabolic signaling pathways, all of which engage in strong crosstalk, generating a highly complex system that underlies the cancer-obesity link (O'Rourke, 2014). For example, postmenopausal breast cancer is thought to be due to increased levels of estrogen in obese women. After menopause, when the ovaries stop producing estrogen, fat tissue becomes the most important source of estrogen and since obese women have more fat tissue, their estrogen levels become higher, potentially leading to more rapid growth of estrogen-responsive breast tumors (Rose and Vona-Davis, 2010).

1.4. Obesity and Type 2 diabetes mellitus

Diabetes mellitus is a chronic metabolic disease that, in 2013, by global estimates has a worldwide prevalence of 382 million people. The number is expected to increase to 592 million by 2035, most having type 2 diabetes mellitus (Guariguata *et al*., 2014; Liao and Tsai, 2014). Type 2 diabetes is the leading co-morbidity of obesity, and is predicted to affect 300 million people worldwide by 2020 (Muoio and Newgard, 2008). It is an emerging health challenge all over the world as a result of urbanization, high prevalence of obesity, sedentary lifestyle, high calorie and diabetogenic diets and other stress related factors compounded with the genetic prevalence (Ghoshal and Bhattacharyya, 2015).

Type 2 diabetes mellitus is a group of metabolic diseases caused by chronic hyperglycemia with disturbances of fat, carbohydrate, protein metabolism, and defective regulation of cell growth, resulting from defects in insulin secretion or activity (Li *et al*., 2014). It is a multifactorial metabolic disease characterized by defects in beta-cell function and insulin action and increased hepatic glucose production. Central to this metabolic condition is altered glucose and lipid metabolism resulting from the combined effects of insulin resistance in skeletal muscle, pancreatic beta-cells, hepatic, renal, and adipose tissue (Mackenzie and Elliott, 2014).

The major risk factor for type 2 diabetes mellitus is obesity, in which the chronic overconsumption of food leads to hyperglycemia, insulin resistance, and impaired metabolic function (International Diabetes Federation, 2013). Deleterious cellular effects of nutrient excess (particularly glucose and fatty acids) include impaired inflammatory signaling, endoplasmic reticulum stress, excess production of reactive oxygen species, mitochondrial dysfunction, and accumulation of ceramides, triglycerides and/or fatty acyl intermediates, and activation of serine-threonine kinases (Thaler and Schwartz, 2010).

Insufficient insulin secretion in response to elevations of plasma glucose, and excessive circulating lipid levels have been suggested to contribute, in conjunction with chronic hyperglycemia, to the progressive deterioration of beta-cell function (Wu *et al*., 2012). Pancreatic beta-cells are key players in the development of type 2 diabetes, as they are required to secrete increasing amounts of insulin so as to compensate for increasing insulin resistance. Consequently, the beta-cells come under increasing metabolic stress and finally their function deteriorates (Rouse *et al*., 2014).

1.5. Treatment of obesity

Obesity is an important risk factor for various metabolic diseases. Therefore, the goals of various obesity treatments are to improve or prevent complications of its metabolic diseases, not simply to induce weight loss. Treatments of obesity which are used currently include lifestyle intervention, pharmacotherapy, and bariatric surgery. Fundamental lifestyle intervention of obesity include nutrition management and physical activity. The amount of ingested fat and total calorie intake are the most important dietary factors to induce obesity (Klop and Cabezas, 2012). However, effects of lifestyle intervention are not always satisfactory in all cases, and pharmacotherapy is used in some patients in conjunction with lifestyle intervention (Joo and Lee, 2014).

Pharmacotherapy agents could be short term agents for their safety issues such as benzphetamine, diethylpropion, phendimetrazine and phentermine, which are appetite suppressants, or long term such as orlistat and lorcaserin. Orlistat partially blocks intestinal digestion of fat through inhibition of lipases, therefore producing weight loss, though oily stools and diarrhea are its unpleasant side-effects (Bray and Ryan, 2014).

Bariatric surgery, aimed at weight reduction, is an option for the treatment of severe obesity. The basic principle of bariatric surgery is to restrict food intake by restricting stomach volume and/ or by decreasing the length of small intestine available for absorption of food (Lee and Almulaifi, 2015). It is designed to alter or interrupt the digestion process so that food is not broken down and absorbed in the usual way. For example, gastric banding isolates a portion of the stomach from accessing ingested food, leaving a functional stomach with a small volume.

Brown adipose tissue is also considered as an interesting target for the treatment of obesity due to its high metabolic capacity. Adult humans have limited and variable numbers of brown fat cells, which play a role in thermogenesis and potentially influence energy expenditure and obesity susceptibility (Frontini and Cinti, 2010). Non-shivering thermogenesis, once activated, can lead to enhanced partitioning and oxidation of fuels in adipose tissues, and reduce the burden of glucose and lipids on other metabolic organs such as liver, pancreas, and skeletal muscle. Sustained long-term activation of brown adipose tissue may also lead to meaningful body weight loss. There are some drugs that have been proposed to regulate brown adipose tissue recruitment or activation, or both, and which have been tested in both rodent and human (Peng *et al*., 2015).

1.6. AMPK activators for treatment of obesity

The biological pathways involved in maintaining energy homeostasis are being targeted for pharmacological manipulation to combat insulin resistance and metabolic dysfunction caused by chronic nutrient excess. One such pathway is that of AMPK (AMP activated protein kinase), an enzyme that has come to be known as a master regulator of metabolism (Coughlan *et al*., 2014). AMPK is a phylogenetically conserved serine/threonine protein kinase, which acts as an energy sensing enzyme in the cells and is activated in response to cellular stresses that deplete ATP levels in cells (Sadasivan *et al*., 2014). The AMPK system is a regulator of energy balance at both the cellular and whole-body levels, in which, once activated by low energy status, affects a switch from ATP consuming anabolic pathways to ATP producing catabolic pathways (Sivajothi and Dakappa, 2014) to restore the cellular energy balance (Gu *et al*., 2014).

In mammalian tissues, AMPK is a heterotrimer consisting of a catalytic α subunit with two regulatory subunits, γ and β. There are also two isoforms of the catalytic subunit: AMPK α1, which is widely distributed and AMPK α2, which is expressed in skeletal muscle, heart, and liver (Srivastava *et al*., 2012). AMPK is converted from an inactive form to a catalytically competent form by phosphorylation of the activation loop within the kinase domain (Xiao *et al*., 2011). Binding of AMP (adenosine mono phosphate) to the γ-regulatory domain promotes phosphorylation by the upstream kinase, binding of ADP (adenosine diphosphate) to the regulatory γ subunit protects AMPK against dephosphorylation and, binding of ATP to the γ subunit operates in an opposing manner by decreasing AMPK activity (Mackenzie and Elliott, 2014).

AMPK activation has numerous effects on a multitude of tissues (Figure 1); it stimulates glucose uptake and fatty acid oxidation in the liver, while inhibiting gluconeogenesis, as well as cholesterol, fatty acid, and protein synthesis **(**Ruderman *et al*., 2013). It stimulates glucose uptake, fatty acid oxidation, GLUT 4 translocation, and mitochondrial biogenesis in skeletal muscle, while inhibiting protein and glycogen synthesis. Similarly, it stimulates glucose uptake, fatty acid oxidation, and glycolysis in cardiac muscle (Srivastava *et al*., 2012). AMPK activation also stimulates fatty acid oxidation and reduces fatty acid synthesis and lipolysis in adipose tissue and inhibits insulin secretion from pancreatic beta-cells. In addition, it causes increased food intake via neurohormonal signals from the hypothalamus. Therefore, the pharmacological activation of AMPK has been a seemingly promising target for drug discovery and development (Coughlan *et al.*, 2014).

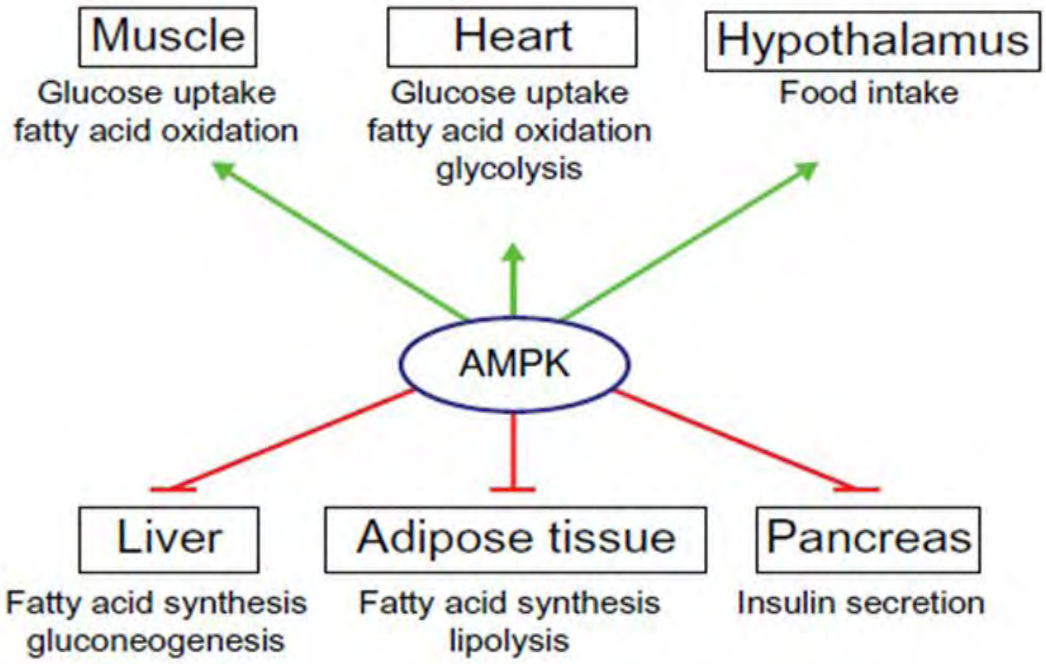


Figure 1. Role of AMPK in the control of whole-body energy metabolism. Activation of AMPK stimulates the energy-generating pathways (green lines) in several tissues while inhibiting the energy-consuming pathways (red lines) (Coughlan *et al*., 2014).

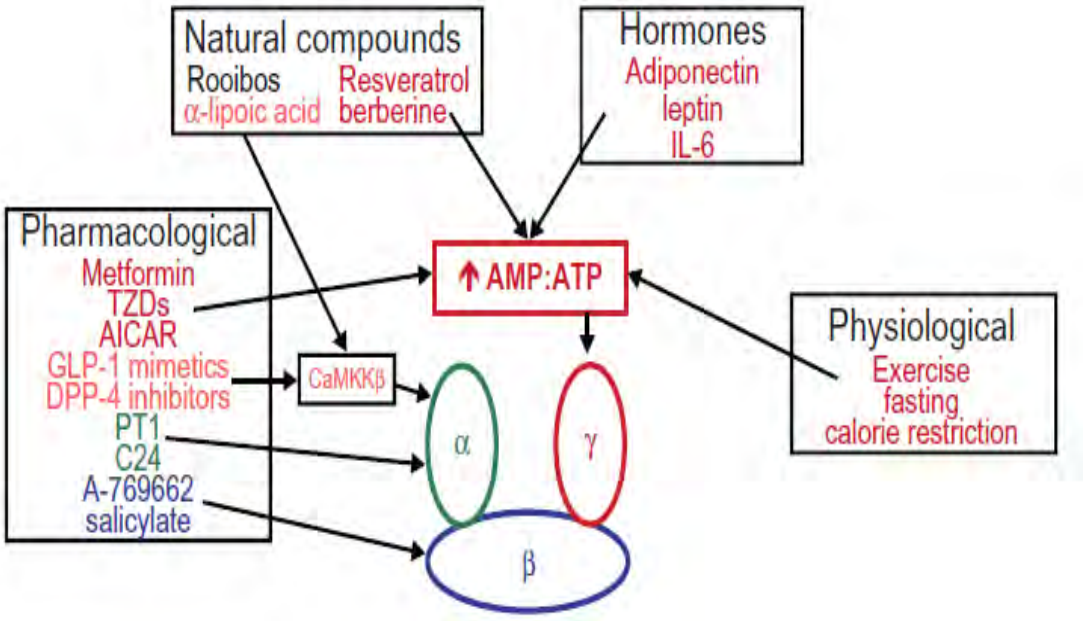


Figure 2. Physiological, pharmacological, natural, and hormonal activators of AMPK (Coughlan *et al*., 2014).

Numerous physiological, pharmacological, natural, and hormonal activators of AMPK are known (Figure 2). Many activators activate AMPK via an increased AMP: ATP ratio, causing AMP to bind to the γ-subunit. However, a subset of compounds stimulate AMPK activation via other mechanisms, such as binding directly to the α-subunit, stimulating phosphorylation of αThr172 through CaMKKβ (Calcium/Calmodulin-Dependent Protein Kinase β) ,or binding directly to the β-subunit (Liu et al., 2014). The effect of resveratrol, metformin and *Eucalyptus* oil as AMPK activators, are discussed in the following sections.

1.6.1. Resveratrol

Resveratrol (3, 4', 5-trihydroxystilbene) is a polyphenolic compound which is found in the skin of red grapes, and other botanical extracts. It is produced by some plants in response to strenuous conditions such as bacteria or fungal infection, injuries or ultraviolet irradiation, stress, and exposure to ozone (Wang *et al*., 2014).With a molecular formula $C_{14}H_{12}O_3$, resveratrol exists naturally as both *cis*- and *trans*-isomers (Figure 3), but due to lack of stability and no commercial availability of *cis*- isomer, as well as greater natural presence and higher biological activity of *trans*- isomer, most researchers have used *trans*-isomer in their studies (Dobrzynska, 2013).

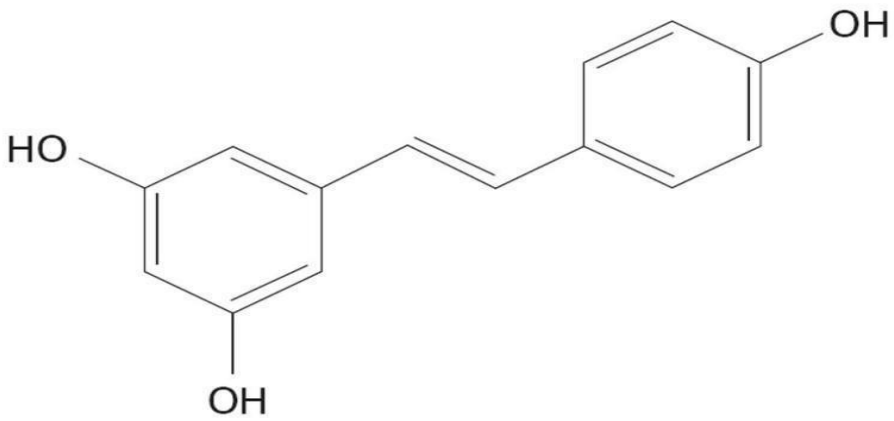


Figure 3. Chemical structure of *trans*-resveratrol (Adelli *et al*., 2013).

Resveratrol was first isolated from the roots of white hellebore in 1924 and later found in many plants, such as grapes, peanuts and *Polygonum cuspidatum* (Japanese knotweed) (Zhang *et al*., 2014) , then identified as the principal active ingredient from the dried roots of *Polygonum cuspidatum*, used in Japanese and Chinese traditional medicine. Resveratrol is present in human diet such as fruits, grapes, peanuts, strawberry, blueberry, cranberry, mulberry, lingberry, and bilberry and in flowers and leaves such as butterfly orchid tree, *Eucalyptus* tree, and spruce. It is also present in wine, especially in red wine, therefore, the skin of red grapes and red wine are considered as a major source of resveratrol in food (Mukherjee et al., 2010) and it has been postulated that it might be the reason for the "French Paradox", the phenomenon in which the French population has significantly lower incidence of cardiovascular diseases in spite of consumption of high-fat diet (Dobrzynska, 2013).

Resveratrol has received attention in recent years because of its various health functions, broad biological activity and its potential for use in medicinal applications. It has shown a variety of beneficial physiological effects including anti-oxidant (Rizvi and Pandey, 2010)**,** anti-atherosclerotic and neuro protective properties and has even been reported to prolong the life span of mice. It has also gained attention as an anti-diabetic agent since it has been shown to increase insulin sensitivity and stimulate glucose uptake in myotubes through AMPK activation (Patel *et al*., 2011). Recent data derived from in vitro, in vivo and ex vivo studies have opened a new, promising perspective for the potential use of resveratrol to prevent serious metabolic disorders such as obesity and diabetes (Zhang *et al*., 2012; Szkudelska and Szkudelski, 2010).

AMPK and the sirtuins, Sirt1 and Sirt3 which are well-known key sensors of energy status and regulators of glucose and lipid metabolism, appears to be a strong target for prevention and control of metabolic diseases such as obesity and diabetes (Bruckbauer and Zemel, 2014; Brenmoehl and Hoeflich, 2013). Resveratrol, has been reported to be a Sirt1 activator, mimicking the effects of caloric restriction on lifespan, oxidative and inflammatory stress, as well as improving insulin sensitivity and reducing adiposity (Timmers *et al*., 2012).

Many studies using adipocytes have demonstrated that resveratrol has an anti-obesity potential by inhibiting pre adipocyte differentiation, decreasing adipocyte proliferation, inducing adipocyte apoptosis, decreasing lipogenesis, and promoting lipolysis and fatty acid β-oxidation. These effects of resveratrol may be mediated by central regulators of adipogenesis, lipogenesis, and fatty acid β-oxidation including AMPK, sirt1, and PGC-1α (peroxisome proliferator-activated receptor gamma coactivator 1-alpha) (Wang *et al*., 2014). Resveratrol increases the intracellular cAMP (cyclic adenosine monophosphate) concentration by inhibiting cAMP phosphodiesterases, which degrade cAMP. Increased cAMP concentrations activate AMPK, then AMPK binds to the promoter of PGC-1α, a transcriptional coactivator and a regulator of mitochondrial biogenesis and function (Park *et al*., 2012; Chung, 2012).

The impact of resveratrol on obesity in animal studies was mainly based on a diet-induced obese animal model (Alberdi *et al*., 2013). In general, authors reported that resveratrol supplementation has shown to decrease body weight, adipose tissue weight, hepatic fat accumulation, and deposition in a dose dependent manner, resulting in improvement of blood lipid profiles and glucose homeostasis (Franco *et al* ., 2013).

1.6.2. Metformin

Metformin (molecular formula $C_4H_{11}N_5$, Figure 4) is widely prescribed for the treatment of type 2 diabetes. The history of metformin dates back to the Middle-Ages, and its structural analogue galegine was isolated from *Galega officinalis* (goat's rue, French lilac, Italian fitch); a plant native to the Middle East that has been used for treatment of diabetes in Europe (Anwar *et al*., 2014).

Metformin is well tolerated and highly efficient in reducing blood glucose in insulin resistant individuals, which is mainly attributed to reductions in hepatic glucose output and increases in peripheral glucose uptake. At the cellular level, metformin activates AMPK (Calixto *et al*., 2013).

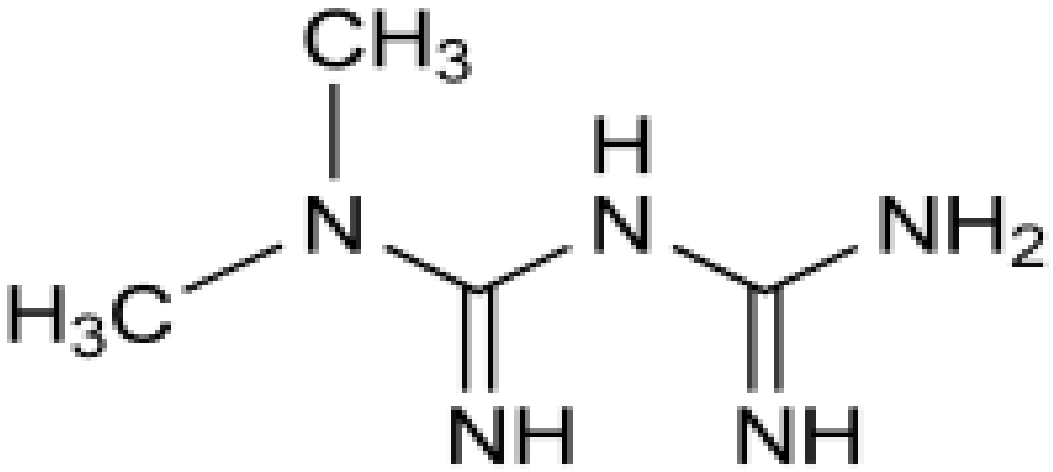


Figure 4. Chemical structure of metformin (Havele and Dhaneshwar, 2010).

Suppression of hepatic glucose production by metformin has been attributed to a block in glucagon stimulated glucose synthesis, which is mediated by a decrease in adenylate cyclase activity resulting from the inhibition of Complex I of the mitochondrial electron transport chain. Metformin also inhibit mitochondrial glycerophosphate dehydrogenase, which suppresses glucose production from lactate and glycerol precursors and increase

GLP-1 (glucagon-like peptide) secretion by the small intestine and increase expression of GLP-1 receptors on adipose and skeletal muscle cells, increasing peripheral glucose absorption and utilization (Anwar *et al*., 2014). In addition, chronic metformin treatment decreases liver lipid accumulation by AMPK-mediated inhibition of acetyl-CoA carboxylase activity, which also contributes to increased insulin sensitivity by reduction of hyperinsulinemia and by enhancing the affinity of insulin receptor for insulin (Jenkins *et al*., 2014). AMPK activation by metformin in cells in the immune system also promotes the switch from a pro-inflammatory to an anti-inflammatory phenotype (Liu *et al*., 2014).

1.6.3. *Eucalyptus* oil

Herbs are rich sources of bio-active compounds with versatile pharmacological uses. From those herbs, more than 800 plants are used as traditional remedies for the treatment of diabetes, but only a few have received scientific analysis (Bokaeian *et al*., 2010).

Eucalyptus is a diverse genus of flowering trees and shrubs, consisting of more than 500 species, taxonomically from the family Myrtaceae, indigenous to Australia, Tasmania and cultivated mostly in sub-tropical and warm temperate regions of the world. The genus of *Eucalyptus* was introduced to East Africa in the late 19th and early 20th century and by the early 1970s the area of *Eucalyptus* growth in Ethiopia, Rwanda, Uganda, Kenya and Sudan had reached 95,684 ha. The largest plantations at that time were in Ethiopia and Rwanda, at 42,300 ha and 23000 ha, respectively (Gessesse and Teklu, 2011).

It contains both volatile and non-volatile fractions; amongst which the terpenoids are one of the major components comprising most of the essential oil of *Eucalyptus* (*Eucalyptus* oil), imparting its characteristic odor and the rest are; the flavonoids, (mostly quercetin,

kaempferol, myricetin), polyphenols (including resveratrol) and phenolics (Dey and Mitra, 2013; Dobrzynska, 2013).

From ancient times, the bark and leaves of different species of *Eucalyptus* have been used as folk medicine for the treatment of illnesses such as cold, fever, toothache+e, diarrhea and snake bites. Aqueous hot *Eucalyptus* leaf decoctions have been also used as a traditional therapy for diabetes mellitus. Studies in streptozotocin–induced diabetic animal models confirmed its anti – hyperglycemic and anti – inflammatory effects (Bokaeian *et al*., 2010).

1.7. Animal obesity models

Great emphasis has been put on establishing treatments for obesity and obesity-related metabolic diseases which represent a growing socioeconomic problem throughout the world. Hence, it is important to test potential new drugs in valid obesity animal models (Nilsson *et al*., 2012). The advantages of mouse models that make them suitable for studying obesity include a short breeding span, access to physiological testing, easy manipulability, short reproductive cycle, much lower cost and possibility of conducting longitudinal studies using large numbers of animals (Karimi, 2012).

In laboratory animals, genetic models of obesity, for example the ob/ob mouse, have been used extensively to study obesity, but these models may not be appropriate for the most common type of acquired obesity found in humans, which is caused largely due to increased calorie intake. The ob/ob mouse, for example, involves mutations in both alleles coding for leptin, and these mice are obese because they lack leptin, which is involved in controlling appetite and body fat stores (Coleman, 2010). Mice lacking leptin gain weight

and develop many features of metabolic syndrome, and these are reversible with administration of leptin. However, although there is a human genetic disease equivalent to the ob/ob mouse, which responds to leptin, it is rare (Mantzoros *et al*., 2011).

The use of hypercaloric or hyperlipidemic diets has been used as a model of obesity induction in animals, because of its similarity to the genesis and metabolic responses caused by most obesity in humans (Tschöp and Heiman, 2000; Barbosa-da-Silva *et al*., 2014). Therefore, induction of obesity by consumption of highly palatable, high-calorie or hyperlipidemic foods may be more appropriate. The use of models of diet-induced obesity in animals has been shown to be effective for the study of the physiopathology of complications associated with obesity (Rosini *et al*., 2012) such as dyslipidaemia, insulin resistance and metabolic syndrome (Panchal and Brown, 2010).

Different types of high fat diets have been used , as fat as either animal-derived fats such as lard or beef tallow, or plant oils such as olive or coconut oil with different fat portions and total caloric energy (Buettner *et al*., 2006). Long-term feeding of rats and mice with high fat diets increased body weight, deposition of liver and plasma triglycerides, free fatty acid concentrations and plasma insulin concentrations compared to standard chow-fed control mice. A large number of investigations also have been carried out in recent years, concerning the therapeutic action of various pharmaceutical and nutraceutical compounds on mouse obesity models (Karimi, 2012).

1.8. Statement of the problem

Obesity is a leading cause of morbidity and mortality in many countries and has reached epidemic proportions globally. It is becoming a prominent health problem also in low- and middle-income countries, including Ethiopia. Previously, obesity was perceived simply as a sign of affluence in low income countries, but nowadays, increasingly recognized as an important health risk. Reduced physical activity and increased dietary calorie intake along with foods that are obesogenic which comes due to globalization and rapid urbanization are a major factors for its epidemicity. From 20 % -50% of urban populations in Africa are classified as either overweight or obese (Teshome *et al*., 2013) and in Ethiopia, 4.6 % of boys, 6.3 % of girls and 4.0 % of men and 8.0 % of women are overweight or obese (Ng *et al*., 2014) and it is shown that obesity is increasing even in Ethiopia. Therefore, more laboratory studies on obesity are needed.

1.9. Significance of the study

This study aimed to develop an animal obesity model using Swiss albino mice fed a high fat diet made from animal fat (lard) obtained from local butchers. High fat diets using lard and other fat sources have been used as a model of obesity induction in animals, because of their similarity to the genesis and metabolic responses caused by obesity in humans. Therefore, the study may provide a system for studying obesity which could lead to development of novel antiobesity agents in the future and improve biochemical knowledge of obesity. In addition, AMPK activators are increasingly being studied as potential anti-obesity and anti-diabetic medications, so in the study, three sources (resveratrol, metformin and *Eucalytpus* oil) considered to have an effect on AMPK activation, were examined for their effects on the mouse obesity model that was developed.

1.10. Objectives of the study

1.10.1. General objectives

To develop an affordable and feasible obesity model in Swiss albino mice using a high fat diet containing lard made from locally obtained animal fat in Ethiopia, and to assess the effect of resveratrol, metformin and *Eucalyptus* oil on fat deposition, blood glucose levels, serum lipid profile and liver function in these mice.

1.10.2. Specific objectives

- To develop a high fat (lard) diet induced obesity model using Swiss albino mice.
- To assess the effect of feeding high fat (lard) diet on body weight, fasting blood glucose, visceral fat deposition, serum lipid profile, liver function and liver histopathology of male Swiss albino mice.
- To assess the effect of resveratrol on body weight, fasting blood glucose, visceral fat deposition, serum lipid profile and liver biomarker enzymes on Swiss albino mice fed a high fat (lard) diet.
- To assess the effect of metformin on body weight, fasting blood glucose, visceral fat deposition, serum lipid profile and liver biomarker enzymes on Swiss albino mice fed a high fat (lard) diet.
- To assess the effect of *Eucalyptus* oil on body weight, fasting blood glucose, visceral fat deposition, serum lipid profile and liver biomarker enzymes on Swiss albino mice fed a high fat (lard) diet.

Chapter 2

Materials and Methods

2.1. Study period

The study was conducted from March 2015 to December 2015.

2.2. Study location

The experiments were done in Addis Ababa University Medical School, Biochemistry Department.

2.3. Treatment protocols

2.3.1. Resveratrol

HPLC purified resveratrol (99.5% pure *trans-resveratrol*) powder with no additives was purchased from CurEase, McEwen, TN, USA. The solution of resveratrol was made by dissolving 50 mg of resveratrol in 1.5 mL of ethanol, then made up to a 0.0075 % solution in drinking water. Mice were given 0.0075 % (75 μg/mL) in their drinking water *ad lib* for 12 weeks. The final ethanol concentration was 0.22 % (v/v). Therefore control, non-treated mice received drinking water containing 0.22 % ethanol. Drinking solutions were freshly made and were changed every 3 to 4 days. An average mice consumes about 5mL of the drinking fluid daily, with the daily consumption of resveratrol therefore being about 12.5 mg/kg/day.

2.3.2. Metformin

Metformin tablets (from DENK PHARMA, GmbH & Co, KG, Germany), containing 500 mg of the actual metformin drug were ground in to a powder using pestle and mortar, dissolved in water and were given to the mice by once-daily intraperitoneal injections of

0.5 mL of a 16 mg/mL solution of metformin in water. Therefore each mouse received a dose of 8 mg of metformin daily, amounting to a dose of about 250 mg/kg/day for 8 weeks.

2.3.3. *Eucalyptus* oil

Eucalyptus oil was chosen because it is believed to contain natural resveratrol (Dobrzynska, 2013) and also it is available readily in Ethiopia. The *Eucalyptus* oil used here was made from both leaves and twigs of *Eucalyptus globulus*. However, the exact amount of resveratrol in the oil used here is not known.

Steam distilled, 100% pure, therapeutic grade *Eucalyptus* oil was purchased from Plant Therapy Essential Oils, Twin Falls, Idaho, USA. A pilot experiment was carried out to determine a safe dose of *Eucalyptus* oil. First, 100 µL of the oil in 0.5 mL emulsion with water was given intraperitoneally and all mice died in a few minutes, noticeably becoming ataxic and having difficulty maintaining balance and walking. This was followed by death within minutes. Then 1/5 of this dose (equivalent to 20 µL of *Eucalyptus* oil) was used, but this also made mice acutely ataxic and ill though the mice survived. Finally, a dose of 10 µL of *Eucalyptus* oil in 0.5 mL emulsion with water, was chosen for the experiment because it did not cause any obvious acute or chronic observable complications. Therefore, each mouse received 10 µL of *Eucalyptus* oil in 0.5 mL emulsion with water intraperitoneally daily for 8 weeks.

2.4. Diets

Standard mouse food was obtained from Kality Agriculture and Reproduction Center, Ethiopia as a powdered chicken food, and then compressed into pellets in Ethiopian Public Health Institute, Addis Ababa.

Many experiments have used mice fed with commercial lard (animal fat) as an obesity model (Brainard *et al*., 2013 and Louwe *et al*., 2012). However, commercial lard is not easily available in Ethiopia. Therefore, a system was developed to prepare lard from bovine fat (choma) from local butchers in Addis Ababa, Ethiopia. In this system, three different methods of preparing fat for feeding mice were tried, as follows:

Method 1. Initially, the fat directly purchased from the butcher was given to mice without melting or further processing, as a non-mixed combination consisting of 40% animal fat and 60 % standard pellet (by weight). This preparation caused mice to die within 3 to 5 days, possibly because it was contaminated with bacteria that was lethal to the mice.

Method 2. To circumvent the possibility that unheated animal fat might cause fatal infections in mice, it was melted in a pan on a stove, then solid, non-fat components (including connective tissue and meat) were removed and the liquid fat cooled down to form solid lard (Figure 5). Then this solid lard was fed to the mice combined (without mixing) with standard food pellets (again 40% lard, 60% standard pellet by weight). The mice showed no signs of illness with this method of feeding, but it was difficult to determine how much fat they were eating, and whether or not they preferred the solid conventional food pellets, because much of the fat became smeared over the cage and mixed with the sawdust.

Method 3. In the final method of high fat food preparation, animal fat was melted, liquefied, and then non-fat solid material, including connective tissue and meat were removed, and the warm (but not hot) liquefied animal fat was mixed, before it solidified, with powdered

standard pellet and allowed to cool to produce a solid homogeneous mixture of lard/ pellet (40% / 60% w/w) that could be fed to the mice (Figure 6).

Figure 5. Preparation of lard. The choma (left), was liquefied by heating in a pan on a stove (middle), then non-fat material including connective tissue and meat were removed and the liquefied fat was cooled overnight to allow it to solidify into lard (right) .

Figure 6. Powdered standard pellet, lard, and a solidified mixture of lard/ powdered pellet (40% / 60% w/w). The fat content of powdered pellet/lard mixture = 52% fat w/w).

The powdered pellet was prepared by grinding standard pellet food in a pestle and mortar. This homogeneous lard-containing pellet provided the best form of high fat diet without the problems of the prior two methods, and mice ate this food freely, and enthusiastically.

Standard mouse food pellets contained 20% fat, 60 % carbohydrates and 20 % proteins. Therefore, with the addition of 40% lard (which is essentially 100% fat) to the pellets which contains 20 % fat, produced a food mixture containing 52% fat. Therefore, the term, "high fat diet" in this study refers to a diet containing 52% fat by weight (40% from added lard and 12% of the standard pellet), since 20% of the standard pellet is equal to 12%. Assuming that carbohydrates produce 4.2 kcal/g and fats produce 9 kcal/g, this high fat food mixture contains 70% calories as fat, 23% calories as carbohydrate and 7 % calories as protein. The composition of the lard made here was not determined, so the exact lipid constituents were not known. In order to check the effect of duration of treatment on the result, mice were fed a high fat diet for 8 weeks and for 12 weeks in separate experiments.

2.5. Animals

Fifty Male Swiss albino mice were purchased from Ethiopian Public Health Institute at 6-8 weeks of age and weight of 25-28 g, then, housed under natural light/ dark cycle at room temperature. Diets and tap water were provided *ad libitum*. All animals were fed a regular pellet diet before the start of an experiment. After two weeks of adaptation to stabilize their metabolic condition, mice were randomly divided into groups of six or seven mice per cage as a control group which fed a normal diet and experimental groups which fed a 52% (w/w) high fat diet alone, a 52% high fat diet with resveratrol treatment (75 μg/mL in their drinking water for 12 weeks), a 52% high fat diet with metformin treatment (250 mg/kg/day

intraperitoneally for 8 weeks) and a 52% high fat diet with *Eucalyptus* oil treatment (10 µL /mouse/ day intraperitoneally for 8 weeks). Body weight and fasting blood glucose were measured weekly and serum lipid panels and serum liver function tests were determined at the end of the experiments.

2.6. Fasting blood glucose determination

For fasting blood glucose determination, mice were fasted for 6 or more hours, then tail vein blood was obtained by slicing 1 mm from the end of the tails of mice with a scalpel blade and allowing a drop of blood to appear, then directly transferring the blood droplet to a test strip inserted into a Sensocard glucometer, which measures blood glucose levels accurately up to 600 mg/mL. The system is based on the glucose oxidase method (Trinder P, 1969); glucose reacts with oxygen in the presence of the enzyme, glucose oxidase, which is present in the test strip, and which oxidizes glucose to gluconolactone. This enzyme is temporarily reduced by electrons transferred from glucose (2 electrons per glucose molecule). This reduced glucose oxidase next reacts with an oxidized mediator, transferring electrons to an electrical system that creates electric current. The strength of the created current is directly proportional to the concentration of glucose in the blood sample, and is displayed digitally on the glucometer.

2.7. Blood and tissue collection and serum preparation

Blood samples for tests other than blood glucose were collected from mice at the end of the study, from the facial-temporal vein, which can be done without anesthetic and allows 0.5mL to 1.5 mL of blood to be obtained without killing the mouse (Francisco *et al*., 2015). The hairless freckle is located on the side of the jaw, a scalpel blade was used to make an

incision about one third the way between the freckle and the inferior part of the ear, and drops of blood were collected by gravity into an Eppendorf tube. Mice were then humanely killed by neck dislocation; the abdominal white adipose tissue deposits (visceral fat pads) and liver were excised immediately. The liver was sliced and stored in 10% formalin solution for histopathological studies, and visceral fat pads were pooled and weighed. For serum preparation, blood was allowed to clot at room temperature for about 30 min and centrifuged at 4000 rpm for 10 min, then serum was pipetted off using a micropipette and stored at -40^{o}C for determination of serum lipid profile and serum liver enzymes.

2.8. Tissue histopathology

The specimens were treated with paraffin wax, then washed using standard histological procedures, dehydrated sequentially in 70%, 80%, 95%, and 100% ethanol for 1 hour at each step. Tissue sections were then treated with xylene to remove ethanol from the tissue. The tissues were embedded in paraffin wax with the help of an Electro-thermal Wax Dispenser to form tissue blocks in squared metallic plate block molds. A rotary microtome was used for sectioning tissue blocks manually at a thickness of 5µm. Microtome sections of tissue were then stained using hematoxylin and eosin standard histological stains (Cardiff *et al*., 2014). Tissues from mice treated with high fat diets were compared with mice that fed normal pellet diet.

2.9. Serum lipid profile determination

Measurement of serum samples such as serum triglyceride, total cholesterol and HDL were assessed using an Auto Lab 18 fully automated clinical chemistry analyzer (Italy).

2.9.1. Serum triglyceride determination

Triglycerides in the sample are hydrolyzed by lipase to glycerol and fatty acids. The glycerol is then phosphorylated by ATP to glycerol-3-phosphate and ADP in a reaction catalyzed by glycerol kinase. Glycerol-3-phosphate is then converted to DAP (dihydroxyacetone phosphate) and H_2O_2 (hydrogen peroxide) by glycerophosphate oxidase. The H_2O_2 then reacts with 4-AAP (4-aminoantipyrine) and 3,5DHBS (3, 5-dichloro 2 hydroxybenzene) in a reaction catalyzed by peroxidase to yield a red colored quinoneimine dye. The intensity of the color produced is directly proportional to the concentration of triglycerides in the sample (Siedel *et al*., 1993; Shephard *et al*., 1990).

Triglycerides + H_2O → Glycerol + Fatty acids (Lipase)

Glycerol + ATP → Glycerol-3-phosphate + ADP (Glycerol kinase)

Glycerol-3-phosphate → DAP + H_2O_2 (Glycerophosphate oxidase)

H_2O_2 + 4AAP + 3, 5 DHBS → Quinoneimine + $2H_2O$ (Peroxidase)

2.9.2. Total Cholesterol Determination

Cholesterol esters are converted to cholesterol and fatty acids by an enzyme cholesterol esterase. Then cholesterol is oxidized with oxygen by cholesterol oxidase in to cholest-4-en-3-one and hydrogen peroxide. The hydrogen peroxide then reacts with 4-AAP by peroxidase enzyme to yield a red colored quinoneimine dye.

Cholesterol esters → Cholesterol + Fatty acids (Cholesterol esterase)

Cholesterol + O_2 → cholest-4-en-3-one + H_2O_2 (Cholesterol oxidase)

H_2O_2 + 4AAP + 3, 5 DHBS → Quinoneimine + $2H_2O$ (Peroxidase)

The intensity of the red color produced is directly proportional to the total cholesterol in the sample when read at 500 nm.

2.9.3. Serum HDL Determination

The method employs an immune-inhibition reagent method which measures HDL directly in a two reagent format. The first reagent contains anti-human beta-lipoprotein antibody which binds to lipoproteins LDL, VLDL (very low density lipoprotein) and chylomicrons other than HDL. This antibody inhibits the ability of the LDL, VLDL and chylomicrons to react with the pegylated enzymes in the system. The second reagent contains pegylated enzymes (identical to those in the method for total cholesterol determination except that they are pegylated), which then selectively react with the cholesterol present in the HDL particles. Consequently, only HDL cholesterol is subject to cholesterol measurement (Linsel-Nitschke *et al.*, 2005).

2.9.4. Calculation of serum LDL

The fraction of LDL-cholesterol in the serum was calculated by using Friedewald's equation as:

[LDL]= [Total cholesterol] – [HDL] – ([TG]/5),

Where all concentrations are given in mg/dL.

2.10. Serum liver biomarkers determination

The liver biomarker enzymes, AST (aspartate aminotransferase), ALT (alanine aminotransferase), and ALP (alkaline phosphatase) were determined using standard enzymatic assay on an Auto Lab 18 fully automated clinical chemistry analyzer (Italy).

2.10.1. AST (Aspartate aminotransferase) determination

In the serum AST assay, AST catalyzes the reversible transfer of an amino group from aspartate to 2-oxoglutarate forming glutamate and oxaloacetate. The oxaloacetate produced is reduced to malate by malate dehydrogenase, in a coupled enzyme step that involves conversion of NADH to NAD^+ (nicotinamide adenine dinucleotide).

L-aspartate + 2-Oxoglutarate → L-glutamate + Oxaloacetate (*AST*)

Oxaloacetate + NADH + H^+ → L-malate + NAD^+ (*Malate dehydrogenase*)

The rate of decrease in concentration of NADH, measured spectrophotometrically at 340 nm, is proportional to the catalytic concentration of AST present in the sample.

2.10.2. ALT (Alanine aminotransferase) determination

ALT catalyzes the transfer of amino group from alanine to 2-oxoglutarate to form glutamate and pyruvate. The quantity of pyruvate generated is determined by oxidation of NADH to NAD^+, using lactate dehydrogenase in an enzyme-coupled assay.

L-alanine + 2-Oxoglutarate → Pyruvate + L-glutamate (*ALT*)

Pyruvate + NADH + H^+ → NAD^+ + L-lactate (*Lactate dehydrogenase*)

The NAD^+ generated from NADH by the reaction can be quantified by measuring the change in spectrophotometric absorption of the reaction mixture at 340 nm. The rate of decrease in concentration of NADH, measured spectrophotometrically at 340 nm, is proportional to the catalytic concentration of ALT present in the sample.

2.10.3. ALP (Alkaline Phosphatase) determination

ALP hydrolyzes the synthetic chromogenic substrate, pNPP (p-nitrophenyl phosphate), releasing pNP (p-nitrophenol) plus phosphate.

The pNP ionizes at alkaline pH to form the p-nitrophenolate ion, which has strong absorption at 405 nm. The change in absorption at 405 nm is proportional to the activity of ALP in the sample.

pNPP → phosphate + p-nitrophenol → p-nitrophenolate ion (Alkaline phosphatase, ALP).

The change in absorbance at 405 nm was compared with standard enzyme preparations to determine the serum ALP concentrations in the serum.

2.11. Ethical considerations

Ethical approval was obtained from Addis Ababa University Department of Biochemistry Research and Ethics Review Committee (DRERC) by the protocol number 15/2014.

2.12. Statistical analysis

Collected quantitative data was coded, entered to computer, processed, edited, and analyzed using Microsoft excel and exported to SPSS version 21 statistical software for analysis. All data are expressed as means ± standard deviation. Differences among groups were assessed by one-way analysis of variance with a post hoc Tukey's test ($P < 0.05$).

Chapter 3

Results

3.1. Effect of feeding high fat diet on Swiss albino mice

Several different protocols for preparation of lard and lard/standard chow diets were assessed as stated previously in the materials and methods section, and the final proportion of food that was appropriate for developing obesity model using Swiss albino mice was a 52 % high fat diet.

3.1.1. Effect of high fat diet on body weight and visceral fat mass

At the end of each experiment, the body weights of mice were measured and in both, the 8 week and 12 week feeding periods, the high fat diet did not cause any detectable, significant change ($p > 0.05$) in body weight of mice when compared with mice fed a normal diet (Table 1), and no visible obesity was observed grossly, though weight increased equally in mice fed a normal diet or a high fat diet as they grew up.

Although the high fat diet did not induce any grossly visible obesity or statistically significant weight increase above that of a normal diet, dissection of the mice clearly showed that there was visible deposition of fat in the abdominal and pelvic regions, corresponding to gonadal (epididymal) fat pads and fat around the kidneys in mice fed a high fat diet (Figure 7). There was a statistically significant increase ($p < 0.01$) in visceral fat deposition of both the 8-week (862.4 ± 148.7 mg, as compared with 611.7 ± 178.3 mg for mice fed a normal diet) and the 12-week experiment (1140.0 ± 248.8 mg, as compared with 66.5 ± 27.6 mg in normal diet fed mice) (Table1).

Therefore, the result showed that, there is much lower amounts of visceral fat deposition in mice fed regular low-fat pellets. Further dissection showed that this visceral fat deposition was present mainly as intraperitoneal /retroperitoneal fat pads, around the kidneys, in the pelvic region, corresponding with epididymal fat pads in male mice, and no obvious increase in subcutaneous fat was shown. Therefore, mice did not gain significantly measurable weight on high fat diet because the proportion of visceral fat relative to total body weight was not detectable and it was a relatively small proportion of whole body weight.

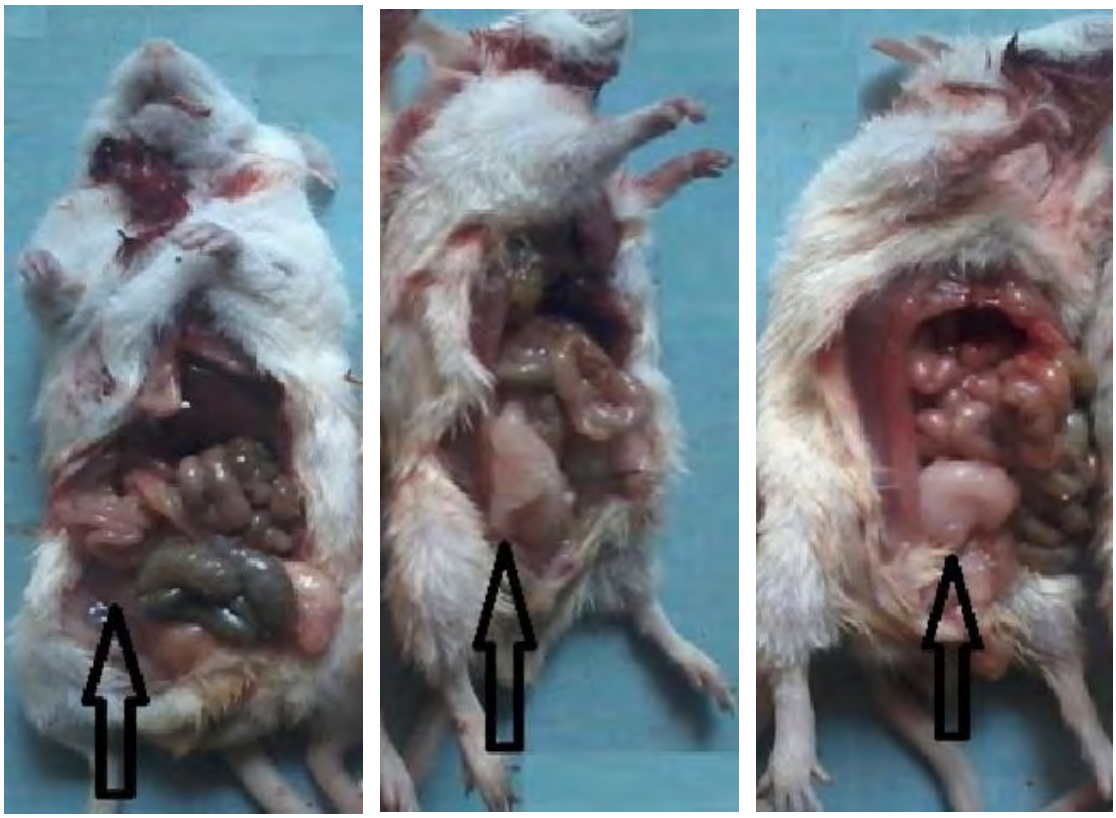


Figure 7. Effect of high fat diet and resveratrol on deposition of visceral fat pads in Swiss albino mice. Mice were fed a normal diet (left), a high fat diet (52% fat, w/w) (middle) and a high fat diet, supplemented with resveratrol (0.0075% in drinking water (right) for 12 weeks.

3.1.2. Effect of high fat diet on fasting blood glucose and serum lipid profiles

Feeding a high fat diet showed a significant increase ($p < 0.05$) in fasting blood glucose level from 98.0 ± 22.5 mg/dL to 156.6 ± 6.6 mg /dL in the 8-week feeding period, and those mice fed a normal diet also showed significant increase in fasting blood glucose level from 120.8 ± 20.9 mg/dL to 156.0 ± 4.8 mg/dL. Feeding a high fat diet for 12 weeks also showed significant increase ($p < 0.05$) in fasting blood glucose level from 105 ± 18.3 mg/dL to 142.6 ± 18.9 mg/dL (Table 1).

Table 1. Effect of high fat diet on body weight, fasting blood glucose, and visceral fat mass of Swiss albino mice.

	12 week experiment		8 week experiment	
	Normal diet	High fat diet	Normal diet	High fat diet
Initial body weight (g)	24.5 ± 1.9	24.4 ± 1.9	23.5 ± 2.6	24.6 ± 3.0
Final body weight (g)	46.3 ± 3.5	47.7 ± 4.5	41.3 ± 1.6	42.6 ± 1.9
Weight gain (g)	21.8 ± 2.7	23.3 ± 3.2	17.8 ± 2.1	18 ± 2.5
Initial fasting blood glucose (mg/dL)	115.7 ± 9.3	105.0 ± 18.3	120.8 ± 20.9	98.0 ± 22.5
Final fasting blood glucose (mg/dL)	124.3 ± 8.9	142.6 ± 18.9 [c]	156.0 ± 4.8 [c]	156.6 ± 6.6 [c]
Visceral fat (mg)	66.5 ± 27.6	1140.0 ± 248.8 [a]	611.7 ± 178.3	862.4 ± 148.7 [b]

[a] indicates $p < 0.001$ compared with normal diet fed group
[b] indicates $p < 0.05$ compared with normal diet fed group
[c] indicates $p < 0.05$ compared with initial value of the same group.
Final body weight and final fasting blood glucose were measured at the end of the experiment. Data are presented as the mean ± SD (n= 5 to 7).

However, all blood glucose values were normal for mice (less than 200 mg/dL), and increased in all mice, regardless of the diet they were fed. Thus, in both the 8-week and 12-week feeding of high fat diet, all blood glucose values were in the normal, non-diabetic range. Therefore, the high fat lard diet (for 8 or 12 weeks) did not induce any obvious prediabetic or diabetic state when compared with regular diet and did not raise fasting blood glucose level above the normal range (60- 200 mg/dL) in mice.

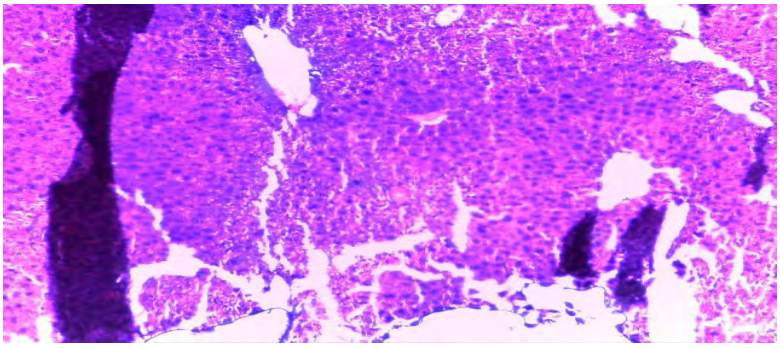
Serum total cholesterol levels of mice fed a high fat diet for 8 weeks (173.6 ± 19.4 mg/dL) increased significantly ($p < 0.05$) when compared with mice fed a normal diet (117.7 ± 7.5mg/dL). Serum LDL level also increased significantly ($p < 0.05$) in mice fed high fat diet (90.6 ± 9.7 mg/dL) when compared with mice fed normal diet (22.6 ± 5.2mg/dL) and serum HDL level decreased significantly ($p < 0.05$) in mice fed a high fat diet (52.0 ± 7.8 mg/dL) when compared with mice fed a normal diet (63.5 ± 7.8 mg/dL) but serum triglyceride level did not show any significant change ($p > 0.05$) (Table 3).

In the 12 week high fat diet feeding, no significant change ($p > 0.05$) in serum total cholesterol level and serum triglyceride level were observed. However, serum HDL level increased significantly ($p < 0.05$) (104.1 ± 14.9 mg/dL) when compared with mice fed normal diet (81.3 ± 10.5 mg/dL) (Table 2). Serum LDL levels of both mice fed a high fat diet and mice fed a normal diet in the 12 week feeding period were low, close to zero, and several were even slightly negative values. Mice have much lower LDL levels (often around 20 mg/dL or less) than humans, so any errors in HDL, total cholesterol and triglyceride determinations might be amplified when the Friedewald equation is used to determine LDL, so use of the direct LDL determination assay might be a better way of determining LDL in mice.

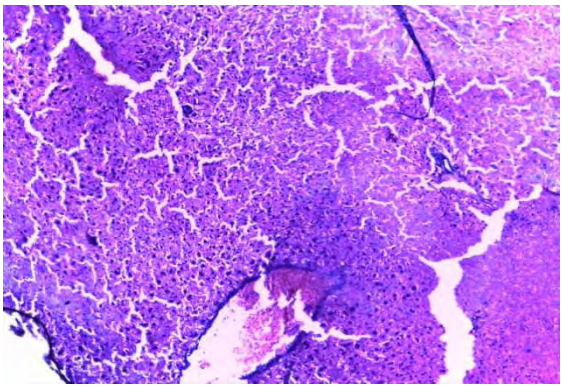
3.1.3. Effect of high fat diet on serum liver enzymes and liver histopathology

High fat diet feeding for 8 weeks did not cause any significant effect ($p > 0.05$) on serum AST level, ALT level and ALP level (Table 3). In the 12 week feeding period, serum AST levels of mice fed a high fat diet increased significantly ($P < 0.05$) (145.4 ± 16.1 U/L) when compared with mice fed normal diet (101.3 ± 34.7 U/L), however, Serum ALT and ALP levels did not show any significant change ($p > 0.05$) (Table 2).

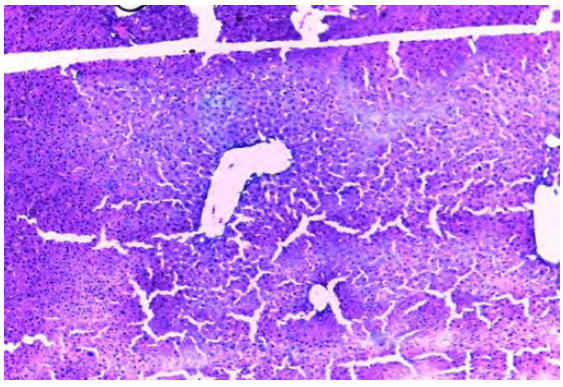
Light microscopic examination of liver histology of Swiss albino mice fed a high fat diet showed normal liver histology with no lipid deposition in the hepatocytes of the liver and no fatty liver was seen in both the 8 week and 12 week feeding period (Figure 8).



(a)



(b)



(c)

Figure 8. Photomicrograph of sections of liver of normal diet fed (a), high fat diet fed for 8 weeks (b), and high fat diet fed for 12 weeks (c); with magnification of 40X.

3.2. Effect of resveratrol on Swiss albino mice fed a high fat (lard) diet

3.2.1. Effect of resveratrol on body weight and visceral fat mass

Both mice fed a high fat diet alone and mice fed a high fat diet supplemented with 75 µg/mL resveratrol in drinking water showed a significant increase ($p < 0.05$) in their final body weights when compared with their initial value. However, there was no significant difference in weight increase between non-treated mice fed a high fat diet (weight gain 23.3 $\pm$ 3.2 g) and mice fed a high fat diet treated with resveratrol (weight gain 21.4 ± 3.2g), showing that resveratrol did not affect body weight of mice fed a high fat diet (Table 2).

There was also no significant change ($p > 0.05$) in visceral fat mass between mice fed a high fat diet only (1140.0 ± 248.8 mg) and mice fed a high fat diet with resveratrol in their drinking water (1076.8 ± 173.4 mg) (Figure 9 and Table 2; also see Figure 7).

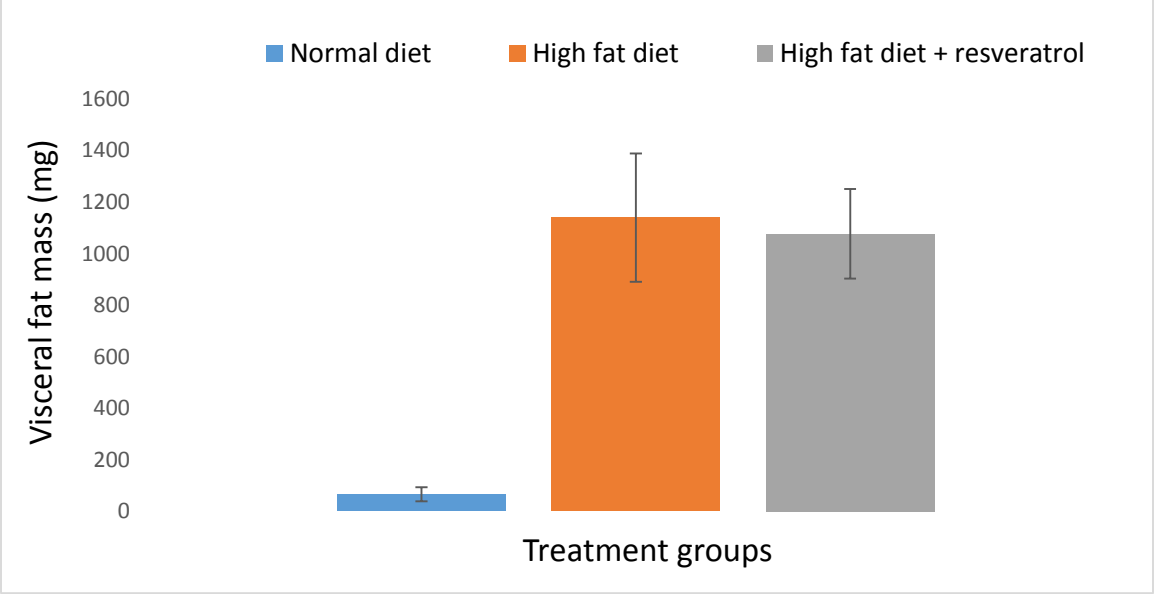


Figure 9. Effect of resveratrol on visceral fat mass in Swiss albino mice fed a high fat diet. Mice fed either standard pellets, a high fat diet (52% fat) alone, or a high fat diet supplemented with resveratrol (75 µg/mL in drinking water) for 12 weeks. Visceral fat was removed by dissection, pooled, and weighed.

3.2.2. Effect of resveratrol on fasting blood glucose, serum lipid profiles and serum liver enzymes

Resveratrol treatment did not cause any significant change ($p > 0.05$) on fasting blood glucose level of mice fed a high fat diet (144.0 ± 20.9 mg/dL), when compared with non-treated mice fed a high fat diet (142.6 ± 18.9 mg/dL), showing that resveratrol did not affect fasting blood glucose level of mice fed a high fat diet (Figure 10, Table 2).

Resveratrol treatment did not cause any significant effect ($p > 0.05$) on serum total cholesterol level of mice fed a high fat diet (144.6 ± 20.5 mg/dL) when compared with mice fed a high fat diet without treatment of resveratrol (142.7 ± 19.6mg/dL).

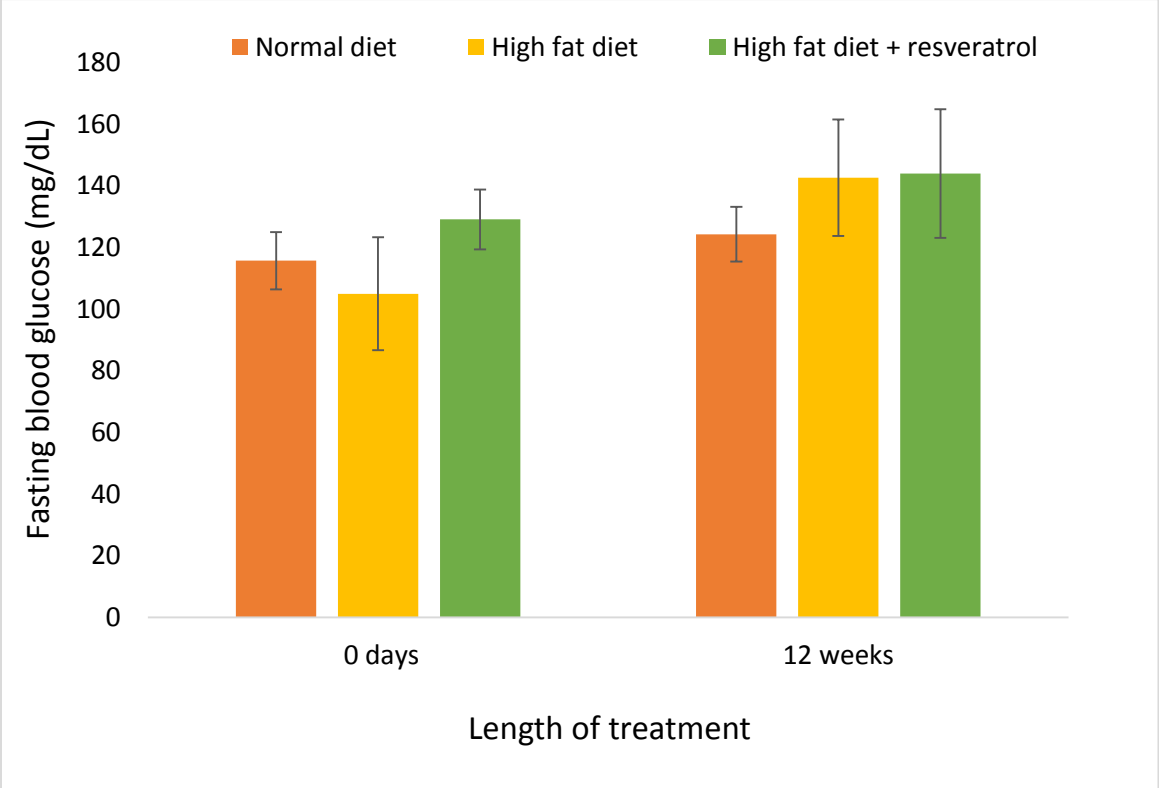


Figure 10. Effect of resveratrol on fasting blood glucose level in Swiss albino mice fed a high fat diet. Mice fed either standard pellets, a high fat diet (52 % fat) alone, or a high fat diet supplemented with resveratrol (75 µg/mL in drinking water) for 12 weeks.

Resveratrol treatment did not cause any significant change ($p > 0.05$) on serum triglyceride level (187.3 ± 68.6 mg/dL) when compared with mice fed high fat diet that did not receive resveratrol (177.6 ± 60.1 mg/dL). No significant effect ($p > 0.05$) of resveratrol treatment on serum HDL levels of mice fed high fat diet (102.6 ± 12.9 mg/dL) also observed, when compared with non-treated mice fed a high fat diet only (104.1 ± 14.9 mg/dL) (Table 2).

Serum LDL levels of both mice fed a high fat diet and mice fed high fat diet with resveratrol treatment were low, close to zero, and several were even slightly negative values. Therefore, the LDL values were not meaningful in this experiment, and ideally it would have been better if direct LDL had been determined.

Resveratrol treatment of mice fed a high fat diet did not cause significant effect ($P > 0.05$) on serum AST level (162.3 ± 30.9 U/L) when compared with mice fed high fat diet only (145.4 ± 16.1U/L). No significant change ($p > 0.05$) also showed on serum ALT level of mice fed a high fat diet with resveratrol treatment (59.4 ± 14.8 U/L) when compared with mice fed high fat diet only (52.0 ± 3.9 U/L) and again no significant difference ($p > 0.05$) observed on serum ALP level of mice fed high fat diet with resveratrol treatment, when compared with mice fed high a fat diet only (Table 2).

In summary, resveratrol administration (75 μg/mL in drinking water) for 12 weeks on Swiss albino mice fed a high fat diet, did not have any significant effect on body weight, visceral fat mass, fasting blood glucose, serum AST level, serum ALT level and serum ALP level, and also on serum total cholesterol level, serum triglyceride level and on serum HDL level (Table 2).

Table 2. Effect of resveratrol on body weight, fasting blood glucose, visceral fat mass, serum liver enzymes and fasting lipid profiles in Swiss albino mice fed a high fat diet.

	Treatment		
	Normal diet	High fat diet	High fat diet + resveratrol
Initial body weight (g)	24.5 ± 1.9	24.4 ± 1.9	25.9 ± 1.7
Final body weight (g)	46.3 ± 3.5	47.7 ± 4.5	47.3 ± 4.7
Weight gain (g)	21.8 ± 2.7	23.3 ± 3.2	21.4 ± 3.2
Initial fasting blood glucose (mg /dL)	115.7 ± 9.3	105.0 ± 18.3	129.1 ± 9.7
Final fasting blood glucose (mg/ dL)	124.3 ± 8.9	142.6 ± 18.9	144.0 ± 20.9
Visceral fat (mg)	66.5 ± 27.6	1140.0 ± 248.8 [a]	1076.8 ±173.4 [a]
AST (U/L)	101.3 ± 34.7	145.4 ± 16.1 [b]	162.3 ± 30.9 [b]
ALT (U/L)	49.7 ± 4.9	52.0 ± 3.9	59.4 ± 14.8
ALP (U/L)	108.8 ± 48.1	290.6 ± 226.7	420.0 ± 297.1 [b]
Total cholesterol (mg/dL)	123.3 ± 12.1	142.7 ± 19.6	144.6 ± 20.5 [b]
Triglyceride (mg/dL)	129.5 ± 25.8	177.6 ± 60.1	187.3 ± 68.6
HDL (mg/dL)	81.3 ± 10.5	104.1 ± 14.9 [b]	102.6 ± 12.9 [b]

[a] indicates $p < 0.001$ compared with normal diet fed group,
[b] indicates $p < 0.05$ compared with normal diet fed group.
Final body weight and final fasting blood glucose were measured at the end of the animal experiment. Data are presented as the mean ± SD (n= 5 to 7), Resveratrol was given in drinking water at 75 μg/mL for 12 weeks.

3.3. Effect of metformin on Swiss albino mice fed a high fat (lard) diet

3.3.1. Effect of metformin on body weight and visceral fat mass

Metformin treatment (250 mg/kg/day intraperitoneally for 8 weeks) did not cause any significant effect ($p > 0.05$) on body weight of mice fed a high fat diet (42.6 ± 0.5g) when compared with mice fed a high fat diet only ((42.6 ± 1.9g) (Table 3). In addition, metformin treatment on visceral fat mass of mice fed a high fat diet (646.4 ± 254.9 mg) did not cause any significant effect ($p > 0.05$) when compared with mice fed high fat diet only (862.4 ± 148.7 mg) (Figure 11, Table 3).

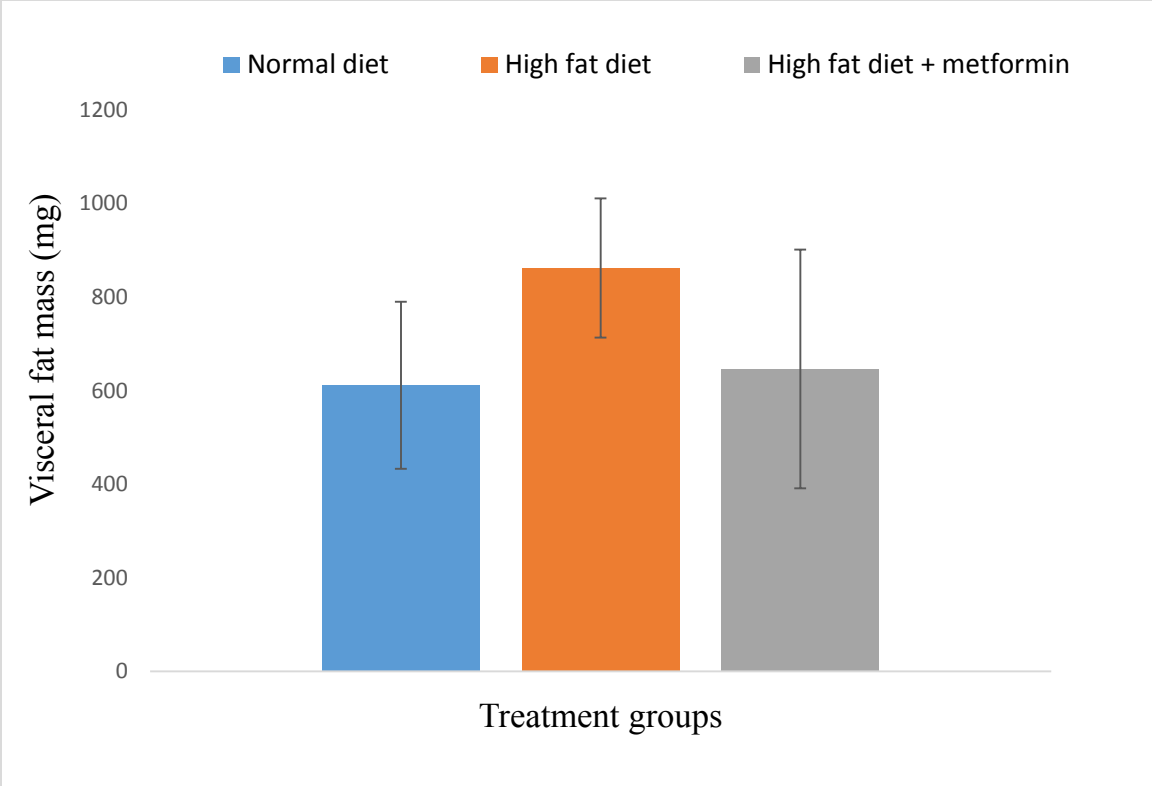


Figure 11. Effect of metformin on visceral fat mass in Swiss albino mice fed a high fat diet. Mice were fed either standard pellets, a high fat diet (52% fat) alone, or a high fat diet with metformin treatment (250 mg/kg/day intraperitoneally) for 8 weeks. Visceral fat was removed by dissection, pooled and weighed.

3.3.2. Effect of metformin on fasting blood glucose, serum lipid profile and serum liver enzymes

Fasting blood glucose level of both mice fed a high fat diet only (156.6 ± 6.6 mg/dL) and mice fed high fat diet with intraperitoneal treatment of metformin (146.8 ± 14.5 mg/dL) were in the normal range for mice and there was no significant change ($p > 0.05$) in blood glucose levels (Figure 12, Table 3).

Metformin treatment on mice fed a high fat diet, did not cause any significant effect ($p > 0.05$) on serum total cholesterol level and serum triglyceride levels when compared with non-treated mice fed a high diet only (Table 3).

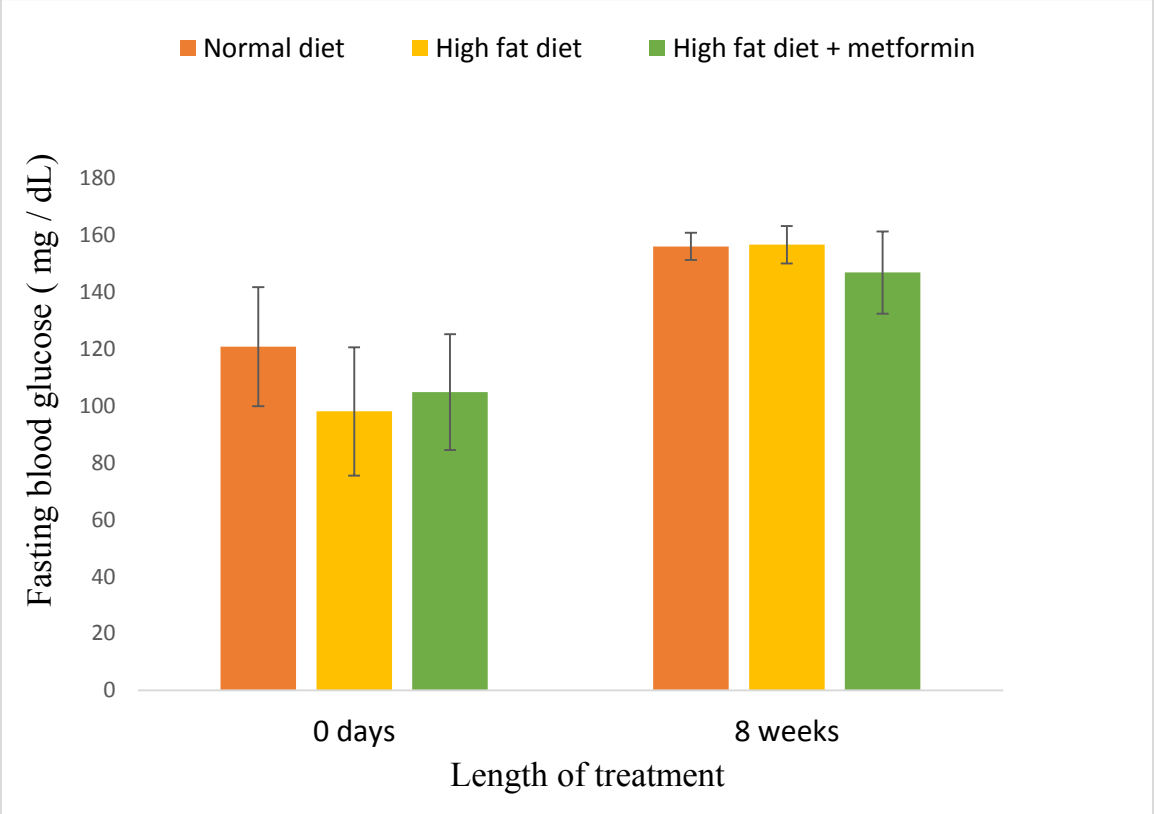


Figure 12. Effect of metformin on fasting blood glucose level in Swiss albino mice fed a high fat diet. Mice were fed either standard pellets, a high fat diet (52% fat) alone, or a high fat diet treated with metformin (250 mg/kg/day intraperitoneally) for 8 weeks.

However, serum HDL level increased significantly ($P < 0.05$) in mice fed high fat diet with metformin treatment (100.0 ± 17.0 mg/dL) when compared with mice fed a high fat diet only (52.0 ± 7.8 mg/dL). Metformin treatment on mice fed high fat diet also caused significant lowering ($p < 0.05$) of serum LDL level (31.6 ± 12.0 mg/dL) when compared with mice fed a high fat diet only (90.6 ± 9.7 mg/dL) (Figure 13, Table 3).

Serum AST levels of mice fed a high fat diet with metformin treatment (219.8 ± 30.0 U/L) were slightly higher when compared with non-treated mice fed a high fat diet (172.2 ± 34.1U/L) which is statistically significant ($p < 0.05$), but these values were essentially in the normal range and not abnormal. Serum ALT and ALP levels did not show significant change ($p > 0.05$) with metformin treatment when compared with mice fed a high fat diet only (Figure 14, Table 3).

In summary, treatment with metformin (250 mg/kg/day intraperitoneally for 8 weeks) of Swiss albino mice fed a high fat diet did not show any significant effect ($p > 0.05$) on body weight, fasting blood glucose level, visceral fat mass, serum ALT level and serum ALP level, on serum total cholesterol level and on serum triglyceride level when compared with non-treated mice fed a high fat diet.

However, metformin treatment slightly raised serum AST level, strongly elevated serum HDL level and strongly lowered serum LDL level when compared with non-treated mice fed a high fat diet ($p < 0.05$) (Table 3).

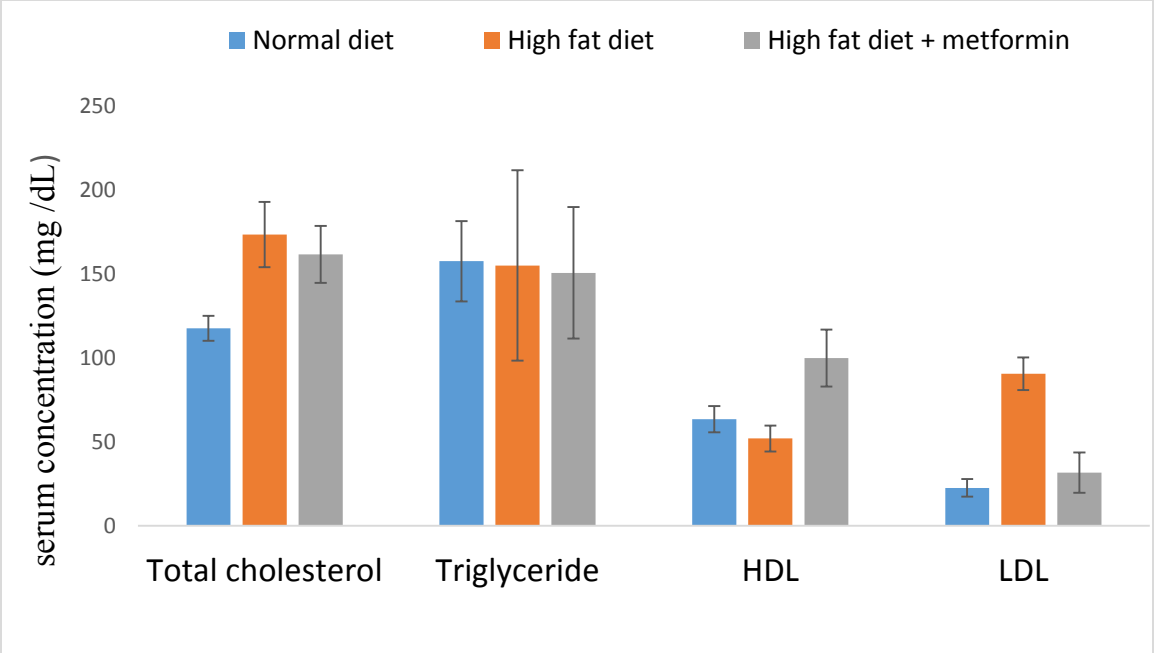


Figure 13. Effect of metformin on serum lipid profiles in Swiss albino mice fed a high fat diet. Mice were fed either standard pellets, a high fat diet (52% fat) alone, or a high fat diet supplemented with metformin (250 mg/kg/day intraperitoneally) for 8 weeks.

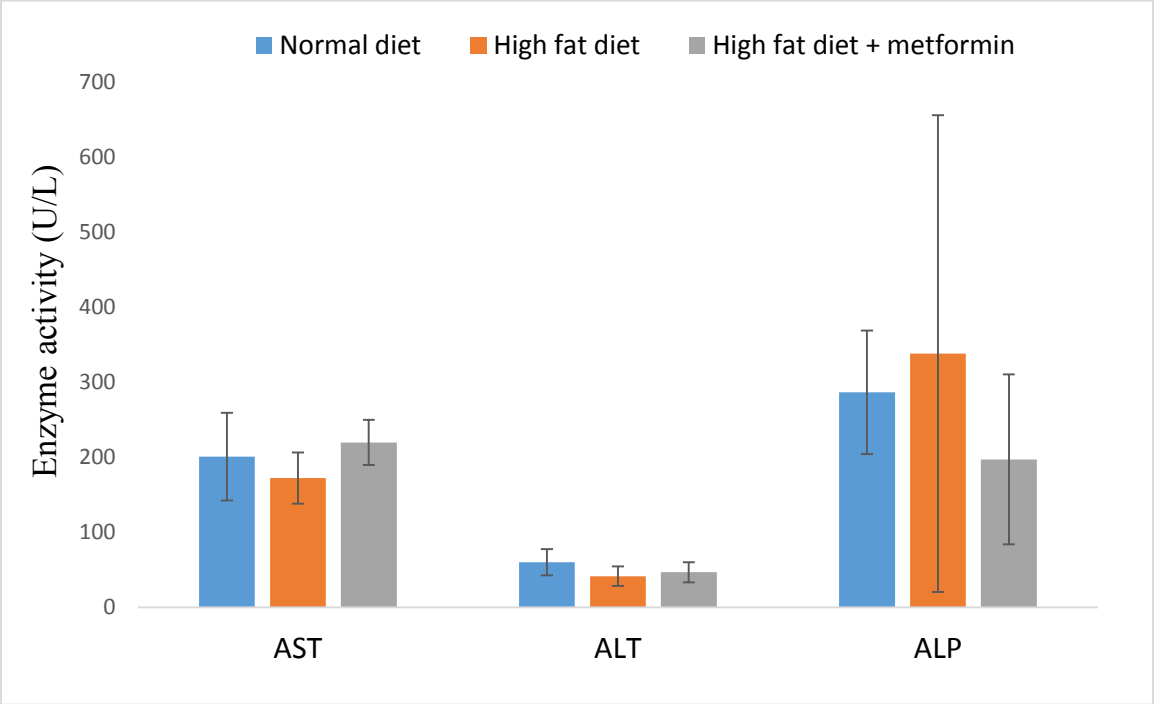


Figure 14. Effect of metformin on serum AST, ALT and ALP level in Swiss albino mice fed a high fat diet. Mice were fed either standard pellets, a high fat diet (52 % fat) alone, or a high fat diet treated with metformin (250 mg/kg/day intraperitoneally) for 8 weeks.

Table 3. Effect of metformin on body weight, fasting blood glucose, visceral fat mass, liver enzymes and serum lipid profile in Swiss albino mice fed a high fat diet.

	Treatment		
	Normal diet	High fat diet	High fat diet + metformin
Initial body weight (g)	23.5 ± 2.6	24.6 ± 3.0	24.6 ± 2.3
Final body weight (g)	41.3 ± 1.6	42.6 ± 1.9	42.6 ± 0.5
Weight gain (g)	17.8 ± 2.1	18.0 ± 2.45	18.0 ± 1.4
Initial fasting blood glucose (mg/dL)	120.8 ± 20.9	98.0 ± 22.5	104.8 ± 20.3
Final fasting blood glucose (mg/dL)	156.0 ± 4.8	156.6 ± 6.6	146.8 ± 14.5
Visceral fat (mg)	611.7 ± 178.3	862.4 ± 148.7 [c]	646.4 ± 254.9
AST (U/L)	200.8 ± 58.5	172.2 ± 34.1	219.8 ± 30.0 [b]
ALT (U/L)	60.0 ± 17.5	41.6 ± 12.9	46.8 ± 13.4
ALP (U/L)	286.7 ± 82.4	338.2 ± 317.7	197.2 ± 113.3
Total cholesterol (mg/dL)	117.7 ± 7.5	173.6 ± 19.4 [c]	161.8 ± 16.9 [c]
Triglyceride (mg/dL)	157.7 ± 23.9	155.2 ± 56.8	150.8 ± 39.2
HDL (mg / dL)	63.5 ± 7.8	52.0 ± 7.8 [c]	100.0±17.0 [a, c]
LDL (mg/ dL)	22.6 ± 5.2	90.6 ± 9.7 [c]	31.6 ± 12.0 [a]

[a] $p < 0.001$ compared with high fat fed group,
[b] $p < 0.05$ compared with high fat fed group,
[c] $p < 0.05$ compared with normal diet fed group,
Final body weight and final fasting blood glucose were measured at the end of the animal experiment. Data are presented as the mean ± SD (n= 5-7), metformin was given intraperitoneally at 250 mg/kg/day, all treatments were for 8 weeks.

3.4. Effect of *Eucalyptus* oil on Swiss albino mice fed a high fat (lard) diet

3.4.1. Effect of *Eucalyptus* oil on body weight and visceral fat mass

There was no significant difference ($p > 0.05$) in final body weight between mice fed a high fat diet with daily intraperitoneal *Eucalyptus* oil treatment (39.0 ± 4.2g) and mice fed a high fat diet only (42.6 ± 1.9 g) (Table 4). In addition, *Eucalyptus* oil treatment of mice fed a high fat diet did not cause any significant effect ($p > 0.05$) on visceral fat mass (678.6 ± 122.1 mg) when compared with mice fed high fat diet without any treatment (862.4 ± 148.7 mg) (Figure 15, Table 4).

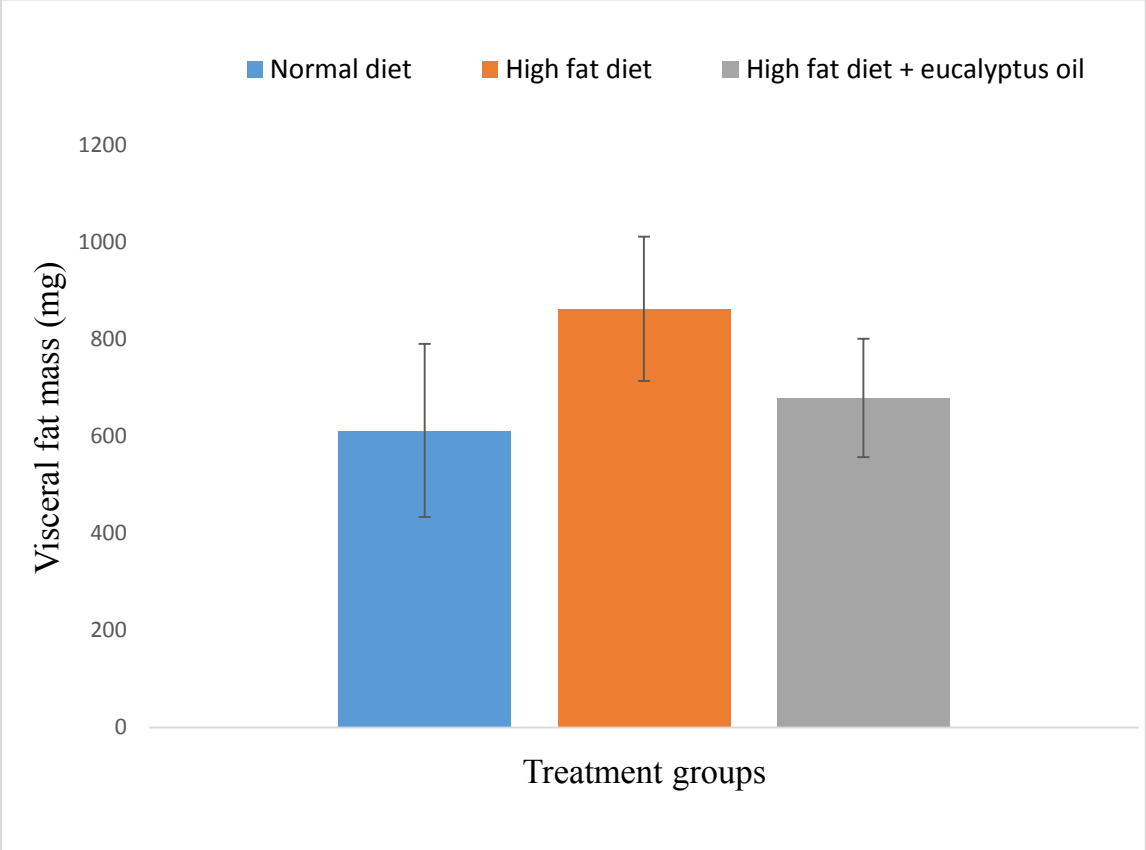


Figure 15. Effect of *Eucalyptus* oil on visceral fat mass in Swiss albino mice fed a high fat diet. All treatments were for 8 weeks. *Eucalyptus* oil was given intraperitoneally at 10 µL /mouse/ day as an emulsion in 0.5 mL of water.

3.4.2. Effect of *Eucalyptus* oil on fasting blood glucose, serum lipid profile and serum liver enzymes

Eucalyptus oil treatment on mice fed a high fat diet, caused significant lowering ($p < 0.05$) of fasting blood glucose (133.8 ± 15.5 mg/dL), when compared with mice fed a high fat diet only (156.6 ± 6.6 mg/dL), although all fasting blood glucose in all mice were in the normal range (Figure 16, Table 4).

Treatment of *Eucalyptus* oil on mice fed a high fat diet did not cause any significant effect ($p > 0.05$) on serum total cholesterol level and also on serum triglyceride level, when compared with mice fed a high fat diet only (Table 4).

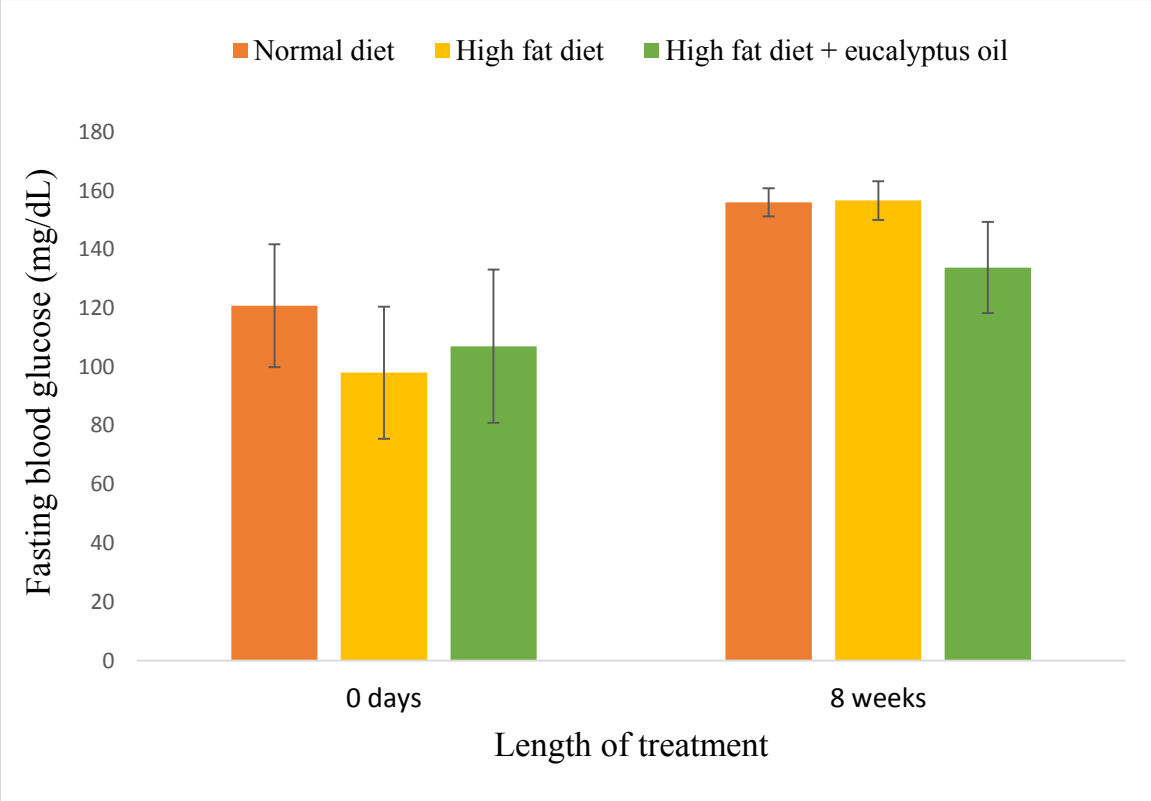


Figure 16. Effect of *Eucalyptus* oil on fasting blood glucose level in Swiss albino mice fed a high fat diet. All treatments were for 8 weeks. *Eucalyptus* oil was given intraperitoneally at 10 µL /mouse/ day as an emulsion in 0.5 mL of water.

However, treatment of *Eucalyptus* oil on mice fed a high fat diet caused significant elevation ($p < 0.05$) of serum HDL level (104.6 ± 22.8 mg/dL) when compared with mice fed a high fat diet only (52.0 ± 7.8 mg/dL). *Eucalyptus* oil treatment also caused a significant lowering ($p < 0.05$) of serum LDL (40.2 ± 7.9 mg/dL) when compared with mice fed high fat diet only (90.6 ± 9.7 mg/dL) (Figure 17, Table 4).

Eucalyptus oil treatment on mice fed a high fat diet caused significant elevation ($p < 0.05$) of serum AST level (246.8 ± 61.9 U/L) when compared with mice fed high fat diet only (172.2 ± 34.1 U/L), although these were mild increases. However, serum ALT and ALP levels of mice fed a high fat diet, did not show any significant change with *Eucalyptus* oil treatment (Figure 18, Table 4).

In summary, intraperitoneal treatment of *Eucalyptus* oil (10 μL /mouse /day) on Swiss albino mice fed a high fat diet did not cause any significant effect ($p > 0.05$) on body weight, visceral fat mass, serum total cholesterol, serum triglyceride level, serum ALT level and serum ALP levels when compared with non-treated high fat fed mice that did not receive *Eucalyptus* oil (Table 4).

However, *Eucalyptus* oil did have a significant effect ($p < 0.05$) on lowering of fasting blood glucose (but were nevertheless in the normal range to begin with), and on lowering of serum LDL level and significantly elevated serum AST level (but within the normal range) and serum HDL level when compared with non-treated high fat fed mice that did not receive *Eucalyptus* oil (Table 4).

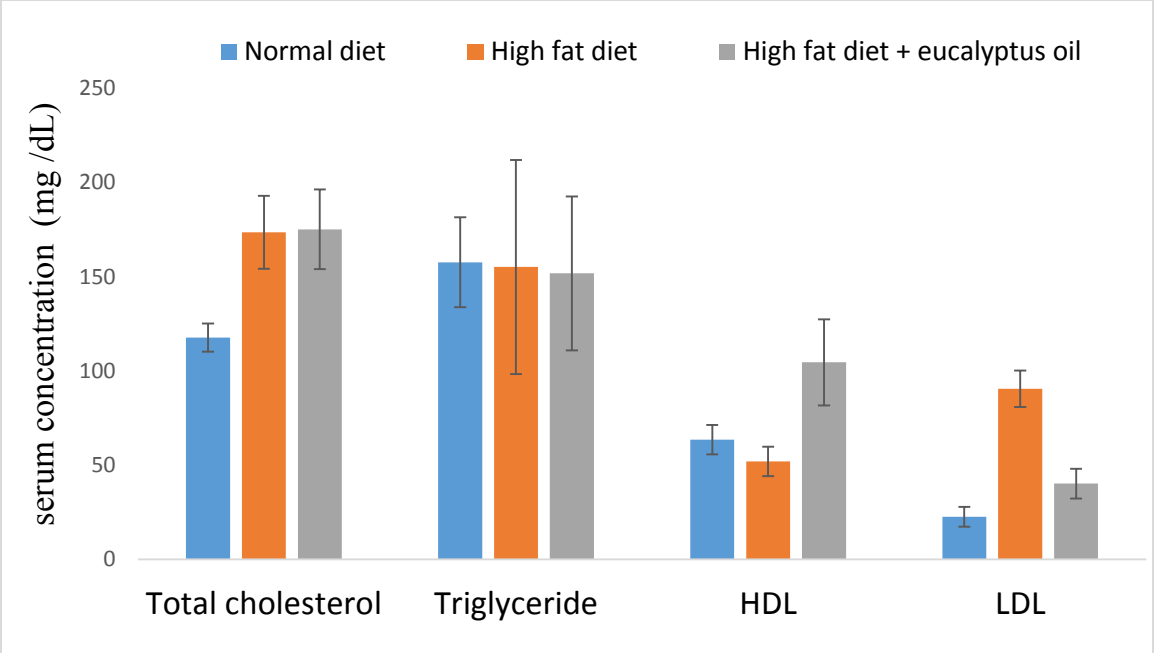


Figure 17. Effect of *Eucalyptus* oil on lipid profiles of Swiss albino mice fed a high fat diet. All treatments were for 8 weeks. *Eucalyptus oil* was given intraperitoneally at 10 µL /mouse/ day as an emulsion in 0.5 mL of water.

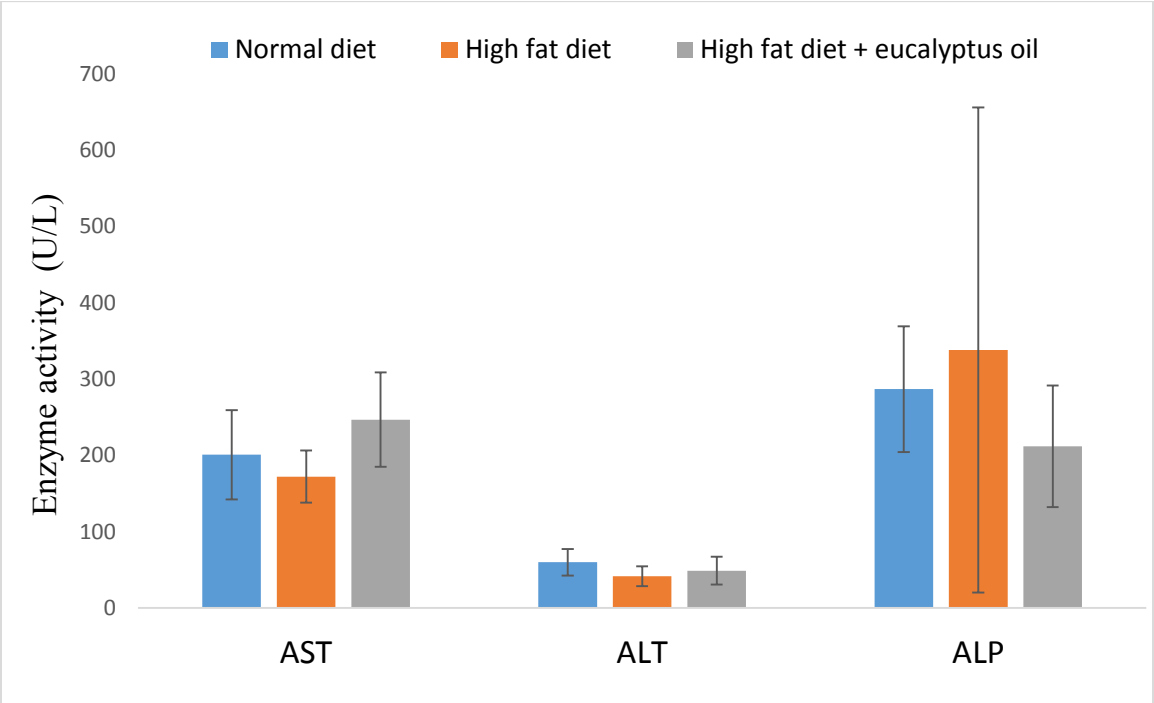


Figure 18. Effect of *Eucalyptus* oil on serum AST, ALT and ALP level in high fat fed Swiss albino mice. All treatments were for 8 weeks. *Eucalyptus* oil was given intraperitoneally at 10 µL /mouse/ day as an emulsion in 0.5 mL of water.

Table 4. Effect of *Eucalyptus* oil on body weight, fasting blood glucose, visceral fat mass, serum lipid profile and serum liver enzymes in Swiss albino mice fed a high fat diet.

	Treatment		
	Normal diet	High fat diet	High fat diet + eucalyptus oil
Initial body weight (g)	23.5 ± 2.6	24.6 ± 3.0	24.6 ± 2.9
Final body weight (g)	41.3 ± 1.6	42.6 ± 1.9	39.0 ± 4.2
Weight gain (g)	17.8 ± 2.1	18 ± 2.45	14.4 ± 3.55
Initial fasting blood glucose (mg/dL)	120.8 ± 20.9	98.0 ± 22.5	107.0 ± 26.1
Final fasting blood glucose (mg/dL)	156.0 ± 4.8	156.6 ± 6.6	133.8 ± 15.5 [a, b]
Visceral fat (mg)	611.7 ± 178.3	862.4 ± 148.7 [a]	678.6 ± 122.1
AST (U/L)	200.8 ± 58.5	172.2 ± 34.1	246.8 ± 61.9 [b]
ALT (U/L)	60.0 ± 17.5	41.6 ± 12.9	49.0 ± 18.3
ALP (U/L)	286.7 ± 82.4	338.2 ± 317.7	211.8 ± 79.5
Total cholesterol (mg/dL)	117.7 ± 7.5	173.6 ± 19.4 [c]	175.2 ± 21.1 [c]
Triglyceride (mg/dL)	157.7 ± 23.9	155.2 ± 56.8	151.8 ± 40.8
HDL (mg / dL)	63.5 ± 7.8	52.0 ± 7.8 [a]	104.6 ± 22.8 [d, e]
LDL (mg /dL)	22.6 ± 5.2	90.6 ± 9.7 [c]	40.2 ± 7.9 [d, e]

[a] indicates $p < 0.05$ compared with normal diet fed group,
[b] indicates $p < 0.05$ compared with high fat fed group,
[c] indicates $p < 0.001$ compared with normal diet fed group
[d] indicates $p < 0.001$ compared with high fat diet fed group
[e] indicates $p < 0.01$ compared with normal diet fed group
Final body weight and final fasting blood glucose were measured at the end of the animal experiment. Data are presented as the mean ± SD (n= 5 to 7), all treatments were for 8 weeks. *Eucalyptus* oil was given intraperitoneally at 10 µL /mouse/ day as an emulsion in 0.5 mL of water.

Chapter 4

Discussion

4.1. Association of increased fat intake and obesity

Maintaining a sedentary lifestyle and consuming excessive portions of foods rich in calories, particularly dietary fats and carbohydrates, have been identified as major contributing factors in the growing epidemic of obesity and its comorbidities. Energy from dietary fat has a larger effect on body-weight gain than has energy from non-fat sources, because fat has more calories per gram than other macronutrients (Saris, 2006). Epidemiological studies have shown a positive relationship between dietary fat intake and obesity (Hariri and Thibault, 2010). Increased carbohydrate calorie intake (for example in baked foods, desserts and sodas), reduced physical activity, and genetic factors also play a role in the development of obesity.

In general, a high fat diet accounts for the largest incidence of metabolic syndrome in the world (Jimoh *et al.*, 2015). Human studies also showed that increased fat intake is associated with body weight gain and obesity, with its associated metabolic diseases, including insulin resistance, diabetes, dyslipidemia, and cardiovascular disease. High fat diets are also widely used to study the development of obesity and insulin resistance in rodent models. Animal models are useful tools for studying obesity as they will readily gain weight when fed high-fat diets (Buettner *et al.*, 2007). In animal models, as in humans, obesity can be assessed by criteria based on gain of body weight and/or increase of body fat content.

However, standard thresholds for obesity have not been developed in mice as they have, for example with BMI, in humans. In most studies, the degree of obesity has been evaluated by comparing body weight, or percentage of body fat composition, or visceral obesity content, of the experimental group fed a high-fat diet with control animals that show normal growth while fed chow or low-fat diet (Hariri and Thibault, 2010).

In this study, male Swiss albino mice were fed a 52% high fat diet, where 40% by weight of the fat comes from lard. No body weight gain and no obvious excessive subcutaneous fat deposition was observed in mice fed a high fat diet when compared with mice fed normal diet. However, there was a significant deposition of visceral fat in mice fed a high fat diet in intraperitoneal/ retroperitoneal areas, with little or no subcutaneous fat deposition, therefore the model could be a good model for visceral obesity. According to the new obesity classification criteria based on body fat composition, rather than on body weight and BMI alone (De Lorenzo et al., 2016), these mice may reflect a type of human obesity which have normal BMI but having excess fat especially those depositing excess visceral fat.

Generally mice may increase their body fat on high-fat diets, but there can be variable responses in weight gain, blood glucose level, serum triglycerides, serum lipoproteins and other metabolic findings, depending on the mouse strain, age and environment. Some inbred strains are more susceptible to obesity when fed a high-fat diets such as the C57BL/6J or AKR mouse. Different mouse strains that exhibit similar levels of weight gain may also show different patterns of metabolic changes (Gajda, 2009).

Significant higher body weight and epididymal fat deposits were found in a study of male C57BL/6J lard-fed mice, in a 34-day period when compared with mice fed a normal diet (Libinaki *et al*., 1999). In another study, significant increase in body weight, body fat percentage and serum total cholesterol concentrations were found in a study of a 5 week old male C57BL/6J mice fed a high-fat diet with 60% of calories derived from lard for 30 weeks when compared with mice fed normal diet (Hoffler *et al.*, 2009).

Significant increase in body weight and retroperitoneal fat deposition, elevation in Total triglyceride, total cholesterol level and serum LDL level, and lowering of serum HDL found in a study of 5 week old male Swiss albino mice that were fed a high fat diet (40g lard + 60g food), in an 8 week period of time when compared with that of mice fed a normal diet (Rani *et al*., 2012). This agrees partly with the results of this study, in which serum LDL level increased and serum HDL decreased, but total cholesterol and triglycerides showed no change in Swiss albino mice fed the high fat lard diet for 8 weeks, when compared with mice fed a normal diet.

Generally, the physiological mechanisms involved in high-fat diet induced obesity involve storage of excess fat calories as body fat, low satiating effects involving alterations in the hormones involved in hunger and satiety as well as alteration in energy balance (Hariri and Thibault, 2010). Elevated plasma insulin levels and insulin resistance are also associated with obesity and independent of obesity, high-fat feeding itself plays a role to impaired glucose tolerance and insensitivity to the blood glucose-lowering effect of insulin (De Ferranti and Mozaffarian, 2008). However, the high fat diet used in this MSc study showed no obvious diabetogenic features but, it is possible that feeding the mice for periods longer than 8 to 12 weeks might eventually produce significant blood glucose elevations.

4.2. Effects of resveratrol

Resveratrol is a type of natural phenol, and a phytoalexin, which is produced naturally by several plants in response to injury and found in various food sources such as skin of grapes. It has received attention in recent years, because of its broad biological activity to prevent or slow the progression of a wide variety of illnesses. In addition, recent data derived from animal and in vitro studies have opened a new, promising perspective for the potential use of resveratrol to prevent serious metabolic disorders such as obesity and diabetes (Zhang et al., 2012; Szkudelska and Szkudelski, 2010).

Although its exact mechanism of action is still controversial, resveratrol-induced Sirt1 activation appears to be dose- and time-dependent, and may be dependent upon AMPK activation by two mechanisms: first, high doses of resveratrol inhibit cAMP-phosphodiesterase, thus increasing cellular cAMP concentrations and activating the upstream kinase CaMKKβ, and second, moderate doses of resveratrol lead to Sirt1-dependent activation of AMPK (Park *et al*., 2012; Bruckbauer and Zemel, 2013). The anti-obesity effect of resveratrol in animals seems to be mediated through stimulation of fat oxidation and metabolism or suppression of adipogenic gene expression (Wang *et al*., 2014).

In this study, 12 weeks of resveratrol administration (75 μg/mL in drinking water) in Swiss albino mice fed a high fat diet, did not cause any significant effect on body weight gain, visceral fat mass, fasting blood glucose, serum total cholesterol level, serum triglyceride level, serum HDL level, serum AST level, serum ALT level and serum ALP level when compared with mice fed normal diet without resveratrol treatment.

Resveratrol (25 mg/kg body weight) treatment on streptozotocin induced diabetic Wistar rats for 40 days ameliorated dyslipidemia and hyperglycemia (Shahi *et al*., 2011)

There were also exceptions where resveratrol supplementation failed to induce changes in body weight, lipogenesic enzymes, blood lipid profiles and glucose (Alberdi *et al*., 2013). In agreement with the result of the present study, neither low-dose (2g/kg food) nor high dose (4g/kg food) resveratrol treatment influenced body weight gain, body fat percentage, or metabolic performance of 7-week male C57BL/6J mice fed a high fat diet (60% of energy from fat) (Tauriainen *et al*, 2011).

In addition, no effect on ectopic or visceral fat content was observed on a 4 week supplementation of resveratrol (using tablets containing 500mg trans-resveratrol three times per day) on obese human subjects, in a randomized, placebo-controlled, double-blinded, and parallel-group, extensive metabolic study (Poulsen *et al*., 2013).

The difference in the outcome of different studies including this study could be due to the duration of study, dosage of resveratrol, and age and strain of animals. There is no enough information about the necessary concentrations of resveratrol needed to achieve physiological effects. Therefore, there is a possibility that higher resveratrol dosage will be needed for preventing weight gain, since supplementation of 75µg/mL resveratrol in a drinking water was not sufficient to produce any metabolic effects. Also longer treatment period will be necessary to get significant metabolic effect.

4.3. Effects of metformin

In this MSc study, intraperitoneal treatment of Swiss albino mice fed a high fat diet with metformin did not cause any significant effect on body weight, fasting blood glucose level, visceral fat mass, serum total cholesterol level, serum triglyceride level, serum ALT level and serum ALP level. However, metformin treatment, strongly elevated serum HDL level and strongly lowered serum LDL level when compared with non-treated mice fed a high fat diet. Therefore, metformin would be promising drug for improvement of lipid profile and the lack of effect on fasting blood glucose could be since metformin is not a hypoglycemic drug and also the mice used in this study were not diabetic.

In agreement with the results of this study, Thamer found significantly lowered level of serum LDL, and elevated serum HDL concentration after treatment of obese rats with 500 mg/kg twice per day metformin for 4 weeks. However, it also caused decreased fasting plasma glucose, decreased serum total cholesterol and triglycerides (Thamer, 2014).

The primary action of metformin is to lower blood glucose concentration by inhibiting hepatic glucose production and stimulating glucose disposal in skeletal muscle (Viollet *et al*., 2012). Metformin activates AMPK, although it is not clear whether this is by direct or indirect stimulation, though by inhibiting Complex I of the electron transport chain, metformin transiently lowers AMP concentrations and activates AMPK. It also acts by inhibition of AMP deaminase, thereby inhibiting AMP catabolism and increasing the cellular AMP/ATP ratio, resulting in phosphorylation and activation of AMPK. In addition, metformin may interact directly with the γ-subunit of the AMPK complex, producing a structural change that promotes phosphorylation and activation by upstream kinases (Bruckbauer and Zemel, 2013).

4.4. Effects of *Eucalyptus* oil

Medicinal plants have been used in almost all cultures as a source of medicines for thousands of years. It has been estimated that about 80 to 85% of the world's population depend on traditional medicine for their primary health care needs and a major part of traditional therapy involves the use of plant extracts or their active principles and *Eucalyptus* is also one of those herbs (Prakash *et al*., 2015).

Eucalyptus globulus which has been used in the traditional treatment of diabetes, is the major medicinal plant having one of the highest polyphenols content and maximum concentration of flavonoids. The most effective parts of *Eucalyptus globulus* are its leaves and oil derived from these leaves (Asgharpour *et al*., 2013).

In this study, intraperitoneal treatment of *Eucalyptus* oil (10 µL /mouse /day) on Swiss albino mice fed a high fat lard diet, did not cause any significant effect on body weight, visceral fat mass, serum total cholesterol , on serum triglyceride level, serum ALT level, and serum ALP level. However, *Eucalyptus* oil treatment showed significant effect on lowering of fasting blood glucose (although it was in the normal range), and lowering of serum LDL level and significantly elevate serum HDL level when compared with non-treated mice fed a high fat diet.

In agreement with the result of this MSc study, glucose-lowering activities with concurrent improvements in serum lipid profiles were shown in treatment of *Eucalyptus globulus* in alloxan-induced diabetic rats (Arjun *et al*., 2009). In addition, significantly improved blood glucose levels were obtained in 4 week treatment of aqueous extract of *Eucalyptus* leaf on streptozotocin induced diabetic rats (Bokaeian *et al*, 2010).

Shahraki and Shahraki also found decreased blood glucose levels in the treatment of streptozotocin -induced diabetic male rats with *Eucalyptus* leaf aqueous extracts (Shahraki A and Shahraki M, 2013).

Reduced hyperglycemia, enhanced glucose oxidation and enhanced incorporation of glucose into glycogen were seen in the abdominal muscle of diabetic mice in the incorporation of an aqueous extract of *Eucalyptus* leaf in the diet (62.5 g/kg) and drinking water (2.5 g/L) of diabetic mice (Nayak and De , 2013).

In another study, increased peripheral glucose utilization, increased glucose oxidation and improved insulin secretion from a pancreatic beta cell line in the abdominal muscle of mice were found by treatment of an aqueous extract of *Eucalyptus* leaf (Panicker and Gopalakrishnan, 2014).

Alcoholic extracts of leaves of *Eucalyptus globulus* were also found to ameliorate the diabetic state by partial restoration of pancreatic beta cells and repair of streptozotocin - induced damage in rats (Chauhan *et al*., 2010).

Generally, the effect of *Eucalyptus* oil on glucose-lowering, elevation of serum HDL and lowering of serum LDL on mice fed a high fat diet may be due to water soluble compounds present in the *Eucalyptus* oil including resveratrol which affect glucose and fat metabolism in fat or skeletal muscle cells and by increasing the glucose influx in the cells. Most studies on the effects of *Eucalyptus* comes from streptozotocin and/or alloxan induced diabetic animals and further studies on the effect of *Eucalyptus* on high fat diet induced hyperglycemia are necessary.

4.5. Conclusions

A high fat diet (52% fat w/w, 70% by calories) which is fed to Swiss albino mice, is shown to induce significant visceral obesity and the model was potentially useful for producing and studying visceral obesity, and may be used for studies on pathophysiology and development of new treatment. The high fat lard diet also caused changes in lipid profiles, namely increasing LDL and decreasing HDL. However, the high fat diet did not induce any obvious prediabetic or diabetic state compared with regular diet and did not raise fasting blood glucose level above the normal range (60- 200 mg/dL) in mice, so, as it stands, was not suitable for inducing and studying prediabetes or type 2 diabetes.

Resveratrol administration in Swiss albino mice fed a high fat lard diet did not cause a significant effect on weight, fat deposition and other metabolic profiles, and might be highly dependable on dose, and mode of administration to come with any effect. Metformin treatment showed elevation of HDL and lowering of LDL level, but had no effect on visceral fat deposition. *Eucalyptus* oil showed a significant effect on lowering of fasting blood glucose, although it was in the normal range, and also it lowered serum LDL levels and significantly elevated serum HDL levels. *Eucalyptus* have a promising effect on lowering of blood glucose and also on normalizing lipid profile, but further studies should be undertaken. A mild elevation of AST, but not of ALT or ALP, was also seen with *Eucalyptus* oil treatment, but the significance of this was unclear.

4.6. Limitations of the study

- The type and amount of fat used in this study (lard) differs from those containing vegetable oils, and may produce different results.
- Small numbers of mice were used in this study due to limited resources, so the statistical power of the study was somewhat limited.
- Routes of administration of resveratrol (drinking water), metformin (intraperitoneally) and *Eucalyptus* (intraperitoneally) may also affect the significance of the results.
- The exact content of resveratrol in the *Eucalyptus* oil used was not known
- *Eucalyptus* oil is heterogeneous and contains many chemicals, therefore, it is unclear which constituents were responsible for the effect of the *Eucalyptus* oil.

4.7. Recommendations

- Further studies needed on the lard-feeding model of obesity in Swiss albino mice using higher numbers of mice and longer periods of time.
- Evaluation of satiety hormone levels (for example, leptin, ghrelin) and serum adipokine levels should be done.
- Further studies of resveratrol on this obesity model, using higher doses of resveratrol and different routes of administration should be done.
- Comprehensive biochemical and pharmacological investigations of active components of the *Eucalyptus*, in particular, those which are responsible for improving lipid profiles of mice should be done.
- Efforts are needed to develop definitive dosage formulations and safety issues of *Eucalyptus* components in humans.
- Further biochemical studies of resveratrol, metformin and *Eucalyptus* oil on their effect on AMPK activities in various tissues are recommended.

References

Adelli, G. R., Srirangam, R., & Majumdar, S. (2013). Phytochemicals in ocular health: Therapeutic potential and delivery challenges. *World*, 2(1): 18-34.

Alberdi G., Rodríguez V. M., Macarulla M. T., Miranda J., Churruca I, & Portillo M. P. (2013). Hepatic lipid metabolic pathways modified by resveratrol in rats fed an obesogenic diet. *Nutrition*, 29(3): 562-567.

Anwar M. A., Kheir W. A., Eid S., Fares J., Liu X., Eid A. H, & Eid A. A. (2014). Colorectal and Prostate Cancer Risk in Diabetes: Metformin, an Actor behind the Scene. *Journal of Cancer*, 5(9): 736.

Arjun P., Shivesh J, & Sahu A. N. (2009). Antidiabetic activity of aqueous extract of Eucalyptus citriodora Hook in alloxan induced diabetic rats. *Pharmacognosy Magazine*, 5(19 suppl.): 51-54.

Asgharpour F., Pouramir M., Khalilpour A., Lamar S. A, & Rezaei M. (2013). Antioxidant Activity and Glucose Diffusion Relationship of Traditional Medicinal Antihyperglycemic Plant Extracts. *International Journal of Molecular and Cellular Medicine*, 2(4): 169.

Barbosa-da-Silva, S., Sarmento, I. B., Bringhenti, I., Bargut, T. C., Souza-Mello, V., Aguila, M. B., & Mandarim-de-Lacerda, C. A. (2014). Animal Models of Nutritional Induction of Type 2 Diabetes Mellitus. *Int. J. Morphol*, 32(1): 279-293.

Bokaeian M., Nakhaee A., Moodi B, & Khazaei H. A. (2010). *Eucalyptus globulus* (eucalyptus) treatment of candidiasis in normal and diabetic rats. *Iranian Biomedical Journal*, 14(3): 121.

Brainard R. E., Watson L. J., De Martino A. M., Brittian K. R., Readnower R. D., Boakye A, & Jones S. P. (2013). High fat feeding in mice is insufficient to induce cardiac dysfunction and does not exacerbate heart failure. *PLoS One,* 8(12): e83174. doi: 10.1371/journal.pone.0083174. eCollection 2013.

Bray, G. A., & Ryan, D. H. (2014). Update on obesity pharmacotherapy. *Annals of the New York Academy of Sciences*, 1311(1): 1-13.

Brenmoehl J, & Hoeflich A. (2013). Dual control of mitochondrial biogenesis by sirtuin 1 and sirtuin 3. *Mitochondrion*, 13(6): 755-761.

Bruckbauer A, & Zemel M. B. (2013). Synergistic effects of metformin, resveratrol, and hydroxymethyl butyrate on insulin sensitivity. *Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy*, 6: 93.

Bruckbauer A, & Zemel M. B. (2014). Synergistic effects of polyphenols and methylxanthines with leucine on AMPK/Sirtuin-mediated metabolism in muscle cells and adipocytes. *PLoS ONE* 9(2): e89166. doi:10.1371/journal.pone.0089166

Buettner R., Schölmerich J, & Bollheimer L. C. (2007). High-fat diets: Modeling the metabolic disorders of human obesity in rodents. *Obesity*, 15(4): 798-808.

Buettner, R., Parhofer, K. G., Woenckhaus, M., Wrede, C. E., Kunz-Schughart, L. A., Schölmerich, J., & Bollheimer, L. C. (2006). Defining high-fat-diet rat models: metabolic and molecular effects of different fat types. *Journal of Molecular Endocrinology*, 36(3): 485-501.

Calixto M. C., Lintomen L., André D. M., Leiria L. O., Ferreira D., Lellis-Santos C, & Antunes E. (2013). Metformin attenuates the exacerbation of the allergic eosinophilic inflammation in high fat-diet-induced obesity in mice. *PLoS One* 8(10): e76786.

Cardiff, R. D., Miller, C. H., & Munn, R. J. (2014). Manual hematoxylin and eosin staining of mouse tissue sections. *Cold Spring Harbor protocols*, 2014(6), pdb-prot073411.

Castro, A. V. B., Kolka, C. M., Kim, S. P., & Bergman, R. N. (2014). Obesity, insulin resistance and comorbidities? Mechanisms of association. *Arquivos Brasileiros de Endocrinologia & Metabologia,* 58(6): 600-609.

Chauhan A., Sharma P. K., Srivastava P., Kumar N., & Dudhe R. (2010). Plants having potential antidiabetic activity: a review. *Der Pharmacia Lettre*, 2(3): 369-387.

Chen S., Wasserman D. H., MacKintosh C, & Sakamoto K. (2011). Mice with AS160/TBC1D4-Thr649Ala knockin mutation are glucose intolerant with reduced insulin sensitivity and altered GLUT4 trafficking. *Cell Metabolism*, 13(1): 68-79.

Chung J. H. (2012). Using PDE inhibitors to harness the benefits of calorie restriction: Lessons from Resveratrol. *Aging (Albany NY)*, 4(3): 144.

Cinti, S. (2002). Adipocyte differentiation and transdifferentiation: plasticity of the adipose organ. *Journal of Endocrinological Investigation*, *25*(10): 823-835.

Coleman, D. L. (2010). A historical perspective on leptin. *Nature Medicine*, 16(10): 1097-1099.

Coughlan K. A., Valentine R. J., Ruderman N. B, & Saha A. K. (2014). AMPK activation: a therapeutic target for type 2 diabetes. *Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy*, 7: 241-253.

De Ferranti, S., & Mozaffarian, D. (2008). The perfect storm: obesity, adipocyte dysfunction, and metabolic consequences. *Clinical Chemistry*, 54(6): 945-955.

De Lorenzo, A., Soldati, L., Sarlo, F., Calvani, M., Di Lorenzo, N. & Di Renzo, L. (2016). New obesity classification criteria as a tool for bariatric surgery indication. *World J Gastroenterol,* 22(2): 681-703.

Dey B, & Mitra A. (2013). Chemo-profiling of eucalyptus and study of its hypoglycemic potential. *World Journal of Diabetes*, 4(5): 170.

Dobrzynska M. M. (2013). Resveratrol as promising natural radioprotector. A review. *Roczniki Państwowego Zakładu Higieny*, *64*(4).

Fabbrini E., Sullivan S, & Klein S. (2010). Obesity and nonalcoholic fatty liver disease: biochemical, metabolic, and clinical implications. *Hepatology*, 51(2): 679-689.

Francisco, C.C., Howarth, G.S. and Whittaker, A.L. (2015). Effects on animal wellbeing and sample quality of two techniques for collecting blood from the facial vein of mice. *Journal of the American Association for Laboratory Animal Science*, 54(1): 76-80.

Franco J. G., Lisboa P. C., Lima N. S., Amaral T. A., Peixoto - Silva N., Resende A. C , & Moura E. G. (2013). Resveratrol attenuates oxidative stress and prevents steatosis and hypertension in obese rats programmed by early weaning. *The Journal of Nutritional Biochemistry*, 24(6): 960-966.

Frontini A, & Cinti S. (2010). Distribution and development of brown adipocytes in the murine and human adipose organ. *Cell Metabolism*, 11(4): 253-256.

Fuentes, E., Fuentes, F., Vilahur, G., Badimon, L., & Palomo, I. (2013). Mechanisms of chronic state of inflammation as mediators that link obese adipose tissue and metabolic syndrome. *Mediators of Inflammation*, *2013*.

Gajda A. M. (2009). High fat diets for diet-induced obesity models. *J Res Diets, 2009.* Research Diets, Inc.

Gebremichael, B., & Chere, A. (2015). Prevalence of Childhood Overweight and Obesity and its Determinant Factors among Elementary School Children in Addis Ababa, Ethiopia: A Cross Sectional Study. *J Nutr Disorders Ther* S1:002. doi: 10.4172/2161-0509.1000S1002

Gessesse D, & Teklu E. (2011). *Eucalyptus* in East Africa, socio-economic and environmental issues. *Planted forests and trees working papers.* Working paper 46/e, forest management team, forest management division.

Ghoshal K, & Bhattacharyya M. (2015). Adiponectin: Probe of the molecular paradigm associating diabetes and obesity. *World Journal of Diabetes*, 6(1): 151.

Gu X. S., Wang Z. B., Ye Z., Lei J. P., Li L., Su D. F, & Zheng X. (2014). Resveratrol, an activator of SIRT1, up regulates AMPK and improves cardiac function in heart failure. *Genet Mol Res*, 13(1): 323-335.

Guariguata L., Whiting D. R., Hamblin I., Beagley J., Linnenkamp U, & Shaw J. E. (2014). Global estimates of diabetes prevalence for 2013 and projections for 2035. *Diabetes Research and Clinical Practice*, 103(2): 137-149.

Hariri N., & Thibault L. (2010). High-fat diet-induced obesity in animal models. *Nutrition Research Reviews*, 23(02): 270-299.

Haththotuwa, R. N., Wijeyaratne, C. N., & Senarath, U. (2012). Worldwide Epidemic of Obesity. *Obesity: A Ticking Time Bomb for Reproductive Health*, 1.

Havele, S. & Dhaneshwar, S. (2010) "Development and validation of a HPLC method for the determination of Metformin Hydrochloride, Gliclazide and Pioglitazone Hydrochloride in multicomponent formulation," *Webmed Central Pharmaceutical Sciences*, vol. 1, no. 10, Article ID WMC0001078, 2010.

Hoffler U., Hobbie K., Wilson R., Bai R., Rahman A., Malarkey, D, & Ghanayem B. I. (2009). Diet-induced obesity is associated with hyperleptinemia, hyperinsulinemia, hepatic steatosis, and glomerulopathy in C57Bl/6J mice. *Endocrine*, 36(2): 311-325.

International Diabetes Federation (2013). IDF diabetes atlas. Brussels*: International Diabetes Federation.*

Janssen I., Shields M., Craig C. L, & Tremblay M. S. (2011). Prevalence and secular changes in abdominal obesity in Canadian adolescents and adults, 1981 to 2007–2009. *Obesity Reviews*, 12(6): 397-405.

Jenkins Y., Sun T. Q., Li Y., Markovtsov V., Uy G., Gross L, & Hitoshi Y. (2014). Global metabolite profiling of mice with high-fat diet-induced obesity chronically treated with AMPK activators R118 or metformin reveals tissue-selective alterations in metabolic pathways. *BMC Research Notes*, 7(1): 674.

Jimoh A., Tanko Y., Ahmed A., Mohammed A, & Ayo J. O. (2015). Protective Effect of Resveratrol Co-Administration with Cholesterol Diet on Erythrocyte Osmotic Fragility and Malondi aldehyde Concentration in Rabbits. *British Journal of Pharmaceutical Research*, 6(1): 14.

Joo, J. K., & Lee, K. S. (2014). Pharmacotherapy for Obesity. *Journal of menopausal medicine*, 20(3): 90-96.

Jung, U. J., & Choi, M. S. (2014). Obesity and its metabolic complications: the role of adipokines and the relationship between obesity, inflammation, insulin resistance, dyslipidemia and nonalcoholic fatty liver disease. *International Journal of Molecular Sciences*, 15(4): 6184-6223.

Kalupahana N. S., Moustaid-Moussa N, & Claycombe K. J. (2012). Immunity as a link between obesity and insulin resistance. *Molecular Aspects of Medicine*, 33(1): 26-34.

Karimi, I. (2012). Animal models as tools for translational research: focus on atherosclerosis, metabolic syndrome and type-II diabetes mellitus, Chapter 21, in *Biochemistry, Genetics and Molecular Biology » "Lipoproteins - Role in Health and Diseases."* Eds. Frank, S. and Kostner, G. ISBN 978-953-51-0773-6. DOI: 10.5772/47769.

Klop, B., & Cabezas, M. C. (2012). Chylomicrons: a key biomarker and risk factor for cardiovascular disease and for the understanding of obesity. *Current Cardiovascular Risk Reports*, 6(1): 27-34.

Klop, B., Elte, J. W. F., & Cabezas, M. C. (2013). Dyslipidemia in obesity: mechanisms and potential targets. *Nutrients*, 6(4): 1218-1240.

Kopelman, P. G. (2000). Obesity as a medical problem. *Nature*, 404(6778): 635-643.

Lee, W. J., & Almulaifi, A. (2015). Recent advances in bariatric/metabolic surgery: appraisal of clinical evidence. *Journal of Biomedical Research*, 29(2): 98.

Lementowski, P. W., & Zelicof, S. B. (2008). Obesity and osteoarthritis. *American Journal of Orthopedics -Belle Mead*, 37(3): 148.

Li W., Li Y., Wang Q, & Yang Y. (2014). Crude extracts from Lyciumbarbarum suppress SREBP-1c expression and prevent diet-induced fatty liver through AMPK activation. *BioMed Research International*. Volume 2014, Article ID 196198.

Liao W. L, & Tsai F. J. (2014). Personalized medicine in Type 2 Diabetes. *Biomedicine,* 4: 1-8.

Libinaki R., Heffernan M., Jiang W. J., Ogru E., Ignjatovic V., Gianello R, & Ng F. (1999). Effects of Genetic and Diet-Induced Obesity on Lipid Metabolism. *IUBMB Life*, 48(1): 109-113.

Linsel-Nitschke, P., & Tall, A. R. (2005). HDL as a target in the treatment of atherosclerotic cardiovascular disease. *Nature Reviews Drug Discovery*, 4(3): 193-205.

Liu Y., Tang G., Li Y., Wang Y., Chen X., Gu X, & Yang G. Y. (2014). Metformin attenuates blood-brain barrier disruption in mice following middle cerebral artery occlusion. *J Neuro Inflammation*, 11: 177.

Louwe M. C., van der Hoorn J. W., van den Berg S. A., Jukema J. W., Romijn J. A., Willems van Dijk K, & Steendijk P. (2012). Gender-dependent effects of high-fat lard diet on cardiac function in C57Bl/6J mice. *Applied Physiology, Nutrition, and Metabolism*, 37(2): 214-224.

Mackenzie R. W, & Elliott B. T. (2014). Akt/PKB activation and insulin signaling: a novel insulin signaling pathway in the treatment of type 2 diabetes. Diabetes, metabolic syndrome and obesity: *Targets and Therapy*, 7: 55.

Mantzoros, C. S., Magkos, F., Brinkoetter, M., Sienkiewicz, E., Dardeno, T. A., Kim, S. Y, & Koniaris, A. (2011). Leptin in human physiology and pathophysiology. *American Journal of Physiology-Endocrinology and Metabolism*, 301(4): E567-E584.

Maury E, & Brichard S. M. (2010). Adipokine dysregulation, adipose tissue inflammation and metabolic syndrome. *Molecular and Cellular Endocrinology,* 314(1): 1-16.

Mukherjee S., Dudley J. I, & Das D. K. (2010). Dose-dependency of resveratrol in providing health benefits. *Dose-Response*, 8(4): 478-500.

Muoio D. M, & Newgard C. B. (2008). Molecular and metabolic mechanisms of insulin resistance and β-cell failure in type 2 diabetes. *Nature Reviews Molecular Cell Biology*, 9(3): 193-205.

Nagao K., Jinnouchi T., Kai S, & Yanagita, T. (2013). Effect of dietary resveratrol on the metabolic profile of nutrients in obese OLETF rats. *Lipids Health Dis*. 2013; 12: 8. doi: 10.1186/1476-511X-12-8

Nayak A, & De S. (2013). Anti-diabetic potential medicinal plants. *Journal of Pharmacy Research*, 6(1): 32.

Ng, M., Fleming, T., Robinson, M., Thomson, B., Greet, N., Margono, C, & Abraham, J. P. (2014). Global, regional, and national prevalence of overweight and obesity in children and adults during 1980–2013: a systematic analysis for the Global Burden of Disease Study 2013. *The Lancet*, 384 (9945): 766-781.

Nilsson, C., Raun, K., Yan, F. F., Larsen, M. O., & Tang-Christensen, M. (2012). Laboratory animals as surrogate models of human obesity. *Acta Pharmacologica Sinica*, 33(2): 173-181.

Nishino, N., Tamori, Y., Tateya, S., Kawaguchi, T., Shibakusa, T., Mizunoya, W, & Hiramatsu, R. (2008). FSP27 contributes to efficient energy storage in murine white adipocytes by promoting the formation of unilocular lipid droplets. *The Journal of Clinical Investigation*, *118*(8): 2808-2821.

O'Rourke, R. W. (2014). Obesity and cancer: at the crossroads of cellular metabolism and proliferation. *Surgery for Obesity and Related Diseases*, 10(6): 1208-1219.

Ogihara T. and Mirmira R. G. (2010). An islet in distress: β cell failure in type 2 diabetes. *Journal of Diabetes Investigation*, 1(4): 123-133.

Panchal, S.K. and Brown, L., 2010. Rodent models for metabolic syndrome research. *Journal of Biomedicine and Biotechnology*, doi:10.1155/2011/351982.

Panicker V. P, & Gopalakrishnan A. (2014). Anti-diabetic medicinal plants of India-A review. *World Journal of Pharmacy and Pharmaceutical Sciences*, 3(7): 482-495.

Park S. J., Ahmad F., Philp A., Baar K., Williams T., Luo H, & Chung J. H. (2012). Resveratrol ameliorates aging-related metabolic phenotypes by inhibiting cAMP phosphodiesterases. *Cell*, 148(3): 421-433.

Patel M. I., Gupta A, & Dey C. S. (2011). Potentiation of neuronal insulin signaling and glucose uptake by resveratrol: the involvement of AMPK. *Pharmacological Reports*, 63(5): 1162-1168.

Peng, X. R., Gennemark, P., O'Mahony, G., & Bartesaghi, S. (2015). Unlock the thermogenic potential of adipose tissue: pharmacological modulation and implications for treatment of diabetes and obesity. *Frontiers in Endocrinology*, 6. doi: 10.3389/fendo.2015.00174

Pereira P. F., de Almeida C. D. G, & Alfenas R. D. C. G. (2014). Glycemic index role on visceral obesity, subclinical inflammation and associated chronic diseases. *Nutricion Hospitalaria*, 2(30): 237-243.

Poulsen M. M., Vestergaard P. F., Clasen B. F., Radko Y., Christensen L. P., Stødkilde-Jørgensen H, & Jørgensen J. O. L. (2013). High-dose resveratrol supplementation in obese men an investigator-initiated, randomized, placebo-controlled clinical trial of substrate metabolism, insulin sensitivity, and body composition. *Diabetes,* 62(4): 1186-1195.

Prakash O., Kumar R., Srivastava R., Tripathi P, & Mishra S. (2015). Plants Explored with Anti-diabetic Properties: A Review. *American Journal of Pharmacological Sciences*, 3(3): 55-66.

Rani, N., Vasudeva, N., & Sharma, S. K. (2012). Quality assessment and anti-obesity activity of Stellaria media (Linn.) Vill. *BMC Complementary and Alternative Medicine*, 12(1): 145.

Rezaee, F., & Dashty, M. (2013). Role of Adipose Tissue in Metabolic System Disorders Adipose Tissue is the Initiator of Metabolic Diseases. *J Diabetes Metab S*, *13*: 2.

Rizvi S. I, & Pandey K. B. (2010). Activation of the erythrocyte plasma membrane redox system by resveratrol: a possible mechanism for antioxidant properties. *Pharmacological Reports*, 62(4):726-732.

Rose, D. P., & Vona-Davis, L. (2010). Interaction between menopausal status and obesity in affecting breast cancer risk. *Maturitas*, *66*(1): 33-38.

Rosini, T. C., Silva, A. S. R. D., & Moraes, C. D. (2012). Diet-induced obesity: rodent model for the study of obesity-related disorders. *Revista da Associação Médica Brasileira*, 58(3): 383-387.

Rouse M., Younès A, & Egan J. M. (2014). Resveratrol and curcumin enhance pancreatic β-cell function by inhibiting phosphodiesterase activity. *Journal of Endocrinology*, 223(2): 107-117.

Ruderman N. B., Carling D., Prentki M, & Cacicedo J. M. (2013). AMPK, insulin resistance, and the metabolic syndrome. *The Journal of Clinical Investigation*, 123(7): 2764-2772.

Sadasivan S., Vasamsetti B., Singh J., Siddaraju N., Khan K., Oommen A, & Rao R. P. (2014). Modulation of de novo purine biosynthesis leads to activation of AMPK and results in improved glucose handling and insulin sensitivity. *J Diabetes Metab Disord*, 13: 1-9.

Saris, W. H. M. (2006). Macronutrient Intake Balance and the Problem of Obesity-Old Recipes and Some New Spices. *Aktuelle Ernährungsmedizin*, 31(S1): 49-54.

Shahi Majid, Mohamad, Haidari Fatemeh, shiri Mohamad Reza. (2011). Comparison of effect of resveratrol and vanadium on diabetes related dyslipidemia and hyperglycemia in streptozotocin induced diabetic rats. *Advanced Pharmaceutical Bulletin*, 1(2): 81-86

Shahraki A, & Shahraki M. (2013). The Comparison of Eucalyptus Aqueous Extract and Insulin on Blood Sugar, and Liver Enzymes in Diabetic Male Rats. *Zahedan Journal of Research in Medical Sciences*, 15(6): 25-28.

Shegaze M , Wondafrash M , Alemayehu A.A, , Mohammed S, Shewangezaw Z, Abdo M, Gendisha G. (2015) .Magnitude and Determinants of Overweight and Obesity Among High School Adolescents in Addis Ababa, Ethiopia. *Journal of Food and Nutrition Sciences* 3(5): 166-173.

Shephard, M. D., & Whiting, M. J. (1990). Falsely low estimation of triglycerides in lipemic plasma by the enzymatic triglyceride method with modified Trinder's chromogen. *Clinical Chemistry*, 36(2): 325-329.

Siedel, J., Schmuck, R., Staepels, J., & Town, M. H. (1993). Long term stable, liquid ready-to-use monoreagent for the enzymatic assay of serum or plasma triglycerides (GPO-PAP method). AACC Meeting Abstract 34. *Clin Chem*, 39: 1127.

Siriwardhana N., Kalupahana N. S., Cekanova M., LeMieux M., Greer B, & Moustaid-Moussa N. (2013). Modulation of adipose tissue inflammation by bioactive food compounds. *The Journal of Nutritional Biochemistry*, 24(4): 613-623.

Sivajothi V, & Dakappa S. S. (2014). In vitro and in silico antidiabetic activity of pyran ester derivative isolated from Tragia cannabina. *Asian Pacific Journal of Tropical Biomedicine,* 4: S455-S459.

Srivastava R. A. K., Pinkosky S. L., Filippov S., Hanselman J. C., Cramer C. T, & Newton R. S. (2012). AMP-activated protein kinase: an emerging drug target to regulate imbalances in lipid and carbohydrate metabolism to treat cardio-metabolic diseases. Thematic Review Series: New Lipid and Lipoprotein Targets for the Treatment of Cardiometabolic Diseases. *Journal of Lipid Research*, 53(12): 2490-2514.

Szkudelska K, & Szkudelski T. (2010). Resveratrol, obesity and diabetes. *European Journal of Pharmacology,* 635(1): 1-8.

Tauriainen E., Luostarinen M., Martonen E., Finckenberg P., Kovalainen M., Huotari A, & Mervaala E. (2011). Distinct effects of calorie restriction and resveratrol on diet-induced obesity and fatty liver formation. *J Nutr Metab.* 2011:525094. doi: 10.1155/2011/525094.

Teshome, T., Singh, P., & Moges, D. (2013). Prevalence and associated factors of overweight and obesity among high school adolescents in urban communities of Hawassa, Southern Ethiopia. *Current Research in Nutrition and Food Science Journal*, 1(1): 23-36.

Thaler J. P, & Schwartz M. W. (2010). Inflammation and obesity pathogenesis: The hypothalamus heats up. *Endocrine Reviews*, 31(4): 600-600.

Thamer S. J. (2014). The Effect of Orlistat and Metformin Treatment on Body Weight, Liver Steatsis, Leptin and Insulin Sensitivity in Obese Rats Fed High Fat Diet. *Journal of American Science*, 10(4): 107-114.

Tharp K.M. & Stahl A. (2015). Bioengineering beige adipose tissue therapeutics. *Frontiers in Endocrinology*. 6:164. doi: 10.3389/fendo.2015.00164.

Timmers S., Auwerx J, & Schrauwen P. (2012). The journey of resveratrol from yeast to human. *Aging (Albany NY)*, 4(3): 146.

Trinder P. (1969). Determination of glucose in the blood using glucose oxidase with an alternative oxygen acceptor. *Ann Clinical Biochem*, 6: 24-27.

Tschöp, M., & Heiman, M. L. (2000). Rodent obesity models: an overview. *Experimental and Clinical Endocrinology & Diabetes: Official Journal, German Society of Endocrinology [and] German Diabetes Association*, 109(6): 307-319.

Viollet B., Guigas B., Garcia N. S., Leclerc J., Foretz M , & Andreelli F. (2012). Cellular and molecular mechanisms of metformin: an overview. *Clinical Science*, 122(6): 253-270.

Wajchenberg, B. L. (2000). Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome. *Endocrine Reviews*, 21(6): 697-738.

Wang S., Moustaid-Moussa N., Chen L., Mo H., Shastri A., Su R, & Shen C. L. (2014). Novel insights of dietary polyphenols and obesity. *The Journal of Nutritional Biochemistry*, 25(1): 1-18.

World Health Organization. (2000). Obesity: preventing and managing the global epidemic. *Report of a WHO Consultation*, 894: i-xii, 1-253.

World Health Organization. (2010). Obesity and Overweight. Fact Sheet No. 311.

World Health Organization. (2012). Global database on body mass index. 2012. *Global Database on Body Mass Index*.

Wu L., Zhou L., Lu Y., Zhang J., Jian F., Liu Y, & Li G. (2012). Activation of SIRT1 protects pancreatic β-cells against palmitate-induced dysfunction. *Biochimica Biophysica Acta (BBA)-Molecular Basis of Disease*, 1822(11): 1815-1825.

Xiao B., Sanders M. J., Underwood E., Heath R., Mayer F. V., Carmena D, & Gamblin S. J. (2011). Structure of mammalian AMPK and its regulation by ADP. *Nature*, 472 (7342): 230-233.

Zagotta I., Dimova E. Y., Debatin K. M., Wabitsch M., Kietzmann T, & Fischer-Posovszky P. (2015). Obesity and inflammation: reduced cytokine expression due to resveratrol in a human in vitro model of inflamed adipose tissue. *Frontiers in Pharmacology*, (6): 79.

Zhang X. H., Huang B., Choi S. K, & Seo J. S. (2012). Anti-obesity effect of resveratrol-amplified grape skin extracts on 3T3-L1 adipocytes differentiation. *Nutrition Research and Practice*, 6(4): 286-293.

Zhang Y., Song H., Shang Z., Chen A., Huang D., Zhao H, & Du H. (2014). Amino Acid-PEGylated Resveratrol and Its Influence on Solubility and the Controlled Release Behavior. *Biological and Pharmaceutical Bulletin*, 37(5): 785-793.