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Carnosic Acid Enriched Rosemary Extract Prevents Obesity And Metabolic Syndrome In High-Fat Diet-Fed Mice

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Carnosic Acid Enriched Rosemary Extract Prevents Obesity and Metabolic Syndrome in High-Fat Diet-Fed Mice

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Biographical Sketch

Rashin Sedighi's first nutritional research experience began in 2008 after she started her Master's degree in Maternal and child nutrition at University of California, Davis. Her experience as a registered nurse for more than 10 years combined with her full time involvement in health care clinics and fostered a strong believe in her about the importance of a proper diet to prevent diseases. Through her graduate programs, she was trying to find the best ways to use food as a defense mechanism against chronic and acute disease such as gastrointestinal diseases, and also as a preventive against obesity and diabetes. She worked as a Research Assistant during her PhD program at Center for Excellence in Post-harvest Technologies, North Carolina Agricultural and Technical State University in Kannapolis. Her primary goal in this field is to strengthen nutritional supplements such as rosemary extract for prevention of obesity and diabetes.

"**The doctor of the future will give no medicine, but will instruct his patient in the care of the human frame, in diet and in the cause and prevention of disease"**, **Thomas Edison**

Dedication

I would like to dedicate this dissertation to my family, especially my husband, Mehrdad Tajkarimi, and my son Ali Tajkrimi, who gave me their unwavering support and love throughout graduate school, they have given me the strength to complete this part of my life. Also I would like to dedicate this dissertation to my beloved country, IRAN.

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Abstract

The objective of this study was to investigate the preventive effects of rosemary leaf extract (RE) and its active components on prevention of weight gain and associated metabolic disorders in high-fat-fed mice; determine the effective doses of the chief compound in RE to reduce weight gain and hyperglycemia; and try to understand the underlying anti-obesity mechanism. For this purpose, commercial RE with 45% carnosic acid (CA) was enriched with CA to 80%. Animals were given low-fat diet (LFD), high-fat diet (HFD), high fat with 0.50% RE (enriched with 45% CA, RE#1), high fat with 0.28% w/w RE (enriched with 80% CA, RE#2H), and high fat with 0.14% w/w RE (enriched with 80% CA, RE#2L) for 16-week periods. Physiological and biochemical parameters were monitored. Treatment with CA, dose dependently, induced a significant reduction of weight and fat mass gain which it was associated with an increase of fecal lipid excretion. Likewise, hypercholesterolemia, fasting glycaemia, and insulin resistant were improved by CA treatments. Hepatic triglyceride levels, correspondingly, were decreased in CA-treated mice. In conclusion, the results from this study demonstrate that consumption of the RE that is rich in CA, dose dependently can limit weight gain induced by a high-fat diet and protect against obesity-related metabolic disorders.

CHAPTER 1

Introduction

Metabolic syndrome is a global emerging health concern. Endocrine and metabolic diseases are problematic modern human disorders, primarily in the United States and other countries with extensive nutrition. Their risk factors have capability of acting as an indicator for many chronic diseases such as cardiovascular disease, kidney disease, and type 2 diabetes mellitus (Woolston, Tu, Baxter, & Gilthorpe, 2012). Effective strategy against obesity and hyperglycemia, two important risk factors of metabolic syndrome, is critical for reducing the possibility of cardiovascular disease. Side effects of the currently existing methods such as pharmacological and surgical interventions have impeded their usefulness. This awareness has led to nonstop effort to discover effective agents such as new medications as well as natural products extracted from herbal plants. Many active compounds extracted from herbal plants possess healing values, i.e. antiobesity and hypoglycemic activity, etc.(Joyal, 2004). Using botanicals provides safer pharmaceutical and nutritious products for customers and less expensive approval process under the guidelines of to the US Food and Drug Administration (M. Romo Vaquero et al., 2012).

Therefore, the present research has been performed to investigate effects of REs and their major active component CA, dose dependently, in high-fat-fed mice.

1.1 Metabolic Syndrome and Obesity

Metabolic syndrome is a combination of conditions related to obesity that includes excess central adiposity, insulin resistance, hyperlipidemia, and hypertension. The syndrome may affect more than 5% of the elderly in the United States and even higher percentages in different racial groups around the world (Golden, Robinson, Saldanha, Anton, & Ladenson,

2009). A rapid increase in metabolic syndrome prevalence and severity has paralleled the dramatic increase in rates of obesity and type 2 diabetes (F. M. Elisa Fabbrini, B. Selma Mohammed, Terri Pietka, Nada A. Abumrad, Bruce W. Patterson, Adewole Okunade, Samuel Klein, 2009).

Metabolic syndrome profoundly affects the quality of life and represents a life-long burden on a patient's social support system. To the extent that the component risk factors of the metabolic syndrome contribute to cardiovascular disease with long time treatment, the financial impact on the Medicare program may be substantial. Alyssa et al. estimated that people with metabolic syndrome cost $259 more per month than those comparable individuals without the syndrome (Alyssa B. Schultz., 2009). Meanwhile another research showed the medical costs are 20% higher rates among patients with the metabolic syndrome than among those without in 10 years (Curtis et al., 2007). The unsustainable growth in health care spending in the United States increases importance of prevention investment. However, costs to the society related to excess metabolic disorders, component risk factors, and their complications are not limited to health care costs. Losses attributable to reduced job productivity such as absenteeism, disability, labor productivity reduction, have also been found to be associated with these health risks. Studies suggest that the costs of lost productivity from metabolic syndrome and its complications could far go above the costs of medical care (Schultz AB, 2009).

Today, obesity is a global concern because of its fast growing rates and its comorbidities, such as type 2 diabetes and cardiovascular disease. In addition, obesity is also associated with increased risk of various cancers, including endometrial, breast, and colon cancer (Strain et al., 2012). It has been predicted that obesity will be the number one health problem globally by the

year 2025 and thought to be overtaking cigarette smoking soon to become the leading cause of death in the United States.

1.2 Adipose Tissue and Obesity

Obesity is related to an abnormal large amount of body fat, specifically adipose tissue. The major component of adipose tissue, adipocyte, plays an important role as an energy storage site in the form of triglyceride (Brännmark et al., 2013). Adipose tissue maintains the free fatty acid levels and triglycerides in circulation. It has been demonstrated that an amplified amount of adipose tissue related to obesity is caused by increase in the number of adipocytes, hyperplasia, or by increase in the size of adipocyte which it is called hypertrophy (Wolfram, Wang, & Thielecke, 2006).

Visceral adipose tissue is around the internal organs and releases free fatty acids and adipokines into the portal vein for direct transport to the liver. This role makes visceral adipose tissue count as an important and independent predictor of metabolic risk factors for coronary heart disease, particularly diabetes and dyslipidemia (F. M. Elisa Fabbrini, B. Selma Mohammed, Terri Pietka, Nada A. Abumrad, Bruce W. Patterson, Adewole Okunade, Samuel Klein, 2009; S. S. Elisa Fabbrini, Samuel Klein, 2010). Moreover, the results from a study on human subjects with metabolic syndrome demonstrated that an increase in visceral adipose tissue is associated with glucose tolerance reduction, insulin resistance, and increased very-low-density lipoprotein– triglyceride secretion (Adiels et al., 2006). These findings, have led to the thought that visceral adipose tissue is in charge for many of the metabolic defects associated with abdominal obesity (Bays, Blonde, & Rosenson, 2006). In conclusion, a reduction in visceral fat has become a key therapeutic goal in the controlling of obesity and metabolic syndrome.

1.3 Dietary Prevention of Weight Gain and Metabolic Syndrome by Rosemary and Rosemary Constituents

In the last decade, nutritional, pharmacological, and surgical strategies have been developed to prevent the metabolic effect of high fat diet. According to the adverse side effect of the pharmacological and surgical intervention, nutritional intervention maybe is the safest and most cost effective option for those who have moderate metabolic syndrome. Hence, nutritional intervention, in particular the use of herbs in the prevention of metabolic syndrome and its complications has been growing tremendously during these years.

Rosemary *(Rosmarinus officinalis)* is an herb widely used in the flavoring of food, folk medicine, cosmetics, and phytopharmacy. Previous studies have revealed that the biological activities of rosemary (*Rosmarinus officinalis L*.) are credited to its components such as CA, carnosol, rosmarinic acid, and ursolic acid content (Debersac, Vernevaut, Amiot, Suschetet, & Siess, 2001; Del Campo, Amiot, & Nguyen-The, 2000; Ibarra et al., 2011; Karpinska, Borowski, & Danowska-Oziewicz, 2000; Koga, Nomoto, Shibata, & Yoshino, 2006; Kosaka & Yokoi, 2003; Lo, Liang, Lin-Shiau, Ho, & Lin, 2002; Moreno, Scheyer, Romano, & Vojnov, 2006; Moss, Cook, Wesnes, & Duckett, 2003; Naemura, Ura, Yamashita, Arai, & Yamamoto, 2008; Pengelly et al., 2012; Perez-Fons, Garzon, & Micol, 2010; Steiner et al., 2001; Takahashi et al., 2009). One of the most interesting food additive compound in rosemary is CA through it strong antioxidant effect. CA is an abietane diterpene found in the popular Labiatae herbs sage and rosemary and it is considered as an originator of other diterpenoid constituents in the herbs. Many studies have determined the antioxidant activities of CA and related diterpenes such as carnosol and rosmanol. The results have shown CA has the most powerful antioxidant power among these diterpenes. Easily oxidization of CA has been related to its typical *o*- diphenol

structure (Bozin, Mimica-Dukic, Samojlik, & Jovin, 2007; Jordan, Lax, Rota, Loran, & Sotomayor, 2012; Masuda et al., 2002; Moreno et al., 2006).

CA has a good safety profile which does not pose a health concern for the European Food Safety Authority (European Food Safety Authority, 2008). The Panel notes that the margin between the not observable adverse effect level of carnosol plus CA, as calculated in 90 days rat studies, is equivalent to 20–60 mg/Kg body weight (mpk), and the mean intake of CA rich REs is estimated to be 500–1500 mg/day in adults and 182–546 mg/day in pre-school children (European Food Safety Authority, 2008). It is assumed that at the concentrations are used as a food additive, CA levels would not cause significant hepatotoxicity. There are couple *in vivo* researches studies have been published for evaluating weight loss and antiadipogenic effects with different doses of CA. Even the capacities of rosemary to control obesity and hyperglycemia have been observed *in vivo*, whether these effects are due to CA or other constituents is currently unknown. Moreover, little is known about the safety, and the mechanisms of CA at doses that are needed to achieve therapeutic effect for weight loss and control hyperglycemia. It is important to identify the major active compound in rosemary, and to confirm its efficacy and safety in relevant biological models before conducting clinical trial research.

1.4 Specific Aims and Central Hypothesis of the Research

The prevalence of metabolic syndrome is dramatically increased in developing and industrialized countries (Miranda, DeFronzo, Califf, & Guyton, 2005). Excess metabolic disorders, component risk factors, and their complications affect the health care costs and also to the social productivity (Alyssa B. Schultz., 2009). Obesity is reaching epidemic and an established risk factor for various comorbidities, such as type 2 diabetes, metabolic disorders and cardiovascular disease worldwide (Golden, Robinson, Saldanha, Anton, & Ladenson, 2009).

Although, Rosemary (*Rosmarinus officinalis L.*) extracts is shown to be an effective treatment to prevent the metabolic effect of high fat diet (Ibarra et al., 2011), it is not fully understood whether the antiobesity and antidiabetic effect is due to CA or other active components. Moreover, the mechanisms of action and effective therapeutic doses for its active components are also unclear.

Our long-term goal is to develop rosemary and its active components as a safe and effective approach for high-fat diet-induced metabolic syndrome. The objectives of this study were to identify the economic way to prepare CA enriched RE from the commercial RE (45% CA), and determine the preventive effects of RE (45% CA), and its active compound on weight gain, glucose levels and lipid homeostasis in high-fat-fed mice; identify the effective doses of the active component and RE to reduce weight gain and type 2 diabetes; and understand the underlying mechanisms. These studies provided useful information as to the effective doses of CA enriched RE on the risk for chronic disease, and they were also served as foundation for understanding the important mechanisms by which CA may help prevent this disease.

Central hypothesis: The central hypothesis was that CA as the major active component of rosemary can dose dependently prevent the development of metabolic syndrome induced by high fat diet.

We expected to test our hypothesis by pursuing the following three specific aims:

1. To determine whether CA is the major active component in RE.
2. Investigate the dose depending effects of dietary CA enriched RE on high fat diet induced metabolic syndrome.
3. Investigate the underlying fat limiting mechanisms of RE and CA.

CHAPTER 2

Literature Review

2.1 Obesity, Type 2 Diabetes, and Metabolic Syndrome

Obesity is a health condition that is characterized by a body mass index (BMI), 30 or higher which is associated to an abnormal large amount of body fat, specifically adipose tissue (J. James, 2004). The rates of obesity have increased dramatically in the United States in the last 20 years and even more rapidly in the last 10 years. The studies have shown consumption of a high fat diet and sedentary lifestyle lead to obesity. Numerous studies in the last 50 years have suggested a significant relation between obesity and the incidence of diseases such as type 2 diabetes, cardiovascular disease, and hypertension (W. P. James, Rigby, & Leach, 2006). However the mechanism(s) responsible for the interrelationship among obesity, insulin resistance, and hypertriglyceridemia is not fully known.

A study by Fabbrini et.al demonstrated that obesity reflected as an increased storage of triacylglycerides in adipose tissues (S. S. Elisa Fabbrini, Samuel Klein, 2010). Their data show that increased visceral adipose tissue and intrahepatic triglyceride level are important risk factors for the metabolic complications associated with obesity, particularly insulin resistance and dyslipidemia. There is ample evidence that an impaired non-esterified fatty acid metabolism could contribute to the insulin-resistant state observed among individuals with visceral obesity (Despres & Lemieux, 2006). Moreover, modifications in tissue fatty acid transport could be involved in the pathogenesis of ectopic triglyceride accumulation by redirecting plasma fatty acid uptake from adipose tissue toward other tissues (F. M. Elisa Fabbrini, B. Selma Mohammed, Terri Pietka, Nada A. Abumrad, Bruce W. Patterson, Adewole Okunade, Samuel Klein, 2009).

Obesity is considered as one of the primary risk factors for type 2 diabetes in many studies. It has been demonstrated that obesity is often accompanied by chronic, low-grade inflammation that is characterized by increased release of inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), monocyte chemotactic protein-1 (MCP-1) and adiponectin in blood and accumulation of macrophages in the adipose tissue and the liver (Curat et al., 2006; Kawano & Arora, 2009; Kim & Lee, 2012). A recent study has also showed that MCP-1 plays an important role in insulin resistance that causes type 2 diabetes (Kim & Lee, 2012). As a result, inflammation reduction may be an important strategy in reducing the risk of obesity and associated type 2 diabetes (Bruun, Lihn, Pedersen, & Richelsen, 2005) . Both the altered non-esterified fatty acid metabolism and the endocrine function hypotheses imply that visceral adipose tissue is causally involved in the pathophysiology of the metabolic syndrome that is often found in patients with visceral obesity. Therefore, visceral adipose tissue is an important risk factor for obesity related metabolic disorders and its reduction has become a significant aim in obesity management (F. M. Elisa Fabbrini, B. Selma Mohammed, Terri Pietka, Nada A. Abumrad, Bruce W. Patterson, Adewole Okunade, Samuel Klein, 2009).

Type 2 diabetes is a heterogeneous disorder. It has been estimated that the risk of developing type 2 diabetes is higher in those who are severely obese rather than of healthy weight (93-fold in women and 42-fold in men) (Dixon et al., 2011). In 2013, the total prevalence for either type of diabetes was 25.8 million Americans, 8.3% of the population in United States, which approximately 90-95% of them have type 2 diabetes (Association, 2013).

Type 2 diabetes is generally characterized as failure to appropriate respond to insulin (Insulin resistance). As a result, glucose access to key organs such as skeletal muscle is reduced and blood glucose levels remains high. This uncontrolled condition could cause blindness, nerve

damage, kidney disease, cardiovascular disease, and other potentially fatal complications (Bose et al., 2008; Mlinar, Marc, Janez, & Pfeifer, 2007). Muscle, liver, and the adipose tissue are three major organs in the body that are affected by insulin resistance. Under normal conditions, these tissues produce a response to elevated level of blood glucose, via the action of insulin. Elevated blood glucose can cause insulin release into the bloodstream from the β-cells in the pancreas. Phosphoinositide-3-kinase (PI3K) signaling cascade that activates the protein AKT is the major response of insulin binding to cell surface receptors in the muscle and the adipose tissue. Translocation of the glucose transporter type 4 (GLUT4) from the cytosol to the cell surface and consequently, glucose entrance to cell is caused by activation of AKT that play an important role in returning blood glucose to normal levels (Bose et al., 2008). Inhibition of gluconeogenic enzymes and suppression of hepatic glucose output in the liver is also activated by the PI3K cascade (Mlinar et al., 2007).

Under insulin resistant condition, the response of muscle, adipose tissue, and liver to glucose uptake is reduced regardless of insulin availability in blood glucose. Therefore, the pancreas is producing more insulin to maintain normal blood glucose with the eventual development of pancreatic β-cell dysfunction. In the result, achieving glycemic control is a critical metabolic goal because hyperglycemia contributes to the progression of type 2 diabetes by adversely affecting both β-cell function and insulin sensitivity (Mlinar et al., 2007).

Metabolic syndrome is a global emerging health concern (Vinluan, Zreikat, Levy, & Cheang, 2012). This syndrome has been defined by clinical symptoms such as insulin resistance, dyslipidemia, central obesity, hypertension, impaired glucose tolerance or diabetes mellitus (Miranda et al., 2005). Current research show individuals with metabolic syndrome have much higher risk for cardiovascular diseases. Based on the National Cholesterol Education Program's

Adult Treatment Panel III (NCEP/ATP III) guidelines, a little more than one-third of the adults in the United States (34% of the US population) could be characterized as having metabolic syndrome (Ervin, 2009). Adults aged between 20 to 29 year have increased prevalence of 6.7% and ages from 60 to 69 have prevalence of 43.5% (Golden et al., 2009). According to NCEP/ATP III, a diagnosis of the metabolic syndrome is made when three or more of the risk factors shown in table 1 are present (Ervin, 2009). Metabolic syndrome increased with age but increased even more dramatically as BMI increased.

Table 1

ATP III clinical identification of the metabolic syndrome

Risk factor Defining level	
Abdominal obesity (waist circumference)	Men >102 cm (>40 in), Women >88 cm (>35 in)
Triglycerides	≥150 mg/ dL
HDL cholesterol	Men <40 mg/dL , Women <50 mg/dL
Blood pressure	≥135/≥85 mm Hg
Fasting glucose	≥100 mg/dL

Metabolic disorders, including obesity and diabetes, can be linked to an increased oxidative stress (Ibarra et al., 2011). Administration of plant extracts with antioxidant ability can decrease this stress (Seifried, Anderson, Fisher, & Milner, 2007). These plant-based compounds could stimulate expression of the nuclear peroxisome proliferator-activated receptors (PPAR). One of these receptors, nuclear peroxisome proliferator-activated receptor gamma (PPARγ) directs genes that control fatty acid uptake and storage, inflammation and glucose homeostasis which are transcription factors that regulate energy homeostasis. This transcription factor is

known to promote a late-stage adipocyte marker and play important roles in the regulation of lipid metabolism (M. Romo Vaquero et al., 2012).

2.2 Rosemary and Its Effects on Obesity and Related Metabolic Syndrome

Diet rich in fruits, vegetables and herbs has been known for decades that may help to reduce cardiovascular and metabolic complications, but there is limited knowledge about components of these foods that confer health benefits (Miranda et al., 2005). Biological activities of plant-derived phenolic compounds have been recently considered as an excellent source to control these diseases, however, there are still unsolved safety and efficacy issues in humans (Beltrán-Debón et al., 2011).

Recently, rosemary is one of the appreciated sources for its health benefits including hepatoprotective, antibacterial, antithrombic, antiulceratic, diuretic, antidiabetic, antinociceptive, anti-inflammatory, antitumor and antioxidant properties (al-Sereiti, Abu-Amer, & Sen, 1999; Borras Linares et al., 2011; Ibarra et al., 2011; Karamadoukis, Shivashankar, Ludeman, & Williams, 2009). Rosemary, *Rosmarinus officinalis L*. (Lamiaceae), is an aromatic evergreen shrubby herb highly distributed in the Mediterranean region (Jalali-Heravi, Moazeni, & Sereshti, 2011). Traditionally, rosemary has been used as a food flavoring spice and folk medicine in curing or managing of a wide range of diseases such as respiratory disorders, stomach problems and inflammatory diseases (Borras Linares et al., 2011). The water decoction of rosemary leaves has been traditionally used to treat diabetic patients, especially in the western part of Turkey, without much scientific evidence of its utility (Bakirel, Keles, Ulgen, & Yardibi, 2008). Rosemary is a good source for microelements and vitamins such as iron, calcium, and vitamin B6.

Application of rosemary has been verified by European Food Safety Authority as a natural preservative in foods and beverages. Rosemary can be found in northern African countries such as Morocco, and Tunisia, in southern Europe countries especially in Spain, France, Italy, the area of former Yugoslavia, Iran and also in America (Jalali-Heravi et al., 2011).

Based on the studies in various cell line systems and animal models, different mechanisms of action have been proposed for the observed beneficial effects of RE, which could be attributed to its components. Though it is not clear which component is the major agent against obesity and metabolic syndrome (Baba et al., 2005; Bel-Rhlid et al., 2009; Hosny, Johnson, Ueltschy, & Rosazza, 2002).

2.2.1 Major components in rosemary. Up to now, almost one hundred components in RE have been identified by using HPLC–MS or GC-MS (Borras Linares et al., 2011; Jalali-Heravi et al., 2011). These compounds belong mainly to the classes of phenolic diterpenes (CA, carnosol, rosmadial, rosmanol and its isomers epirosmanol and epiisorosmanol), flavonoids (genkwanin, homoplantaginin, scutellarein and cirsimaritin), triterpenes (ursolic acid, oleanolic acid,betulinic acid,botulin), and phenolic acids (rosmarinic acid, caffeic acid) (Bel-Rhlid et al., 2009; Borras Linares et al., 2011; Razborsek, Voncina, Dolecek, & Voncina, 2007). The levels of rosemary compounds vary significantly based on the parts of the plant (leaves, stems, sepals, petals, seeds, roots), seasonal variation, and the extraction method (Almela, Sanchez-Munoz, Fernandez-Lopez, Roca, & Rabe, 2006; Razborsek, 2011).

The principal bioactive components of the extracts are the phenolic diterpenes specifically CA (molecular formula $C_{20}H_{28}O_4$) (Fig. 1). It is a constituent of the species *Salvia* and *Rosmarinus* where it is mainly to be found in the leaves of rosemary and sage. Dried leaves of rosemary contain between 1.5 – 2.5% CA. Carnosol, rosmanol, epirosmanol and

methoxyepirosmanol are often present in less percentage than CA and also in some extent a result of degradation of CA with antioxidant properties (Thorsen & Hildebrandt, 2003). Some studies suggested that CA is therefore the only phenolic diterpene present in native state in rosemary and, accordingly, has the sole right to be called a natural product (Song et al., 2014).

The molecule of CA has two physic-chemical properties; the molecule contains apparently polar functions, such as carboxylic acid and phenol function. On the other hand, the remainder of its skeleton, made up essentially of hydrocarbons, provides a relatively nonpolar character by contrast with all other phenolic compounds, such as the flavonoids or hydroxybenzoic which are present in abundance in plant.

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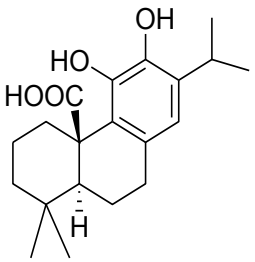


(4a*R*,10a*S*)-1,2,3,4,4a,9,10,10a-octahydro-5,6-dihydroxy-7-isopropyl-1,1-dimethylphenanthrene-4a-carboxylic acid

Figure 1. Molecular structure of carnosic acid

CA can be oxidize to carnosol by oxidation, and carnosol can degrade further to produce other phenolic diterpenes (Saenz-Lopez, Fernandez-Zurbano, & Tena, 2002). The highly oxidized abietane diterpenes rosmanol, isorosmanol, and dimethyl isorosmanol are formed from CA by dehydrogenation and the participation of activated oxygen (Bonoli, Pelillo, & Lercker, 2003) (Fig.2). Some methods for the preparation of CA by extraction have been proposed in literatures and patents in which the processes are long, complex. Due to economic reasons, these methods cannot be applied to an industrial process (Herrero, Plaza, Cifuentes, & Ibanez, 2010;

Okamura, Fujimoto, Kuwabara, & Yagi, 1994). Aeschbach et al. invented a preparation process according to these two physic-chemical properties of CA, extraction by using first nonpolar then polar solvent. This process gives a residue contains between 65 to 90% CA (Robert Aeschbach, 1993).

Several different analytical methods have been developed in order to determine the content of CA in samples with comparatively simple matrices. The high-performance liquid chromatography (HPLC) coupled with UV and/or MS detection has been the most widely used methods (Sanchez-Escalante, Djenane, Torrescano, Beltran, & Roncales, 2001; Haixia Yan et al., 2009).

This phenolic diterpene, and its phenolic hydroxyl group is easily oxidized and degraded under alkaline conditions (pH > 7) and is stable at acid conditions (pH < 4). CA can easily be converted into carnosol by air oxidation (Mulinacci et al., 2011; Schwarz, Ternes, & Schmauderer, 1992). However, the results of a study by Yan et al., 2009, show carnosol was not found in rat plasma samples after intragastric administration of CA in their study, indicating that CA could not be oxidized *in vivo* to generate carnosol as a metabolite (Haixia Yan et al., 2009). There have been only few reports for CA bioavailability and tissue distribution in rats (Doolaege, Raes, De Vos, Verhe, & De Smet, 2011; Haixia Yan et al., 2009), and no information for its metabolic profiles. Analysis of CA in rat plasma after intravenous and intragastric administration by Yan et al. reported a bioavailability of 65% (Haixia Yan et al., 2009). Doolaege et al. investigated the absorption of CA into the bloodstream after oral administration in rats. CA was found in the rat's intestinal content, liver and muscle tissue of abdomen and legs. It was found that CA *in vivo* present in its free form and that the main elimination route of CA is the fecal route (Doolaege et al., 2011).

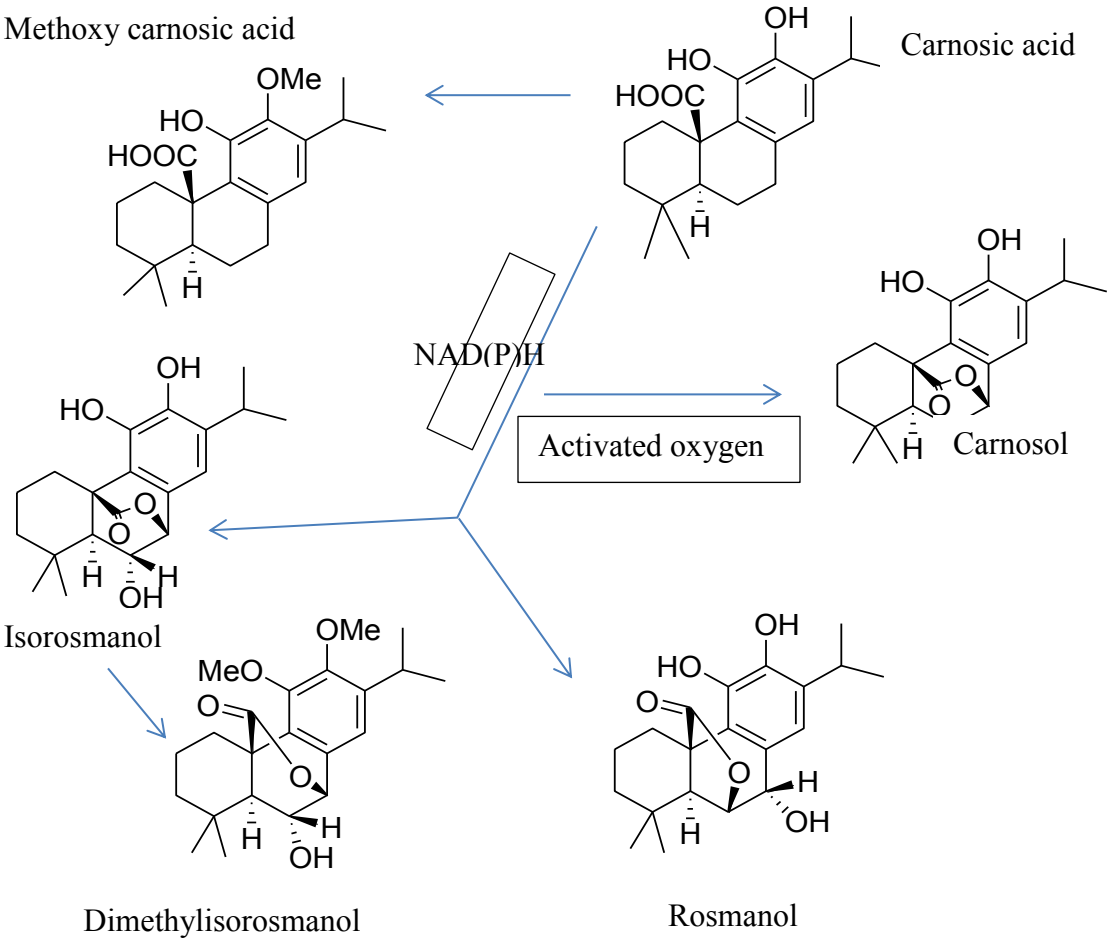


Figure 2. Formation of abietane diterpenes in rosemary plants (Munne-Bosch, Schwarz, & Alegre, 1999).

2.2.2 Antiobesity and antidiabetic effects of rosemary active components

2.2.2.1 In-vitro studies. Takahashi et al. found that CA and carnosol inhibited the differentiation in mouse preadipocytes (3T3-L1 cells) into adipocytes. In this study, these compounds induced phase 2 enzymes which are involved in metabolism of glutathione (GSH), activated nuclear factor E2-related factor-2 (Nrf2) and antioxidant-response element (ARE), and increased intracellular GSH. However, it remains unknown as yet which pathway mainly contributes to the inhibition by CA (Takahashi et al., 2009).

In 2012, Cui et al. demonstrated that abietane-type diterpenes from rosemary possessed moderate diacylglycerol acyltransferase (DGAT) inhibitory activity (Long Cui, 2012). Triglycerides (TG) are one of the main forms of stored energy. Excess accumulation of TG in certain tissues leads to serious diseases such as obesity, type 2 diabetes, and hypertriglyceridemia. TG synthesis has been assumed to occur primarily through (DGAT), which catalysis the final and only committed step in the glycerolphosphate pathway. DGAT activity can be promising strategy for the potential treatment of Type 2 diabetes, obesity and other related diseases that contain metabolic syndrome. In this study additionally, carnosol, one of the major components, exhibit a very effective inhibition property on *de novo* intracellular triglyceride synthesis on human hepatocyte HepG2 cells (Long Cui, 2012). CA and carnosol were used in an inhibitory test for pancreatic lipase activity *in vitro.* The inhibitory effect of CA was comparable to that of orlistat, and carnosol showed stronger lipase inhibitory activity than CA (Ninomiya et al., 2004). Tu et al. investigated the mechanisms of RE on metabolism in HepG2 cells. They found that RE significantly increased glucose consumption and glycolysis in HepG2 cells and activated the AMP-activated protein kinase (AMPK) and PPAR signaling pathways. They concluded that rosemary potential increased liver glycolysis and fatty acid oxidation by activating AMPK and PPAR pathways (Tu, Moss-Pierce, Ford, & Jiang, 2013).

2.2.2.2 In-vivo studies. It has been reported that RE has significant beneficial effect on metabolic disorders, such as obesity, hyperlipidaemia and hyperglycaemia in many animal models. Harach et al. (Harach et al., 2010) demonstrated male C57BL/6J mice fed on a HFD (60 Kcal% fat) supplemented with RE (200mg/kg BW per day, not 20mg/kg BW per day) for 50 days were protected against weight gain induced by the HFD. Their results show RE (contains 2.5% CA, 4% rosmarinic acid and 5.6% carnosol) was able to reduce fat mass gain and liver TG

content, and increase fecal fat excretion in compare to control group. The effects of RE might be related to the inhibition of pancreatic lipase activity (demonstrated *in vitro*) and caused a limitation of lipid absorption. In this study, the dose of CA (5mg/kg BW) was lower than that (20mg/kg BW) in the study of Ninomiya et al.(Ninomiya et al., 2004). They also found that administration of 20 or 200 mg/kg BW of RE in mice fed a HFD did not improve glucose tolerance, fasting plasma glucose or fasting plasma insulin level (Harach et al., 2010).

Moreover, Ibarra et al. reported that 16-week dietary intake of RE with 20% CA (500mg/kg BW/day) reduced the body weight gain, as well as epididymal fat weight in HFD-fed (44·92% Kcal fat) male C57BL/6J mice. The plasma cholesterol level was decreased by the RE administration, but there was no significant difference in plasma TG and non-ester fatty acid (NEFA) levels. RE treatment in this study was associated with increased fecal fat excretion. RE supplement was able to inhibit pancreatic lipase activity and activate PPAR-γ *in vitro* (Ibarra et al., 2011). Together, with reports of Ninomiya et al. (Ninomiya et al., 2004) and Harach et al. (Harach et al., 2010), the effects of limiting lipid absorption is a potential mechanism by which RE limits weight gain, at least partially.

In another study, 64-day administration of RE (0.5% w/w, supplemented in standard chow diet) with 40% CA also reduced body weight gain and improved the plasma lipids profile in obese (*fa/fa*) and lean (*fa/+*) female Zucker rats through inhibiting of gastric lipase (M. Romo Vaquero et al., 2012). However, the decrease of levels of TG and cholesterol was only significant in the lean female rats. There were the same results of reducing body weight and increasing fecal weight as those of Harach et al. (Harach et al., 2010) and Ibarra et al. (Ibarra et al., 2011) studies. In this study, the levels of CA, carnosol and methyl carnosate in the gastrointestinal tract with RE supplement were quantified, and the highest levels of CA and

carnosol were found in the stomach. The methylated derivative of CA was found to be the most abundant in the jejunum, ileum, and liver, may be due to the action of cathecol-*O*-methyl transferases (COMTs) in the intestine and liver. The highest and most significant inhibition of lipase activity (70-80%) was found in the stomach of the RE treated rats, which is responsible for the reduction in the absorption of dietary fat.

Few studies demonstrated positive effects of RE on improving the lipid profile in some animal models fed with hypercholeterolemic diet (Afonso et al., 2013; Al Sheyab, Abuharfeil, Salloum, Bani Hani, & Awad, 2011). Al Sheyab et al. (Al Sheyab et al., 2011) reported that the levels of plasma total cholesterol, low density lipoprotein (LDL), and TG were significantly reduced by rosemary water soluble extract administration (100 mg/kg BW) for 15 days in female BALB/c mice. In another study, an aqueous extract (AQ, 70 and 140 mg/kg BW) and non-esterified phenolic fraction (NEPF, 70 and 140 mg/kg BW) were studied in rats by gavage for 4 weeks. AQ contains 1.87% rosmarinic acid and NEPF contains 5.71% CA. However, only the AQ at 70 mg/kg significantly reduced the serum total cholesterol and non-HDL-c levels, maybe due to the amount of total phenolic compounds found in AQ was almost twice as much as in NEPF. Both AQ and NEPF have significant antioxidant activities in different tissues, but neither was able to ameliorate the alterations in the hypercholesterolemic diet-induced fatty acid composition in the liver (Afonso et al., 2013).

Limited number of studies applied CA alone. CA was carried out in HFD fed mouse model for its anti-obese effects by Ninomiya et al. In this study, CA was given orally to male ddY mice fed HFD (40 Kcal % fat) for 14 days. The results show CA (20 mg/kg BW/day) limited body weight gain and the accumulation of epididymal fat weight. Interestingly, although

with three different doses of CA (5, 10, 20 mg/kg BW/day), only 10 mg/kg BW/day of CA could suppress the serum TG elevation. Further studies are needed to clarify this observation.

Wang et al. analyzed the effects of CA (0.05% w/w, added in standard chow diet) in male *ob/ob* mice (obese leptin-deficient mice). After administration of CA for 5 weeks, the weight gain of mice was significantly inhibited and the visceral adiposity was reduced by CA administration. Meanwhile, the serum TG and cholesterol levels were reduced and the glucose tolerance was improved significantly. CA also had significantly protective effect by inhibiting hepatic fat accumulation and decreasing serum alanine aminotransferase (ALT) levels. CA also inhibited adipocyte hypertrophy of white adipose tissue (WAT) by decreasing adipocyte size (T. Wang et al., 2011). In good according with the results of Ninomiya et al. (2004), CA has lipid-lowering and body weight reducing effects, and is recognized to be a novel therapeutic agent for obesity and obesity-related diseases. The further mechanism was studied by Wang et al. (Wang et al., 2012). They found the preventing effects of CA on hepatocyte lipid accumulation was associated with repressed PPARγ levels and activated EGFR, MAPK, AMPK and ACC, which regulate lipid metabolism. They concluded that EGFR/MAPK signaling pathway plays an important role in the effects of CA. In this study, insulin resistance itself was not actually examined and unexpectedly the fasting glucose in CA+ mice was higher than control group.

In a previous study in Dr. Sang's lab, fifty mice were categorized into 3 groups according to the following design: negative control group on a low-fat diet (10 kcal% fat) (n=20); control group on a high-fat diet (60 kcal% fat) (n=20); and a high-fat diet with 0.5% of CA enriched RE (40% CA) (n=10) (unpublished data). This study showed that 0.5% of RE with 40% CA in the diet limited weight gain and hyperglycemia in mice that were fed a HFD over 16- week. The RE reduced body weight gain by 54.9 %, omental fat gain by 38.9%, and retroperitoneal fat gain by

25.1%. It also reduced fasting blood glucose levels by 71.2% and plasma insulin levels by 92.5% after 16-week . Moreover the incidence of fatty livers has also been inhibited.

Currently, substantial scientific interest is focused toward the effects of rosemary on obesity and obesity-related diseases. With regard to the above-mentioned, RE has been shown to be effective on body weight gain, fat mass gain, lowering serum lipid and glycemia in some animal models. However, form and mechanism of action of them have not been fully understood. The previous studies in this area provide a foundation for future studies that will identify the mechanistic bases for the metabolic effects of them. More *in vivo* studies are warranted to find weather CA is the major active component in rosemary to prevent obesity and related metabolic syndrome, and to further understand the underlying mechanism.

2.3 The High-Fat Diet-Fed Mouse as an Appropriate Model for Obesity and the Metabolic Syndrome

To understand metabolic syndrome and nutritional interventions related to its prevention or treatment, it is important to have appropriate animal models. Due to different technical skills, the availability of equipment, and the substances, it is unlikely metabolic experiments would be accomplished in the exact same way in different laboratories. Therefore the results will hardly be consistent from one laboratory to another. However, certain parameters can be standardized, regardless of where tests are conducted. This will help researchers the necessary information for interpreting results that might differ as a result of differences in methodology. One of the important parameter in metabolic studies is choosing appropriate animal model. Differences in these parameters can affect the results found from metabolic tests. Obesity, adipose tissue, glucose tolerance, Insulin resistant, and lipid levels are important elements in the metabolic disorders studies. One hypothesis for growing of metabolic syndrome in humans is the

possibility that our regulatory systems become overwhelmed by high-fat high-density foods, which have become increasingly available over the past 20–30 years. We chose high-fat diet-induced obesity and diabetes mouse model because among environmental factors, the high-fat content of the typical Western diet is considered a major cause of obesity and related type 2 diabetes. This mouse model was originally introduced by Surwit et al. in 1988 (Collins, Martin, Surwit, & Robidoux, 2004). They demonstrated that the C57BL/6J mouse would develop severe obesity, hyperglycemia, and hyperinsulinemia if weaned onto a high-fat diet.

Table 2

In vivo studies showing the effects of Rosemary on obesity and diabetes

Year	References	Treatment	Dosage /administration route	Animal model	Time	Outcomes	Conclusion
1994	Al-Hader, A. A., Hasan, Z. A., & Aqel, M. B. "Hyperglycemic and insulin release inhibitory effects of *Rosmarinus officinalis*."	Volatile oil extract of Rosemary	i.m. 25mg/kg BW, 6h	Alloxan-induced male diabetic rabbits	6h	-Serum glucose: Increased ~17%, Increased in normal rabbits after IPGTT -Serum insulin:~30%Decreased after IPGTT 30min in normal rabbits	The volatile oil extract has significant hyperglycemic and insulin release inhibitory effects.
2004	Ninomiya, K., Matsuda, H., Shimoda, H., Nishida, N., Kasajima, N., Yoshino, T., Yoshikawa, M. "CA, a new class of lipid absorption inhibitor from sage."	CA	Oral, 5, 10 and 20mg/kg BW	High fat(Olive oil)-fed male ddY mice	14 d	-BW Reduced by ~7% -Serum TG: No difference for 14days, but reduced at 2, 4, 6h after administration - Serum glucose: level was reduced only at 10mg/kg dose for 14days treatment and at 5, 10mg/kg at 2, 4, 6h, -increased weight of Epidydimal fat pad. - Inhibited pancreatic lipase activity.	Inhibited pancreatic lipase activity.

Table 2

Cont.

2006	Koga, K., Nomoto, K., Shibata, H., & Yoshino, K. "Effects of 50% ethanol extract from rosemary on [alpha]-glucosidase inhibitory activity and the elevation of plasma glucose level in rats, and its active compound. "	Rosemary 50% ethanol extract	As drinking water at 0.01% dose Maltose or sucrose was orally administerewith or without rosemary-distilled extract, to mice at a dose of 20 mg/mouse.	STZ-induced male ddY diabetic mice		Serum glucose: Decreased	Rosemary may play a role in controlling dietary glucose uptake in the small intestinal tract, because of RE's effects of inhibiting a-Glucosidase activity, In part.
2007	Nusier, M. K., Bataineh, H. N., & Daradkah, H. M. "Adverse effects of rosemary on reproductive function in adult male rats."	Rosemary 70% ethanol extract	As drinking water at 250 and 500 mg/kg BW	Male Sprague-Dawley rats	63days	-Body weight: No difference -Serum TG: No difference -Serum cholesterol: No difference - Serum glucose: No difference -No difference in ALT and AST	

Table 2

Cont.

2008	Bakirel, T., Keles, O. U., Ulgen, S. G., & Yardibi, H. "In vivo assessment of antidiabetic and antioxidant activities of rosemary in alloxan-diabetic rabbits."	Rosemary 95% ethanol extract	Oral, 50, 100, 200 mg/kg BW, Normal , acute at 1, 2, 6h, and at Subacute blood samples were collected at 1st, 3rd, 5th, and 8th days after each treatment.	Alloxan-induced diabetic adult rabbits of either sex	7days	-Body weight: increased - Serum glucose: Acute: decreased only in 200mg/kg at 2 and 6h. Subcute: decreased in 8th day with 100mg/kg and decreased in 200mg/kg from 3rd day. -Serum insulin: Acute: increased only in 200mg/kg at 2 and 6 h. Subcute: increased in 8th day with 100mg/kg and increased in 200mg/kg from 3rd day - Normal: decreased in 100 mg/kg after 2h of oral administration and recovered at 6h. Decreased in 200 mg/kg after 2 and 6h. No change in insulin. And decreased in 100 mg/kg at 6h and in200mg/kg at 2, 6h after OGTT. No insulin change.	Hypoglycaemic action was observed to be dose dependent. The RE exerts remarkable hypoglycaemic and antihyperglycaemic activity due to its possible multiple effects involving in pancreatic and extra-pancreatic and antioxidant mechanism.

Table 2

Cont.

2010	Harach, T., Aprikian, O., Monnard, I., Moulin, J., Membrez, M. "Rosemary leaf extract limits weight gain and liver steatosis in mice fed a high-fat diet."	RE(contains 4% rosmarinic acid, 2.5% CA, and 5.6% carnosol)	Oral, 20 mg/kg BW and 200mg/kg BW(0.25% and 0.025% W/W diet)	HFD fed male C57BL/6J mice	50 days	-Body weight: Reduced by ~11% with 200mg/kg dose -Liver TG: Reduced with 200mg/kg -Serum TG: No difference -Serum cholesterol: No difference -Serum glucose: No difference -Serum insulin: No difference - RE induced a higher fecal fat excretion	The results suggest that RE did not regulate glucose homeostasis in diet-induced obesity mice but RE induced a higher faecal fat excretion inhibited pancreatic lipase activity in vitro.
2011	Wang, T., Takikawa, Y., Satoh, T., Yoshioka, Y., Kosaka, K., Tatemichi, Y., & Suzuki, K. "CA prevents obesity and hepatic steatosis in ob/ob mice."	CA	Oral, 0.05% (w/w)	Male obese leptin-deficient (ob/ob) mice	5 weeks	-Body weight: Decreased - Serum TG: Reduced by ~ 60% - Serum cholesterol: Reduced by ~24% - Liver TG: N -Serum glucose: Reduced by ~18% -Serum insulin: Decreased by 47% - No difference in food intake. -Plasma FFA decreased.	CA inhibited adipocyte hypertrophy of WAT by a decrease of adipocyte size.

Table 2

Cont.

2011	Ibarra, A., Cases, J., Roller, M., Chiralt-Boix, A., Coussaert, A., & Ripoll, C. "CA-rich rosemary leaf extract limits weight gain and improves cholesterol levels and glycaemia in mice on a high-fat diet."	RE (20% CA)	Oral, 500mg /kg BW	HFD fed male C57BL/6 J mice	16-week	-Body weight: Decreased - Serum TG: No difference - Serum cholesterol: Reduced -Serum glucose: Decreased - Serum insulin: No difference - RE increased fecal total lipid excretion and fecal energy excretion, and decreased pancreatic lipase activity.	Anti-hyperglycemic activity in a HFD mouse model.
2012	Romo Vaquero, M., Yanez-Gascon "Inhibition of gastric lipase as a mechanism for body weight and plasma lipids reduction in Zucker rats fed a RE rich in CA."	RE (40% CA)	Oral, 0.5% (w/w) (RE 350-700 mg/kg; CA, 140 - 280 mg/kg)	Female Zucker lean(*fa/+*) and obese (*fa/fa*) rats	64 days	-Body weight: Reduced by 15% -Serum TG : Decreased in lean rats, but no difference in obese rats -Serum cholesterol: Decreased in lean rats, but no difference in obese rats -Liver TG: No difference -Serum glucose: No difference -Serum insulin: reduced in leans.	-RE significantly inhibited gastric lipase activity in the stomach , which may cause a moderate reduction of fat absorption.

Table 2

Cont.

2012	Al Sheyab, F. M., Abuharfeil, N., Salloum, L. "The effect of rosemary plant extracts on the immune response and lipid profile in mice."	RE (water soluble extract)	Oral, 100mg/kg BW d	High cholesterol diet fed female BALB/c mice	15days	- Serum TG: Reduced -Serum cholesterol: Reduced - LDL decreased and HDL significantly increased by RE supplement.	- Hypolipidemic activity of RE
2012	Aljamal, A. "Effects of rosemary on lipid profile in diabetic rats."	RE hot water extract	Oral, 550 mg/mouse/day	STZ-induced male albino rats	4 weeks	- Serum TG : Decreased by 24% - Serum cholesterol: Decreased by 22% . - Serum glucose: Decreased by20%LDL decreased by 27% and HDL increased by18%.	-RE had a Hypoglycemic effect and improved the lipid profile.

Table 2

Cont.

2013	Milessa S Afonso, A. M. d. O. S., Eliane BT Carvalho, Diogo P Rivelli, Sílvia BM Barros,, & Marcelo M Rogero, A. M. L., Rosângela P Torres and Jorge Mancini-Filho. "Phenolic compounds from Rosemary attenuate oxidative stress and reduce blood cholesterol concentrations in diet-induced hypercholesterolemic rats."	Rosemary aqueous extract (AQ, contain 1.87% Rosmarinic acid)and non-esterified phenolic fraction (NEPF, contain 5.71% CA)	AQ 70 and 140 mg/kg BW, NEPF 7 and 14 mg/kg BW	Hypercholesterolemic diet fed male Wistar rats	4 weeks	-Body weight: No difference -Serum TG: No difference -Serum cholesterol: Only decreased in 70mg AQ/kg dose. -No difference in food intake. -70mg AQ/kg dose decreased non-HDL-cholesterol.	

CHAPTER 3

Methodology

3.1 Materials

Rosemary Extract: The RE with 45% CA used in this study was purchased from China.

Mice: Seventy-five male C57BL/6J mice, aged 5 weeks were purchased from Jackson Laboratories (Bar Harbor, ME, USA).

- Low-fat diet (10 Kcal%, 4.3 % fat w/w), high-fat diet (60 Kcal%, 35% fat w/w), high fat with 0.50% w/w RE with 45% CA, high fat with 0.28% w/w RE with 80% CA, high fat with 0.14% w/w RE with 80% CA were made by Research Diets, Inc. (New Brunswick, NJ) (Table 3).

3.2 Experimental Methods

3.2.1 Experiment 1- Preparation of RE with 80% CA and CA (95%) standard. RE with 80% CA and CA standard were freshly prepared from the RE (45% CA) in our lab.

3.2.1.1 Extraction and isolation. CA was prepared according to the U.S. patent 5,256,700 (Robert Aeschbach, 1993) in our lab with modification (Fig. 3). In brief, 50 g RE (45% CA) was subjected to column chromatography over silica gel (230-400 mesh, Sorbent Technologies Inc., Atlanta, GA) and was eluted with a gradient of hexane-acetone (50:2, 50:3, 50:4, 50:5, 0:100; 1000 mL each) to give 5 fractions (Fr. A-E).

Purification of fraction B (23.3g) by reverse phase C_{18} silica gel medium pressure column chromatography (35 mm × 400 mm, 60 Å, Sigma) eluted with 75% methanol /water containing 0.05% formic acid, gave four sub fractions (Fr. B_1-B_4). Fraction B_2 (6.85 g) was identified as the CA enriched fraction (80% CA) by HPLC-MS. In order to get enough samples for the mouse study, this procedure was repeated five times. For further purification, 5.0 g of fraction B_2 was

loaded to Sephadex LH-20 column chromatography using ethanol as eluent, and only 0.5 g pure CA (98%) was obtained.

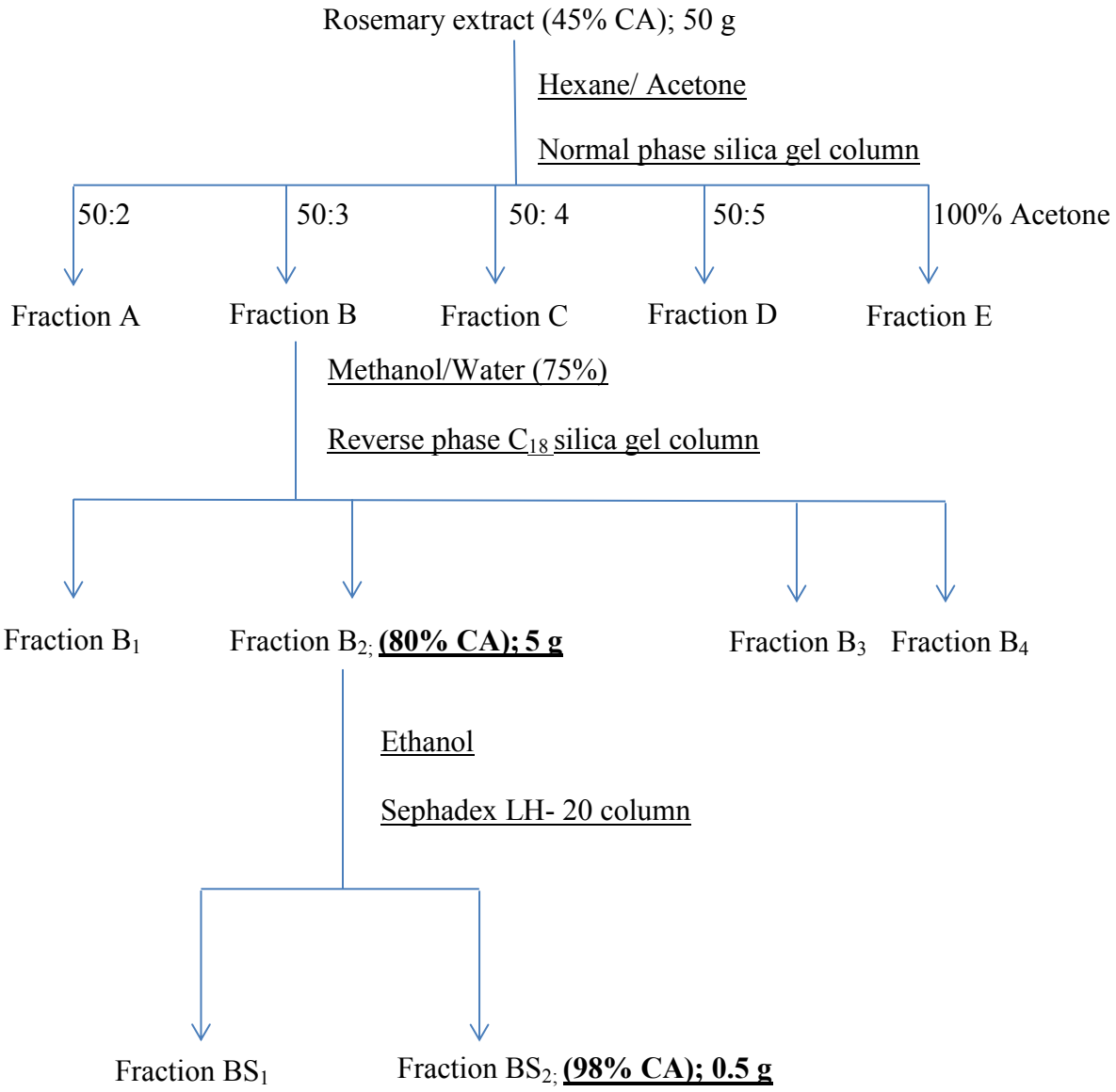


Figure 3. Separation scheme to prepare RE with 80% CA and purified CA (98%).

3.2.1.2 Analysis of CA by NMR. Nuclear magnetic resonance (NMR) was used for confirming the CA structure. ^{1}H and ^{13}C NMR spectra (analyzed in $CDCL_3$) were recorded on a Bruker AVANCE 700 MHz spectrometer (Brucker, Silberstreifen, Rheinstetten, Germany).

3.2.1.3 Analysis of the major components in RE by HPLC/ECD and LC/ESI-MS.

3.2.1.3.1 HPLC/ECD analysis. A high-performance liquid chromatography/ESA electrochemical detector (HPLC-ECD) (ESA, Chelmsford, MA) consisting of an ESA model 584 HPLC pump, an ESA model 542 autosampler, an ESA organizer, and an ESA CoulArray detector coupled with two ESA model 6210 four sensor cells, was used in the current study. A Gemini C18 column (150 × 4.6 mm, 5 μm; Phenomenex, Torrance, CA) was used for chromatographic analysis at a flow rate of 1.0 mL/min. The mobile phases consisted of solvent A (30 mM sodium phosphate buffer containing 1.75% acetonitrile and 0.125% tetrahydrofuran, pH 3.35) and solvent B (15 mM sodium phosphate buffer containing 58.5% acetonitrile and 12.5% tetrahydrofuran, pH 3.45). The gradient elution had the following profile: 5% B from 0 to 3 min; 5 to 55% from 3 to 10 min; 55 to 100% from 10 to 40 min; 100% B from 40 to 45 min; and 5% B from 45.1 to 50min. The cells were then cleaned at a potential of 1000 mV for 1 min. The injection volume of the samples was 10 μL. The eluent was monitored by the Coulochem electrode array system (CEAS) with potential setting at -50, 100, 200, 300, 400, 500, and 600 mV.

3.2.1.3.2 LC/ESI-MS analysis. LC/MS was carried out with a Thermo-Finnigan Spectra System, which consists of an Accela high-speed MS pump, an Accela refrigerated auto sampler, and an LTQ Velos ion trap mass detector (Thermo Electron, San Jose, CA) incorporated with an electrospray ionization (ESI) interface. A Gemini C18 column (150 × 4.6 mm, 5 μm; Phenomenex, Torrance, CA) was used for separation at a flow rate of 200 μL/ min. The column was eluted with 100% solvent A (5% aqueous methanol with 0.2% acetic acid) for 3 min, followed by linear increases in B (95% aqueous methanol with 0.1% acetic acid) to 60 % from 3 to 8 min; then to 75 % from 8 to 30 min, to 82 % from 30 to 48 min; and then with 100% from

48 to 55 min. The column was then re-equilibrated with 100 % A for 5 min. The injection volume was 10 µL for each sample. Nitrogen gas was used as the sheath gas and auxiliary gas. The collision-induced dissociation (CID) was conducted with an isolation width of 2 Da and normalized collision energy of 35 for MS^2 and MS^3. The mass range was measured from 50 to 1000 *m/z*. Data acquisition was performed with Xcalibur version 2.0 (Thermo Electron, San Jose, CA, USA).

3.2.2 Experiment 2 –Animal study and biological analysis. All animal experiments were conducted according to protocol (ID: 12-012) approved by the Institutional Animal Care and Use Committee at the North Carolina Research Campus (NCRC) in Kannapolis.

3.2.2.1 Animal and diets. All mice were housed on a 12-hour light/ dark cycle in a temperature-controlled environment during 1-week acclimatization, with ad libitum access to water and diet. After acclimatization, mice were randomized into 5 groups (n=15). Each group was fed the following experimental diet for 16-week : low-fat diet (10 Kcal%, 4.3 % fat w/w, LFD), high-fat diet (60 Kcal%, 35% fat w/w, HFD), high fat with 0.50% RE (enriched with 45% CA, RE#1), high fat with 0.28% w/w RE (enriched with 80% CA, RE#2H), high fat with 0.14% w/w RE (enriched with 80% CA, RE#2L) (Table 4).

Body weight and drink intake were measured weekly. Food intake was recorded every day. Signs of abnormality and possible toxicity were monitored. At the end of the study, liver, omental fat, and retroperitoneal fat were harvested, rinsed, and weighed. In addition, for measuring fecal lipid levels, from each group 5 mice were placed at a metabolic cage and 24-hour fecal samples were collected at week 16. All samples were stored at -80 ºC. Fasting blood glucose was measured at 0, 10, and 16-week of treatment. Food was removed 6 h prior to blood glucose measurements. Blood was collected from the tail vein, and glucose levels were measured

with One Touch Ultra 2 glucose monitor (Life scan, Inc. Milpitas, CA, USA). Mice were food-deprived for 8 hours and sacrificed by isophorone inhalation after 16-week of treatment. Whole blood was obtained by cardiac puncture. Blood samples were centrifuged at 5,000 rpm for 15 min.

3.2.2.2 Biochemical analysis of plasma samples. Biochemical levels were measured using commercial kits. Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were colorimetrically measured using Infinity ALT and AST Reagent provided by Thermo Scientific (Waltham, MA). Plasma triglyceride and cholesterol concentrations were measured by Infinity Triglyceride and Infinity Cholesterol Reagent (Thermo Scientific), respectively. Plasma free fatty acid was determined with a Free Fatty Acid Quantification Kit (BioVision, Milpitase, CA). Insulin levels were determined using Ultrasensitive Insulin ELISA kit (ALPCO, Salem, NH). Glucose levels in tail vein blood samples were measured using an OneTouch Ultra2 Blood Glucose Meter (LifeScan, Milpitase, CA).

3.2.2.3 Determination of lipid concentrations in liver. Quantitative assay of lipids was conducted by measuring triglyceride, cholesterol and FFA concentrations in the liver. In brief: hepatic lipids are extracted by homogenizing liver tissue in chloroform using 1% Triton X-100. The organic extracts were air dried, vacuumed, and dissolved in Trition X-100. Triglyceride, cholesterol, and FFA concentrations in extracts were determined using commercial kits (see Blood Biochemical Markers).

3.2.2.4 Body composition. A nuclear echo magnetic resonance imaging whole-body composition analyzer (Echo MRI System, Kannapolis, NC, USA) was used to assess body fat and lean mass in conscious mice. One cage was chosen from each group in week 15.

3.2.2.5 Fecal lipid measurements. The feces harvested during a 24-hour period, were collected at week 16; frozen at -80°C; and pulverized. Total lipids were extracted from 100 mg of dried feces using the following method (Folch, Lees, & Stanley, 1957): The feces were cleaned and dried for 24 hours by freeze dryer, incubated with 2 mL of chloroform-methanol (2:1) for 30 min at 60°C with constant agitation, and then centrifuged. Water (1 mL) was added to the supernatant, and following vortexing, phase separation was induced by low-speed centrifugation (2,000 rpm for 10 min). The lower chloroform phase was then removed and transferred to a new tube, and the sample was evaporated to dryness by nitrogen evaporator and weighed.

3.2.2.6 Statistical analysis. The data from the *in vivo* studies were expressed as mean ± standard deviation (x ± SD). One-way analysis of variance (ANOVA one-way, Newman-Keuls multiple comparison test) and student *t*-test were performed to compare groups using Sigma Plot 11.0 (2008) (Systat Software, Inc.). Statistical significance was considered at $p<0.05$.

Table 3

Composition of the experimental diets.

Macronutrient	**LFD**		**HFD**		**HFD + RE#1 (0.5%)**		**HFD + RE#2H (0.28%)**		**HFD + RE#2L (0.14%)**	
	g %	**Kcal %**	**g %**	**Kcal %**	**g %**	**kcal %**	**g %**	**kcal %**	**g %**	**kcal %**
Protein	19.2	20	26.2	20	26.1	20	26.2	20	26.2	20
Carbohydrate	67.3	70	26.3	20	26.2	20	26.3	20	26.3	20
Fat	4.3	10	34.9	60	34.7	60	34.8	60	34.8	60
Total		100		100		100		100		100
Kcal/g	3.85		5.24		5.22		5.23		5.24	
Ingredient	g	kcal	g	kcal	g	kcal	g	kcal	g	kcal
Casein/80 mesh	200	800	200	800	200	800	200	800	200	800
L-Cystine	3	12	3	12	3	12	3	12	3	12
Corn starch	506.2	2024.8	0	0	0	0	0	0	0	0
Maltodextrin 10	125	500	125	500	125	500	125	500	125	500
Sucrose	68.8	275.2	68.8	275.2	68.8	275.2	68.8	275.2	68.8	275.2
Cellolose,BW200	50	0	50	0	50	0	50	0	50	0
Soybean oil	25	225	25	225	25	225	25	225	25	225
Lard	20	180	245	2205	245	2205	245	2205	245	2205
Mineral mix,S10026	10	0	10	0	10	0	10	0	10	0
D Calcium Phosphate	13	0	13	0	13	0	13	0	13	0
Calcium carbonate	5.5	0	5.5	0	5.5	0	5.5	0	5.5	0
Potassium citrate, 1 H2o	16.5	0	16.5	0	16.5	0	16.5	0	16.5	0
Vitamin mix, V10001	10	40	10	40	10	40	10	40	10	40
Cholin Biartrate	2	0	2	0	2	0	2	0	2	0
RE#1	0	0	0	0	3.9	0	0	0	0	0
RE#2	0	0	0	0	0	0	2.18	0	1.09	0
Dye	0.05		0.05		0.05		0.05		0.05	
Total	1055.05	4057	773.85	4057	777.75	4057	776.03	4057	774.94	4057

CHAPTER 4

Results

4.1 Experiment 1- Characterization of the Major Components in REs.

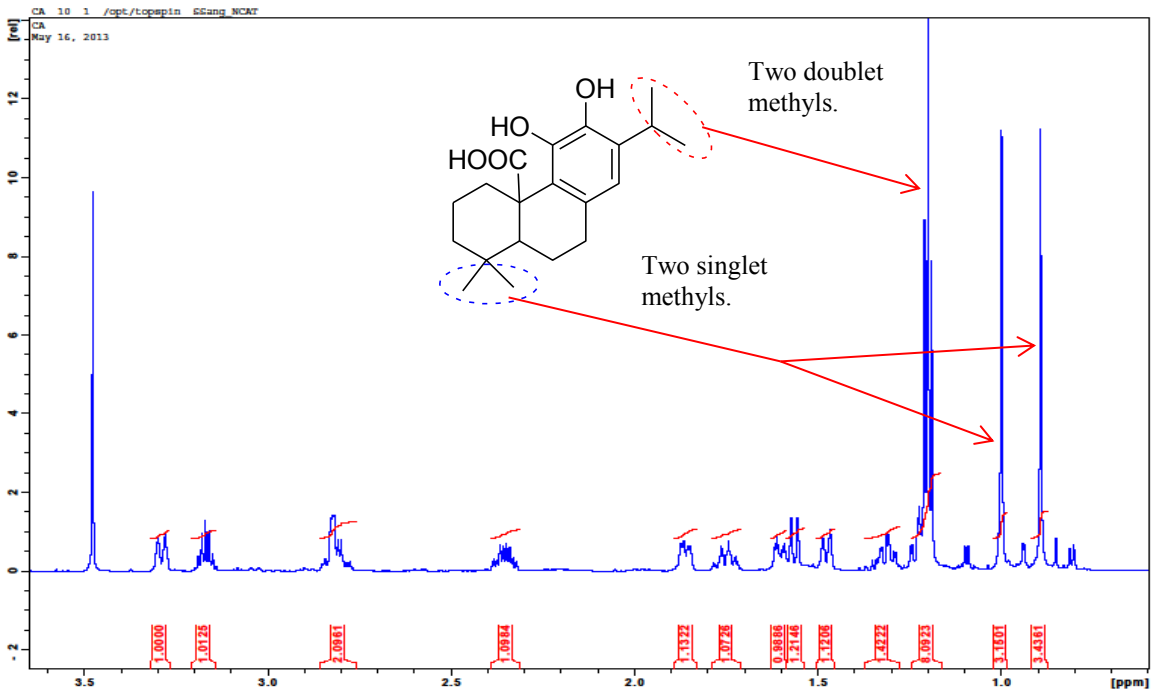
4.1.1 Structure elucidation of purified CA from commercial RE by NMR. The structure of CA was identified using NMR techniques and confirmed by comparison of its NMR data with those already published (Masuda, Inaba, & Takeda, 2001; H. Yan et al., 2009). Multiplicities are indicated by s (singlet), d (doublet), and brd (broad doublet) (Table 4, Fig. 4 A, B).

4.1.2 HPLC/ECD and LC/MS analysis.

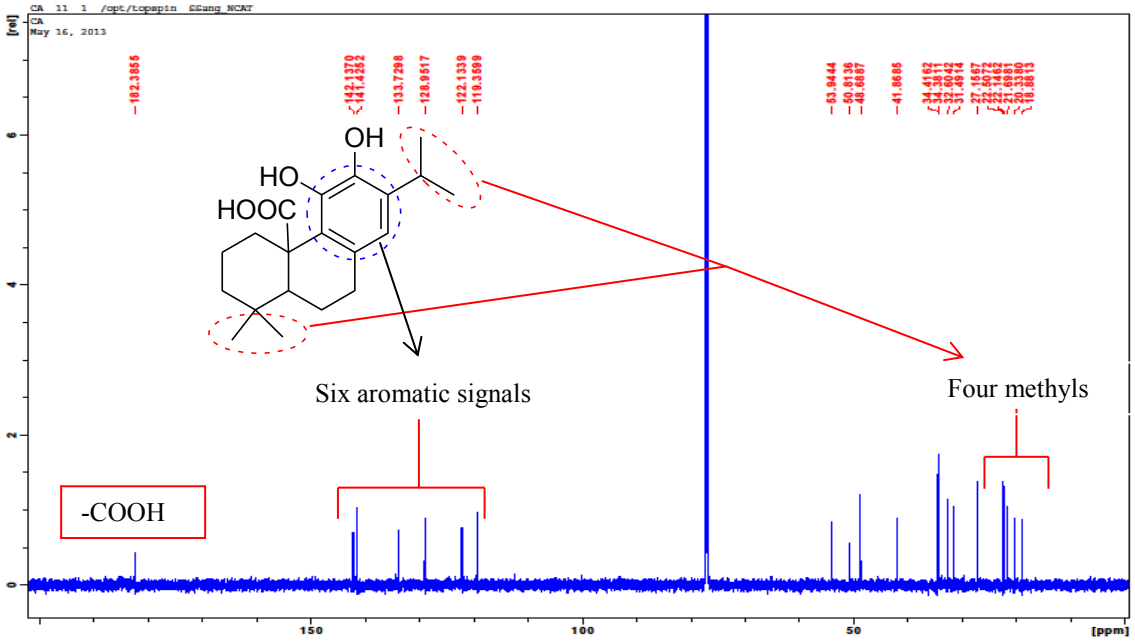
4.1.2.1 Determination of the purity of CA standard by HPLC/ECD. CA standard was purified by the combination of different column chromatography techniques in our lab. The purity of CA standard was obtained based on the analysis of peak areas generated automatically from CoulArray Data Station 3.00 (ESA Biosciences, MA). In a same manner, the content of CA in RE was also quantified.

4.1.2.2 LC/MS analysis. LC/MS analysis of the prepared REs revealed a total of three major diterpene phenolic compounds present in the extracts. The compounds were characterized by their retention times, UV-Vis and mass spectrum, and identified by comparison with published data (H. Yan et al., 2009) or commercial standards. Figure 5 A shows, the chromatographic profile of the commercial RE with 45% CA. CA was shown in the middle and the highest peak. After extraction of the commercial product, the figure 5 B shows the chromatographic profile of the RE with 80% CA. LC/ESI-MS results demonstrated that for CA related compounds, the neutral loss of a CO_2 molecule (44 Da) was regarded to be a characteristic fragmentation pathway (María Romo Vaquero et al., 2013; Song et al., 2014). Take

CA (Peak Ⅰ, at retention time of 36.5 min) for example, the MS spectrum of CA exhibited and deprotonated ion at m/z 331 $[M-H]^-$ (data not shown), corresponding to a molecular weight of 332 amu, MS^2 spectrum (Fig. 5D) of the precursor ion at m/z 331 $[M-H]^-$ afforded a predominant product ion at m/z 287, generating by the neutral loss of a CO_2 (44 Da) from the carboxyl moiety. Likewise, the other two major peaks (Peak Ⅱ and Ⅲ, at retention times of 32.7 and 40.1 min, respectively) were tentatively identified as carnosol and 12-O-methyoxy CA by comparing their MS/MS data with the reference data from literature(Song et al., 2014). The stability of extracts was tested by monitoring the content of CA using HPLC-DAD-MS/MS. The levels of CA in the experimental diets remained stable throughout the study period.



(A)



(B)

Figure 4. ^{1}H and ^{13}C NMR spectra of CA

Table 4

NMR spectra data of CA

	CA	
position	δ_H multi (J)	δ_C
1	1.63 m	41.9
2	α: 1.90 m β: 2.39 m	18.9
3	2.86 m	34.4
4		34.4
5	1.78 m	53.9
6	α: 1.52 m β: 1.60 brd (11.6)	20.3
7	α: 1.34 m β: 3.32 brd (13.9)	31.5
8		129.0
9		122.1
10		48.7
11		142.1
12		141.4
13		133.7
14	6.57 s	119.4
15	3.21 m	27.2
16	1.23 d (7.0)	21.7
17	1.23 d (7.0)	22.2
18	1.04 s	32.6
19	0.93 s	22.5
20		182.4

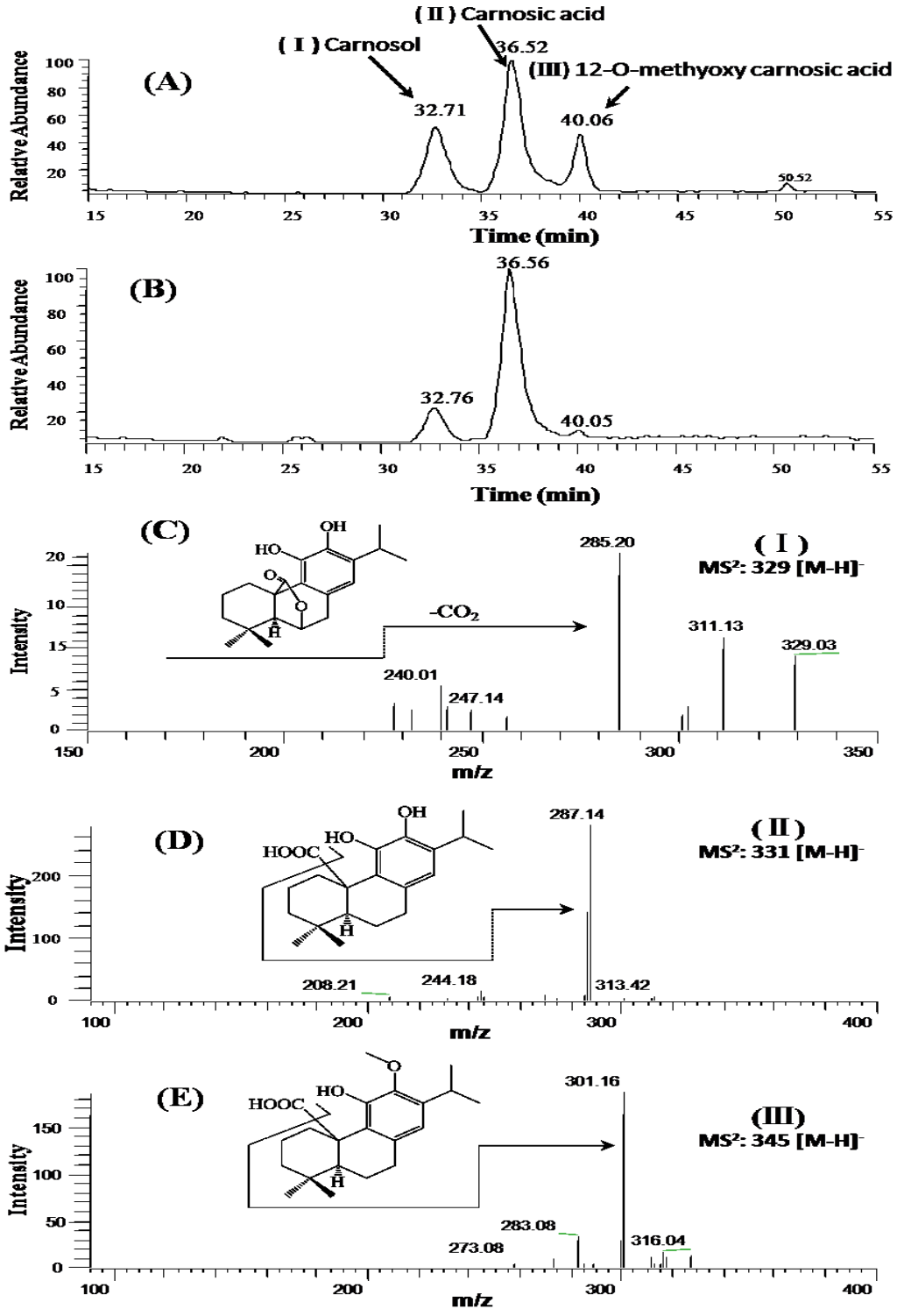


Figure 5. TIC chromatograms of commercial RE (A), CA enriched RE (B), and MS/MS (negative) spectra of peak Ⅰ(C), Ⅱ(D)and Ⅲ(E).

4.2 Experiment 2- Animal Study and Biological Analysis

4.2.1 Effects of REs on body and organ weight and food intake in mice fed a high-fat diet.

Body weight between the HFD and LFD groups began to differ significantly after the first week of treatment ($p<0.01$). Likewise, body weight between the HFD and the three RE treated groups started to differ significantly ($p<0.05$) after the first week of experiment (Fig. 6). At the end of the study, LFD group had 58% less weight gain than HFD. Weight gain for all treatment groups (RE#1, RE#2H and RE#2L) were less than that of HFD at 48, 52 and 32% ($p<0.05$) at the end of the study, respectively (Fig. 7).

After 16-week treatment, LFD group had 72% mesenteric fat, 70% perirenal fat, 56% total fat and 57 % liver weight less than HFD group. The three treated groups (RE#1, RE#2H, and RE#2L) had 67, 69 and 54 % ($p<0.05$) less than HFD group in mesenteric fat; 41, 50 and 26 % less than HFD group in perirenal fat; 23.5, 32 and 5 % ($p<0.05$) less than HFD group in total fat , 54, 57and 53 % less than HFD group in liver weight, respectively. No significant changes were observed between groups in epididymal fat (Table 5). An obvious difference between the mouse abdominal cavities has been observed at the necropsy room.

The above results were further supported by the MRI results. The figures generated from the MRI demonstrate that the weight gain reduction by RE#1 and RE#2H groups are due to decrease the fat mass (Fig. 8).

The HFD was very soft and easily crushed and dumped into the cage. Therefore, it is not possible for us to accurately measure the food intake even we tried to measure it every day. Most of the literature studies reported that between RE treated group and HFD group there was no significant difference in food intake(Harach et al., 2010; T. Wang et al., 2011). However, it is unclear to us how they could measure the food intake in an accurate way.

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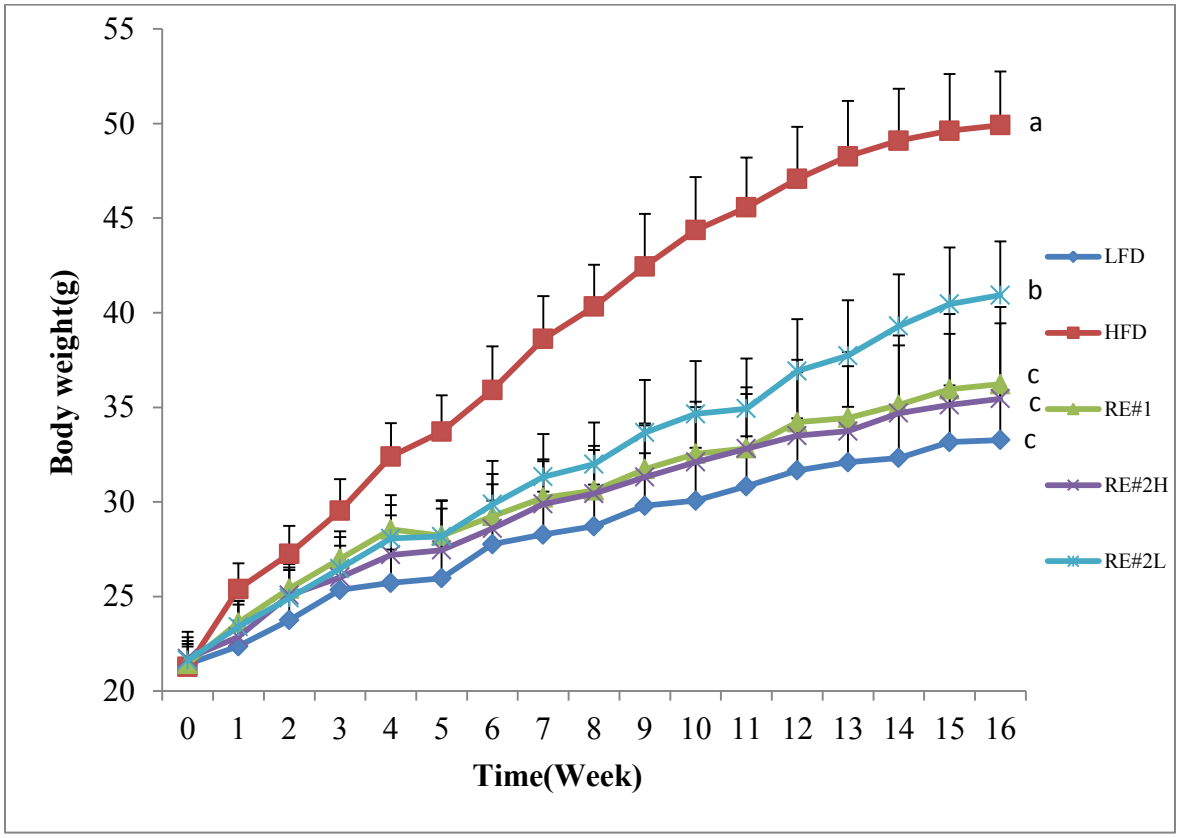


Figure 6. Effects of low fat diet (LFD), high fat diet (HFD), 0.5% RE with 45% CA (RE#1), 0.28% RE with 80% CA (RE#2H), and 0.14% RE with 80% CA (RE#2L) on body weight of mice during 16-week experiment. Values are means, with standard deviations represented by vertical bars. Dis similar letters are significantly different at the $P<0.05$ level, n=15.

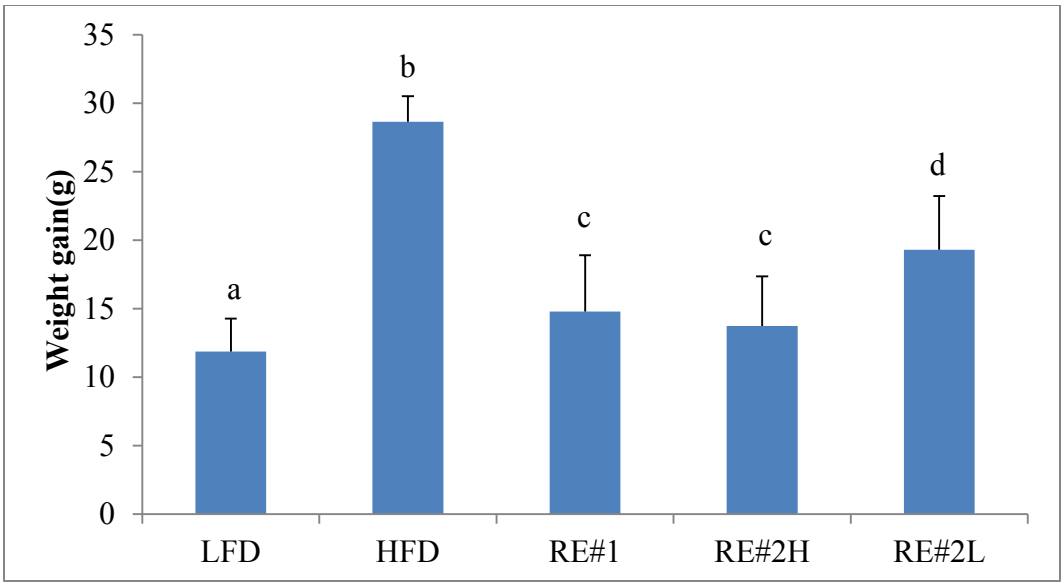


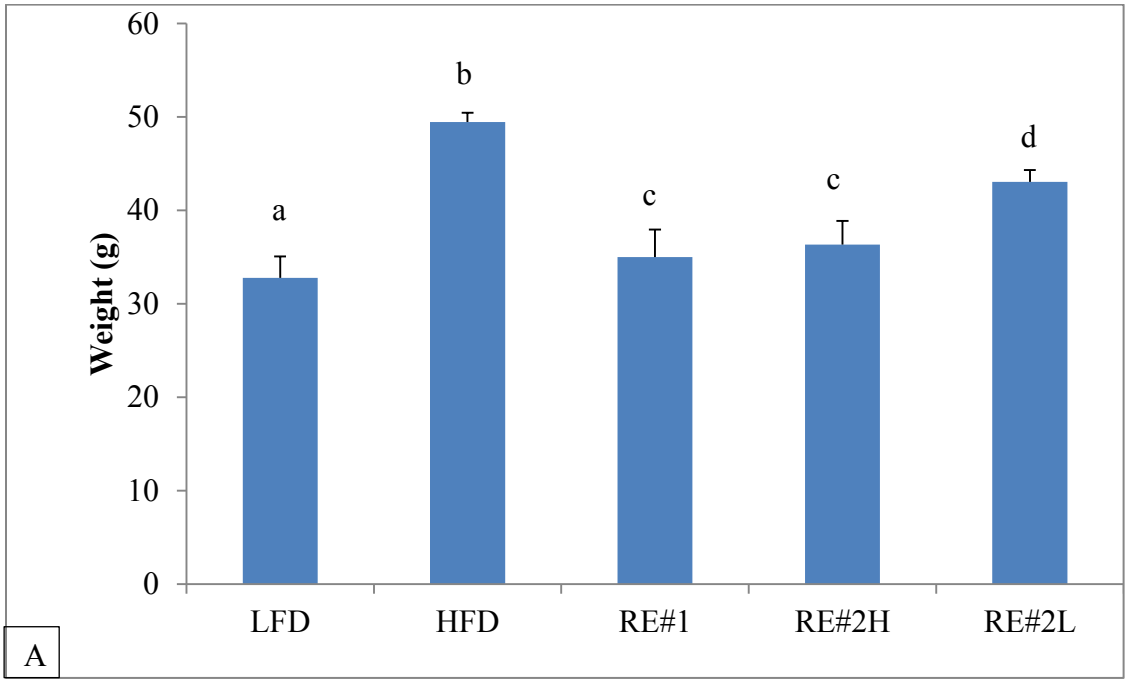
Figure 7. Effects of low fat diet (LFD), high fat diet (HFD), 0.5% RE with 45% CA (RE#1), 0.28% RE with 80% CA (RE#2H), and 0.14% RE with 80% CA (RE#2L) on weight gain after 16-week. Values are means, with standard deviations represented by vertical bars. Values with different letter are significantly different at the $P<0.05$ level, n=15.

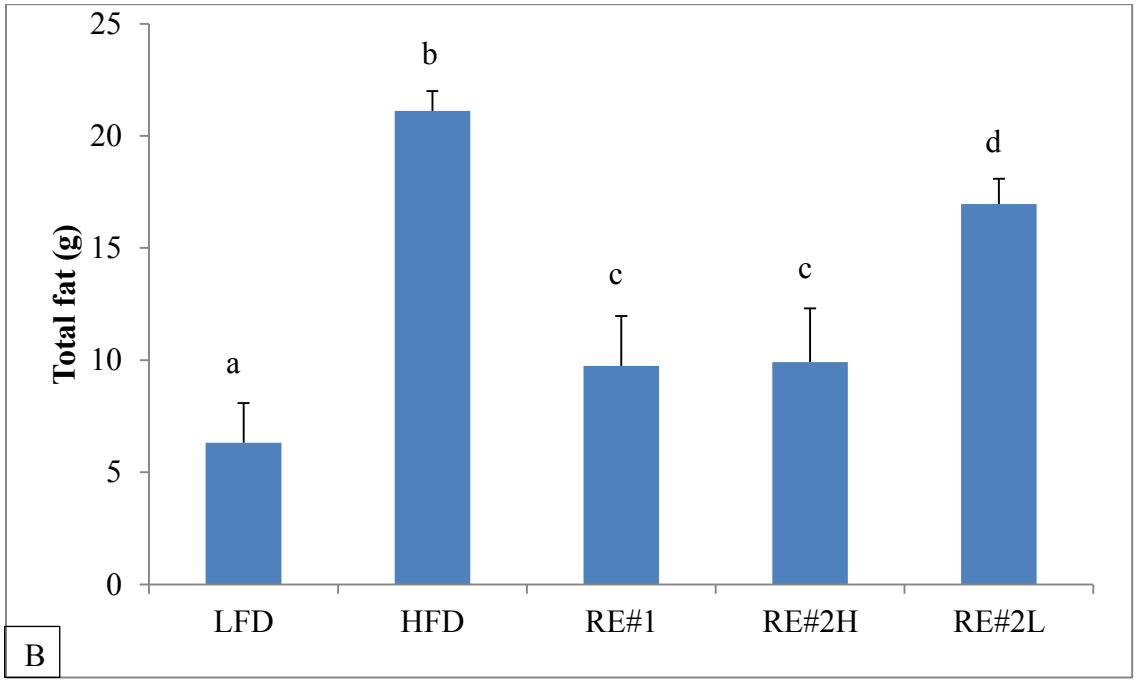
Table 5

Effects of low fat diet (LFD), high fat diet (HFD), 0.5% RE with 45% CA (RE#1), 0.28% RE with 80% CA (RE#2H), and 0.14% RE with 80% CA (RE#2L), on weight parameters (mesenteric fat, perinenal fat, epididdymal , total fat and liver after 16-week.

Parameter	LFD	HFD	RE#1	RE#2H	Re#2L
Mesenteric fat	0.48 ± 0.15	1.71 ± 0.24	0.55 ± 0.21	0.54 ± 0.13	0.78 ± 0.22
Perirenal fat	0.48 ± 0.21	1.58 ± 0.33	0.93 ± 0.28	0.79 ± 0.31	1.16 ± 0.24
Epididdymal fat	1.16 ± 1.16	1.54 ± 1.54	2.21 ± 2.21	1.96 ± 1.96	2.63 ± 2.63
Total fat	2.13 ±0.82	4.84 ± 0.46	3.7 ± 1.14	3.3 ± 1.12	4.59 ± 1.12
Liver weight	1.18 ± 0.26	2.78 ± 0.6	1.27 ± 0.15	1.19 ± 0.14	1.3 ± 0.27

The results are expressed as the mean± SD (n=15)


60
50
40
30
20
10
0
Weight (g)
a
b
c
c
d
LFD
HFD
RE#1
RE#2H
RE#2L
A


25
20
15
10
5
0
Total fat (g)
a
b
c
c
d
LFD
HFD
RE#1
RE#2H
RE#2L
B

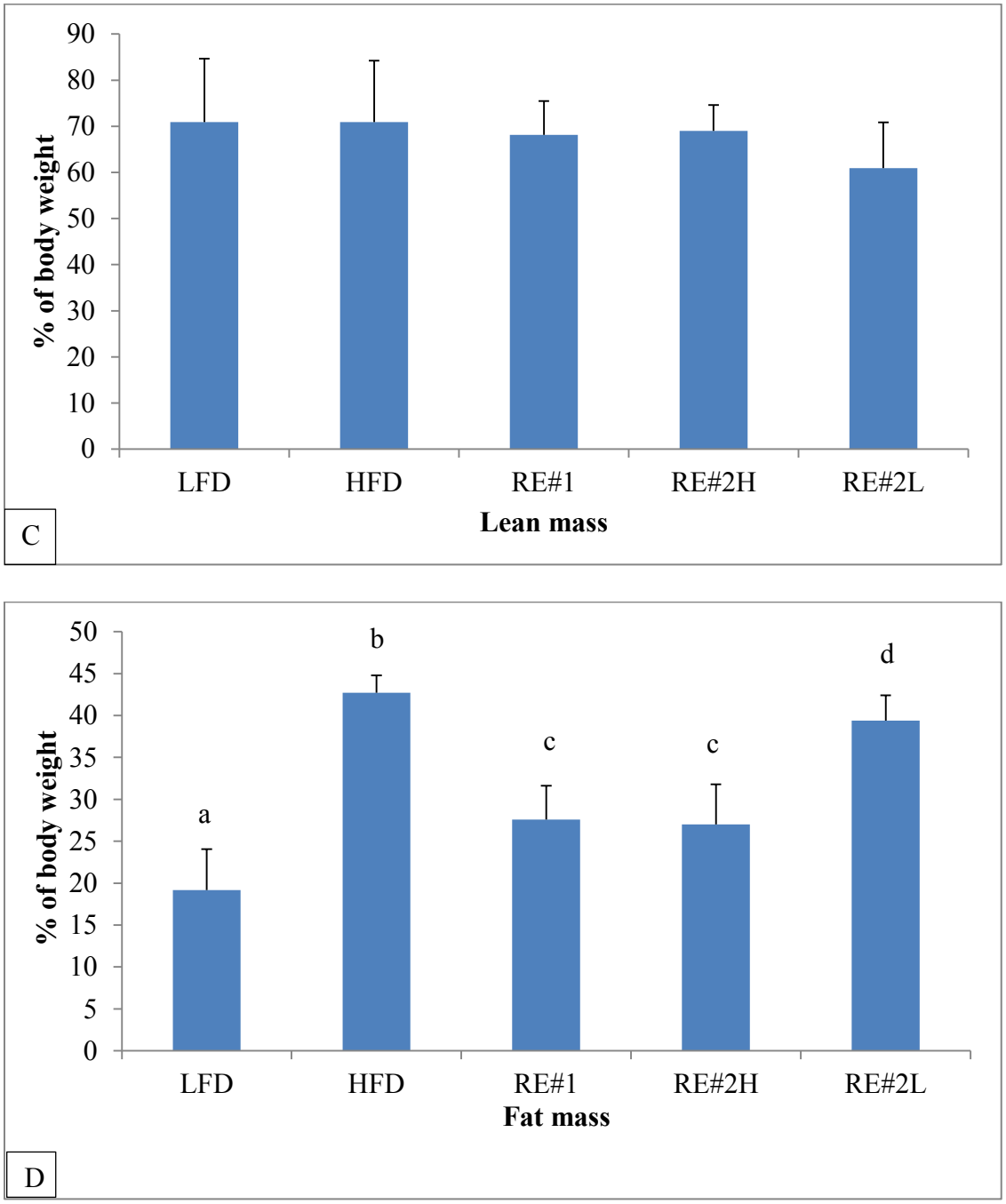


Figure 8. Body composition results of Eco-MRI. The Results of the total weight (A), total fat (B), lean mass% of the body weight (C) and fat mass% of the body weight (D) after 16-week of treatment by LFD, HFD, RE#1, RE#2H and RE#2L. Values are mean ± SD. Values with different letter are significantly different at the $P<0.05$, n=5.

4.2.2 Effects of RE on serum biochemical parameters. After 16-week of treatment, total plasma cholesterol level rose significantly in the HFD group compared with LFD animals. Plasma cholesterol in LFD compare to HFD is 49% less; in treatment groups, (RE#1, RE#2H and RE#2L) are 19, 34 and 18% less than HFD, respectively (Fig. 9). However, Plasma triglyceride and plasma free fatty acids in the HFD group compared with LFD animals are not significantly different (Fig. 10, Fig. 11).

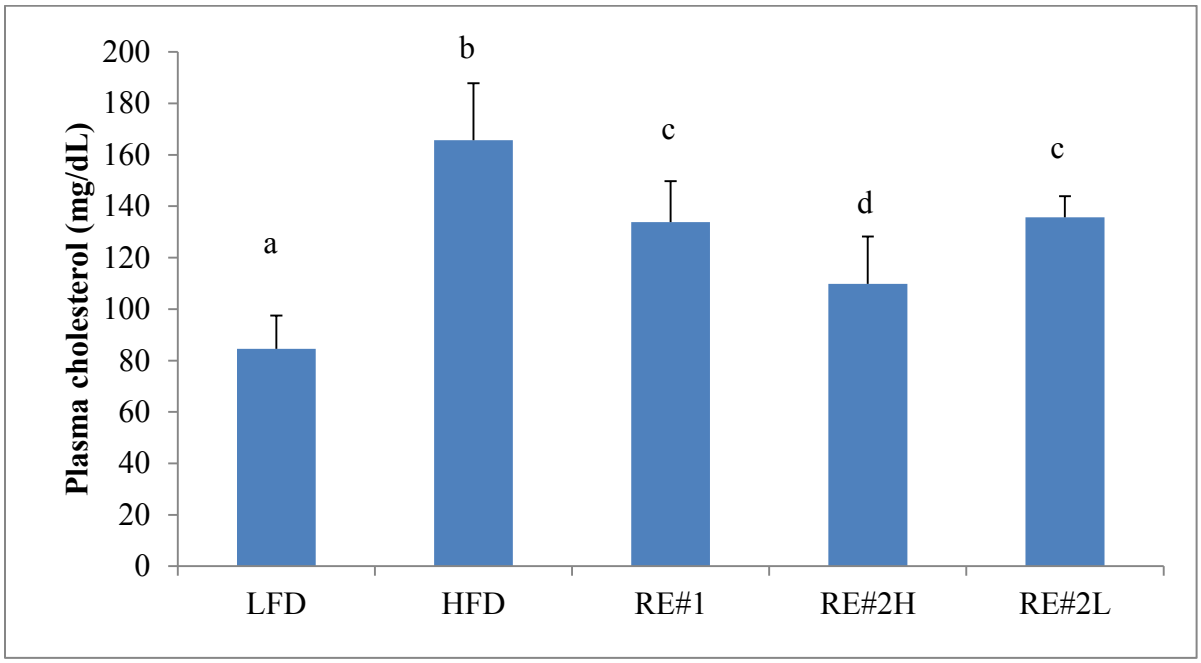


Figure 9. Effects of low fat diet (LFD), high fat diet (HFD), 0.5% RE with 45% CA (RE#1), 0.28% RE with 80% CA (RE#2H), and 0.14% RE with 80% CA (RE#2L) on plasma cholesterol after 16-week. Values are means, with standard deviations represented by vertical bars, $P<0.05$, n= 6.

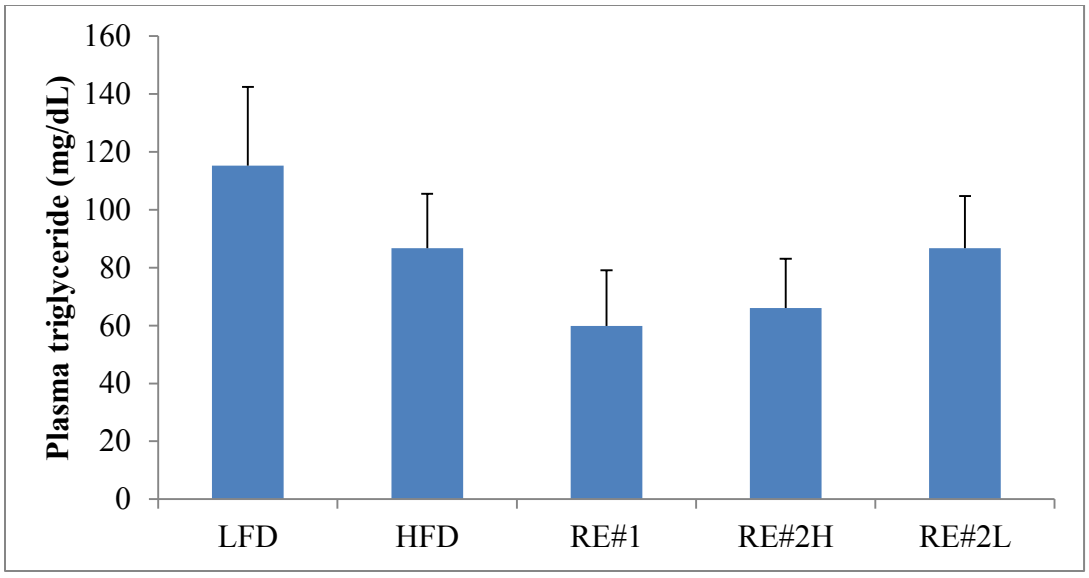


Figure 10. Effects of low fat diet (LFD), high fat diet (HFD), 0.5% RE with 45% CA (RE#1), 0.28% RE with 80% CA (RE#2H), and 0.14% RE with 80% CA (RE#2L) on plasma triglyceride after 16-week. Values are means, with standard deviations represented by vertical bars, n= 6.

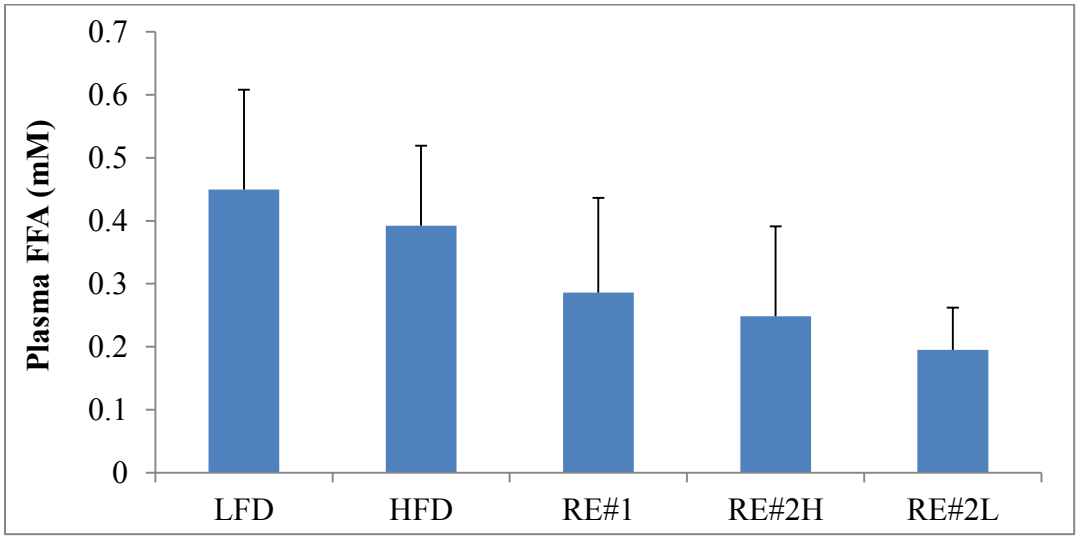


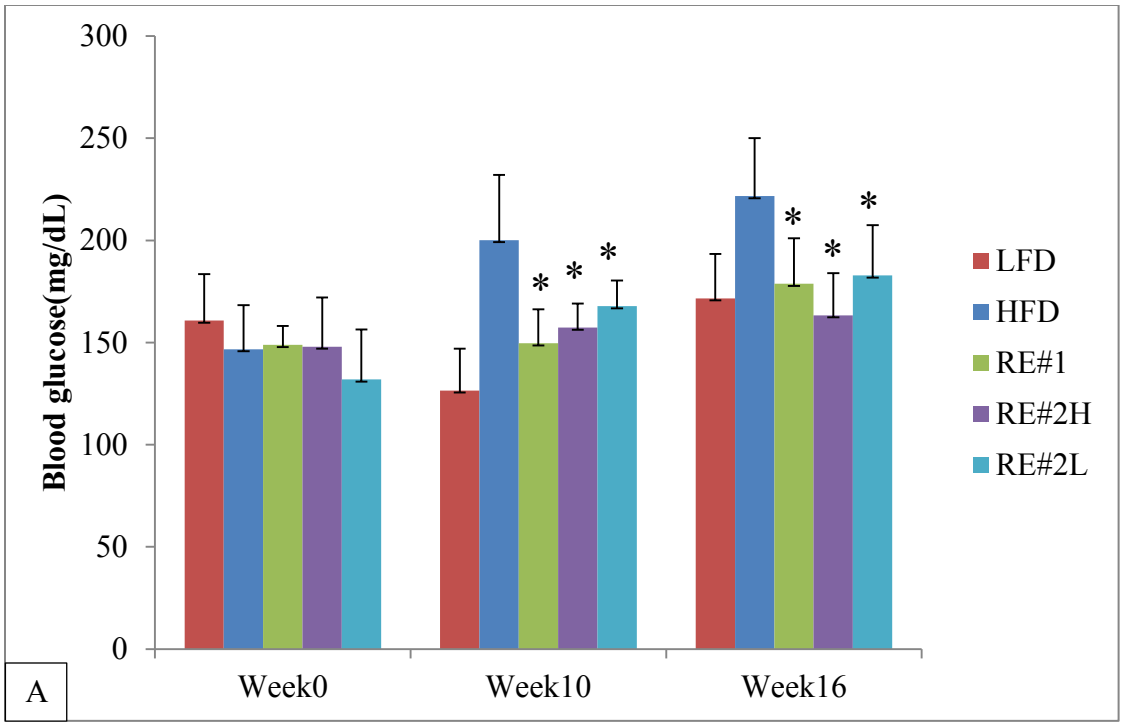
Figure 11. Effects of low fat diet (LFD), high fat diet (HFD), 0.5% RE with 45% CA (RE#1), 0.28% RE with 80% CA (RE#2H), and 0.14% RE with 80% CA (RE#2L) on plasma free fatty acid after 16-week. Values are means, with standard deviations represented by vertical bars, n= 6.

Total glucose levels in LFD group are 21% less than those in HFD group. Mice in treatment groups (RE#1, RE#2H, and RE#2L) experienced 15, 22 and 17 % plasma glucose levels ($p<0.001$) less than those in HFD group (Fig. 12 A). Fasting blood glucose levels in all the groups were recorded at week 0, 10, and 16 as reported in Figure 12A.

Animals in the LFD group had normal fasting blood glucose level after the 16-week treatment (160.73±22.72 mg/dl at Week 0; 126.10±20.54 mg/dl at Week 10; 171.6±21.76 mg/dl at Week 16). In contrast, HFD animals increased fasting blood glucose levels progressively from the beginning of the study (146.73±21.54 mg/dl) until Week 16 (221.66±28.40 mg/dl).

In the RE-treated groups, fasting blood glucose rose 19.36% in RE#1, 26.31% in RE#2H, and 17.53% in RE#2L (178.73±22.22 md/dl , 163.33±20.70 md/dl, 182.8±24.67 md/dl , $p<0.001$) less than those in HFD mice at the end of the study. There is no significant difference between blood glucose levels between the two of the treatment groups (RE#1 and RE#2H) and the LFD group after 16 week.

Figure 12B shows the levels of fasting plasma insulin at the end of the study, week 16. Reduced fasting plasma insulin levels was significantly different between LFD and HFD, and also between treatment groups (RE#1, RE#2H, Re#2L) and HFD group ($p<0.001$). Insulin level in LFD compare to HFD is 69% less; in treatment groups (RE#1, RE#2H and RE#2L) are 66, 59 and 50% less than HFD, respectively. There is no significant difference of the fasting plasma insulin levels between two of the treatment groups (RE#1 and RE#2H) and LFD group after 16 week.



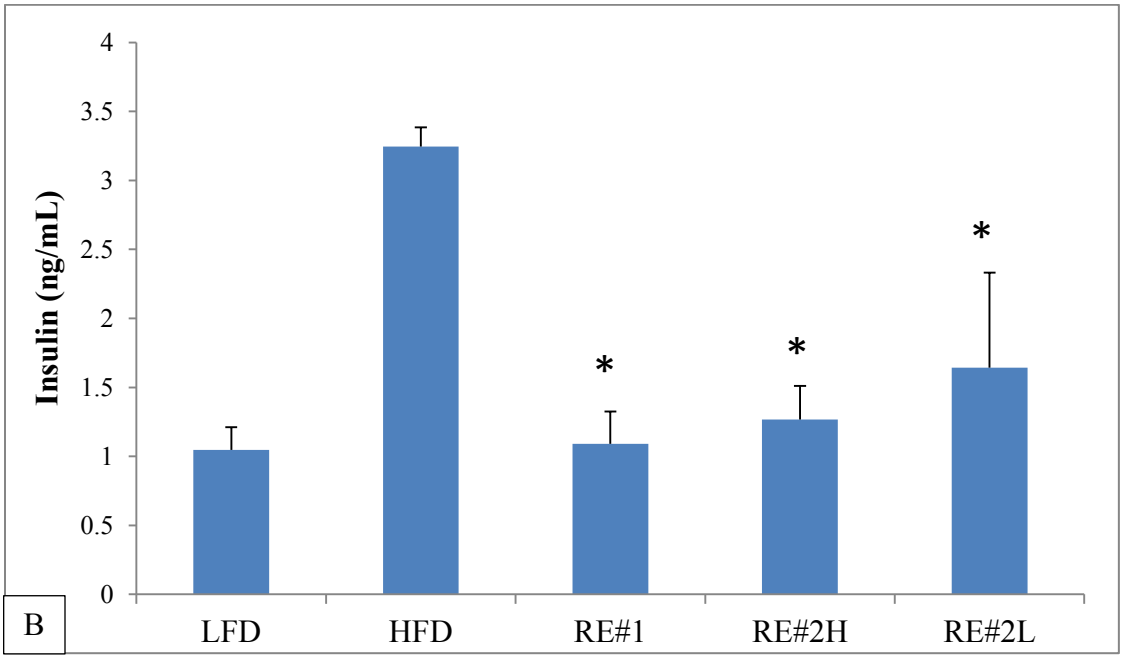


Figure 12. (A) Fasting blood glucose levels in low fat diet (LFD), high fat diet (HFD), 0.5% RE with 45% CA (RE#1), 0.28% RE with 80% CA (RE#2H), and 0.14% RE with 80% CA (RE#2L) treated mice at week 0, 10, and 16. (B) Fasting plasma insulin levels in LFD, HFD, RE#1, RE#2H and RE#2L treated mice after 16-week study. Values are mean ± SD, * = $P<0.05$, n= 6.

4.2.3 Changes in liver weight, incidence of fatty liver, and plasma ALT and AST levels. After 16-week of treatment, the average liver weight reached 1.18±0.26 g in the LFD group, 2.78 ±0.60 g in the HFD group, and less liver weight 54.1, 57 .21 and 53.11% in RE#, RE#2H, Re#2L compare to HFD group($P<0.05$) (Fig. 13).

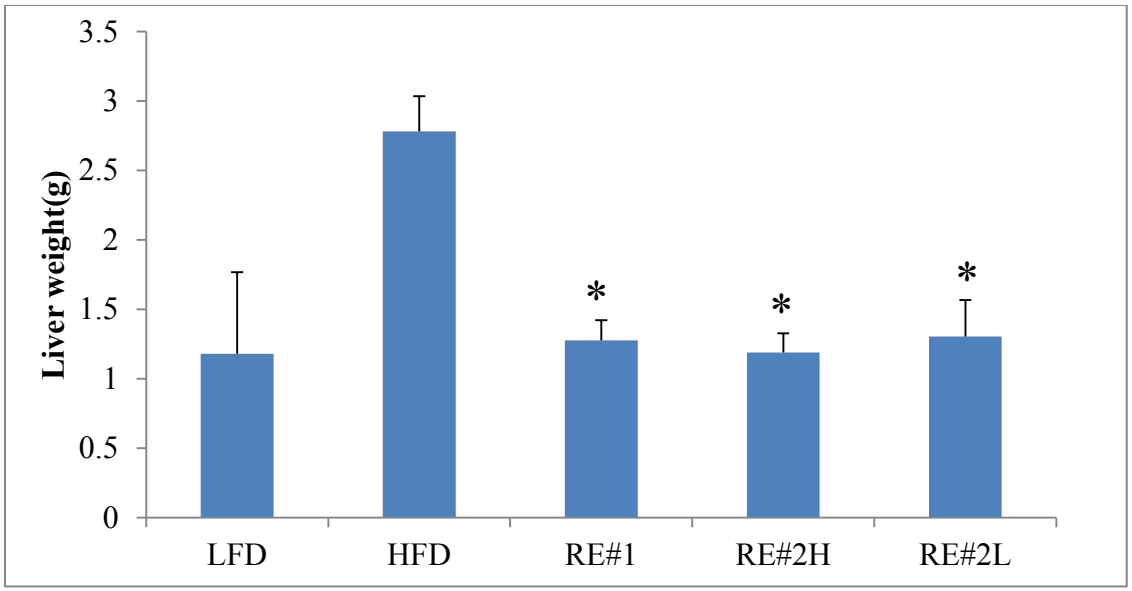


Figure 13. Liver weight in low fat diet (LFD), high fat diet (HFD), 0.5% RE with 45% CA (RE#1), 0.28% RE with 80% CA (RE#2H), and 0.14% RE with 80% CA (RE#2L) groups after the 16-week study. Values are mean ± SD, * = $P<0.05$, n= 6.

Liver triglyceride level in LFD compare to HFD is 85% less; and in treatment groups (RE#1, RE#2H and RE#2L) are 92, 93 and 78% less than that in HFD, respectively (Fig. 14). Hepatic cholesterol increased significantly in LFD and all treatment groups compare to HFD group ($P<0.05$) (Fig. 15). .Free fatty acid in liver level in LFD compare to HFD is 77% less, in treatment groups, (RE#1, RE#2H and RE#2L) are 83, 82 and 73% less than HFD, respectively (Fig. 16).

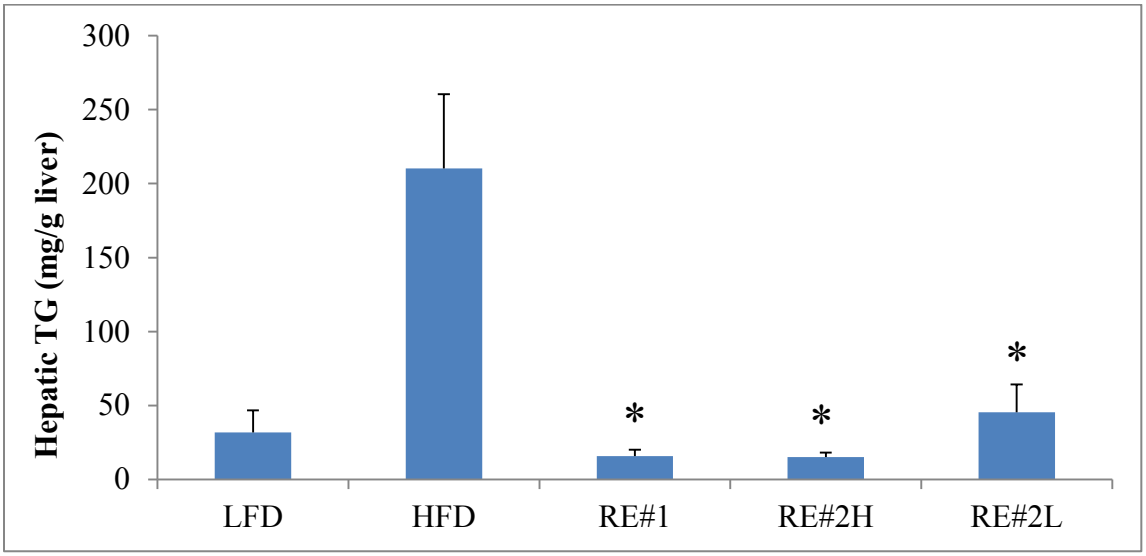


Figure 14. Hepatic triglyceride levels in low fat diet (LFD), high fat diet (HFD), 0.5% RE with 45% CA (RE#1), 0.28% RE with 80% CA (RE#2H), and 0.14% RE with 80% CA (RE#2L) treated mice after 16-week study. Values are mean ± SD, * = P<0.05, n= 6.

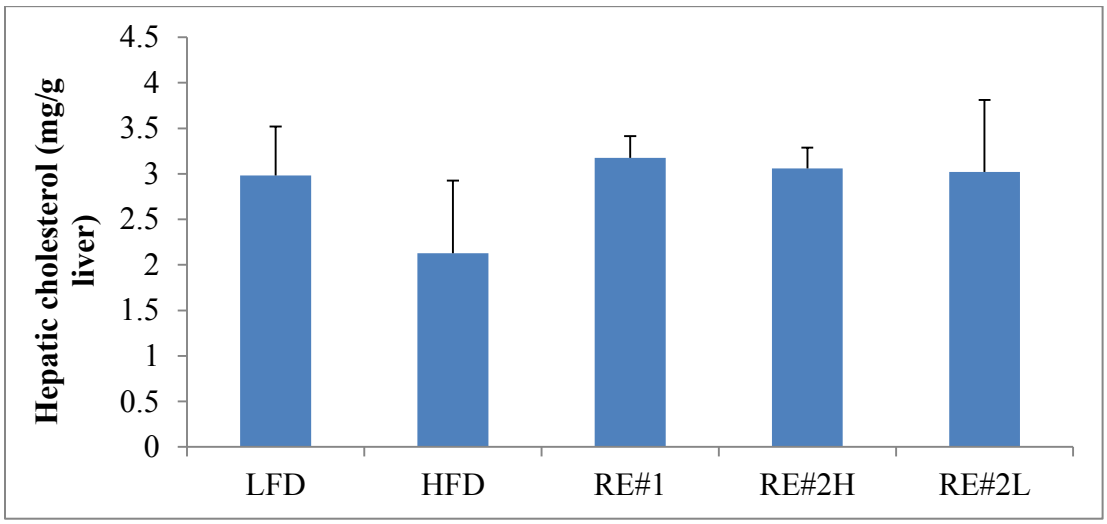


Figure 15. Hepatic cholesterol levels in low fat diet (LFD), high fat diet (HFD), 0.5% RE with 45% CA (RE#1), 0.28% RE with 80% CA (RE#2H), and 0.14% RE with 80% CA (RE#2L) treated mice after 16-week study. Values are mean ± SD, n= 6.

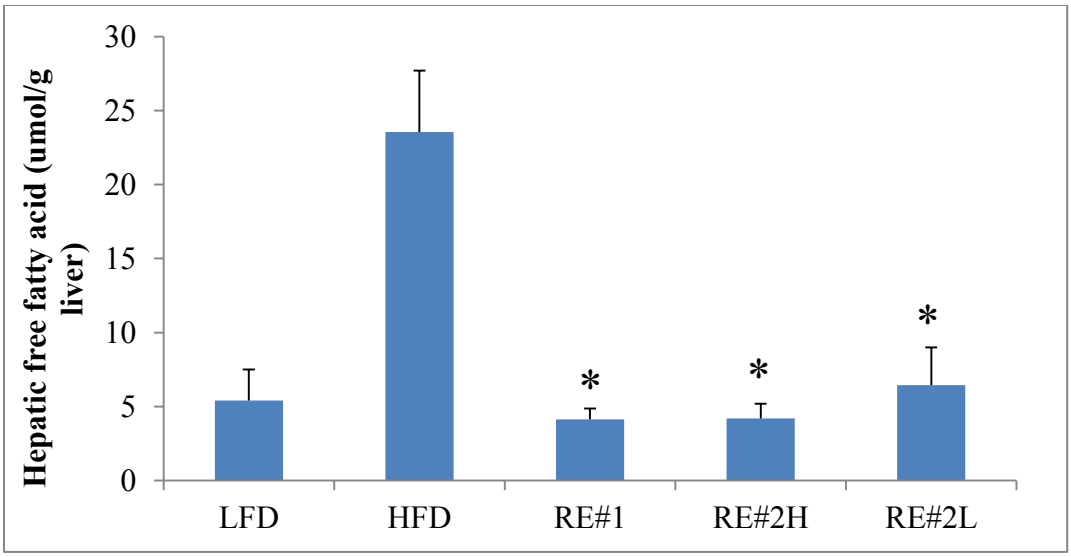


Figure 16. Hepatic free fatty acid levels in low fat diet (LFD), high fat diet (HFD), 0.5% RE with 45% CA (RE#1), 0.28% RE with 80% CA (RE#2H), and 0.14% RE with 80% CA (RE#2L) treated mice after 16-week study. Values are mean ± SD,* = $P<0.05$, n= 6.

In addition, plasma ALT levels in LFD, RE#1, RE#2H, RE#2L treated mice were significantly different compare to the HFD group ($P<0.001$). In LFD group, plasma ALT level compare to HFD is 74% less and in treatment groups, (RE#1, RE#2H and RE#2L) are 74, 71 and 76% less than HFD, respectively. There are no significance differences between LFD and the treated groups (Fig. 17A).

Plasma AST levels in LFD, RE#1, RE#2H, RE#2L animals were significantly different compare to the HFD group ($P<0.05$). AST level in LFD compare to HFD is 40% less, in treatment group, (RE#1, RE#2H and RE#2L) are 47, 49 and 39% less than HFD, respectively. There are no significance differences between LFD and the treated groups (Fig. 17B).

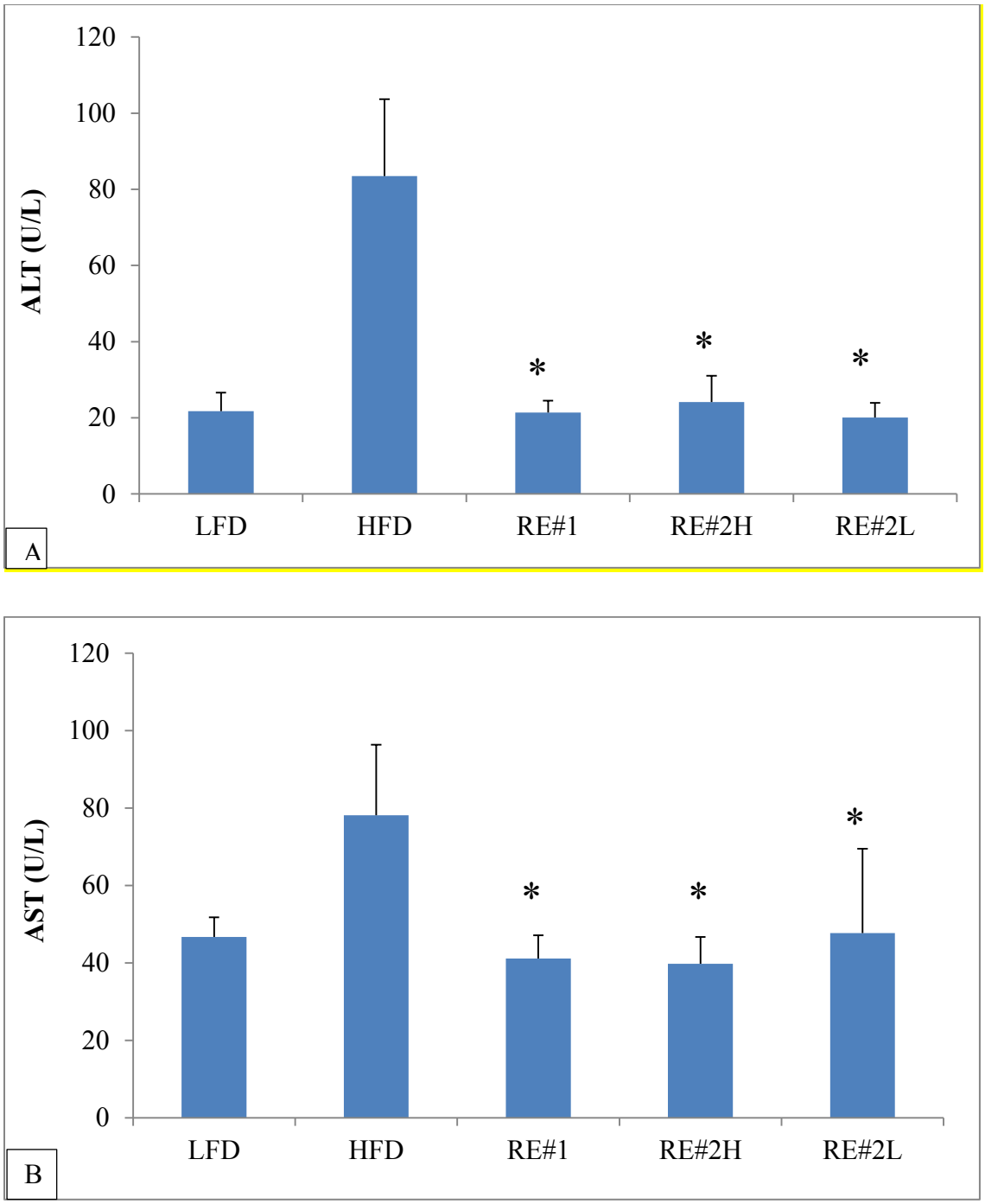


Figure 17. Plasma ALT (A) and AST (B) levels in low fat diet (LFD), high fat diet (HFD), 0.5% RE with 45% CA (RE#1), 0.28% RE with 80% CA (RE#2H), and 0.14% RE with 80% CA (RE#2L) groups. Values are mean ± SD, * = $P<0.001$, n= 6.

4.2.4 Effect of RE on fecal fat excretion. Animals in HFD group had higher fecal fat excretion values (2.58±0.41 mg/100 mg) compared with those in LFD group (1.39 ± 0.17 mg/100 mg, P<0.05). Lipid excretion among HFD and animals with three RE treatment groups were significantly different, RE#1 (5.06 ± 0.82 mg/100 mg, P<0.05), RE#2H (4.09 ± 0.27 mg/100 mg, P<0.05), RE#2 L (3.94 ± 0.49 mg/100 mg, P<0.05) in the last week of the experiment (Fig. 18).

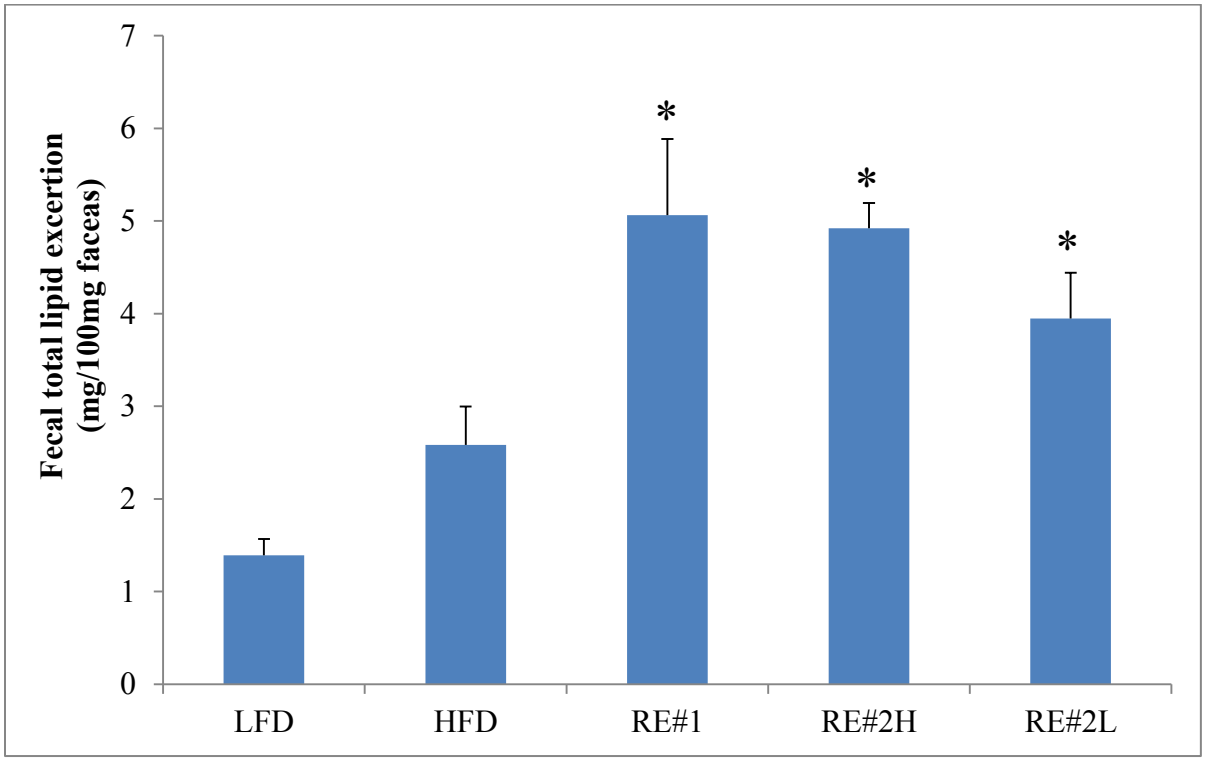


Figure 18. Effects of RE on lipid excretion in low fat diet (LFD), high fat diet (HFD), 0.5% RE with 45% CA (RE#1), 0.28% RE with 80% CA (RE#2H), and 0.14% RE with 80% CA (RE#2L) groups. Results are presented as means ± S.D, * = p<0.05, n=3.

CHAPTER 5

Discussion and Future Research

In this doctoral thesis, we developed column chromatography techniques to prepare RE with 80% CA from commercial available RE (with 45% CA). Moreover, their capacity to prevent metabolic disorders, such as obesity, hyperlipidemia, and hyperglycemia in different dosage was evaluated as well as their limit fat absorption mechanism.

Rosemary is one of the most widely consumed spices worldwide. From its origin in Southeast Asia and its spread to Europe, rosemary is a well-known and greatly valued medicinal herb that is widely used in pharmaceutical products and folk medicine as a digestive, tonic, diuretic. More recently, it was reported that rosemary also possessed anticancer, antioxidant, antibacterial, and anti-inflammatory activities (Aljamal, 2012; Cheung, Hasman, Tai, & Wu, 2012). However, there is less emphasis on the major responsible compound, effective dosage and underlying mechanisms of rosemary in management of metabolic diseases and their complications. Thus, it can be difficult to conclude the health benefits of a major component in RE to another exclusively on the basis of published data. This study has developed a new method to prepare CA and its enriched RE (80%), and evaluated their anti-obesity and anti-diabetic effects with different doses.

5.1 Experiment 1- Preparation of CA and Its Enriched RE

In this experiment, we prepared the CA enriched RE (80%) to identify the impact of CA and the dose effect of CA on weight gain, blood glucose and lipid hemostasis. There has been emergent interest in the employment of CA in food industry, for its usefulness as preservatives, as well as for its beneficial effects on human health (Song et al., 2014). CA, like any molecule of the catechol (or-tho-diphenol) type, is a reactive compound. It is highly sensitive to oxidation

and, hence, to all the operations typically carried out to isolate natural substances (extraction, liquid- liquid separation, chromatographic fraction, etc.) (Khalafi, Rafiee, Shahbak, & Shirmohammadi, 2013). This characterization of CA during its purification makes it an expensive component (85$/10 mg from Sigma). The problem addressed by the present experiment is to provide a process for enriching RE with 80% CA which would be economical and practical for the current study.

5.2 Experiment 2- Study of the Effect of RE Enriched With CA on Body Weight Gain and Development of Metabolic Syndrome in HFD Treated C57BL/6J Mice

5.2.1 Long term HFD induces obesity and related metabolic syndrome in the C57BL/6J mice. In animal studies which demonstrate metabolic syndromes to explore antiobesity and antidiabetes treatments, the choice of the animal model, diet, and experiment's duration are critical points that need to be considered. In the present study we investigated the effects of the daily consumption of a HFD (60 Kcal % fat) on the development of obesity and related metabolic syndrome in C57BL/6J mice for 16-week. The results demonstrated that the HFD caused remarkably increased weight gain, fat accumulation, hyperglycemia, insulin resistance, and increased liver fat in our animal model. In contrast to the most previous similar studies dietary induced metabolic disorders models, the C57BL/6J mice phenotype becomes visible within 3 weeks of starting the HFD.

5.2.2 CA is the major active component in RE. Our data indicate treatment of mice with 0.5 % w/w RE with 45% CA (~224 mg/Kg BW/day) reduced the gains in weight that were induced by the HFD, which are only 17.8 and 13.1% more than weight gain in LFD group. It also significantly lowered total fat tissue weight, hepatic TG, liver weight, fasting plasma insulin, fasting blood glucose compared with HFD mice. In addition, total fecal lipid content increased

significantly in RE#1 mice compared with the HFD group. Based on the present study, there was not any significant difference between the results from RE#1 and RE#2H (~225 mg/Kg BW/day). Since the quantities of CA contents were equal in these two groups, it appears that the CA plays an important role against in the metabolic disorders and is the major active compound in RE. Although various biological activities with preventive nutritional intervention against metabolic disorders has been developed for RE; nevertheless, the chemical description of applied extracts in studies, is not always available. Thus, it can be difficult to conclude the health benefits of a unique compound in RE on the basis of published data. Few studies have been conducted on CA as an effective compound in rosemary (Ninomiya et al., 2004; T. Wang et al., 2011). Ninomiya et al. have observed that after 2-week of treatment with 20 mpk of CA alone, the weight of ddY (Deutschland,Derker, Yoker) mice fell by 7.6% compared with the control group (HFD 40% w/w fat) and the similar results demonstrated by Wang et al., whereas no results for fasting blood glucose effect has been observed in both studies. Our results together with the published data on CA in different animal models indicate that CA is the major active component in RE for obesity and related metabolic syndrome.

5.2.3 Dose dependent effects of CA enriched RE. The results presented in Chapters 4 show that RE with 80% CA could dose dependently reduce HFD induced obesity and metabolic syndrome by decreasing weight gain, fat mass accumulation, insulin resistance, and hypercholesterolemia. Furthermore, total fecal lipid content increased in groups with RE with 80% CA dose dependently, compared with the HFD group. Based on the present study and other evidences (Harach et al., 2010; Ibarra et al., 2011), limiting lipid absorption in the intestine is a possible mechanism by which CA prevents weight gain.

In our study, for the first time, we demonstrated the dose-dependent effect of CA enriched RE on obesity and related metabolic syndrome in the HFD treated mouse model. At week 3, the RE treated mice began to experience significant reductions in weight gain. The mice in both RE#1 and RE#2H groups, which contained the same amount of CA (0.22% of diet w/w), showed significantly less weight gain than those in the RE#2L group (0.11% of diet w/w) after 14-week treatment. These results are consistent with findings from Ibarra and Harach (Harach et al., 2010; Ibarra et al., 2011). In addition to its effects on weight gain, our results also demonstrated that CA significantly reduced elevated cholesterol levels that were induced by the HFD. Furthermore, they found no difference in serum TG and insulin level between control and treatment group; when our results demonstrated the reduction in liver TG, serum cholesterol, and FFA in compare to HFD. These findings demonstrate that CA, due specifically to fat mass reduction, has anti-obesity effects *in vivo*. Moreover, in the present study, fasting glycaemia and plasma insulin level were reduced in animals in the treatment groups dose dependently compared with the HFD control group. There are limited studies that have evaluated the effect of rosemary on diabetes. Anti-hyperglycaemic effects can be induced by an ethanolic RE in the alloxan diabetic rat model (Bakirel et al., 2008), however, there was not any information about RE compound.

5.2.4 Toxicity. In the present study, chronic consumption of the high and low doses (28% and 14% of diet w/w) of CA enriched RE for 16-week did not show any toxicity to the treated mice. In contrast, CA significantly reduced the ALT and AST levels that induced by the high-fat diet which have not been observed in other studies on CA.

5.2.5 Mechanism of RE enriched with CA on obesity and metabolic syndrome. There are many mechanisms that have been suggested by previous studies for RE against obesity and

metabolic syndrome. Some of these studies have been accomplished *in vitro* such as pancreatic lipase inhibition (Ninomiya et al., 2004), peroxisome proliferators-activated receptor gamma (PPARγ) activation, and the differentiation of mouse pre-adipocytes into adipocytes prevention (Takahashi et al., 2009). Some *in vivo* studies have investigated the ability of rosemary to control weight gain and glycaemia (Harach et al., 2010; H. Wang et al., 2011). Vaquero et al. in 2012 demonstrated RE (40% CA) significantly inhibited gastric lipase activity in the stomach, which may cause a moderate reduction of fat absorption (M. Romo Vaquero et al., 2012). Several studies have found that limit fat absorption is the key mechanism for RE to limit weight gain (Harach et al., 2010; Ibarra et al., 2011; Ninomiya et al., 2004). The present study further confirms this mechanism by observing the increase of fecal fat excretion in RE treated mice.

5.2.6 Future research. The present study and literature studies found that RE could prevent weight gain and metabolic syndrome induced by HFD treatment. However, it is unclear whether RE has treatment effect to obese mice. In addition, it is also worthwhile to study the detail mechanisms that RE limits fat absorption. Furthermore, whether RE has antiobesity effect in human is another topic of future research.

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