EFFECTS OF GALACTOMANNAN AND DIOSGENIN ON BIOMARKERS OF OBESITY AND EXPRESSION OF GENES RELATED TO LIPID METABOLISM

THESIS SUBMITTED TO THE
ICAR-NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
(DEEMED UNIVERSITY)
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

ANIMAL BIOCHEMISTRY

BY

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M.Sc. (Animal Biochemistry)

ANIMAL BIOCHEMISTRY DIVISION
ICAR-NATIONAL DAIRY RESEARCH INSTITUTE
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KARNAL-132001 (HARYANA), INDIA

2017

Regn. No. 1051208

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to

Almighty

&

Beloved Family

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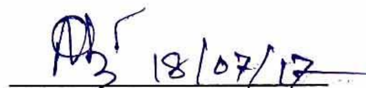

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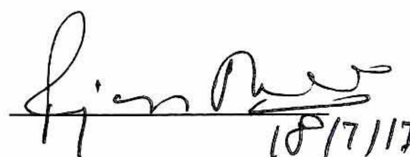

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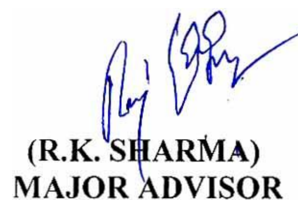
This is to certify that the thesis entitled **"EFFECTS OF GALACTOMANNAN AND DIOSGENIN ON BIOMARKERS OF OBESITY AND EXPRESSION OF GENES RELATED TO LIPID METABOLISM"** submitted by **M. ASWANI** in partial fulfillment of the requirement for award of the degree of **DOCTOR OF PHILOSOPHY** in **ANIMAL BIOCHEMISTRY** of the ICAR-National Dairy Research Institute (Deemed University), Karnal (Haryana) is a bonafide research work carried out by her under my supervision and guidance and no part of the thesis has been submitted for any other degree or diploma**.**



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Abbreviations:

°C	degree Celsius
@	at the rate of
ANOVA	Analysis of Variance
BAT	Brown Adipose Tissue
BMI	Body Mass Index
bp	base pair
cDNA	complementary DNA
CL	Cellulose
CLA	Conjugated Linoleic Acid
cpt1	Carnitine Palmitoyl Transferase 1
Ctrl	Control
DEPC	Diethyl pyrocarbonate
DIO	Diet Induced Obesity
DPP-IV	Dipeptidyl peptidase-IV
E. fat	Epididymal fat
FFAs	Free Fatty Acids
fiaf	Fasting Induced Adipose Factor
FOS	Fructooligosaccharide
foxa2	Forkhead box protein A2
g	gravitational force
g	gram
GG	Guar gum
GIT	Gastro intestinal tract
GLP-1	Glucagon Like Peptide-1
GLUT	Glucose transporter
h	hour
HDL	High Density Lipoprotein
HF	High Fat
HFD	High Fat Diet
HFD-FGM	High Fat Diet + Fenugreek galactomannan
HFD-LGM	High Fat Diet + Locust bean galactomannan
HFD-D	High Fat Diet + Diosgenin
HFD-FGMD	High Fat Diet + Fenugreek galactomannan + Diosgenin
HFD-LGMD	High Fat Diet + Locust bean galactomannan + Diosgenin
HOMA-IR	Homeostasis Model Assessment of Insulin Resistance
HFS	High fat high sucrose diet
HPMC	Hydroxypropyl methylcellulose
GM	Galactomannan

IL	Interleukin
ITF	Inulin Type Fructans
kg	Kilogram
LDL	Low Density Lipoprotein
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
mg/dl	milligram/deciliter
min	Minute
ml	milliliter
mM	Millimolar
ng	Nanogram
nm	Nanometer
mU	milliunits
NTPs	Nucleoside triphosphates
OFS	Oligofructose
OGTT	Oral Glucose Tolerance Test
PCR	Polymerase Chain Reaction
Pgc1β	Peroxisome proliferator-activated receptor Gamma Coactivator 1β
PPAR	Peroxisome Proliferative Activated Receptor
PUFA	Polyunsaturated fatty acid
qRT-PCR	quantitative Real Time PCR
RPM	Revolution Per Minute
SCFAs	Short Chain Fatty Acids
SEM	Standard Error of Mean
T2D	Type 2 diabetes
Taq	*Thermus aquaticus*
TC	Total cholesterol
TG	Triglycerides
ME	Metabolizable energy
TZD	Thiazolidinedione
UCP	Uncoupling protein
v/v	volume/volume
VLDL	Very Low Density Lipoprotein
w/v	weight/volume
w/w	weight/weight
WAT	White Adipose Tissue
WHO	World Health Organization
wk	Week
TNF	Tumor Necrosis Factor

MCP	Monocyte chemoattractant protein
GPCR	G-Protein coupled receptors
SREBP	Sterol Regulatory Element Binding Proteins
ZO	Zonula Occludens
CPT	Carnitine palmitoyltransferase
AI	Atherogenic index
foxa2	Forkhead box protein A2
Pgc1β	Peroxisome proliferator-activated receptor Gamma Coactivator 1β
HK	Hexokinase
GK	Glucokinase
PFK	Phosphofructokinase
PK	Pyruvatekinase
FBPase	Fructose 1,6-bisphosphatase
PEPCK	Phosphoenolpyruvate carboxykinase
NADP	Nicotinamide adenine dinucleotide phosphate

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Abstract

Effects of galactomannan and diosgenin on biomarkers of obesity and expression of genes related to lipid metabolism

Dietary interventions involving different types of fibers and phytosterols have gained much interest in management of obesity and related disorders. The present study was conducted to examine effects of two types of functional fibers viz., fenugreek galactomannan (FGM) & locust bean galactomannan (LGM), and a sapogenin (diosgenin), alone or in combination on biomarkers of obesity and expression of genes related to lipid metabolism in C57BL/6 mice fed high fat diet. Body weight and E.fat were significantly lower in galactomannan/diosgenin fed groups as compared to animals fed high fat diet (HFD) only. Mean adipocyte size indicated anti-adiposity effects of functional ingredients. Fasting blood glucose levels appeared to be normalized by FGM/LGM/diosgenin. Both types of galactomannans were equally effective in resisting hyperinsulinemia & improvement in HOMA-IR, and the effect was better than diosgenin. FGM/LGM were more effective than diosgenin in normalizing serum TG, however, no differences were observed in TC level. Whereas increase in hepatic TG was resisted to the same extent by FGM/LGM/diosgenin, significant effect on TC was with diosgenin alone and in combination with fiber. Activities of key enzymes in liver (regulating glycolysis and gluconeogenesis) showed a trend towards decrease in activities of enzymes related to glycolysis and increased activities of gluconeogenesis enzymes due to HFD. Significantly higher pyruvate kinase activities were measured in FGM/LGM fed group compared to HFD group. PEPCK activity revealed positive effects of all dietary treatments. mRNA expression of adiponectin, leptin, GLUT-4, PPARγ & GPCR43 (E. fat); PPARα & CPT-1 (liver); FIAF (small intestine) evinced anti-adiposity effects of FGM/LGM > diosgenin. MCP-1 & TNFα expression in adipose tissue also showed protective effects of functional components. ZO-1 and occludin indicated positive effects on intestinal integrity. Findings of present investigation suggest that dietary incorporation of galactomannans and diosgenin exhibit anti-obesity effects. Synergistic effects of co-administration were indicated by expression of certain genes.

गलाक्टोमंनन और डिओसजेनिन का मोटापे और लिपिड चयापचय बिओमर्कर
जिनो की अभिव्यक्ति परप्रभाव

सारांश

विभिन्न प्रकार के फाइबर और फ़यटोस्टरोल ने शामिल आहार उपायों मोटापा और संबंधित विकारों के प्रबंधन में ज्यादा दिलचस्पी अर्जित किया है। वर्तमान अध्यन कार्यात्मक फाइबर के दो प्रकार के प्रभाव की जाँच करने के लिए आयोजित की गयी थी अर्थातमेथी(गलाक्टोमंनन) FGM और टिड्डी सेम गलाक्टोमंनन और इक सपोजेनिन (डिओसजेनिन), अकेले या संयोजन का मोटापेके बिओमर्कर और लिपिड चयापचय से संबंधित जीनो की अभिव्यक्ति पर उच्च वसा आहार खिलाए गए चूहों मै| शरीर का वजन और E.fat (HFD) गलाक्टोमंनन/इक सपोजेनिन (डिओसजेनिन) फीड समूह में HFD की तुलना में काफी कम था| वसा कोशिका आकार का वसा विरोधी कार्यात्मक अवयवों प्रभावकारिता की तरफ संकेत देती है| उपवास रक्त शकरा स्तर FGM/LGM/डिओसजेनिन से सामान्यीकृत दिखाई दिया| दोनों प्रकार के गलाक्टोमंनन हाइपरइन्सुलिनियामा और होमा आई आर स्कोर की वृद्धि का समान रूप से विरोध करने में प्रभावि रहे और प्रभाव डिओसजेनिन से बेहतर था। FGM/LGM सीरम तीजी स्तरों को सामान्य करनेमें डिओसजेनिन की तुलना में अधिक प्रभावी प्रतीत होता है| हालाँकि, सीरमटीसी पर कोई प्रभाव नहीं दिखाई दिया| जबकि यकृत तीजी में वृद्धि का समान रूप से FGM/LGMडिओसजेनिन ने विरोध किया, यकृत टीसी का डिओसजेनिन नेअकेले या किसी भी कार्यात्मक फाइबर के संयोजन के साथ काफी हद तक विरोध किया| जिगर के महत्वपूर्ण एन्जिएमो (ग्लैकोल्यसिस और ग्लुकोनेयोजेनेसिस) के माध्यम से प्रवाहका नियंत्रण की गतिविधियों की परख ग्लैकोल्यसिस से संबंधित और वृद्धि की उच्च वसा वाले आहार खिलने के परिणाम के रूप में ग्लुकोनेयोजेनिसिस अन्ज़िमो की गतिविधियों की कमी की दिशा में एक प्रवर्तित देखी गयी | HFD जानवरों की तुलना में FGM/LGM खिलाए गए समूहों में उच्च प्यूवत काइनेज गतिविधियां मापी गयी| PEPCK की गतिविधि पे सभी आहार उपचार का सकरात्मक प्रभाव का पता चलता है| इ वसा ऊतक मे अदिपोनेक्टिन, GLUT-4, PPARϒ और GPCR43(E.fat), जिगर मे Ppara और CPT-1 आंत मे FIAF मे mRNA अभिव्यक्ति FGM\LGM>डिओसजेनिन मे विरोधी वसा को अधिकपर्दर्शन किया| वसा ऊतको (MCP और TNF-α) की अभिव्यक्ति पर सुरक्षात्मक परभाव दिखाया| ZO-1 और Occludin कि अभिव्यक्ति आंतो की अखंडता पर सकारात्मक प्रभाव का संकेत दिया| वर्तमान जाच के निष्कर्ष बताते है कि गलाक्टोमंनन और डिओसजेनिन के आहार समावेश मोटापा विरोधी प्रभाव को दर्शाता है| स्य्नेर्गिस्टिक सह प्रशासन प्रभाव विशेष जीनो की अभिव्यक्ति के साथ दिखाई दे रहे ह|

CHAPTER - 1

Introduction

1. INTRODUCTION

Obesity is a crucial problem of public health in both developed and developing countries, associated with a cluster of metabolic disorders such as insulin resistance, type 2 diabetes and fatty liver disease (Delzenne et al., 2013). It develops when energy intake exceeds energy expenditure, resulting in abnormal or excessive fat accumulation that may impair health. Environmental factors such as increased availability of high calorie food or decreased physical activity contribute to its development and their influence is amplified by genetic predisposition. In addition, overfeeding is often associated with inadequate nutrition, leading namely to a low intake of n-3 PUFA and of dietary fibers. In United States 68.5% of adults are overweight (BMI >25 kg/m^2), 34.9 % are obese (BMI >30 kg/m^2) (Food Research and Action Centre., 2012). India, with 41 million obese people, ranks third after the US and China in having the highest number of overweight people (The Economic Times, 6 June 2014). A variety of programs and treatments including drug therapeutics, surgical intervention and dietary control for obesity management or prevention have been developed; however, these are often associated with safety issues. Therefore, the development of a safe and effective dietary supplement to assist in body weight management is essential. In that respect, some dietary habits related to an increase in bioactive food components present in whole grain cereals could be helpful in prevention of chronic diseases.

Dietary fiber simply is a carbohydrate fraction present in plant foods (cereals, fruits, vegetables and pulses) and not degraded by digestive enzymes into absorbable units in stomach and small intestine. Depending on solubility, dietary fibers are of two types: 1. Soluble fibers or fermentable fibers which are soluble in water and also fermented by the gut microflora, produce short chain fatty acids (SCFA), 2. Insoluble fibers, which are not soluble in water and not fermentable (cellulose, hemicellulose, lignin). Soluble fiber has gel-forming capacity, which gives volume to feces (inulin, pectins, galactomannans and fructo-oligosaccharides). The benefits of consuming foods rich in fiber are numerous, ranging from improved large bowel function to slowed digestion and absorption of carbohydrate and fat and a reduced risk of diseases (Ali et al., 1982; Schneeman and Tietyen., 1994). As summarized by Howarth et al., (2001) increased dietary fiber intakes have been associated with a body weight loss of 1.9 kg over 3.8 months, with greater weight loss in more obese subjects. A thirty gm of fenugreek

dietary fiber a day and appropriate physical activity, gradual and significant weight-loss can be easily achieved without adverse protein-calorie malnutrition and other ill effects of dieting (Chanda., 1985).

There are many plants which have long history of medical uses in Ayurvedic and Chinese medicine. The seed (leguminosae) spices influence various systems in the body such as gastrointestinal, cardiovascular, reproductive and nervous systems resulting in diverse metabolic and physiologic actions. Seed spices have a diverse array of natural phytochemicals that have complementary and overlapping actions, including antioxidant effects, anticancer, antidiabetic, antimicrobial activity, hypolipidemic effect, helping in digestion, hypertension, modulation of detoxification enzymes, stimulation of immune system, reduction of inflammation, modulation of steroid metabolism (Srinivasan et al., 2006). The seeds of leguminosae contain mainly polysaccharides or gums which can be used as dietary fiber, texture modifiers, gelling agents, thickeners, emulsifiers, stabilizers, coating agents and packaging films. In dairy, galactomannan is used as thickener for ice cream and milk desserts; stabilizer in sorbets, ice cream; in processed cheese moisture retention and flavor enhancement; fat substitutes, in diabetic products - Coffee whiteners, baby milk formulations and in beverages (Garti et al., 1997; Taylor & Francis., 2006).

Galactomannans are energy reserve polysaccharides of all leguminous plant seeds (Reid., 1985), mainly in fenugreek, guar gum, locust bean gum, tara gum etc. These are integral components of cell wall of the endosperm and the structure composed of linear β-(l→4)-mannan backbone attached to single D-galactopyranosyl residues via α-(l→6) linkages. Galactomannans from different sources differ in their mannose to galactose ratio (M :G) (Mathur and Mathur., 2005). The M:G ratio in galactomannans determines the functional properties of the gum. A gum with higher percentage of galactose has higher solubility, dispersability and viscosity (Whister & BeMiller., 1993).

Galactomannans from the seeds of fast-growing guar (guar gum), carob or locust bean (locust bean gum) (Patmore *et al*, 2003), Tara gum and fenugreek (methi or *Trigonella foenum-graceum*) (Garti et al., 1997) are used widely in the food industry. The broad range of applications reflects a number of different functional characteristics including high solution viscosity (guar), stabilization of frozen systems (guar and locust bean gum), and mixed gel formation with other polysaccharides and proteins (locust bean gum). The selection of a gum is

based on the desired function and food application. The gums of fenugreek, locust bean, guar and tara gum also have potential health benefits. They are hypoglycemic (Mowla et al., 2009), hypocholesterolemic, antidiabetic, hypolipidemic (Vijayakumar et al., 2010). Diosgenin, a steroidal saponin (phytosterol) found in many plants such as fenugreek, *Costus speciosus*, and different *Dioscorea* species has been reported to exhibit many biological functions like maintenance of blood cholesterol levels, act as a precursor giving rise to few of the steroidal hormones like dehydroepiandrosterone and activate neurotrophic molecules like nerve growth factor (NGF) (Uemura et al., 2011). Further, it has been reported to be very effective against gastrointestinal disorders and inflammation (Yamada et al., 2009).

The literature available indicates that the whole seed or aqueous or ethanolic or methanolic extracts preparation of fenugreek seed have antidaibetic, antihyperglycemic, hypolipidemic hypocholestemic, insulinotrophic effects. These extracts contain carbohydrates (galactomannan) and also other components like steroids (saponin), proteins, alkaloids. Evans et al. (1992) found that rat fed high cholesterol diet containing gums of fenugreek, LBG and guar gum lowered the cholesterol concentration in both liver and blood plasma. It is of great significance to examine how fibers with potential prebiotic properties are interacting with host metabolism in the context of obesity. Prebiotics are mostly soluble dietary fibers, which are good for health. A few reports are available on hypoglycemic and hypolipidemic effects of steroidal sapogenin extract from Jamaican bitter yam (*Dioscorea polygonoides*) or commercial diosgenin.

Soluble fibers can act through different mechanisms. This may be due to differences in viscosity, gel formation, delay in gastric emptying, effect on gut microbiota or fermentation pattern affecting host metabolism. Galactomannans from different sources may give different physiological effects. Very little work is available on locust bean gum so it was thought to be exciting to compare two types of galactomannans from different sources (differing in M:G ratio). Diosgenin, being a phytosterol, is also expected to work through a different mechanism. Therefore, it was also considered to examine the effects of pure form of diosgenin alone and on co-administration with galactomannan. Obesity prone C57BL/6 mice model was considered where high fat/calorie rich diet was fed which is the cause of alterations in metabolism and metabolic disorders

Therefore, the present study was planned with the following objectives:

1. To study the effects of galactomannan and diosgenin on progression of obesity in high fat diet fed mice.
2. Analysis of expression pattern of genes related to lipid metabolism as affected by galactomannan and diosgenin feeding.

CHAPTER - 2

Review of Literature

2. REVIEW OF LITERATURE

The growing epidemic of obesity is threatening the health of millions of people around the world. Obesity is defined as abnormal or excess fat accumulation in the body due to an imbalance between energy intake and expenditure. Obesity is largely viewed as a lifestyle-disease with two primary causes: excessive energy intake and insufficient physical activity. Today, it is considered to be a major problem in the developed as well as developing countries. Western type diets high in fat content as well as refined cereals and sugars, combined with an increasingly sedentary lifestyle, are the causes of health threats in the form of increasing obesity and associated metabolic disorders, including insulin resistance (IR), nonalcoholic fatty liver disease (NAFLD), type 2 diabetes mellitus (T2DM), and cardiovascular disease(CVD).

2.1 OBESITY

Body mass index (BMI) is a simple index of weight-for-height that is commonly used to classify overweight and obesity in adults. It is defined as a person's weight in kilograms divided by the square of his/her height in meters (kg/m^2). BMI greater than or equal to 25 is considered as overweight while greater or equal to 30 is considered as obese. BMI provides the most useful population-level measure of overweight and obesity as it is the same for both sexes and for all ages of adults.

Although obesity stems from the interactions of genetic and environmental factors, its root cause is an increased consumption of more energy-dense, nutrient-poor foods containing high levels of sugar and saturated fats in combination with reduced physical activity over a relatively long period of time. In addition, overfeeding is often associated with inadequate nutrition, leading to a low intake of n-3 PUFA and dietary fibers. Plenty of literatures have been generated worldwide to understand the intricate mechanisms of human/rodents metabolism in general, and obesity & its associated disorders e.g. insulin resistance, type 2 diabetes mellitus etc. in particular. The casual role of obesity in type 2 diabetes (T2D) is highlighted by the fact that preventive weight reduction is associated with a decreased incidence of diabetes (Tuomilehto et al., 2001; Taylor, 2008). Moreover, weight loss following bariatric surgery in morbidly obese patients with T2Dleads to a rapid reversal of the pathophysiology and subsequent resolution of diabetes mellitus (Taylor, 2008). It is now widely accepted that obesity

is associated with low grade inflammation and that inflammation contributes to the risk of insulin resistance and type 2 diabetes as well as other detrimental health consequences linked to obesity (Lee et al., 2009; Greenbergand Obin, 2006). Circulating and adipose derived cytokines such as tumor necrosis factor α (TNFα) or interleukins 6 (IL6) have been shown to be elevated in obese humans (Kern et al., 2001; Syrenicz et al., 2006) and this can be reversed with weight loss (Dandona et al., 1998). Multiple studies in animal models have demonstrated that diet-induced obesity (DIO) is associated with increased expression of a number of proinflammatory cytokines or biomarkers of inflammation in adipose tissue (Xu et al., 2003; Brake et al., 2006). But the mechanisms underlying obesity associated inflammation are not fully understood. Brake et al. (2006) reported that HFD induces expression of adhesion molecules in adipose tissue which are associated with leukocyte migration and adherence. A number of studies support the concept that inflammation may derive from accumulation of activated macrophages within adipose tissue in obese animals or humans (Weisberg et al., 2003; Xu et al., 2003). Gastrointestinal tract is now being considered potential source of inflammation associated with diet or obesity (Ding et al., 2010).

2.1.1 Treatment for obesity

The startling rise in the number of obese people, together with the inability of most individuals to comply with treatment regimes that require sustained lifestyle changes, has stimulated efforts to identify new therapeutic agents for the treatment and prevention of this pervasive disorder. Diet and lifestyle changes remain the cornerstones of therapy for obesity, but the resultant weight loss and long-term success is extremely uncommon and disappointing. Anti obesity agents can be useful to some patients in achieving and maintaining meaningful weight loss, yet these agents are of limited effectiveness in the face of the magnitude of the problem (Zanella et al., 2009). At present, only two drugs, orlistat and sibutramine, are approved for long term use in the treatment of obesity but each of these typically promotes 5% to 10% of loss of body weight (Padwal et al., 2007; Rucker et al., 2007). Rimonabant, a cannabinoid-1 receptor antagonist, was withdrawn from market, although very effective in promoting meaningful weight loss, reduction in waist circumference and improvement in several metabolic risk factors, because of concerns about its safety, including risk of suicidal and seizures (Zanella et al., 2009). Considering the lack of successful weight-loss treatments and public-health implications of the obesity pandemic state, the development of safe and effective agents (dietary fiber/phytosterols)

which results in significant weight loss either by a reduction in food intake or by increasing energy expenditure should be a priority.

Bioactive functional components:

Functional foods or functional food ingredients exert beneficial effects on host health and/or reduce the risk of chronic diseases due to certain physiologically active components, beyond their nutritive value. According to this definition, functional foods must demonstrate their effects at levels that can be expected to be consumed in the diet. A food can be made functional by addition of a potential health promoting entity, reducing or removing concentrations of harmful components, and/or modifying the nature or the bioavailability of one or more components. The first generation of functional food was based on deliberate supplementation with minerals (mainly calcium) and vitamins. However, the concept has recently moved towards food additives exerting a positive effect on the gut microbiota, introducing pro- and prebiotics. Dietary fibers and phytochemicals also have health benefits. Fiber plays an essential role in digestive, heart, and skin health, and may improve blood sugar control, weight management. Phytosterols are the chemical substances, increase fecal fat excretion by decreasing lipase activity and involve in body weight management.

Cani et al. (2007c) found that feeding of oligofructose (OFS) to Wistar rats for 4 weeks with normal diet reduced body weight gain, food and energy intake significantly and this was associated with lower epididymal, inguinal and visceral adipose tissue weight. Markers of fermentation i.e. caecum enlargement, caecum weight and caecal tissue weight were higher in OFS fed group. Portal GLP-1 content, proglucagon expression and number of GLP-1 positive L-cells in proximal colon increased significantly. Authors did not observe any modulation of proglucagon mRNA and GLP-1 content in ileum, caecum and distal colon. Moreover, doubling of the number of L-cells in the proximal colon upon OFS feeding was found to be associated with a significant increase in expression of Neuro D and NGN3 mRNA (differentiation factors).

Wang et al. (2007) demonstrated that feeding of same level of fiber content (10%; but differing in quantity of soluble vs. insoluble fiber) may have different effects on body weight gain and carbohydrate metabolism. They observed that incorporating sugar cane and psyllium (higher percentage of soluble fiber) in high fat diet attenuated weight gain, lowered hyperglycemia and insulinemia significantly. Ghrelin mRNA levels were reduced, plasma GLP-1 levels increased while leptin levels reduced significantly by psyllium and sugarcane feeding.

However, cellulose feeding (10%) had no effect on any of the parameters. Isken et al. (2010) showed that long term (45 weeks) supplementation of western style diet with soluble, highly fermentable guar fiber resulted in an obese phenotype in C57BL/6J mice compared to insoluble, moderately fermentable cereal fiber. Weight gain and insulin resistance was significantly different between groups, although dietary energy intake was comparable. Significantly lower energy loss via feces in the soluble fiber group together with comparable dietary energy intake in all mice suggested that more metabolizable energy was extracted from the soluble, highly fermentable fiber, without affecting satiety and feeding behavior. Also, mice fed insoluble fiber showed significantly increased expression levels of foxa2 and pgc1β in liver tissue. These transcription factors are known to play an important role in the regulation of hepatic lipid homeostasis. Co-expression of foxa2 and pgc1β has been reported to concomitantly increase gene expression of key enzymes of mitochondrial β-oxidation (Wolfrum and Stoffel, 2006). Significantly increased expression levels in liver of pparα and dgat-2 in insoluble fiber-fed mice potentially contributed to increased beta-oxidation and reduced fat mass in mice fed insoluble cereal fibers. Thus, increased energy digestion with soluble, highly fermentable fiber consumption and increased expression of SCFA target genes might unfavorably affect energy homeostasis in long term (Isken et al., 2010).

Although influence of gum arabic (GA) on energy intake and body weight regulation remains controversial, a growing body of scientific evidence indicates that GA ingestion causes significant reduction in caloric intake with an increased subjective feeling of satiety (Calame et al., 2011). Babiker et al. (2012) reported a significant reduction in BMI and fat percentage due to regular ingestion of gum acacia (30g/day) for 6 weeks. Side effects caused by GA ingestion were experienced only in the first week. They included unfavorable viscous sensation in the mouth, early morning nausea, mild diarrhea and bloating abdomen. Nasir (2014) found that ingestion of 10% (w/v) GA dissolved in tap water significantly improved oral glucose tolerance while intra-peritoneal glucose tolerance was unaffected in C57BL/6 mice, indicating that effects of GA on the glucose metabolism are related to interaction with the intestine. Under prolonged treatment for 4 weeks with a 20% glucose solution, glucose-treated mice gained significantly more body weight compared to group which received 20% glucose solution together with 10% GA despite similar food and fluid intake. Fasting blood glucose concentrations were increased significantly following challenge with a 20% glucose solution which was blunted by simultaneous treatment

with GA. The total body weight gain induced by high fat diet was significantly decreased in GA treated as compared to non-treated mice. The fasting blood glucose was also blunted by simultaneous treatment with GA as compared with other group followed by a significant decrease in fasting insulin concentrations in GA treated mice.

Ushida et al. (2011) observed no significant difference in food consumption between gum acacia fed and control mice, but the body weight was lower in gum acacia fed group, however, the difference in body weight was significant after 40th day of experiment. Weight of mesenteric, paranephric and periovarian fat was found to be significantly lower in GA-fed mice and there was no difference in expression of fatty acid synthesis genes (srebp-1c, fas, glut1 and glut4) and adipose tissue remodeling genes (icam 1, pecam, selp and sell) between GA fed and control group.

Arabinoxylan (AX) supplementation (@10%) for 4 weeks along with high fat diet restored the number of bacteria that were decreased upon HF feeding, i.e. Bacteroides-Prevotella spp. and Roseburia spp. Importantly, AX treatment markedly increased caecal bifidobacteria content, in particular *Bifidobacterium animalislactis*. This effect was accompanied by improvement of gut barrier function and a lower circulating inflammatory marker. AX treatment decreased adipocyte size and HF diet-induced expression of genes mediating differentiation (PPARγ), fatty acid uptake (C/EBPα, FAT/CD36, aP2, LPL), fatty acid oxidation (CPT1, ACO) and inflammation (IL6, MCP-1, F4/80), and decreased a key lipogenic enzyme activity (FAS) in the subcutaneous adipose tissue. Furthermore, AX treatment significantly decreased HF-induced adiposity, body weight gain, serum and hepatic cholesterol accumulation and insulin resistance. Correlation analysis revealed that Roseburia spp. and Bacteroides/Prevotella levels inversely correlated with these host metabolic parameters. The study supported a role for wheat AX as interesting nutrients with prebiotic properties related to obesity prevention (Neyrinck et al., 2011).

Arabinoxylan oligosaccharides (AXOS) supplementation @7.5% in high fat diet to C57BL/6J mice for 8 weeks induced caecal and colon enlargement associated with an important bifidogenic effect. It increased the level of circulating satietogenic peptides produced by the colon (peptide YY and GLP-1), and coherently counteracted HF-induced body weight gain and fat mass development. HF-induced hyperinsulinemia and the Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) were decreased upon AXOS feeding. In addition, AXOS reduced

HF-induced metabolic endotoxemia, macrophage infiltration (mRNA of F4/80) in the adipose tissue and interleukin 6 (IL6) in the plasma. The tight junction proteins (zonula occludens-1 and claudin-3) altered upon HF feeding were upregulated by AXOS treatment suggesting that the lower inflammatory tone was associated with the improvement of gut barrier function. Together, these findings suggested that specific non-digestible carbohydrates produced from cereals such as AXOS constitute a promising prebiotic nutrient in the control of obesity and related metabolic disorders (Neyrinck et al*.,* 2012).

Recently, Adam et al. (2014) showed that sustained daily intake of diverse types of soluble fermentable dietary fibre (beta glucan, pectin and FOS for 4 weeks @ 10% level), decreased voluntary food intake, weight gain and adiposity in normal healthy young adult male rats, and was associated with increased circulating GLP-1 and PYY levels. It was inferred that significant satiety-inducing effects of dietary fibre only manifest themselves after several days of consistently increased fibre intake and it was tempting to speculate that this reflects a minimum exposure time for the underlying chronic changes in the gut environment and in tonic gut satiety hormone secretion to develop. Although the three different soluble fibres differ from each other in their physicochemical attributes (pectin and beta glucan are highly viscous whereas FOS has very low viscosity). However, all fibres had similar effect on food intake. The common feature of the three fibres was their fermentability in the large intestine, resulting in greatly increased total concentrations of fermentation products in the caecum and colon contents. Surprisingly, there was no increase in concentrations of the main three SCFAs in caecum and colon contents of rats fed the fermentable fibres compared with controls, and indeed concentrations were unexpectedly lower for acetate in FOS and beta glucan groups, and for propionate in the FOS group. Authors concluded that measuring concentrations at a single time point would have given no indication of biologically significant changes in rates of turnover. Nonetheless, the volume of contents and hence the total pool of SCFAs would have been increased in the fermentable fibre groups and the large intestine was visibly enlarged. Moreover, although ffar3 gene expression was not increased but indeed there was even a decrease in expression in the proximal colon of FOS and pectin groups. Similarly little evidence was found for increased gene expression for ffar2, the one exception being an increase in the distal ileum of FOS fed rats. The absence of increased gene expression for PYY and GLP-1 in distal ileum and proximal colon, despite the greatly increased plasma concentrations of these hormones, was another apparently anomalous

finding and gene expression for both was even decreased in the pectin group. Since PYY and GLP-1 are secreted from intestinal L-cells, the increased plasma concentrations must reflect either increased secretary activity per cell or increased number of L-cells.

Phytosterols are steroid compounds present in plants which are similar to cholesterol in structure and functions. Several animal and human studies show that phytosterols lower plasma total and LDL-cholesterol levels. It is generally accepted that cholesterol-lowering effect of phytosterols is due to direct inhibition of cholesterol absorption, through displacement of cholesterol from mixed micelles (Raphael et al., 2015). Limited data from cell culture and animal studies suggest that phytosterols may attenuate the inflammatory activity of immune cells, including macrophages and neutrophils. Thus, they may have anti-inflammatory activities in living systems (Awad et al., 2003). Phytosterols may produce health benefits in animals/humans such decreased risk of coronary heart diseases, anti-inflammatory activities, induction of apoptosis in cancer cells, disease prevention and treatment.

2.2 DIETARY FIBER

Dietary fiber is the name given to a group of components present in foods of vegetable origin (cereals, fruit, vegetables and pulses) which is not broken down by human digestive enzymes (De Vries et al., 1999; Dietary Fiber Definition Committee of the American Association of Cereal Chemists, 2001). Dietary fiber can be classified into insoluble and soluble fiber. As its name suggests, soluble fiber is composed of water-soluble elements with a gel-forming capacity, which gives volume to feces (inulin, pectins, gums, and fructo-oligosaccharides). These substances, which predominate in pulses, cereals (oats and barley) and some fruits, are used by intestinal micro-organisms, especially colon flora. This type of fiber is therefore known as fermentable fiber. Insoluble fiber is not dissolving in water (cellulose, hemicellulose, lignin, and resistant starch). Its components resist the action of intestinal microorganisms, and this fiber can therefore be classed as non-fermentable. The benefits of consuming foods rich in fiber are numerous, ranging from improved large bowel function to slowed digestion and absorption of carbohydrate and fat and a reduced risk of diseases (Ali et al., 1982; Schneeman and Tietyen., 1994). Howarth et al. (2001) reported that increased dietary fiber intake have been associated with a body weight loss of 1.9 kg over 3.8 months, with greater loss in more obese subjects.

Recommended intake of dietary fibers

Current recommendations for dietary fiber intake are related to age, gender, and energy intake, and the general recommendation for adequate intake (AI) is 14 g/1000 kcal (USDA, 2005). This AI includes non-starch polysaccharides, analogous carbohydrates (e.g., resistant starches), lignin, and associated substances (Witwer, 2008). Using the energy guideline of 2000 kcal/day for women and 2600 kcal/day for men, the recommended daily dietary fiber intake is 28 g/day for adult women and 36 g/day for adult men. However, there have been no studies on evaluating the dietary fiber requirements in Indians (NIN, 2009).

2.2.1 SOLUBLE FIBER AS PREBIOTIC

Prebiotics are "nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon" by fermentation (Gibson and Roberfroid., 1995). Most identified prebiotics are carbohydrates and oligosaccharides with different molecular structures from plant sources. Dietary carbohydrates such as fibers, are candidate prebiotics, but most promising are nondigestible oligosaccharides (Gaggia et al., 2010).

An important mechanism of action for dietary fiber and prebiotics is fermentation in the colon and changes in gut microflora. First by, dietary fiber resists digestion in the small intestine, and thereby allowing it to enter the large intestine where it is fermented by the colonicmicrobiota leading to production of short chain fatty acids (SCFA). These SCFAs produced in the colon are acetic, propionic and butyric acid. Among these, 90% of the butyric acid is utilized by the colonocytes and very little reaches to visceral tissues (Al-Lahham et al., 2010). The majority of acetic acid and propionic acid is a ligand for the GPCR41 and GPCR43 (G-Protein coupled receptors41/43). G-Protein coupled receptors (GPCR) stimulate the production of anorexigenic hormones such as peptide-YY (PYY) and glucagon like peptide-1 (GLP-1), the two gut hormones known to reduce the food intake and increase the energy expenditure. SCFAs are water-soluble and are absorbed into the blood stream. The brain, muscles, and tissues metabolize acetate systemically whereas propionate is cleared by the liver and may lower the hepatic production of cholesterol by interfering with its synthesis. Transport to and further metabolism of SCFAs in the liver, muscle, or other peripheral tissues is thought to contribute about 7%–8% of host daily energy requirements (Cummings and Macfarlane, 1991). Fermentation and SCFA production also inhibit the growth of pathogenic organisms by reducing luminal and fecal pH.

Low pH reduces peptide degradation and the resultant formation of toxic compounds such as ammonia, amines, and phenolic compounds, and decreases the activity of undesirable bacterial enzymes (Salvin, 2013).

Second, dietary fiber may decrease a diets metabolizable energy (ME), which is gross energy minus the energy lost in the feces, urine and combustible gases. Baer et al. (1997) observed that an increased consumption of dietary fiber resulted in a decrease in the ME of the diet. This may be attributed to the fact that fat digestibility decreased as dietary fiber increased. Also, as dietary fiber intake increases, the intake of simple carbohydrates tends to decrease. Third, dietary fiber/prebiotics are capable of re-establishing the equilibration of gut microbiota ecosystem. The human fetus is microbiologically sterile and is colonized at birth by bacteria from the mother and the surrounding environment. It is dominated by bacteria belonging to three major groups (phyla): firmicutes, bacterioidetes, and actinobacteria that together represent>95% of the microbiota. The microbiota can be viewed as a metabolic ''organ'' exquisitely tuned to our physiology that performs functions that we have not had to evolve on our own. These functions include the ability to process otherwise indigestible components of our diet, such as xenobiotic compounds, amino acids and carbohydrates (Gill et al*.,* 2006; Kurokawa et al*.,* 2007; Turnbaugh et al., 2009a). Several factors such as diet, genetic background, and immune status affect the composition of gut microbiota (Turnbaugh et al*.,* 2009b; Benson et al., 2010). The prebiotic approach prevents the over-expression of several host genes that are related to adiposity and inflammation (Delzenne et al., 2011).

2.2.1.1 Effects on body weight and adiposity

Prebiotic treatment changes the gene expression pattern in the white adipose tissue of obese mice (by acting on PPARγ and G-coupled receptors protein 43) leading to an increased lipolysis, a decreased adipogenesis and an increased metabolic response to hormones such as leptin, all phenomenon contributing to a lower adiposity (Cani & Delzenne., 2009; Delzenne N.M & Cani P.D., 2010; Neyrinck & Delzenne., 2010; Dewulf et al., 2011; Neyrinck et al., 2011; Everard et al., 2011; Parnell & Reimer., 2012). Three months of treatment with fructans also decreased body weight gain and fat mass in adult obese subjects (Parnell and Reimer, 2009). Ingestion of inulin-type fructan prebiotic (8 g/d) for 1 year had a significant benefit in the maintenance of BMI and fat mass in non-obese young adolescents (Abrams et al., 2007).

2.2.1.2 Effects on gut peptides and appetite regulation

Soluble fiber, when fermented in the large intestine, produces glucagon-like peptide (GLP-1) and peptide YY (PYY). These gut hormones play a role in inducing satiety (Keenan et al*.,* 2006). A randomized, double-blind, placebo-controlled clinical study in 100 overweight healthy Chinese adults investigated the effect of different dosages of dietary supplementation with wheat dextrin, on satiety over time. Wheat dextrin increased short-term satiety, which was time and dosage correlated. The hunger feeling decreased significantly from day 5 to the end of the evaluation for the group 24 g and from day 7 for the groups 14 and 18 g. By day 5, the group 24 g showed significantly longer time to hunger between meals compared with placebo. A significant decrease in caloric intake was seen from week 2 to the end of the 9 week study for the groups 14 g, 18 g and 24 g of wheat dextrin (Guerin-Deremaux et al., 2011). In obese animals fed inulin type fructans, an increase in anorexigenic peptides (peptide YY and glucagon-like peptide 1 (7–36) amide) and a decrease in the orexigenic peptide (ghrelin) occurs, which contributes to the satietogenic effect of the peptide (Cani and Delzenne., 2011). In addition, the supplementation of fructans in high-fat diet-fed mice modulates the neuronal activation within the arcuate nucleus, which can contribute to the control of food intake (Anastasovska et al., 2012).

2.2.1.3 Effect on Gut microbiota

The human gut contains an immense number of microorganisms, collectively known as the microbiota. This community consists of at least 10^{13} citizens, is dominated by anaerobic bacteria (Backhed et al*.,* 2004). Although the intestinal microbiota is complex and the role of most of the bacteria in providing benefit to the host is not clear, bacterial species of the genera *Lactobacillus* and *Bifidobacterium* have been shown to supply protection against enteric infections. An important mechanism of action for dietary fiber and prebiotics is fermentation in the colon and changes in gut microflora. Addition of prebiotics to the diet has brought positive aspects to the gut microbial balance. Delzenne et al. 2013 have summarized how highly fermentable carbohydrates (prebiotics) are able to counteract several types of metabolic alterations linked to obesity (Fig. 2.1).

Arora et al. (2012b) fed C57BL/6 male mice for 8 weeks on a high fat diet containing 10% (w/w) inulin or 10% (w/w) β-glucan. A significant modulation of bacterial populations was observed in both the cecal and fecal contents. Bacterial groups, *Lactobacillus-Enterococcus* and

Bifidobacterium showed a significant increase in both inulin and β- glucan. Inulin and β- glucan fed mice also exhibited a significant increase in total bacterial MIB and EREC counts compared to control.

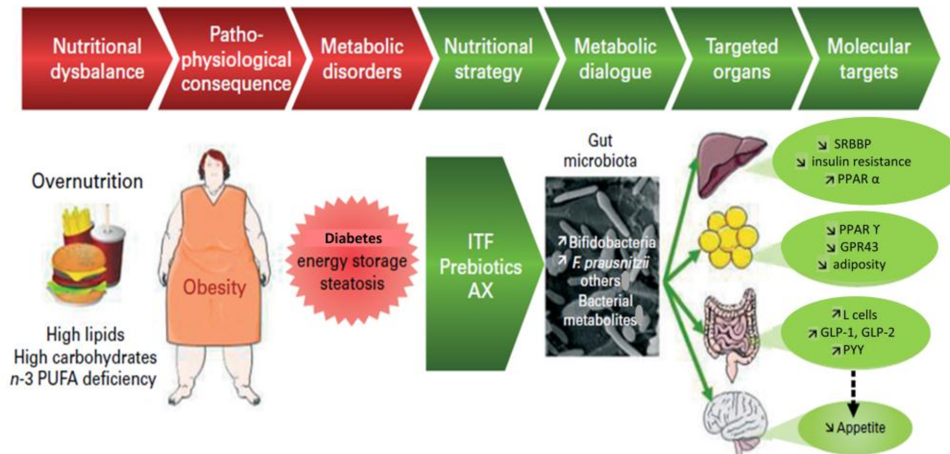


Fig 2.1. Effect of dietary carbohydrates with prebiotic properties on host pathophysiology related to obesity (Delzenne et al., 2013).

Cani et al. (2007) fed C57BL/6J mice HFD diet and examined the effects of different experimental diet. a fermentable dietary fiber fructooligosaccharides (OFS) (HFOFS) and a mixture of HF diet & a non-fermentable dietary fiber (microcrystalline cellulose [HF-Cell]). They observed that high fat diet resulted in a significant modulation of population levels of the dominant members of the intestinal microbiota. The major modifications observed were a dramatic reduction in quantities of bifidobacteria in HF-fed mice compared with control and the Gram-negative *Bacteroides* MIB. Quantities of bifidobacteria in HF-OFS fed mice were restored and were even higher than those of control mice, confirming the role of OFS as a prebiotic. Together with the *E. rectale–C. coccoides* group, they constitute the dominant members of the mouse intestinal microbiota. Numbers of the *E. rectale–C. coccoides* group were also significantly lower in animals fed the HF diet compared with control and even more so in HF-Cell mice.

Dewulf et al. (2011) fed male C57bl6/J mice a standard diet or an HF diet without or with ITF (Inulin type fructan) (0.2 g/day per mouse) for 4 weeks. Administration of the HF diet induced a decrease in total bacterial counts and in the counts of most of the analyzed bacterial groups, except for bifidobacteria. The co-administration of the HF diet with ITF significantly increased bifidobacterial counts by 100-fold. However, ITF treatment also led to a drop in

Roseburia spp. and of *Clostridium cluster XIVa.*

2.2.1.4 Effects on glucose and lipid metabolism

Several studies in human subjects also show an improvement in postprandial glycaemia, or, in some studies, in triglyceridaemia, upon prebiotic treatment, but those data are really not numerous enough to draw any conclusion on a potential benefit for diabetic or dyslipidaemic patients (Delzenne et al., 2011). Cani et al. (2007) fed C57BL/6J mice for 14 weeks a HF diet containing 49.5% fat w/w corresponding to 72% of the total energy content, a mixture of HF diet and a fermentable dietary fiber oligo fructooligosaccharides (OFS) (HFOFS) and HF diet containing a non-fermentable dietary fiber (microcrystalline cellulose [HF-Cell]. They found that HF-OFS improved glucose tolerance compared with HF and HF-Cell mice. Fasted insulinaemia was significantly increased in HF and HF-Cell mice compared with control. Insulin secretion following glucose load was almost absent in HF and HF Cell mice. In contrast, HF-OFS mice showed normal fasting plasma insulin levels and restored glucose induced insulin secretion. In most experimental studies, prebiotics are able to decrease the hepatic accumulation of TAG and/or cholesterol in the liver tissue, defined as steatosis. This effect could be particularly interesting, as the occurrence of non-alcoholic fatty liver disease is present in 25–75% of the obese individuals. There again, mechanistic studies in animals reveal changes in hepatic host gene expression upon prebiotic treatment that could implicate, depending on the experimental conditions, a decrease in sterol-response-element-binding protein-dependent cholesterogenesis and/or lipogenesis, and/or of changes in PPARα- driven fatty acid oxidation (Delzenne et al., 2011; Delzenne & Cani., 2011; Pachikian et al., 2011).

2.2.2 GALACTOMANNAN

Galactomannan represents the major polysaccharide found in seeds like methi or fenugreek, locust bean, guar etc. It is an integral component of the cell wall which is found concentrated around the seed coat (Spyropoulos, 2002). Galactomannans are structurally composed of a 1→4 β-D-mannosyl backbone substituted by a single galactose unit α-linked at the C-6 oxygen (Bhaumick, 2006). Galactomannans from different sources differ in their mannose to galactose ratio (M:G) (Mathur & Mathur., 2005). Different M:G ratio have different properties like solubility and viscosity etc. The high degree of galactose substitution in fenugreek (M:G 1:1) renders molecule relatively more soluble compared to galactomannans from guar or locust bean, which has a galactose to mannose ratio of 1:2 and 1:4, respectively (Reid and Meier,

1970; Brummer et al., 2003). Fenugreek galactomannans are unique relative to other commonly used galactomannans such as those found in guar and locust beans.

Table 2.1 Chemical structure of galactomannans

Galactomannan	Structure
Galactomannan-1, 4 (LGM	-M-M-M-M-M-M-M-M-M- 			 G G G
Galactomannan-1, 1 (FGM)-	-M-M-M-M-M-M-M-M-M-M-M |									| G G G G G G G G G G G

M = Mannose; G = Galactose

2.2.2.1 Health benefits of galactomannan

The soluble nature of galactomannan fiber from fenugreek has been linked to numerous human health benefits, mainly in the reduction of plasma glucose levels which has an antidiabetic effect (Sharma, 1986; Madar et al., 1988; Madar and Shomer, 1990). Hannan et al. (2003) also have demonstrated that the soluble dietary fiber (SDF) portion of fenugreek can significantly improve glucose homeostasis in type 1 and type 2 diabetes by delaying carbohydrate digestion and absorption. They have also suggested that the SDF fraction may enhance insulin action in type 2 diabetes as indicated by the improvement of oral glucose tolerance in these test subjects.

Lin et al. (2011) studied *in vitro* assessment of dietary fibers (oat β-glucan, flaxseed gum, and fenugreek gum) in pig intestinal digesta and concluded that lactate in the β-glucan-grown cultures had the highest level among SCFAs produced, whereas flaxseed or fenugreek gum-containing cultures generated a significant amount of acetate, propionate and butyrate over three subcultures tested. Yoon et al. (2006) reported on the effects of partially hydrolyzed galactomannan (guar gum) on the fecal microbiota of nine healthy human volunteers administered at a dose of 7 g of product/volunteer/day for 14 days and concluded that both the *bifidobacterium spp*. counts and percentage of these species with respect to the total counts

increased significantly. Dietary galactomannans increased caecal volatile fatty acids particularly propionic acid, increased the weight of the caecum and its contents and increased the amount of water in the feces (Evans et al., 1992).

2.2.2.2 Weight regulation

Ethanolic extract of fenugreek seed significantly reduced the body weight gain induced by high fat diet in obese mice model (Handa et al., 2005). Feeding C57BL/6J mice with thermostable extract of fenugreek (15mg/kg body weight for 15 days) significantly reduced the body weight (Vijayakumar et al., 2010). STZ induced diabetic rats treated with aqueous extract of fenugreek orally for 6 weeks results in weight gain, while untreated diabetic rats showed progressive reduction in body weight (Xue et al., 2007; Eidi et al., 2007). Diet supplemented with 5% fenugreek powder fed to obese Zucker rats for 8 weeks had no effect on body weight gain, eating habits or any behavioral changes in obese and lean rats (Raju and Bird., 2006). However the percent body weight gain has been reported to be significantly low in obese rats fed 2.5% and 5% fenugreek seed extract for 9 weeks (Ramulu et al., 2011). A slight reduction in body weight has also been reported fenugreek (2g/kg BW) containing HFD diet fed to C57BL/6J mice for 35 weeks (Hamza et al., 2012).

2.2.2.3 Hypoglycemic effects

A significant number of reports are available on the antidiabetic property of fenugreek seeds. Hypoglycemic effects of whole fenugreek seed powder, ethanolic/methanolic and aqueous extracts have been reported in diabetic rats (Raju., 2001; Vats et al., 2002; Mondal., 2004), in diabetic mice (Ajabnoor et al., 1988). Srichamroen et al. (2009) reported that galactomannan (ether extract) reduce the intestinal glucose uptake dose dependently in genetically obese JCR rats. The uptake of low or high glucose was significantly reduced by increasing concentrations of galactomannan in both obese and lean rats. Besides many animal studies, several human trials have unequivocally demonstrated the beneficial hypoglycemic potential of this spice in both type-1 and type-2 diabetes. Antihyperglycemic compound GII from fenugreek has also been found to reduce blood glucose and AUC of GTT significantly (Moorthy et al., 2010; Puri et al., 2012).

2.2.2.4 Hypolipidemic and hypocholesterolemic effects

Dietary fenugreek or extract has been found to be hypolipidemic and hypocholesterolemic in a number of animal studies as well as a few clinical trials. Vijayakumar

et al. (2010) reported the thermostable extract of fenugreek seeds (TEFS) inhibited accumulation of fat in differentiating and differentiated 3T3-L1 cells via decreased expression of SREBP-1, PPAR-gamma, and c/EBP-alpha. They also showed significantly decreased cellular triglycerides and cholesterol concentrations via reduced expression of SREBP-1 at mRNA and protein level and TEFS also up regulated the LDR receptor in HepG2 cells. In C57BL/6J mice administered TEFS (1.5mg/kg/day) oral gavage for 15 days and found decreased serum TG, LDL-C. In another study (Ramulu et al., 2011) diet containing galactomannan extract of fenugreek (2.5 and 5%) significantly reduced body weight gain, triglycerides, total cholesterol and increased HMG CoA reductase activity in WNIN/GR-ob rats. Raju and Bird (2006) fed 5% fen supplemented AIN-93G diets for 8 weeks to obese zucker rats and showed significantly reduce liver weight, plasma insulin, lactate, cholesterol, triglyceride and TNF alpha, increased TNF II receptor in liver. Feeding of Canadian grown fenugreek seeds to high sucrose fed Sprague Dawley rat has also been reported to significantly reduce the glycemic response, plasma insulin level and reduced plasma TG, cholesterol, VLDL, LDL, HDL and also reduce abdominal fats in normal rats (Srichamroen et al., 2008). The plasma total cholesterol and triglycerides in liver were significantly lower in STZ induced rats fed 3% guar gum (3G), 3% locust bean gum (3L), 3% xanthan gum (3X), mixture of 1:2 G+ X and L+X for 4 weeks and has been concluded that mixture of xanthan and guar gum has an improved hypolipidemic effects on non diabetic and STZ diabetic rats (Yamamoto et al., 2000).

2.2.2.5 Effects on enzyme activities

Fenugreek galactomannan (extract) in diabetic rats significantly lowered the activities of intestineal disaccharidase (Hannan et al., 2007), maltase, lactase, sucrase and lipase (Hamden et al., 2010). Galactomannan also has been found to decrease AST, ALT and LDH in serum of diabetic rats (Eidi et al., 2007; Hamden et al., 2010). In other reports, Muraki et al. (2011) observed no significant differences in AST, ALT or AST/ALT levels in liver in high fat-high sucrose diet containing fenugreek seed powder. Raju and coworkers (2001) examined the effect of oral administration of *Trigonella* whole seed powder (5% in the diet) for 21 days on glycolytic, gluconeogenic and $NADP^+$ linked lipogenic enzymes in liver and kidney in alloxan-induced diabetic Wistar rats and concluded that the activities of the glycolytic enzymes (Hexokinase isozymes, Phosphofructokinase, Pyruvate kinase, Lactate dehydrogenase) were significantly higher in liver and lower in kidney of fenugreek treated diabetic rats. The

gluconeogenic enzymes (Glucose 6-phosphatase, Fructose 1,6-bisphosphatase) and NADP dependent lipogenic enzymes (G-6-P dehydrogenase , Malic enzyme) were lowered significantly in both liver and kidney of fenugreek treated diabetic rats.

2.3 DIETARY PHYTOCHEMICALS

Dietary phytochemicals might be employed as anti-obesity agents, because they may suppress growth of adipose tissue, inhibit differentiation of preadipocytes, stimulate lipolysis, and induce apoptosis of existing adipocytes, thereby reducing adipose tissue mass (Gonzalez-Castejon and Rodriguez-Casado, 2011). The different types of dietary phytochemicals are:

2.3.1 POLYPHENOLS

Polyphenols are the more relevant family of phytochemicals endowed with health benefits (Bravo, 1998). Numerous preclinical studies reveal that selected polyphenols exhibit strong protective actions on many pathological conditions particularly those triggered by oxidative stress such as cardiovascular disease (CVD) and metabolic disorders. Moreover, dietary polyphenols may suppress growth of the adipose tissue through their anti angiogenic activity and by modulating adipocyte metabolism (Badimon et al., 2010; Mulvihill et al., 2010). Others benefits of polyphenols are related to infections, cancer, and autoimmune and neurodegenerative processes (Ramassamy, 2006; Schaffer et al., 2006; Nobili et al., 2009; Jew et al., 2009). Polyphenols, including their functional derivatives – esters and glycosides – have one to various phenol groups with one hydroxylsubstituted aromatic ring (Dey et al., 1989). According to their structure, number of phenol rings and the type and number of structural elements binding- polyphenols are grouped into different classes. They are 1. simple phenolic acids, e.g. ferulic, caffeic, p-coumaric, vanillic, gallic, ellagic, p-hydroxybenzoic, chlorogenic acids; 2. stilbenes, e.g. resveratrol; 3. curcuminoids, e.g. curcumin; 4. chalcones, e.g. phlorizin, naringenin chalcone; 5. lignans, e.g.matairesinol, secoisolariciresinol; and 6. flavonoids, composed of seven subclasses: flavonols, e.g. quercetin, flavanols (monomeric, e.g. catechin, epicatechin, oligomeric, and polymeric compounds, e.g. proanthocyanidins, also called condensed tannins), anthocyanins, e.g. cyaniding, flavones, e.g. luteolin, apigenin, flavanones, e.g. naringenin, flavanonols, e.g. taxifolin, and isoflavones, e.g. genistein (Bravo, 1998; Harborne et al., 1999; Williams et al., 2004).

2.3.2 TERPENOIDS (ISOPRENOIDS)

Terpenoids (isoprenoids) constitute one of the largest families of natural products, accounting for more than 40,000 compounds of both primary and secondary metabolisms (Goto et al., 2010). Terpenoids can be described as chemically-modified terpenes. Many terpenes are hydrocarbons, but they also have oxygen-containing compounds such as alcohols, aldehydes, and ketones (terpenoids) (Park et al., 2011). The simplest unifying feature present in the structure of all terpenoides is the isoprene unit (CH2 C-(CH3)–CH CH2). Based on the number of carbon atoms, terpenoids can be classified into further groups: hemiterpenoid (C5), monoterpenoids (C10), sesquiterpenoids (C15), diterpenoid (C20), sesterterpenoid (C25), triterpenoids (C30), tetraterpenoid (C40), and politerpenoid (C> 40). Most of the terpenoids are of plant origin and are present in vegetables and fruit. Daily eating of certain terpenoids might be useful for the management for obesity-induced metabolic disorders, such as type 2 diabetes, hyperlipidemia, insulin resistance, CVD, and a lower prevalence of metabolic syndrome (Goto et al., 2010). PPARγ activation attenuates obesity and type-2 diabetes. Geranylgeraniol, farnesol, and geraniol terpenoids are ligands with potential to activate PPARγ, dietary lipid sensors that control energy homeostasis and lipid and carbohydrate disorders (Takahashi et al., 2002; Goto et al., 2010). Several reports have indicated that co-application of PPARα and PPARγ agonists causes more efficient glucose uptake into adipocytes to decrease blood glucose levels without a concomitant increase in body weight (Tsuchida et al., 2005). In this regard, farnesol and geranylgeraniol isoprenols have the effects of dual activation of PPARα and PPARγ (Takahashi et al., 2002). The monoterpene derivative auraptene, a citrus fruit compound mainly contained in the peel, is also a PPARα/γ dual agonist (Takahashi et al., 2008; Kuroyanagi et al., 2008). In adipocytes, auraptene regulates the transcription of PPARγ target genes, induces the expression of adiponectin, and inhibits those of MCP-1 (Kuroyanagi et al., 2008). It was also observed that auraptene suppresses the inflammatory signaling exchange between adipocytes and macrophages and the macrophagic infiltration into obese adipose tissues (Tsuchida et al., 2005).

2.3.3 ORGANOSULFURS

Organosulfur compounds are particularly abundant in Allium vegetables including garlic, onion, scallion, chive, shallot, and leek that contain bioactive substances such as allicin, allixin and allyl sulfides (Sahu, 2002). These molecules account for the distinctive flavor and aroma as well as the many purported medicinal effects of these vegetables. Organosulfurs provide

glucosinolates, which are converted in the human body in thiosulfonates, indoles (indole-3-carbinol), an isothiocyanates (Cartea and Velasco, 2008). Organosulfurs from garlic and onions have been reported to exert various physiological obesity related effects. They decrease the synthesis of cholesterol by hepatocytes through inhibition of HMG-CoA reductase, a critical enzyme in the cholesterol biosynthesis pathway. Organosulfurs also lower blood pressure and stimulate non-specific immunity (Benkeblia et al., 2007). They are heralded as powerful anti-thrombic, hypoglycemic, and lipid-lowering agents. Also, they prevent platelet aggregation and are attributed with liver protection and immune system strengthening activities (Sahu, 2002; Cartea and Velasco, 2008). Garlic-derived organosulfur compounds have been found to inhibit the activity of the inflammatory enzymes, cyclooxygenase and lipoxygenase, in vitro and to decrease the expression of inducible nitric oxide synthase (iNOS) in macrophages (Cartea and Velasco, 2008). Organosulfur compounds have also been found to decrease the production of inflammatory signaling molecules in cultured macrophages and human whole blood (Cartea and Velasco, 2008). Finally, although the beneficial effects of organosulfurs have been mostly ascribed to their antioxidant and anti-carcinogenic properties (Sahu, 2002), the adipocyte-specific effects of ajoene, a garlic derivative, were also reported (Yang et al., 2006). In particular, garlic extracts may decrease fat cell number, thereby suggesting some therapeutic possibility for obesity.

2.3.4 PHYTOSTEROL

Phytosterols or plant sterols found naturally in plants and the most important natural sources of plant sterols in human diets are cereals, fruits, vegetables, nuts and unrefined vegetable oils (Moreau et al., 2002; Ostlund., 2002). They are structurally similar to cholesterol but vary only in carbon side chains and/or presence or absence of a double bond and are biosynthesized through mevalonate pathway. In plants, these sterols act as structural component of cell wall influencing permeability and, thus cell fluid exchange (Lagarda et al., 2006; Moreau et al., 2002; Piironen et al., 2003). More than 200 different types of plant sterols have been identified and they can be found as free sterols, esterified or glycosylated at the C3 hydroxyl group. Usually regular diet affords around 200 to 300 mg per day of plant sterols and for vegetarian individuals this value is almost two times higher (Moreau et al., 2002; Quilez et al., 2003; Klingberg et al., 2008). Phytosterols have been classified into two: (1) Sterols, which have a double bond in the sterol ring, so are unsaturated compounds and (2) Stanols, which lack a

double bond in the sterol ring, so are saturated molecules. The most abundant sterols in plants and human diets are sitosterols and campesterols. Stanols are also present in plants, but they form only 10% of total dietary phytosterols (Raphael et al., 2015).

Phytosterols with potential effects on obesity are diosgenin, campesterol, brassicasterol, sitosterol, stigmasterol, and guggulsterone. High intakes of these compounds can also protect against atherosclerosis (Schonfeld, 2010) and decrease serum total and LDL-cholesterol levels (Izar et al., 2011). Mechanistically, phytosterols compete with cholesterol for micelle formation in the intestinal lumen and inhibit cholesterol absorption (Izar et al., 2011). Their influence on intestinal genes and transcription factors make phytosterols key regulators in metabolism and cholesterol transport in the expression of liver genes (Gupta et al., 2011; Jesch et al., 2008). In terms of phytotherapy, furostanol saponin is the active ingredient of the rhizomes of Dioscorea gracillima, the protodioscin studied for its anti-hyperlipidemic effect. The administration of protodioscin in hyperlipidemic rats significantly reduced the blood levels of TG, cholesterol, low- and high-density lipoproteins (Wang et al., 2010). Acid hydrolysis of this bisdesmoside forms diosgenin, a phytoestrogen that can be chemically converted into progesterone (Murray et al., 2005). Diosgenin is found in a variety of plants, including fenugreek and the roots of wild yam. Diosgenin has various biological functions, including anti-inflammatory roles via down-regulation of I-κB-α degradation and JNK activation (Hirai et al., 2010), which is independent of PPARγ activation (Uemura et al., 2011). Diosgenin (5 and 10 mol/L) inhibited the accumulation of TG and the expression of lipogenic genes in HepG2 cells (Uemura et al., 2011). Also, diosgenin is used for hypercholesterolemia and diabetes treatments, and it possesses anti-thrombosis effects in vitro and in vivo (Gong et al., 2011). A phytosterol used as an effective non-toxic herbal medicine for obesity, arthritis, cancer, and CVD is guggulsterone, the active agent of the guggul plant (Commiphora mukul) (Yang et al., 2008). Its most studied effects are related to lipid, cholesterol, TG, and glycemia lowering and serum high density lipoprotein rising. Thereby, this plant is considered as a potential anti-obesity agent (Rayalam et al., 2007; Urizar and Moore, 2003; Cui et al., 2003). Guggulsterone is a selective bile acid receptor modulator that regulates expression of a subset of farnesoid X receptor (FXR) targets and decreases the expression of bile acid-activated genes (Cui et al., 2003). In addition, guggulsterone may modulate anti-inflammatory and antioxidant responses mediated through the inhibition of NF- B (Shishodia and Aggarwal, 2004; Meselhy, 2003). Finally, guggulsterone

demonstrated significantly improved PPAR expression and activity in vivo and in vitro, in addition to inhibiting adipocytes differentiation in vitro (Sharma et al., 2009).

Phytosterols has been shown to inhibit the uptake of both dietary and endogenously-produced (biliary) cholesterol from the intestine. This results in a decrease in serum total and low-density lipoprotein cholesterol (LDL-C) levels (Ling et al., 1995), even for those individuals who are already on a low cholesterol diet (Maki et al., 2001). Levels of protective, high-density lipoprotein cholesterol (HDL-C) are typically not decreased by dietary phytosterol consumption. The exact mechanism by which phytosterols decrease serum cholesterol levels is not completely understood but several theories have been proposed (Pollak et al., 1981; Piironen et al., 2000). One theory suggests that cholesterol in the intestine, already marginally soluble, is precipitated into a non-absorbable state in the presence of added phytosterols and stanols. A second theory is based upon the fact that cholesterol must enter bile-salt and phospholipid-containing ‘‘mixed micelles’’ in order to pass through intestinal cells and to be absorbed into the bloodstream. Cholesterol is only marginally soluble in these micelles and it is displaced by phytosterols (and stanols), preventing its absorption. Their influence on intestinal genes and transcription factors make phytosterols key regulators in metabolism and cholesterol transport in the expression of liver genes. Unlike cholesterol, phytosterols and to a greater extent, phytostanols are very poorly absorbed (Heinemann et al., 1993) and the small amount that is absorbed is actively re-excreted in bile. This results in low serum levels of these sterol molecules.

2.3.4.1 DIOSGENIN (A SAPOGENIN)

Diosgenin is a 27-carbon steroidal compound with the chemical name (25R)- spirost-5-en-3-β-ol and is one of the many sapogenins found in several plants namely *Costus speciosus, Smilax menispermoidea*, *Trigonella, Trillium* and many species of *Dioscorea- D.althaeoides, colletti*, *zingiberensis.* It is currently an important raw material for the manufacturing of pharmaceutical hormones and steroids such as estrogen, progesterone, testosterone and glucocorticoids (Skaltsa, 2002). Diosgenin occurs naturally as a glycosylated compound in fenugreek, and can be liberated by acid hydrolysis (which removes three carbohydrate residues) of the steroidal saponin, dioscin. Diosgenin is synthesized as part of the melavonate pathway in the biosynthesis of steroids (C18-C30). Steroids are modified triterpenoids which contain a tetracylic ring structure of lanosterol but lack the three methyl groups at C-4 and C-14 (Dewick, 1997). Lanosterol is a precursor to the biosynthesis of plant cholesterol, which in turn is the most

fundamental structure of a plant steroid. Steroidal diosgenin is formed by modification of the side chain of cholesterol, in which a spiroketal structure is formed at C-22, yielding a non-polar compound with 6 carbon rings**.** Diosgenin has been used in traditional medicine for treatment of urethral and renal infections (Attele et al., 1999) and also minimize post-menopausal symptoms (Marker RE and Krueger J. Sterols 1940). Diosgenin is used to induce apoptosis in cancer cells and to reduce high blood pressure (Higdon et al, 2001).

Diosgenin extracted from *Trigonella foenum graecum* commonly called fenugreek, is a leguminous plant is used as a hypoglycemic agent in type I and type II diabetes (Taylor WG and Elder JL, 2000). Its molecular formula is similar to dehydroepiandrosteron (DHEA) which is a precursor of sex steroid hormones (Fig. 2.2). It has hypoglycemic (Sato et al., 2014), hypolipidemic, hypocholestemic (Son et al., 2007) and insulinotrophic effects (Kalailingam et al., 2014). Recently Huang et al. (2012) reported that other than carbohydrates, a phytosterol diosgenin also has prebiotic effects.

POTENTIAL HEALTH BENEFITS OF DIOSGENIN

Sangeetha et al. (2013) studied the effect of diosgenin on high fat diet-STZ induced rats. Diosgenin was administered orally at two doses (40 & 80mg/kg body weight) for 14 days and was found to reduce the hyperglycemia, hypercholesterolemia and hypertriglyceridemia and also showed improved level of antioxidant enzymes SOD, GPx and minimized the level of lipid peroxidation. Further, analyzing the lipid accumulation by Oil Red O staining in 3T3-L1 preadipocytes in presence and absence of diosgenin showed a dose dependent increase in accumulation of lipid. PPARγ protein level was profoundly increased in diosgenin treated cells whereas a slight decrease in the PPAR α expression also noted.

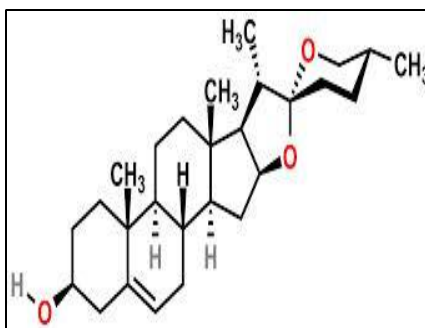


Fig 2.2 Structure of Diosgenin

Uemura et al. (2010) reported that treatment of KK-Ay mice with a high fat diet supplemented with 2% fenugreek ameliorated diabetes. Moreover, fenugreek miniaturized the adipocytes and increased the mRNA expression levels of differentiation-related genes in adipose tissues. In another report (Uemura et al., 2011) they fed KK-Ay mice with high-fat diet containing 0.5% or 2% fenugreek for 4 week and observed significantly lower mRNA expression level of SREBP-1c, FAS, SCD-1, ACC, and GPAT in the liver. The mRNA expression levels of CPT-1 and ACO, not affected by fenugreek and they also reported that the other active components of fenugreek, diosgenin also significantly lowered the mRNA expression level of SREBP-1c, FAS, SCD-1, ACC, GPAT, and also inhibited TG accumulation in HepG2 cell line. Sato et al. (2014) concluded that the blood glucose level was significantly decreased 90–180 min after diosgenin injection, relative to the control group and diosgenin and 5 α reductase inhibitor group. However, injection of diosgenin with the 5 α reductase inhibitor suppressed the diosgenin-induced decrease in blood glucose level in diosgenin injection rats than in STZ control rats. The effect of diosgenin was attenuated in the 5 α reductase inhibito rats. Kalailingam et al. (2014) fed diosgenin (10 mg/kg bw/day) purified from *Costus igneus* extract to STZ induced diabetic wister male rats for 30 days orally result which stimulated the renewal of β-cells in the pancreas or permitted the recovery of partially destroyed β-cells and stimulates pancreatic insulin secretion.

Effect of methanol extract of *Dioscorea nipponica makino* (DN) @ 2% and 5% in HFD fed to male SD rats for 8 weeks showed significant decrease in body weight gain in 5% fed group, and fecal fat excretion was significantly higher in both 2% and 5% DN fed groups. They further purified the DN extract and showed that the extract contained dioscin and its aglycon diosgenin. They also measured porcine pancreatic lipase activity with 4-methylumbelliferyl oleate as a substrate. Diosgenin inhibited the lipase activity in dose dependent manner (Kwon et al., 2003). Son and coworkers (2007), was given diosgenin @ 0.1% and 0.5% in high cholesterol diet to SD rats for 6 weeks and the observed decrease in the plasma and hepatic cholesterol levels and increase in HDL cholesterol level and also increase in oxidative enzymes was also observed in 0.5% diosgenin group. Diabetic male wister rats were fed diet supplemented with 1% bitter yam extract and diosgenin (Sigma) for three weeks shows significantly decreased blood glucose levels towards normal and also alters the intestinal morphology and nuclear dimensions which may be beneficial in the dietary management of diabetes (Mc Anuff et al.,

2003). In another study same workers reported that significantly lowered plasma glucose and total cholesterol in diabetic rats fed bitter yam and diosgenin in diet (Mc Anuff et al., 2002).

Huang et al., (2012) conducted OVA challenge & experimentation made BALB/c mice studied effects of diosgenin. The results showed that diminished LAB density induced by repeated OVA challenges was markedly restored by diosgenin administration. They also isolated *Lactobacillus murinus, L. reuteri, Enterococcus hirae and Enterococcus faecium* from the faeces of diosgenin-administered mice and cultured in MRS broth alone or supplemented with ethanol (1%; as VH control), and/or diosgenin (0.2–2 mM), solasodine, hecogenin, sarsasapogenin, stigmasterol (1 mM of each) or FOS (1 mM) for 0–72 h. and observed that diosgenin (0.4–2.0 mM), in a concentration-dependent manner, enhanced significantly the growth of *L. murinus* and *L. reuteri,* as compared to the VH control. They concluded that diosgenin, a steroidal sapogenin has prebiotic potential in both *in vivo* and *in vitro.*

2.4 ADIPOSE TISSUE- AN ENDOCRINE GLAND

Adipose tissue is the most abundant tissue in humans, representing approximately 10–29% of body weight. It consists of two major forms, white adipose tissue (WAT) and brown adipose tissue (BAT). WAT is the main site for the storage of energy. It stores energy in the form of triglycerides which are located in large lipid droplets. They occupy most of intracellular space of many adipocytes. BAT usually consumes energy to produce heat by catabolizing lipids. Thus brown adipocytes store fewer triglycerides within small lipid droplets (Serrero et al., 1996). The important endocrine function of adipose tissue is emphasized by the adverse metabolic consequences of both adipose tissue excess and deficiency. Adipose tissue excess or obesity, particularly in the visceral compartment, is associated with insulin resistance, hyperglycemia, dyslipidemia, hypertension, and prothrombotic and proinflammatory states.

The efficiency of fat deposition is affected by local secretion of adipocyte-derived factors. Studies suggest that these factors influence insulin signaling, decrease lipogenic gene expression, increase lipolysis, control blood flow to organs, and also regulate intracellular triglyceride synthesis (Chu et al., 2001).

2.4.1 Adiponectin

Adiponectin is a peptide hormone (224 amino acids) produced almost exclusively in adipose tissue. It circulates in the blood and powerfully affects the metabolism of fatty acids and

carbohydrates in liver and muscle via two specific receptors (AdipoR1 and AdipoR2). AdipoR1 is ubiquitously expressed with the highest expression found in skeletal muscle, whereas AdipoR2 is mainly expressed in liver (Yamauchi et al., 2003). Adiponectin increases the uptake of fatty acids from the blood by myocytes and the rate at which fatty acids undergo β-oxidation in the muscle. It also blocks fatty acid synthesis and gluconeogenesis in hepatocytes, and it stimulates glucose uptake and catabolism in muscle and liver. It is well established that circulating adiponectin levels are reduced in patients who are obese and insulin-resistant (Whitehead et al., 2006). Recombinant adiponectin administration in diabetic rodents improves insulin sensitivity (Yamauchi et al., 2001).

2.4.2 Leptin

One of the most important hormone of adipose tissue is leptin, a peptide hormone composed of 167 amino acids that is secreted by adipose tissue as an indicator of body energy status and moves through the blood to the brain, where it acts on receptors in the hypothalamus to curtail appetite. Leptin carries the message that fat reserves are sufficient, and it promotes a reduction in fuel intake and increased expenditure of energy. Leptin receptor interaction in the hypothalamus alters the release of neuronal signals to the region of the brain that affects appetite. Exogenous administration of leptin to rodents decreases body weight, body fat, and food intake, and increases energy expenditure (Pelleymounter et al., 1995; Halaas et al., 1995).

Leptin regulates food intake and energy expenditure primarily through inhibition of NPY/AgRP neurons and direct stimulation of POMC/CART neurons as well as the disinhibition of POMC/CART neurons by decreasing GABA release by NPY/AgRP neurons (Cowley et al., 2001). Neurons expressing the leptin receptor in the nucleus tractus solitarius also seem to play a role in regulating energy balance (Schwartz et al., 2000).

2.5 GASTROINTESTINAL SIGNALS VS. OBESITY

The gastrointestinal tract is body's largest endocrine organ and releases more than 20 different regulatory peptide hormones that influence a number of physiological processes and act on tissues including exocrine glands, smooth muscle and the peripheral nervous system. The dietary macronutrient composition can influence concentrations of gastrointestinal hormones, which could impact sensations of hunger, satiety, and ultimately energy intake. Satiety signals are those arising from the GI tract and related organs during a meal. These signals influence

eating behavior by activating neurons in the nucleus of the solitary tract (NTS) in the hindbrain. Although there are minor variations in the exact pattern, most satiety signals interact with specific receptors on peripheral nerves passing from the GI tract to the hind brain, especially the vagus nerves or else circulate to the hindbrain via the blood and interact with local receptors there. Of particular interest, with respect to weight loss, are the L-cell-derived anorexigenic peptides and peptide YY (PYY), and the X/A-like cell-derived orexigenic peptide ghrelin (Parnell and Reimer, 2012).

In addition to digesting and assimilating nutrients, gastrointestinal (GI) tract plays a key role in sensing and signaling the status of energy to the brain. Several decades of research have identified the key role played by the gastrointestinal peptide hormones (satiety signals) in the control of food intake and hunger/satiety (Woods, 2013). Ghrelin secretedfrom the stomach stimulates food intake while cholecystokinin (CCK), peptide tyrosine tyrosine (PYY) and glucagon like peptide-1 (GLP-1) from the intestine signal satiety and inhibit food intake (Del Prete et al., 2012). It is postulated that the satiating effects of dietary fibre are mediated at least in part by these gut hormones since, for example, FOS feeding is associated with increased circulating GLP-1 and decreased ghrelin in rats (Cani et al., 2004), and oat beta glucan increases PYY secretion in diet-induced obese mice. Short term alterations in energy status are communicated to brain through satiety signals from the GI tract. Understanding the biochemical mechanismsby which fibers modulate satiety or glucose or lipid metabolism is essential to propose a key nutritional advice for specific disorders associated with metabolic syndromes.

2.5.1 Glucagon like peptide-1

The regulation of energy homeostasis requires a specific detection of nutrients by specialized cells of the enteric area when absorbed by the digestive tract. The first actor of energy sensing is the intestinal epithelium where numerous endocrine cells are located. L-cells are second most abundant population of endocrine cells in the human intestine exceeded only by the population of enterochromaffin cells. A high abundance of L-cells is present in the colon (Moody, 1980; Bryant et al., 1983; Sjolund et al., 1983; Eissele et al., 1992). GLP-1 is a key hormone secreted from enteroendocrine L-cells in response to nutrient ingestion (Orskov et al., 1986). It is produced by tissue-specific posttranslational processing of its precursor proglucagon peptide by prohormone convertase-1 enzyme (Dhanvantari et al., 1996), and is released in proportion to the amount of food consumed. GLP-1 plasma levels are reduced in obesity

(Vilsboll et al., 2001) but the mechanism/s leading to reduced GLP-1 secretion in obesity are not explored as yet.It slows down gastric-emptying, reduces glucagon secretion,acts as an insulin secretagogue (Baggio and Drucker, 2007; Massimino et al., 1998; Reimer and Mc Burney, 1996), promotes satiety and β-cell proliferation in the pancreas (Brubaker and Drucker, 2004; Holst, 2004; Knauf, 2005). Recent studies point to the potential importance of GLP-1 in salvaging pancreatic insulin secreting cells (Kim et al., 2008).These actions render GLP-1 highly attractive therapeutic agent, but a rapid enzymatic degradation of the molecule by dipeptidyl peptidase-IV (DPP-IV) makes it unsuitable for injection (Deacon et al., 1998; Meier et al., 2004). Therefore, two pharmacological strategies are being pursued: the development of DPP-IV resistant analogues of GLP-1 and the development of DPP-IV inhibitors. A way to promote endogenous GLP-1 secretion or stability would be useful in that context. It is synthesized in two forms, GLP-1 (1-37) and GLP-1 (1-36) amide which are then subjected to cleavage of first six amino acids to release the active forms, GLP-1 (7-36) amide and GLP-1 (7-37). The beneficial effects of OFS (improved glucose tolerance, insulin secretion and satiety both in animals and humans) are often associated with higher plasma GLP-1 content (Yamashita et al., 1984; Piche et al., 2003; Cani et al., 2004; 2005a& b, 2006b). These effects of OFS were abolished when given in diet to GLP-1 receptor knockout mice, as well as in mice chronically treated with GLP-1R antagonist (exendin 9-39) (Cani et al., 2006a).Thus, nutritional strategies represent alternatives to pharmaceutical approaches for reducing hyperglycemia and body weight.

2.5.2 Peptide YY

The two main endogenous forms of PYY are PYY1-36 and PYY3-36 (Grandt et al., 1994), the latter of which is produced when the first two amino acids at the N-terminus of PYY are removed during post-translational processing (Medeiros and Turner., 1994). Both forms are biologically active (Batterham et al., 2003), but PYY3-36 has high affinity for the inhibitory Y2R, where it acts as an agonist. PYY is secreted by endocrine cells in the lining of the small intestine and colon in response to food. The level of PYY3–36 in the blood rises after a meal and remains high for some hours. PYY3-36 primarily inhibits food intake through its ability to reduce NPY secretion in the ARC. The intermittent intravenous administration of $PYY_{3–36}$ can cause long-term reductions in food intake, body weight and adiposity in rats. However, dosage pattern seems to be crucial in producing sustained reductions in food intake and body weight (Chelikani et al., 2006). Injection of PYY3−36 directly into the ARC, however, inhibits feeding

in rats (Batterham et al., 2002). Peripheral administration of PYY3−36 also reduces food intake in rodents, rabbits, monkeys, and humans (Sileno et al., 2006).

2.5.3 Ghrelin

The gene coding for human prepro-ghrelin, *GHRL*, is located on chromosome 3, consists of 117 amino acids, and the mature ghrelin peptide is constituted of 28 amino acids. It is primarily released in the stomach, and also released by other peripheral tissues, such as the gastrointestinal tract, pancreas, ovary and adrenal cortex (Klok et al., 2007). The secretion of ghrelin by the stomach depends largely on the nutritional state, increases in pre-prandial and decreases in postprandial (Cummings et al., 2001; Ariyasu et al., 2001; Tschop et al., 2001). The effects of ghrelin on energy balance are mediated by the hypothalamus. Korbonits et al. (2001) proposed three different pathways for the appetite inducing effects of ghrelin. First, after release into the bloodstream by the stomach, ghrelin may cross the blood brain barrior and bind to its receptors in the hypothalamus. Second, ghrelin may reach the brain through the vagal nerve and nucleus tractus solitarus. Third, ghrelin is produced locally in the hypothalamus, where it may directly affect the various hypothalamic nuclei. Ghrelin attenuates leptin-induced reduction in food intake and body weight by modulating the expression of various hypothalamic peptides. Thus ghrelin stimulates the food intake by stimulating the activity of neurones expressing NPY, AgRP (agouti-related peptide) and orexin (Nakazato et al., 2001; Kamegai et al., 2001) and inhibitory effect on POMC and CART neurons (Cowley et al., 2003). Its orexigenic effects on food intake, ghrelin is decreased in positive energy balance (Tschop et al., 2001) and increased in the circulation of mice and rats in negative energy balance (Tschop et al., 2000; Cummings et al., 2001).

2.6 KEY GENES/ ENZYMES OF CARBOHYDRATE METABOLISM

Obese and type2diabetic patients, insulin fails to suppress hepatic glucose output, which leads to hyperglycemia via an up regulation of gluconeogenic enzymes glucose 6-phosphatase and phosphoenolpyruvate carboxykinase (Magnusson, et al., 1992). In hepatocytes, glycolysis is involved in the control of hepatic glucose production and dysregulation of this occurs in conditions of insulin deficiency or resistance (Guo et al., 2012). Epigenetic changes such as DNA methylation plays a crucial role in the regulation of glycolytic enzymes in high fat diet induced obesity. Jiang et al. 2011 reported that DNA hypermethylation is correlated with a

decline in hepatic glucokinase and pyruvate kinase expression in high fat diet induced obese rats as compared with the control diet group, suggesting that DNA methylation is involved in the development of metabolic diseases, such as obesity, insulin resistance and type2diabetes mellitus. Another group (Kirchner et al., 2016) conducted experiments in obese and type2 diabetic patients and observed alterations in the hepatic methylome and trancriptome, with hypomethylation of several genes controlling glucose metabolism. Enhancing hepatic glycolysis by over expressing glucokinase and phosphofructokinase reduces body weight and adiposity in obese mice (Wu et al., 2005). Diabetic albino rats were treated with fenugreek seed extract resulted improved glucose homeostasis by reversing the altered glycolytic and gluconeogenic enzyme activities as compared with diabetic control rats (Vats et al., 2003; Raju et al., 2001). Lower doses of vanadate in combination with trigonella restore altered carbohydrate metabolism in alloxan induced diabetic rats (Mohamad et al., 2004).

2.6.1 GLYCOLYTIC ENZYMES

Glycolysis is a first step of metabolic pathway, that extract energy for cellular metabolism from glucose, by splitting it into two three-carbon molecules (pyruvates). Most living organisms carry out glycolysis as a part of their metabolism. It occur in cytosol of cell, under anaerobic conditions. Depending on cell types, rates of glycolysis are determined at various steps of glycolysis that are subjected to the control of key metabolic and regulatory enzyme(s), which include glucokinase, phosphofructokinase, and pyruate kinase. These enzymes are regulated by both nutritional and hormonal signals at the levels of transcription, translation, and post-translational modifications.

Glucokinase is an enzyme that phosphorylates glucose to glucose-6-phosphate in first step of glycolysis, it present in cells of liver, pancreas, gut, and brain. In each of these organs it plays an important role in the regulation of carbohydrate metabolism by acting as a glucose sensor in metabolism or cell function in response to rising or falling levels of glucose, such as occur after a meal or when fasting. Following 48h of starvation, liver glucokinase enzyme activity was reduced nearly by two-thirds and gene expressions nearly zero (Tiedge M and Lenzen S., 1995; Tiedge M and Lenzen S., 1991). Refeeding experiments indicated that the restoration of starvation reduced glucokinase enzyme activity, as well as gene expression, is insulin dependent in the liver (Tiedge M and Lenzen S., 1995). Regulation occurs at several levels and speeds, and is influenced by many factors that affect mainly two general mechanisms,

first glucokinase activity can be amplified or reduced in minutes by actions of the glucokinase regulatory protein (GKRP). The actions of this protein are influenced by small molecules such as glucose and fructose. Secondly, the amount of glucokinase can be increased by synthesis of new protein. Insulin is the principal signal for increased transcription, operating mainly by way of a transcription factor called sterol regulatory element binding protein1c (SREBP1c) except in the liver. This occurs within an hour after a rise in insulin levels, as after carbohydrate meal. Glucagon and its intracellular second messenger cAMP suppress glucokinase transcription and activity, even in the presence of insulin. Glucokinase gene expression has been to shown to be up regulated in response to SREBP-1c over expression (Becard, 2001), other studies have shown that increases in SREBP-1c were neither necessary nor sufficient to induce glukokinase (Hansmannel et al., 2006; Stoeckman A.K and Towel H.C., 2002). Ruiz et al. (2014) in their study they confirms that *in vivo*, SREBP-1c is dispensable for expression of glucokinase gene. Obesity associated insulin resistance is accompanied by impairment in the ability of insulin to increase *in vivo* GK activity (Lam et al., 2003).

One of the most important regulatory enzyme of glycolysis. Because phosphofructokinase (PFK) catalyzes the ATP dependent phosphorylation to convert fructose6phosphate into fructose 1,6-bisphosphate and ADP, it is one of the key regulatory and rate limiting step of glycolysis. PFK is able to regulate glycolysis through allosteric inhibition, and in this way, the cell can increase or decrease the rate of glycolysis in response to the cell’s energy requirements. PFK1 is allosterically inhibited by phosphoenolpyruvate (PEP), citrate, and ATP.

Pyruvate kinase, the enzyme catalyzing the third irreversible step in glycolysis, It catalyzes the transfer of a phosphate group from PEP to ADP, yielding one molecule of pyruvate and one molecule of ATP. Pyruvate kinase is allosterically activated by fructose1,6 bisphosphatse and inactivated by ATP and alanine, glucagon, cyclic AMP and epinephrine.High fat diet feeding decreases the mRNA expression and also the activities of hepatic glucokinase & pyruvate kinase due to hypermethylation of these genes in mice (Jiang et al., 2011).

2.6.2 GLUCONEOGENENSIS

Gluconeogenesis, the synthesis of glucose from noncarbohydrate precursors, occurs in liver, kidney and also in intestine, is subject to dietary and hormonal regulation. It is a key

regulatory gene expressed mainly by liver, kidney and intestine catalyzes the conversion of oxaloacetate to phosphoenolpyruvate and CO_2, in synthesis of glucose from non carbohydrate precursor (gluconeogenesis). Expression of PEPCK is regulated by many factors like type of food and hormones. Rats fed SCFAs ([^{14}C]-propionate, butyrate) and FOS enriched diets increases both mRNA and protein levels of phosphoenolpyruvate carboxykinase and [^{14}C]-propionate carbons incorporated into newly synthesized glucose. [^{14}C]-glucose specific activity after a 24 hr fast was significantly higher in portal venous blood than in arterial blood suggesting that the small intestine is able to efficiently convert propionate into glucose (Vadder et al., 2014).

Fructose 1,6-bisphosphatase is an enzyme that catalyses one of the irreversible step in gluconeogenesis. It converts fructose 1,6-bisphosphate to fructose 6-phosphate. Feeding high fat diet to NZC mice for 12 days resulted in increased activity and protein levels of hepatic FBP (Andrikopolous and proietto 1995) this has also confirmed in rats fed high fat diets (Song et al 2001). In another report, transgenic mice with a specific upregulation of human hepatic FBP in liver had increased glycerol gluconeogenesis (Lamont et al., 2006). Under obese or high fat diet circumstances, FBP may contribute significantly to increased hepatic glucose production.

2.7 KEY GENES RELATED TO LIPID METABOLISM

2.7.1 Sterol Regulatory Element Binding Proteins (SREBPs)

Sterol regulatory element-binding proteins (SREBPs) are transcription factors that bind to the sterol regulatory element DNA sequence. Inactivated SREBPs are attached to endoplasmic reticulum membranes. In cells with low levels of sterols, SREBPs are cleaved to a water-soluble N-terminal domain that is translocated to the nucleus. These activated SREBPs then bind to specific sterol regulatory element DNA sequences, thus up regulating the synthesis of enzymes involved in sterol biosynthesis. Mammalian genomes have two separate SREBP genes (SREBF1 and SREBF2). SREBP-1 expression produces two different isoforms, SREBP-1a and -1c. SREBP-1 is responsible for regulating the genes required for *de novo* lipogenesis. SREBP-2 regulates the genes of cholesterol metabolism.

The levels of SREBP 1 are significantly elevated in obese patients and in animal models of obesity and type2 diabetes, transcription factor that causes hepatic lipid accumulation and insulin resistance. In normal animals, SREBP 1 deficiency increased PEPCK1 (gluconeogenic enzyme) and reduced glycogen deposition during fed conditions (Ruiz et al., 2014). Han et al.

(2015) studied that feeding of high fat/cholesterol (46% calories fat) to C57BL/6J male mice for 24 weeks increase SREBP-1 protien expression by contrast, cereal dietary fiber (OatWell, wheat bran) supplimention reversed this effect. The primary rat hepatocytesd treated with black bean seed coat extract, flavonoid or saponin rich fraction (100 μM) and/or T0901317, a high affinity LXR agonist (10 μM) was added and the hepatocytes were stimulated for 24h for each treatments significantly reduced expression of SREBP-1c,FAS and HMGCR (Chavez-Santoscoy et al., 2014). In another study, Rideout et al. (2010), conducted by feeding control diet(AIN 76A) with 2% plant sterol for 6 weeks reduced plasma and hepatic triglyceride concentration by increasing hepatic lipogenic gene expression SREBP-1c (2.4 fold of control).

2.7.2 Peroxisome proliferator-activated receptors (PPARs)

PPARs are nuclear receptors that belong to the family of transcription factors, whose functions are similar to those of steroid receptors. PPARs respond to changes in dietary lipid by altering the expression of genes involved in fat and carbohydrate metabolism. These transcription factors were first recognized for their roles in peroxisome synthesis, thus their name. Their normal ligands are fatty acids or fatty acid derivatives, but they can also bind synthetic agonists and can be activated in the laboratory by genetic manipulation. They act in the nucleus by forming heterodimers with another nuclear receptor, RXR (retinoid X receptor), binding to regulatory regions of DNA near the genes under their control and changing the rate of transcription of those genes. Evans et al. 2004 have summarized how different PPARs play important roles in integration of metabolism through their coordination activities in different tissues (Fig. 2.3). The three PPAR family members have distinct patterns of tissue distribution.

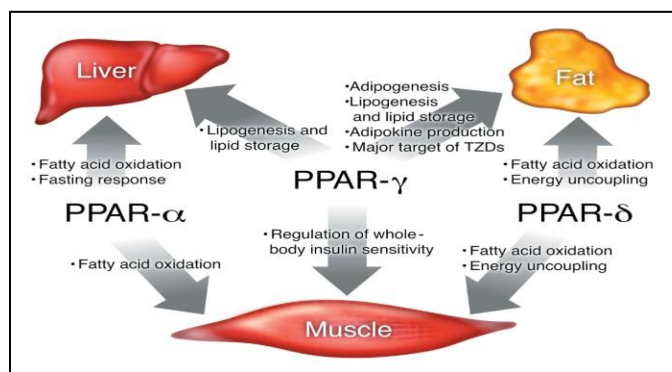


Fig 2.3. Regulation of lipid and glucose homeostasis by PPAR in liver, muscle and adipose tissue (Evans et al., 2004).

2.7.2.1 PPARα

The first genetic sensor for fats was identified in the early 1990s and termed the peroxisome proliferator–activated receptor-α (PPAR-α) because of its ability to bind chemicals known to induce peroxisome proliferation (Issemann and Green., 1990). PPAR-α is expressed predominantly in liver and, to a lesser extent, in heart and muscle, where it has a crucial role in controlling fatty acid oxidation (Reddy and Hashimoto, 2001). An overnight or prolonged fast, adipose tissue released fatty acids are transported to the liver, where PPAR-α is induced and causes the activation of PPAR-α promotes hepatic fatty acid oxidation to generate ketone bodies, providing an energy source for peripheral tissues. PPAR-α-null mice are unable to meet energy demands during fasting and suffer from hypoglycemia, hyperlipidemia, hypoketonemia and fatty liver (Kersten et al., 1999). The induction of fatty acid oxidation by PPAR-α activation improves plasma lipid profiles. In a variety of mouse models, PPAR-α agonists lower plasma triglycerides, reduce adiposity and improve hepatic and muscle steatosis, consequently improving insulin sensitivity (Guerre-Millo et al., 2000)**.**

2.7.2.2 PPARγ

PPARγ is abundant in adipose tissue and is present in low concentrations in the skeletal muscle of humans, and is a master regulator in the formation of fat cells. It is induced during adipocyte differentiation, and forced expression of PPAR-γ in nonadipogenic cells effectively converts them into mature adipocytes (Tontonoz et al., 1994). PPAR-γ knockout mice fail to develop adipose tissue (Rosen et al., 2002; Kubota et al., 1999). Humans with dominant-negative mutations in a single allele *PPARG* (the gene encoding PPAR-γ) have partial lipodystrophy and insulin resistance (Agarwal et al*.,* 2002; Hegele et al., 2002; Savage et al., 2003). *In vitro* studies suggest that the ultimate effector of adipogenesis is PPAR- γ in a transcriptional cascade and also involves members of the C/EBP transcription factor family (Wu et al., 1999; Rosen et al., 2002). Activation of PPAR-γ in adipose tissue improves its ability to store lipids, thereby reducing lipotoxicity in muscle and liver. This model involves activation of genes encoding molecules that promote a combination of lipid storage and lipogenesis, such as aP2 (fatty-acid binding protein), CD36 (receptor for lipoproteins), lipoprotein lipase (hydrolysis of lipoproteins), FATP-1 (fatty acid transporter), glycerol kinase, and SREBP-1 and SCD-1 (regulators of sterol and fatty acid synthesis, respectively). Activation of this metabolic pathway causes body-wide lipid repartitioning by increasing the triglyceride content of adipose tissue and lowering free fatty

acids and triglycerides in the circulation, liver and muscle, thereby improving insulin sensitivity (Yamauchi et al., 2001; Guan et al., 2002). Dewulf et al. (2011) fed inulin type fructans (0.2g/day per mouse) along with high fed diet to C57BL/6J mice for 4 weeks. They observed that PPAR γ expression was not modified by high fat diet but inulin type fructans tended to decrease its expression in comparison to HF group.

2.7.3 Carnitine palmitoyltransferase 1 (CPT1)

CPT 1 is a mitochandrial enzyme related to fat oxidation, it transfers an acyl group from CoA to carnitine, which is then translocated into mitochandria for fatty acid oxidation. Three isoforms of CPT1 are currently known: CPT1A, CPT1B, and CPT1C. CPT1 is associated with the outer mitochondrial membrane. This enzyme can be inhibited by malonyl CoA, the first committed intermediate produced during fatty acid synthesis. Its role in fatty acid metabolism makes CPT1 important in many metabolic disorders. Nomaguchi et al. (2011) in their study C57BL/6J mice fed high fat diet for 4 weeks after they were orally administered 1 ml (1µg/day) aloe vera phytosterol for 12 weeks and they were observed that expression levels of fatty acid oxidation genes (Cpt 1a, Cpt2, Acaa 1a, Acox 1,Cyp4a10 and cyp4a 14) were significantly higher in Aloe vera group than in the control HFD group. The primary rat hepatocytes, FSE, the flavonoid or saponin rich fraction (100 µM) and/or T0901317, a high affinity LXR agonist (10 µM) was added and the hepatocytes were stimulated for 24h for each treatments, resulted that FSE had no effect on the expression of CPT1. However, it inhibited the suppressive effect of T0901317 on genes in primary hepatocyt culture (Chavez-Santoscoy et al., 2014).

2.8 Tight junction proteins

The intestinal epithelium is formed by a single layer of epithelial cells that separates the intestinal lumen from the underlying lamina propria. The space between these cells is sealed by tight junctions (TJ), are complex protein structures comprised of transmembrane proteins such as claudin, occludin and tricullulin, which regulate the permeability of the intestinal barrier. Bischoff et al., (2014) reported that intestinal barrier prevents against loss of water and electrolytes and entry of antigens and microorganisms into the body while allowing exchange of molecules between host and environment and absorption of nutrients in the diet.

2.8.1 Occludin

The first TJ integral membrane protein identified was occludin, expressed predominately

at TJs in epithelial and endothelial cells, but also by astrocytes, neurons and dendritic cells. Occludin (60–82 kDa) is a tetraspanning integral membrane protein with two extracellular loops, a short cytoplasmic N-terminus and a long cytoplasmic C-terminus. The extracellular loops and transmembrane domains of occludin regulate selective paracellular permeability. Intracellularly, the C-terminus interacts with the PDZ-domain containing protein ZO-1, which is required to link occludin to the actin cytoskeleton (Katherine and Hogan, 2009). The study conducted by Cani and group (2008), C57BL/6J mice were fed high diet (72% fat from lard and corn oil) for 4 weeks shows a reduced expression of tight junction genes occludin and ZO1which reflects increased intestinal permiability as compared to control. In another study the C57BL/6J mice were fed control diet containing fermentable dietary fiber(oligofructose) or diet containing non-fermentable dietary fiber (cellulose) resulted that prebiotic treatment increased ZO1 and occludin mRNA (Cani et al., 2009).

2.8.2 Zonula occludens (ZO1)

The ZO proteins were the first TJ-specific proteins identified and three ZO proteins, ZO-1, -2, and -3, have been identified to date and they are membrane associated proteins. The multi-domain structures of ZO, provide an intracellular scaffold in the TJs and are required for regulation and maintenance of TJ structure. Interestingly, many TJ proteins bind to the N-terminal half region of ZO proteins, while the C-terminal region interacts with the actin cytoskeleton and cytoskeleton-associated proteins. Among the ZO proteins, the biochemical function and property of ZO-1 have been well examined. ZO proteins may mediate the early assembly of TJ proteins into cell-cell contacts. To data, intensive efforts have been made to clarify the functional role of ZO proteins, but it has been difficult to obtain clear evidence showing the importance of ZO proteins in TJ regulation. Recent studies have been shown that ZO-1 deficient cells are still able to form normal TJ structures and show normal permeability; however, an obvious delay in the assembly of other TJ proteins including occludin and claudins into the TJ is observed, indicating that the ZO proteins have an important role in the regulation of TJ assembly (Lee, 2015). Evidence from the study of Lam and collaborators (2012) suggested that high fat diet feeding (60%kcal from fat) to female C57BL/6J mice for 12 weeks decreased the mRNA levels of ZO 1 gene in proximal colon as compared to control group fed 10%kcal from fat. Neyrinck et al, (2012) suggested that feeding high fat diet supplemented with arabinoxylan oligosaccharides to C57BL/6J mice reduced the decrease of tight junction proteins

occludin and ZO1 gene expression as compared to high fat diet.

2.9 Inflammatory markers

Obesity generates a state of low-grade inflammation and is associated with the development of type 2 diabetes. MCP1 and TNFα are the most important pro-inflammatory markers.

2.9.1 MCP-1

Monocyte chemoattractant protein-1 (MCP-1) is a potent chemoattractant for monocytes and macrophages in the areas of inflammation (Melgarejo et al., 2009). It is a member of beta chemokine family and is secreted by fibroblasts, endothelial cells, vascular smooth muscle cells, monocytes, T cells, and other cell types that mediate the influx of cells to sites of inflammation (Conti and DiGioacchino, 2001). It triggers chemotaxis and transendothelial migration of monocytes to inflammatory lesions by interacting with the membrane CC chemokine receptor 2 (CCR2) in monocytes (O'Hayre et al., 2008). MCP-1 expression has been observed in a large number of tissues during progression of inflammation-dependent diseases (Shin et al., 2002; Taylor et al., 2000; O'Hayre et al., 2008). Thus, the expression of MCP-1, which is likely to be critical for fighting infectious diseases, must be tightly regulated.

Obesity is a result of expansion in both number and size of adipocytes. The gene expression of CC chemokines and their receptors (such as MCP-1 and CCR2) was found higher in the visceral and subcutaneous adipose tissues of obese patients compared to lean controls (Huber et al., 2008). MCP-1 signaling has a direct role in the development of obesity. For example, Younce et al., (2009) reported that MCP-1- induced protein (MCPIP, a zinc finger protein) induced adipogenesis in 3T3-L1 cells independent of PPAR gamma activation. Weisberg et al. (2006) reported mice lacking with CCR2 (a receptor for MCP-1) had attenuated deposition of visceral fat and insulin resistance when challenged with a high fat diet and they also suggested that the MCP-1/CCR2 pathway to influence the development of obesity and insulin resistance via adipose macrophage accumulation and inflammation. Transgenic mice by utilizing the adipocyteP2 promoter (aP2-MCP-1 mice) showed greater macrophage accumulation in adipose tissue, and the aggregated macrophages were more readily apparent when the transgenic mice were fed high fat diet (Kamei et al., 2006).

‘

2.9.2 TNFα

The proinflammatory cytokine tumor necrosis factor (TNF) plays an important role in diverse cellular events, including cell proliferation, differentiation and apoptosis. TNF is also involved in many types of diseases (Zheng-gang LIU., 2005). The infiltration of adipose tissue by macrophages is believed to lead to increased production of inflammatory mediators that may contribute to the pathogenesis of insulin resistance, including IL-1β, TNF-α, and IL-6 (Wellen and Hotamisligil, 2005). Several hypotheses have been proposed to explain how TNF- α induces insulin resistance in adipocytes. For example, TNF-α inhibited insulin-stimulated IRS-1 phosphorylation. Thus, it might inhibit PI3K and inhibit a pathway that regulates glucose uptake (Medina et al., 2005). In addition, TNF- α up regulates transcription of many preadipocyte genes and proinflammatory cytokines, such as IL-6 and MCP-1. These proteins were elevated in the plasma or adipose tissue of diabetic patients. TNF-α also inhibited adiponectin expression, which may impair insulin action. Furthermore, TNF- α has been reported to directly stimulate lipolysis, which caused increase in plasma free fatty acids (Kern et al., 2003). Three potential sites of TNFα action that might mediate insulin resistance are: regulation of free fatty acid level, leptin production, glucose transporters numbers and insulin sensitivity (Uysal et al., 1997; Uysal et al., 1998; Kirchgessner et al., 1997).

CHAPTER - 3

Materials and Methods

3.0 MATERIALS AND METHODS

3.1 CHEMICALS

RNA*later*®, diethyl pyrocarbonate (DEPC), RNaseZAP™, TRI Reagent®, ethidium bromide, agarose, PCR primers, *Taq* DNA polymerase, phosphoglucoisomerase, dGDP were procured from M/s Sigma-Aldrich. Ultrasensitive mouse insulin ELISA kit was product of Crystal Chem. Nuclease free water, GeneRuler (100 bp) DNA ladder, 6X DNA loading dye, dNTPs, RevertAid first strand cDNA synthesis kit and Maxima SYBR Green qPCR Master Mix (2X) (products of Thermo scientific) were procured from Genetix Asia Pvt. Ltd, New Delhi. Glucose strips used for blood glucose analysis (Accu-Check® active) were product of Roche diagnostics. Diethyl ether, potassium chloride, chloroform, isopropanol, NADP, NADH, glucose 6- phosphate, fructose 1, 6-bisphosphate, phosphoenolpyruvate, malate dehydrogenase, glucose 6-phosphate dehydrogenase, lactate dehydrogenase were purchased from SISCO Research laboratories Pvt, Ltd. Sodium hydroxide pellets and di-sodium hydrogen phosphate were procured from Merck specialities Pvt. Ltd. Lipid analysis kits (based on enzymatic colorimetric method) were purchased from Recombigen laboratories Pvt Ltd., New Delhi. Vitamins and salts for mineral mixture were purchased from CDH, New Delhi. Casein was procured from modern dairy plant, Karnal and maltodextrin (D.E 11.60) was procured from Sukhjit Agro Industries, Himachal Pradesh. Branded refined soybean oil, vitamins A, D & E, corn starch and sugar were purchased from local market. Lard incorporated in diet was purchased from MP Biomedicals (India) Pvt. Ltd. All other chemicals used in the study were of analytical grade (AR) or equivalent.

3.2 SOLUBLE FIBER AND DIOSGENIN

Fenugreek galactomannan (80% pure) was purchased from Parry Nutraceuticals Division of E.I.D. Parry (India) Ltd, Chennai. Locust bean galactomannan (82-85% pure) was purchased from Advance Inorganics, Delhi. Each soluble fiber was incorporated in the experimental diet @ 5% (w/w). Diosgenin (≥93% pure) was procured from M/s Sigma-Aldrich and incorporated in the experimental diet @ 0.5% (w/w).

3.3 ANIMALS

C57BL/6 male mice, were purchased from Indian Institute of Integrative Medicine (IIIM), Jammu.

3.4 TO STUDY THE EFFECTS OF GALACTOMANNAN AND DIOSGENIN ON PROGRESSION OF OBESITY IN HIGH FAT DIET FED MICE

All animal procedures were conducted at Small Animal House of Institute accordance with guidelines of Institutional Animal Ethics Committee (IAEC). Animals were housed in ventilated plastic cages with soft husk bedding under 12 h light/dark conditions at controlled room temperature (22 ± 2 ^{o}C). These conditions were maintained throughout the study period. The animals were acclimatized for two weeks during which they were fed on normal chow *ad libitum* and had free access to water.

3.4.1 Grouping of animals and experimental diets

After acclimatization period, the animals were randomized according to their body weights divided into seven groups (n=9/group, housed individually) and fed on respective experimental diets (Table 3.1). The composition of experimental diets is given Table 3.2.

Table 3.1 Grouping of animals

Group	Diet
GROUP I	Normal Diet (Control-Ctrl)
GROUP II	High Fat Diet (HFD)
GROUP III	HFD-Fenugreek galactomannan (HFD-FGM)
GROUP IV	HFD-Locust bean galactomannan(HFD-LGM)
GROUP V	HFD-Diosgenin(HFD-D)
GROUP VI	HFD -Fenugreek galactomannan-diosgenin (HFD- FGMD)
GROUP VII	HFD-Locust bean galactomannan-diosgenin (HFD- LGMD)

3.4.2 Feeding schedule

Animals in different treatment groups were fed respective diets *ad libitum* and had free access to water. The feeding schedule was followed for 18 weeks. Four animals from each group were sacrificed after 6 weeks of dietary treatments while remaining animals were continued on their respective diets for 18 weeks, and thereafter, sacrificed by cervical dislocation under diethyl ether anesthesia.

Table 3.2 Composition of experimental diets

Ingredient g/100 g	Control	HFD	HFD-FGM/ HFD-LGM	HFD-D	HFD-FGMD/ HFD-LGMD
Casein[1]	19.5	26.5	26.5	26.5	26.5
L-Methionine	0.3	0.4	0.4	0.4	0.4
Sucrose	12.0	9.4	8.15	9.28	8.02
Corn starch	53.3	-	-	-	-
Maltodextrrin[2]	-	17.2	17.2	17.2	17.2
Soyabean oil	5	3.2	3.2	3.2	3.2
Fat (Lard)[3]	-	31.8	31.8	31.8	31.8
Galactomannan[4]	-	-	5.0	-	5.0
Diosgenin[5]	-	-	-	0.5	0.5
Cellulose	5	6.57	2.85	6.22	2.48
Mineral mixture (AIN-76)	3.5	3.5	3.5	3.5	3.5
Vitamin mixture (Teklad)	1.0	1.0	1.0	1.0	1.0
CaCo3	0.4	0.4	0.4	0.4	0.4
Ethoxyquin	0.001	0.004	0.004	0.004	0.004
Energy information					
Fat (% kcal)	11.67	59.54	59.54	59.54	59.54
Total energy (kcal/100 g diet)	385.4	529.1	529.1	529.1	529.1

[1]Casein was procured from Modern dairy plant, Karnal

[2]Matltodextrin was purchased from Sukhjit Agro Industries, Himachal Pradesh

[3]Lard was purchased from MP Biomedicals (India) Pvt Ltd

[4]Galactomannan was purchased from E.I.D Parry (India) Ltd, Chennai

[5]Diosgenin was procured from Sigma –Aldrich.

3.4.3 Parameters studied

3.4.3.1 Body weight and feed intake

The body weights were taken at weekly intervals, and the food intake was measured daily. The cumulative feed intake by mice in each treatment group was also calculated.

3.4.3.2 Fasting blood glucose

The fasting blood glucose levels were determined at 6 and 18 weeks by puncturing tail vein, using glucometer (ACCU-CHECK® active, Roche Diagnostics, Germany), and the comparisons were made among different groups.

3.4.3.3 Collection of blood and tissue samples

The animals from different groups were sacrificed after 6 and 18 weeks by cervical dislocation under diethyl ether anaesthesia after fasting ~ 6 h. Blood samples were collected in sterile 2.0 ml micro-centrifuge tubes by cardiac puncture. Serum was recovered by keeping blood undisturbed at room temperature for 30 min, followed by centrifugation at 3000 x*g* (HERMLE Labortechnik) for 15 min at 4 °C. The serum obtained was used for measurement of lipid profile and insulin levels.

Liver, spleen, kidney and epididymal fat pad were collected and weighed. A portion of epididymal fat tissue was stored in 10% buffered formalin for histopathological analysis to determine adipocyte size and number. A half portion of liver was washed with 1X phosphate buffer saline (PBS; pH 7.2), soaked in tissue to remove PBS and stored at -80 ^{o}C for enzyme assays. A portion of liver tissue was stored in 1X PBS for estimation of TG, TC and protein. The rest of liver, epididymal fat and distal small intestinal tissues were stored in RNA*later*® at -20 ^{o}C for gene expression analysis.

3.4.3.4 Analysis of serum lipid profile

Serum triglyceride (TG) and total cholesterol (TC) level were estimated using kits (Recombigen laboratories Pvt. Ltd., New Delhi) based on enzymatic reactions as per manufacturer's instructions. HDL-cholesterol was determined enzymatically after precipitation of other lipoproteins such as VLDL-cholesterol, LDL-cholesterol and chylomicrons spectrophotometrically using kit as per manufacturer's instructions (Recombigen laboratories Pvt. Ltd., New Delhi).

(a) Triglycerides

For triglyceride assay, 10 µl of serum or 10 µl of triglyceride standard was mixed

with 1.0 ml of triglyceride reagent (Reagent 1), mixed well and incubated at 37 °C for 10 min. The absorbance was measured at 505 nm. Triglyceride levels were calculated as per formula:

$$\text{Triglycerides (mg/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 200$$

(b) Total Cholesterol

For total cholesterol assay, 10 µl of serum or 10 µl of cholesterol standard (Reagent 3) was mixed with 1.0 ml of Enzyme reagent (Reagent 1), mixed well and incubated at 37 °C for 10 min. The absorbance was measured at 505 nm. Total cholesterol levels were calculated as per formula:

$$\text{Total cholesterol (mg/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 200$$

(c) HDL-Cholesterol

For HDL-cholesterol assay, 100 µl of serum was mixed with 100 µl of Precipitating reagent (Reagent 2), mixed well and centrifuged at 3000-4000 rpm for 10 min and clear supernatant was obtained for HDL-cholesterol estimation. Ten microlitre of supernatant or 10 µl of standard (Reagent 3) was mixed with 1.0 ml of Enzyme reagent (Reagent 1), mixed well and incubated at 37 °C for 10 min. Absorbance was measured at 505 nm. HDL-cholesterol levels were calculated as per formula:

$$\text{HDL-Cholesterol (mg/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 200$$

The Friedewald's equation (Friedewald et al., 1972) was used to calculate LDL-C and expressed as mg/dl:

LDL-cholesterol (mg/dl) = {TC - triglycerides/5} - HDL-cholesterol

VLDL-cholesterol (mg/dl) = Triglycrides/5

(d) Atherogenic Index (AI): was calculated as per formula given below:

$$\text{Atherogenic Index (AI)} = \frac{\text{Total cholesterol- HDL-cholesterol}}{\text{HDL-cholesterol}}$$

3.4.3.5 Analysis of liver lipids

Analysis of liver lipids was done by the method as described by Morton et al. (2005). Liver tissue was homogenized in isopropanol (1:10), and then shaken for 45 min. Supernatant was collected by centrifugation at 3000 x*g* for 10 min and was used for analysis using kits based on enzymatic reactions and measurement of coloured end product spectrophotometrically (Recombigen laboratories Pvt. Ltd., New Delhi), as per instructions of the manufacturer. The procedures followed for assay of TG and TC were same as described under section 3.5.3.4.

3.4.3.6 Estimation of serum insulin

Serum insulin levels were measured by using ultrasensitive mouse insulin ELISA kit (Crystal ChemInc, USA).

Principle

Mouse insulin in the sample is bound to the guinea pig anti-insulin antibody coated on the microplate well. The unbound material is removed by washing. The Horse radish peroxidase (POD)-conjugated anti-insulin antibody is then bound to the guinea pig anti-insulin antibody/mouse insulin complex immobilized to the microplate well. Excess POD-conjugate is removed by washing. The bound POD-conjugate in the microplate well is detected by the addition of 3, 3', 5, 5'-tetramethylbenzidine (TMB) substrate solution. The insulin concentration is determined by interpolation using the standard curve generated by plotting absorbance vs. the corresponding concentration of mouse insulin standard.

Preparation of reagents

Prior to use, all reagents were brought to room temperature (18-25 °C) and stored at 2-8 °C immediately after use. Before use, the reagents were mixed thoroughly by gentle agitation or swirling.

(i) Antibody-coated microplate

Plate (Marked "A") was removed from the sealed foil pouch after the pouch had been equilibrated to room temperature.

(ii) Mouse insulin stock solution

Lyophilized mouse insulin standard (Marked "B") was reconstituted by careful addition

of 100 µl of distilled water to the vial. The vial was inverted gently until the contents were completely dissolved. This stock solution contained 25.6 ng/ml of mouse insulin and was stored at 2-8 °C (stable for one week).

(iii) Anti-insulin enzyme conjugate

For six modules, anti-insulin enzyme conjugate solution was prepared by mixing 3.6 ml of anti-insulin enzyme conjugate stock solution (Marked “C”) with 1.8 ml of enzyme conjugate diluent (Marked “D”) and mixed completely to obtain a homogeneous and clear solution. The anti-insulin enzyme conjugate was prepared just before the second reaction and used immediately.

(iv) Enzyme substrate solution

The enzyme substrate solution (Marked “E”) was provided as a ready-to-use preparation. Once bottle was opened, the solution was stable for one week at 2-8 °C. Exposure of enzyme substrate solution to light was avoided.

(v) Enzyme reaction stop solution (1N H_2SO_4)

This was provided as a ready-to-use preparation (Marked “F”).

(vi) Sample diluent

It was Marked “G” (provided as a ready-to-use preparation). Once the bottle is opened, this solution is stable for one week at 2-8 °C.

Table 3.3 Working mouse insulin standards

	Mouse insulin concentration (ng/ml)							
	6.4	**3.2**	**1.6**	**0.8**	**0.4**	**0.2**	**0.1**	**0**
MISS*(µl)	50	--	--	--	--	--	--	--
SD** (µl)	150	50	50	50	50	50	50	50
	--	50	50	50	50	50	50	--
Total (µl)	200	100	100	100	100	100	100	50

MISS*= Mouse Insulin Stock Solution (25.6 ng/ml)

SD** = Sample Diluent

(vii) Wash buffer

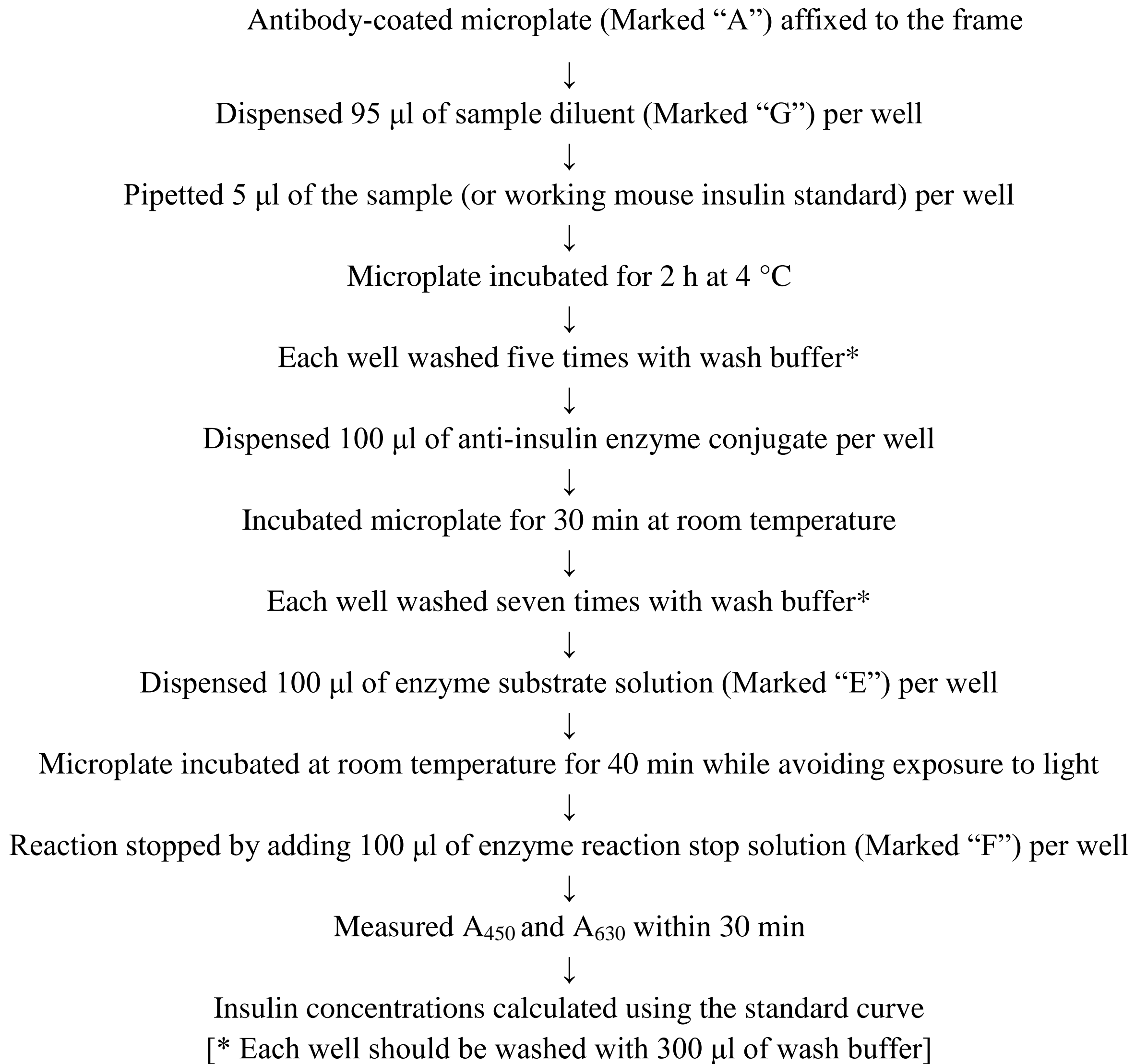
Wash buffer stock solution (Marked ‘H’) was brought to 1 L with distilled water and stored at 2-

8 °C. The solution was mixed well prior to use and was stable for one week at 2-8 °C.

Preparation of working mouse insulin standards

The working mouse insulin standards were prepared as shown in Table 3.3.

Procedure



Mean absorbance values for each set of duplicate standards and samples were determined. The insulin standard curve was constructed by plotting mean absorbance value for each standard on the Y-axis vs. the corresponding standard mouse insulin concentration on the X-axis. The standard curve obtained is presented in Fig 3.1. Mean absorbance values were determined for each set of duplicate samples for all animals in each treatment group and insulin concentration was calculated from standard curve as follows:

Insulin concentration (ng/ml) = concentration obtained from standard curve (ng/ml) X 5/sample volume (µl)

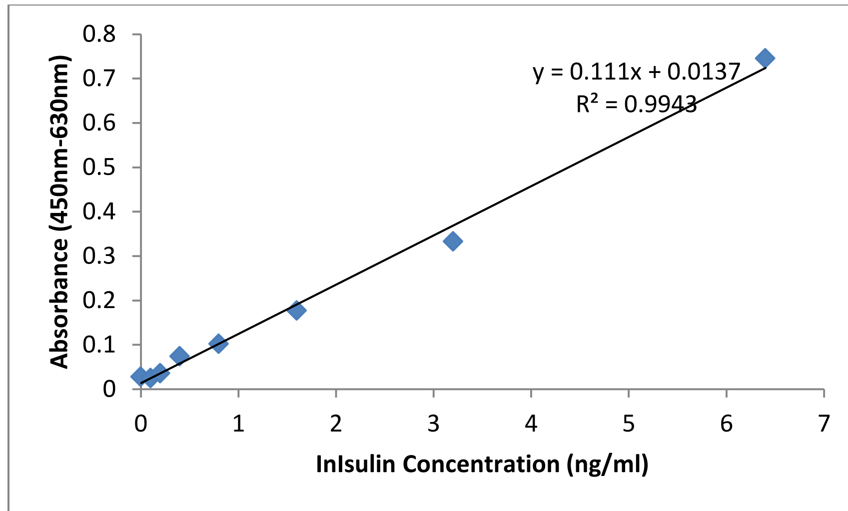


Fig. 3.1 Standard curve for insulin assay

3.4.3.7 HOMA-IR

The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the relation between fasting blood glucose and serum insulin level according to the following equation (Haffner et al., 1997).

$$\text{HOMA-IR} = \frac{\text{fasting insulin } (\mu U/ml) \text{ X fasting blood glucose } (mmol/l)}{22.5}$$

3.4.3.8 Adipocyte size and number

Epididymal fat tissue was removed from animals of all experimental groups, fixed in 10% (v/v) buffered formalin and sent to local pathology lab for further processing. The samples were embedded in paraffin, sectioned (4 µm), and mounted on glass slides. Three replicates were taken for each sample. Hematoxylin-eosin staining was performed using the standard technique. Morphology was examined in bright field microscopy (three fields) under 200x magnification (Olympus BX51 fitted with Olympus DP71 camera). Three epididymal fat tissues per group were analysed. Images were captured and cell size & number were measured using Image J software (National Institutes of Health, Bethesda, MD, USA).

3.4.3.9 Hepatic enzyme activities

The activities of key enzymes of glycolysis and gluconeogenesis in liver were determined by spectrophotometric-coupled enzyme assays.

a. Preparation of liver homogenate

Liver tissues were homogenized (1:10 wt/vol) in appropriate chilled buffers. For assay of glucokinase, phosphofructokinase, fructose 1, 6-bisphosphatase and phosphoenolpyruvate carboxykinase, liver tissue was homogenized in the buffer containing 50 mM triethanolamine (pH 7.2) and 0.1 mM dithiothreotol using tissue homogenizer. The homogenate was centrifuged at 25,000 x*g* for 40 min at 4 ^{o}C. The supernatant was collected and stored at -20 ^{o}C for enzyme assays.

For assay of pyruvate kinase activity, the homogenate was prepared essentially by the method given by Morifuji et al., (2005) with slight modification. Liver tissue was homogenized (1:10 wt/vol) in 0.1 M Tris-HCl buffer (pH 7.4) and the supernatant was obtained after centrifugation at 12,000 x*g* for 1 h at 4 ^{o}C.

The supernatants obtained were used for assay of activities of different enzymes by measuring the change in absorbance at 340 nm/min at 25 ^{o}C and expressed as mU/mg protein.

b. Assays of enzyme activities

i. Glucokinase (GK)

Glucokinase was assayed by the method given by Grossman et al. (1974), with slight modification.

The assay mixture contained in a final volume of 1 ml: 100 mM triethanolamine (pH 7.2), 5 mM $MgCl_2$, 5 mM ATP, 0.4 mM NADP, 100 mM glucose, 0.3 U G6PDH and 100 µl supernatant. Blank was prepared without adding G6PDH and the volume made to 1 ml by adding triethanolamine buffer.

The reaction was started by adding G6PDH. Immediately mixed by inversion, and recorded the increase in A_{340nm} for 3 min at 25 ^{o}C for both test and blank.

ii. Phosphofructokinase (PFK)

Phosphofructokinase was assayed by the method given by Karadsheh et al. (1977), with slight modification.

The assay mixture contained in a final volume of 1 ml: 50 mM triethanolamine (pH 7.2), 2 mM $MgCl_2$, 2.5 mM DTT, 1 mM EDTA, 5 mM $(NH_4)_2SO_4$, 1 mM fructose 6-

phosphate, 1 mM ATP, 0.16 mM NADH, 0.4 U glycerophosphate dehydrogenase, 0.4 U aldolase, 2.4 U triosphosphate isomarase and 100 µl supernatant. Blank was prepared without adding fructose 6-phosphate and the volume made to 1 ml by adding triethanolamine buffer.

The reaction was started by adding fructose 6-phosphate. Immediately mixed by inversion, and recorded the increase in A_{340nm} for 3 min at 25 ^oC for both test and blank.

iii. Pyruvate kinase (PK)

Pyruvate kinase was assayed by following the method given by Harada et al. (1978), with slight modification.

The assay mixture contained in a final volume of 3 ml; 50 mM tris-HCl buffer (pH 7.5), 100 mM KCl, 5 mM $MgSO_4$, 2 mM ADP, 0.18 mM β-NADH, 2mM phosphoenolpyruvate, 24 U lactate dehydrogenase and 0.1 ml supernatant. Blank was prepared without adding PEP and volume made to 3 ml by adding tris-HCl buffer.

The reaction was started by adding PEP. Immediately mixed by inversion, and recorded the decrease in A_{340nm} for 3 min at 25 ^oC for both test and blank.

iv. Fructose 1,6-bisphosphatase

Fructose 1, 6-bisphosphatase was assayed by the method given by Tejwani et al. (1976), with slight modification.

The assay mixture contained in a final volume of 1 ml: 50 mM triethanolamine buffer (pH 7.2), 2 mM $MgCl_2$, 0.1 mM EDTA, 40 mM $(NH_4)_2SO_4$, 0.3 mM NADP, 0.15 mM fructose 1,6-bisphosphate, 0.6 U glucose 6-phosphate dehydrogenase, 2.3 U phosphoglucose isomerise and 100 µl supernatant. Blank was prepared without adding fructose 1,6-bisphosphate, volume made to 1 ml by adding triethanolamine buffer.

The reaction was started by adding fructose 1,6-bisphosphate. Immediately mixed by inversion, and recorded the increase in A_{340nm} for 3 min at 25 ^oC for both test and blank.

v. Phosphoenolpyruvate carboxykinase (PEPCK)

PEPCK was assayed by the following method given by Petrescu et al. (1979), with slight modification.

The assay mixture contained in a final volume of 1 ml: 50 mM triethanolamine buffer (pH 7.2), 1 mM $MnCl_2$, 0.1 mM NADH, 20 mM $NaHCO_3$, 0.5 mM

phosphoenolpyruvate, 0.2 mM deoxyGDP, 2 U malate dehydrogenase and 100 µl supernatant. Blank was prepared without adding $NaHCO_3$, volume made to 1 ml by adding triethanolamine buffer.

The reaction was started by adding $NaHCO_3$. Immediately mixed by inversion, and recorded the decrease in A_{340nm} for 3 min at 25 ^oC for both test and blank.

Calculations of enzyme activity

All the enzyme activities were calculated by using the formula given below:

$$\text{Units/ml supernatant} = \frac{(\Delta A_{340nm}/\text{min Test} - \Delta A_{340nm}/\text{min Blank})(\text{Total assay volume in ml})(\text{df})}{(6.22)(0.1)}$$

df= Dilution factor

6.22= Millimolar extinction coefficient

0.1 = Sample volume used

$$\text{Units/mg protein} = \frac{\text{Units / ml supernatant}}{\text{mg protein / ml supernatant}}$$

Unit of enzyme activity

One unit of enzyme is defined as the amount which catalyses the conversion of 1 µmol substrate/min under the specified conditions

c. Estimation of protein

Protein was estimated in supernatants obtained after centrifugation of liver homogenates according to the Bradford's method (1976).

Reagents:

Preparation of protein reagent:

Coomassie brilliant blue G-250 1(00 mg) was dissolved in 50 ml 95% ethanol to which 100 ml 85% (w/v) phosphoric acid was added. The solution was diluted to the final volume of 1 liter and filtered.

Preparation of standard solution of protein:

A standard solution (2 mg/ml) of Bovine serum albumin (fraction V) was prepared.

Procedure:

Appropriate volumes of supernatants obtained after centrifugation of liver homogenates were added to different test tubes and the final volume was made to 100 µl with distilled water.

Five ml of protein reagent was added in each tube, shaken vigorously and absorbance at 595 nm was measured after 2 min in spectrophotometer (Spectro UV-VIS Double Beam UVD-3500, Labomed, Inc.). Protein concentrations (mg/ml) were calculated using the standard curve prepared with standard solution of bovine serum albumin.

3.5 ANALYSIS OF EXPRESSION PATTERN OF GENES RELATED TO LIPID METABOLISM AS AFFECTED BY GALACTOMANNAN AND DIOSGENIN FEEDING

The relative mRNA expression of selected genes in epididymal adipose tissue, liver and distal small intestine as affected by dietary incorporation of two types of soluble fibers viz., fenugreek galactomannan & locust bean galatcomannan, and diosgenin was studied by Real Time PCR in C57BL/6 mice fed high fat diet for 18 weeks. Relative mRNA abundance was determined with gene specific primers. The details of different steps followed are given below:-

3.5.1 Preparation of reagents

High purity water (Millipore) was made RNase free by adding diethyl pyrocarbonate (DEPC) at the level of 0.1%, stirred for 12 h at room temperature and autoclaved at 15 psi for 15 min to remove traces of DEPC. All reagents for RNA work (75% ethanol for washing RNA pellet and 1x TBE) were prepared in DEPC-treated autoclaved water, and autoclaved if necessary at 15 psi for 15 min. Plastic wares i.e., microtips, microcentrifuge tubes, PCR tubes were made RNase free by overnight soaking and shaking in high purity water containing 0.1% DEPC at room temperature. These were removed, autoclaved and then dried in an incubator at 50 °C. Hands, working platform, and pestle & mortar were made RNase free by spraying RNaseZAP™. RNase-free plasticwares, glasswares and other utilities were stored in clean RNase free working area.

3.5.2 Isolation of total RNA

Epididymal fat/liver/distal small intestine tissue was crushed to powder form in pestle and mortar using liquid nitrogen. Total RNA was isolated using TRI Reagent® method. All the centrifugation steps were performed at 4 °C. One ml of TRI Reagent® was added to 50-100 mg of powdered tissue, vortexed several times by pipetting up and down, and kept at room temperature for 10 min for complete digestion/lysis of the tissue by TRI Reagent®. This was followed by centrifugation at 12,000 x*g* for 10 min at 4 °C to remove excess of fat. The supernatant was collected carefully, and chloroform was added @ 0.2 ml/ml of TRI Reagent®.

This was followed by vortexing vigorously for 15 sec and kept in ice for 10 min. It was then centrifuged at 12,000 x*g* in a refrigerated centrifuge for 15 min. The clear aqueous phase was transferred very carefully to another 1.5 ml microcentrifuge tube and 0.5 ml of chilled isopropanol was added to precipitate the RNA. The contents were mixed gently and incubated again at -20 °C for 15 min, followed by centrifugation at 12,000 x*g* for 15 min. Supernatant was discarded and the RNA pellet obtained was washed with 1 ml of 75% ethanol (prepared in nuclease free water) by centrifugation at 7,500 x*g*, at 4 °C for 5 min. Finally, the RNA pellet was air dried and dissolved in 20 µl of sterile nuclease free water. The RNA samples were stored at -20 °C till further use.

3.5.2.1 RNA quantification and purity determination

Total RNA was quantified by measuring absorbance at 260 nm using nanodrop (NanoQuant M200 pro) and purity was assessed by measuring ratio of absorbance at 260 and 280 nm. The total RNA with A_{260}/A_{280} ratio between 1.7 - 1.9 was acceptable for subsequent use.

3.5.2.2 Integrity of RNA

The integrity of isolated RNA was checked by gel electrophoresis in 1.5% agarose, stained with ethidium bromide using 1x TBE buffer at 100 V for 30 min. Gel was observed under a UV transilluminator (Gel Doc system, Alpha innotech). Samples with two clear bands of 28S and 18S RNA were used for cDNA synthesis.

3.5.3 Synthesis of first strand cDNA

cDNA synthesis was carried out by using RevertAid first strand cDNA synthesis kit (Thermo Scientific). For each reverse transcription reaction, following reagents were added into sterile, nuclease free PCR tubes kept in ice:

Total RNA	:	approx. 500 ng (2 µl)
Primer	:	1 µl of random hexamer primer
Nuclease-free water	:	To make up the total volume to 12 µl
Total volume	:	12 µl

Contents in PCR tubes were mixed gently, centrifuged briefly and incubated in PCR machine as follows:

65 °C	:	5 min
4 °C	:	--

After completion of the above steps, the tubes were removed from PCR machine and kept

quickly on ice. The following remaining components were added into the tubes for completion of first strand cDNA synthesis:

5X Reaction buffer	:	4 µl
RiboLock RNase Inhibitor (20 U/µl)	:	1 µl
10 mM dNTP Mix	:	2 µl
RevertAid M-MuL V RT (200 U/µl)	:	1 µl
Total volume	:	20 µl

The contents in PCR tubes were mixed gently, centrifuged briefly and incubated in PCR machine as follows:

25 °C	:	5 min
45 °C	:	60 min
70 °C	:	5 min
4 °C	:	--

The reverse transcription reaction product was used immediately or stored at -20 °C (not more than one week) for real time PCR to quantitate relative mRNA abundance of selected genes in different tissues.

3.5.4 Real-Time polymerase chain reaction (qRT-PCR)

The first strand cDNA synthesized was directly used as a template for qRT-PCR (Applied Biosystems 7500). The primers for qRT-PCR were designed using primer3 software from inter-exonic regions of respective genes, and got custom synthesized from Sigma-Aldrich. For the purpose of normalization, β-Actin gene was used. Primer sequences used are given in the Table 3.4.

The real time PCR was performed in a total volume of 10 µl. The reaction mixture consisted of the following reagents:

Primers (Forward and Reverse)	:	0.25 µl each
SYBR Green mix (2X)	:	5 µl
Nuclease-free water	:	2.5 µl
cDNA	:	2 µl
Total volume	:	10 µl

Table 3.4 Primer sequences used for real-time quantitative PCR

S.No	Gene	Primer sequence (5'-3')
1	Adiponectin	F-TGT TGG AAT GAC AGG AGC TG R-CGA ATG GGT ACA TTG GGA AC
2	Leptin	F-TGA CAC CAA AAC CCT CAT CA R-TCA TTG GCT ATC TGC AGC AC
3	PPARγ	F-CTG GCC TCC CTG ATG AAT AA R-GGC GGT CTC CAC TGA GAA TA
4	GLUT4	F-GAT TCT GCT GCC CTT CTG TC R-ATT GGA CGC TCT CTC TCC AA
5	GPCR43	F-GGC TTC TAC AGC AGC ATC TA R-AAG CAC ACC AGG AAA TTA AG
6	MCP1	F-GCA GTT AAC GCC CCA CTC A R-CCC AGC CTA CTC ATT GGG ATC A
7	TNFα	F-AGC CGA TGG GTT GTA CCT TGT CTA R-TGA GAT AGC AAA TCG GCT GAC GGT
8	CPT1	F-CCA GGC TAC AGT GGG ACA TT R-GAA CTT GCC CAT GTC CTT GT
9	PPARα	F-CAG TGC CCT GAA CAT CGA GTG T R-TTC GCC GAA AGA AGC CCT T
10	SREBP-1c	F-GGC ACT AAG TGC CCT CAA CCT R-GCC ACA TAG ATC TCT GCC AGT GT
11	FIAF	F-TAG AGT CCC TGA AGG CCA GA R-AAT GAG CTG GGT CAT CTT GG
12	Occludin	F-ATG TCC GGC CGA TGC TCT C R-TTT GGC TGC TCT TGG GTC TGT AT
13	ZO-1	F-ACC CGA AAC TGA TGC TGT GGA TA R-AAA TGG CCG GGC AGA ACT TGT GT
14	G6Pase	F- GCTGGAGTCTTGTCAGGCAT R- ATCCAAGCGCGAAACCAAAC

15	PEPCK1	F- ATGAAAGGCCGCACCATGTA R- GCACAGATATGCCCATCCGA
16	β-actin	F-TGT TAC CAA CTG GGA CGA R-GGG GTG TTG AAG GTC TCA

PPARγ, peroxisome proliferator-activated receptor gamma; MCP-1, monocyte chemoattractant protein-1; TNFα, tumor necrosis factor alpha; FIAF, fasting induced adipose factor; ZO-1, zonula occludens-1; CPT1, carnitine palmitoyl transferase 1; PPARα, peroxisome proliferator-activated receptor alpha; GLUT4, glucose transporter 4; GPCR23, G Protein Coupled Receptor 43; SREBP, Sterol regulatory element-binding protein;G6pase, glucose 6-phosphatase; PEPCK, phosphoenolpyruvate carboxykinase.

3.5.4.1 Thermal cycling conditions

The PCR conditions programmed in qRT-PCR for relative expression analysis are given in Table 3.5.

Melting curves for all the reactions were analysed for the presence of primer dimers or secondary structures (if any) in all the samples. β-actin was used as reference gene for normalization of target gene for relative quantification.

Table 3.5 Real-Time PCR program for expression analysis

Program	Step	Temperature(°C)	Time	Cycles
Pre-incubation	-	95	5 min	1
Amplification	Denaturation	95	15 sec	40
	Annealing	60	30 sec	
	Extension	72	30 sec	
Melting curve	Denaturation	95	5 sec	1
	Renaturation	70	1 min	
	Final denaturation	95	Continuous mode	
Cooling	-	40	30 sec	1

3.5.4.2 Data analysis

The quantitative PCR data were analysed by $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) and represented as fold change.

$\Delta C_{T\ test} = C_{T(target,\ test)} - C_{T\ (reference\ gene,\ test)}$

$\Delta C_{T\ calibrator} = C_{T\ (target,\ calibrator)} - C_{T\ (reference\ gene,\ calibrator)}$

$\Delta\Delta C_{T} = \Delta C_{T\ test} - \Delta C_{T\ calibrator}$

Fold difference in expression = $2^{-\Delta\Delta CT}$

3.6 STATISTICAL ANALYSIS

Statistical analysis was performed by one way analysis of variance (ANOVA) followed by *post hoc* Tukey's multiple comparison test, using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA). Data with different superscript letters are significantly different at $p<0.05$, according to *post hoc* one way ANOVA statistical analysis. Data are expressed as mean ± SEM. The results were considered statistically significant when $p<0.05$.

CHAPTER - 4

Results

4.0 RESULTS

Obesity has emerged as a major health concern in both, the developed as well as developing countries, and is associated with a cluster of metabolic disorders. In the recent past, there has been increased interest in finding nutritional strategies for management of obesity and associated complications among which dietary fibers including galactomannans, phytosterols (terpenoids, flavanoids & sapogenins) and probiotics have become the focus of attention of researchers. Almost no information is available with reference to the role of pure form of galactomannans and sapogenins like diosgenin in reduction of obesity under high fat/energy dense diet fed conditions. The present study was conducted to examine the effects of two types of functional fibers viz., fenugreek galactomannan (FGM) & locust bean galactomannan (LGM), and a sapogenin (diosgenin), alone or in combination on biomarkers of obesity and expression of genes related to lipid metabolism in C57BL/6 mice fed high fat diet for 18 weeks. The results of experiments conducted are presented in the following sections.

4.1 TO STUDY THE EFFECTS OF GALACTOMANNAN AND DIOSGENIN ON PROGRESSION OF OBESITY IN HIGH FAT DIET FED MICE.

Under this objective, C57BL/6 mice were fed different experimental diets for 6 or 18 weeks to assess the effects of dietary incorporation of galactomannan (FGM/LGM, 5% w/w) and diosgenin (0.5%, w/w). Different dietary interventions were tried to examine the effects of galactomannans as well as diosgenin in animals fed high fat diet. The high fat diet (5.29 kcal/g) contained 59.54% energy from fat while the low fat control diet (3.85 kcal/g) contained 11.67% energy from fat. The different experimental groups were designated as already indicated in Table 3.1. The effects on different parameters viz., body weight, feed intake, fasting blood glucose, organ weights, epididymal fat mass accumulation, serum and liver lipid profile, serum insulin level, homeostasis model assessment of insulin resistance (HOMA-IR), adipocyte size & number, and activities of key enzymes of glycolysis & gluconeogenesis in liver were studied.

4.1.1 Body weight and feed intake

The body weights were recorded at different time intervals for all experimental groups viz., Ctrl, HFD, HFD-FGM, HFD-LGM, HFD-D, HFD-FGMD and HFD-LGMD and the data are presented in Fig. 4.1. The initial body weights at the start of experiment (zero day) were

measured to be 27.58 ± 0.51, 27.41 ± 0.40, 27.40 ± 0.49, 27.58 ± 0.38, 27.26 ± 0.50, 27.26 ± 0.40 and 27.18 ± 0.81 g (mean ± SEM) for Ctrl, HFD, HFD-FGM, HFD-LGM, HFD-D, HFD-FGMD and HFD-LGMD groups, respectively. The body weight of C57BL/6 mice was found to be continuously increasing on feeding of high fat diet and was higher than the control group animals (Ctrl) throughout the feeding schedule of 18 weeks. The animals fed high fat diet (HFD) were observed to gain more weight in comparison to the animals fed control diet. The final body weights after 18 weeks in Ctrl, HFD, HFD-FGM, HFD-LGM, HFD-D, HFD-FGMD and HFD-LGMD groups were measured to be 34.82 ± 1.38, 40.34 ± 1.30, 33.74 ± 1.29, 33.50 ± 1.35, 33.86 ± 1.23, 32.60 ± 1.41 & 33.12 ± 1.35 g, respectively. Resistance to gain in body weight as a result of incorporation of different functional ingredients in high fat diet was clearly visible 9th week onwards, however, it was found to be statistically significant in case of HFD-FGM vs. HFD ($p<0.05$). After 18 weeks, the mean body weights in HFD-FGM, HFD-LGM, HFD-D, HFD-FGMD and HFD-LGMD groups were found to be significantly lower than the HFD group. The results also show slight additive effect on co-administration of galactomannan and diosgenin (HFD-FGMD & HFD-LGMD groups), however, not found to be significantly different when compared with HFD-FGM, HFD-LGM and HFD-D groups.

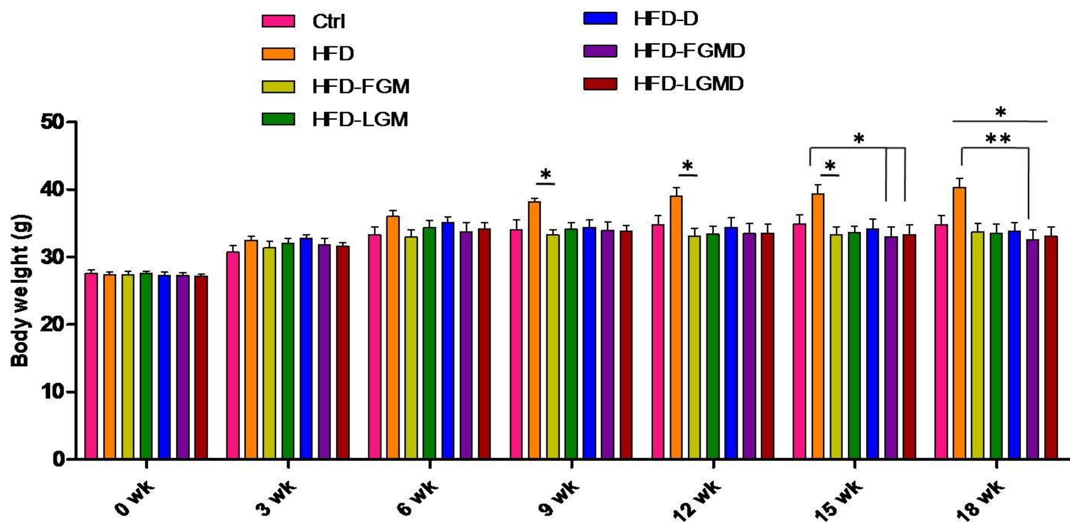


Fig. 4.1 Effect of dietary incorporation of galactomannan (FGM/LGM) and diosgenin alone or in combination on body weight of C57BL/6 mice fed high fat diet. Values are expressed as mean ± SEM ($p<0.05$); (n=9 at 0-6 wk; 5 at 9-18 wk).

The cumulative feed intake of different treatment groups was calculated for the 18 week feeding period, and the data are presented in Fig. 4.2. The results indicate that the animals on high fat/energy dense diets were consuming lesser quantity of solid diet in comparison to the animals fed on normal diet (Ctrl group). The cumulative feed intake in Ctrl, HFD, HFD-FGM, HFD-LGM, HFD-D, HFD-FGMD and HFD-LGMD groups were calculated to be 492.1, 315.30, 327.42, 318.10, 319.38, 333.09 and 321.32 g, respectively. No difference in cumulative feed intake was visible among the different treatment groups fed on isocaloric high fat diet with or without functional ingredients.

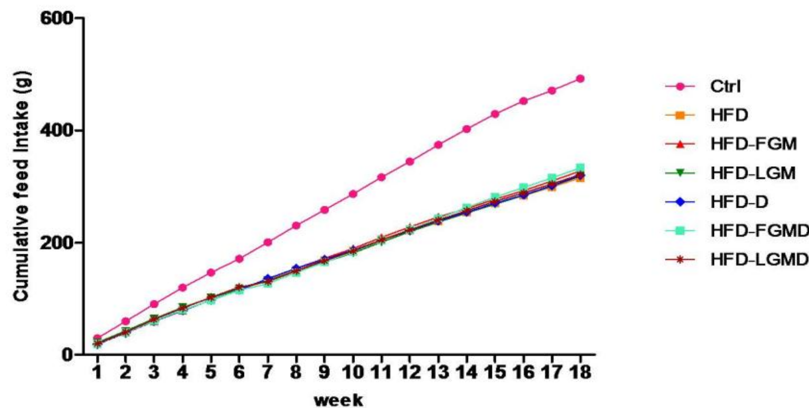


Fig. 4.2 Cumulative feed intake in different dietary treatment groups. Values are expressed as mean (n= 5-9).

4.1.2 Organ weights

The animals from different experimental groups were sacrificed at 6 & 18 week. The epididymal fat mass, liver, kidney & spleen were weighed, and the data are presented in Fig. 4.3 & Fig. 4.4.

As expected, the epididymal fat mass accumulation was higher in animals fed high fat diet (HFD group) in comparison to those fed on control diet (Ctrl). After 6 weeks of feeding, the mean fat weight in HFD group (1.05 ± 0.14 g), though higher than the Ctrl group (0.79 ± 0.07 g) the difference was not found to be statistically significant. Dietary incorporation of either type of galactomannan or diosgenin (alone or in combination) seemed to resist the fat mass accumulation, however, the epididymal fat mass was not significantly lower in HFD-FGM, HFD-LGM, HFD-D, HFD-FGMD and HFD-LGMD groups in comparison to HFD group. After

18 weeks, significantly higher fat mass accumulation could be observed in HFD group (1.72 ± 0.15 g) as compared to the Ctrl group (1.06 ± 0.09 g). Also, significantly decreased fat mass accumulation compared to HFD group was visible in different treatment groups fed galactomannan/diosgenin (HFD-FGM/HFD-LGM/HFD-D) for 18 weeks. The maximum effect could be observed in groups fed fenugreek galactomannan/locust bean galactomannan. The mean values of epididymal fat mass in HFD-FGM and HFD-LGM groups were 1.04 ± 0.13 and 1.06 ± 0.11 g, respectively, and were almost equal to the control group values. Feeding of diosgenin alone or in combination with either type of galactomannan also resulted in significantly decreased epididymal fat mass accumulation compared to HFD group. The mean values of epididymal fat in HFD-D, HFD-FGMD and HFD-LGMD were 1.14 ± 0.14, 1.13 ± 0.10 and 1.07 ± 0.11 g, respectively. The results reveal that the fat mass accumulation was significantly resisted by different dietary treatments involving administration of galactomannan/diosgenin (alone or in combination) as the values were almost comparable to the control group, and no further additive effect was visible over and above that exhibited as a consequence of feeding FGM/LGM/Diosgenin alone.

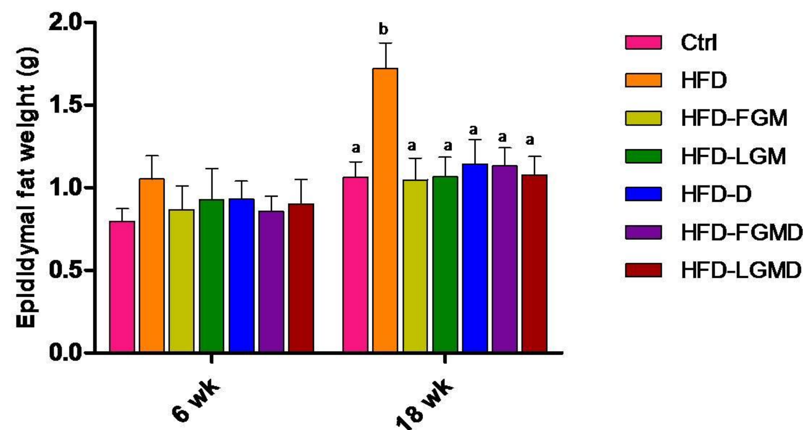


Fig. 4.3 Effect of dietary incorporation of galactomannan (FGM/LGM) and diosgenin alone or in combination on epididymal fat mass in mice fed high fat diet. Mean values with different superscript letters differ significantly (p<0.05). Values are expressed as mean ± SEM (n = 4 at 6 wk; 5 at 18 wk).

The mean liver weights were slightly higher in HFD group compared to the Ctrl group, both at 6 & 18 week. However, the values were not found to be different statistically. The

dietary incorporation of galactomannan (FGM/LGM) or diosgenin in HFD showed a trend towards decreased liver weight but the mean values were not found to be significantly lower compared to HFD group. Further, dietary incorporation of two types of galactomannans and diosgenin (alone or in combination) did not evince any effect on kidney and spleen weights after 6 as well as 18 weeks.

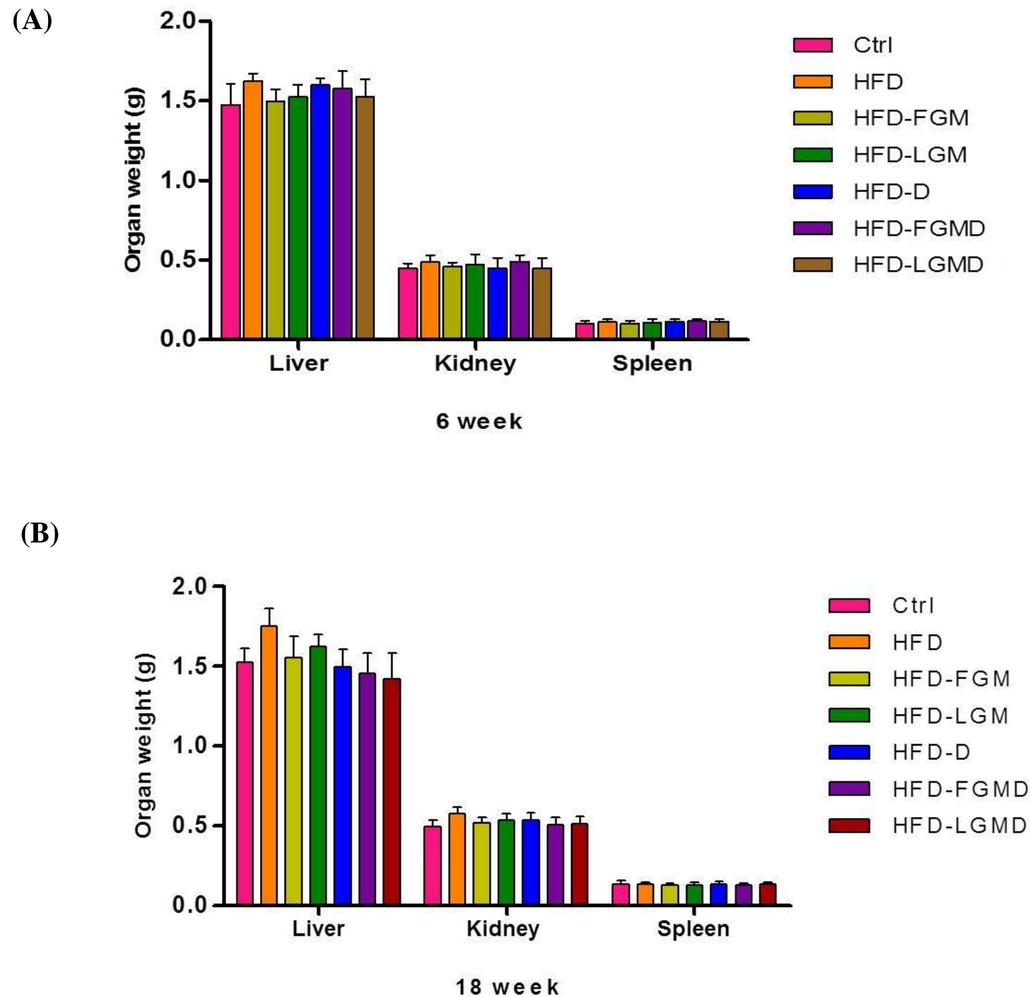


Fig. 4.4A-B Effect of dietary incorporation of galactomannan (FGM/LGM) and diosgenin alone or in combination on liver, kidney and spleen weight (A) at 6 week and (B) 18 week in mice fed high fat diet ($p>0.05$). Values are expressed as mean ± SEM (n = 4 at 6 wk; 5 at 18 wk).

4.1.3 Fasting blood glucose

The results on the fasting blood glucose level at 6 and 18 week are presented in Fig. 4.5.

Feeding of high fat diet (HFD group) for 6 weeks elevated the fasting blood glucose level (156 ± 12.0 mg/dl), however, it was not significantly different (p>0.05) as compared to the level in Ctrl (132.8 ± 15.55 mg/dl). There was a tendency to resist the rise in fasting blood glucose level on feeding of FGM/LGM/diosgenin (alone or in combination), but not found to be statistically different in comparison to HFD group. At the end of study (18 weeks), mean fasting blood glucose level was found to be significantly higher (P<0.05) in HFD group in comparison to Ctrl group (178.4 ± 12.14 vs. 123.6 ± 11.47 mg/dl), exhibiting the induction of hyperglycemic condition as consequence of feeding high fat diet for a long duration. Incorporation of FGM/LGM/diosgenin in high fat diet exhibited protective effect by resisting the increase in fasting blood glucose level due to HFD. The mean blood glucose levels in HFD-FGM, HFD-LGM and HFD-D were measured to be 123.8 ± 12.28, 115.4 ± 12.15 and 122.8 ± 14.25 mg/dl, respectively. Feeding of galactomannan and diosgenin in combination was also effective in normalizing the fasting blood glucose level, the values being 114.2 ± 9.99 and 113.8 ± 12.42 mg/dl for HFD-FGMD and HFD-LGMD groups, respectively. It could be seem that different dietary treatments were almost equally effective in normalizing the fasting blood glucose level.

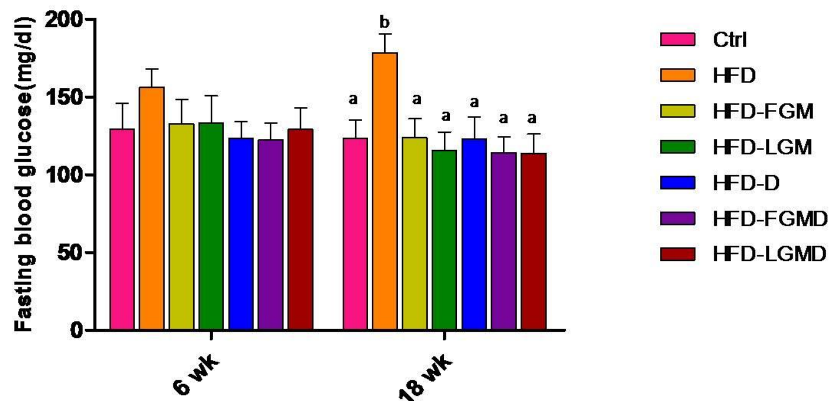


Fig. 4.5 Effect of dietary incorporation of galactomannan (FGM/LGM) and diosgenin alone or in combination on fasting blood glucose in mice fed high fat diet. Mean values with different superscript letters differ significantly (p<0.05). Values are expressed as mean ± SEM (n = 4 at 6 wk; 5 at 18 wk).

4.1.4 Serum insulin and HOMA-IR

As shown in Fig. 4.6A, high fat diet fed mice (HFD) exhibited an increase in serum

insulin reaching to the levels of 1.68 ± 0.16 ng/ml (mean ± SEM) and was found to be significantly higher ($p<0.05$) at 18 wk, as compared to the Ctrl group (0.81 ± 0.09 ng/ml). Protective effect could be seen on administration of galactomannans and diosgenin. Both types of galactomannans were found to be almost equally and significantly ($p<0.05$) effective in resisting the increase in serum insulin level, the levels being 0.90 ± 0.18 and 0.84 ± 0.16 ng/ml in HFD-FGM and HFD-LGM, respectively. Though, diosgenin feeding also seemed to resist the induction of hyperinsulinemia, mean serum insulin level in HFD-D (0.14 ± 0.18 ng/ml) was not found to differ statistically when compared with HFD group ($p>0.05$). The co-administration of either type of galactomannan (FGM/LGM) with diosgenin also exhibited significant resistance to rise in serum insulin level. The mean serum insulin levels in HFD-FGMD and HFD-LGMD were measured to be 0.91 ± 0.20 and 0.90 ± 0.14 ng/ml, respectively, however, no additive effect of co-administration of functional ingredients was visible.

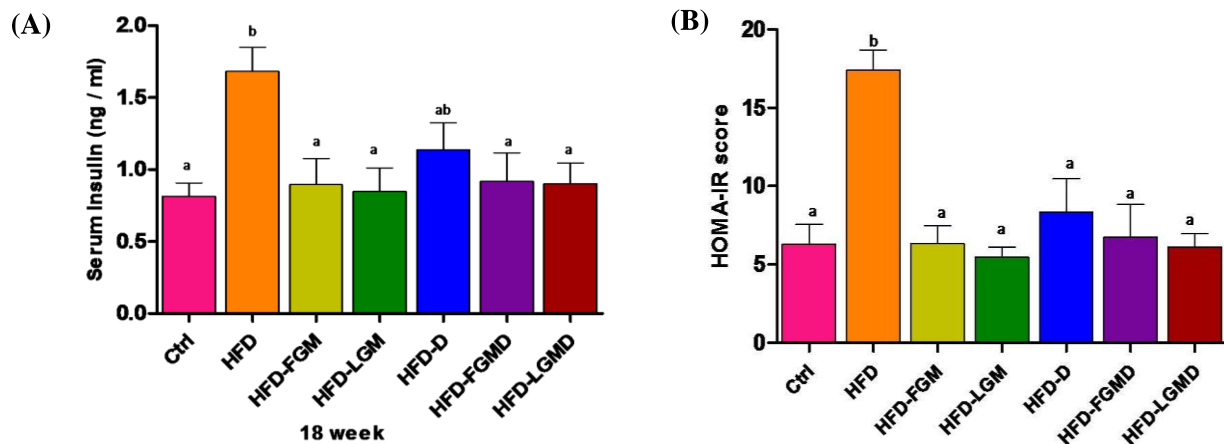


Fig. 4.6 Effect of dietary incorporation of galactomannan (FGM/LGM) and diosgenin alone or in combination on (A) serum insulin levels and (B) HOMA-IR score in mice fed high fat diet. Mean values with different superscript letters differ significantly ($p<0.05$). Values are expressed as mean ± SEM (n = 4).

The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the values of fasting blood glucose (mmol/L) and serum insulin levels (µU/ml) in different treatment groups at 18 week, and the data are shown in Fig. 4.6B. In accordance with the rise in fasting blood glucose as well as serum insulin, the HOMA-IR score was found to increase as a consequence of feeding high fat diet and reached to the level of 17.40 ± 1.30 and found to be

significantly higher ($p<0.001$) as compared to the Ctrl group (6.30 ± 1.26). The increase in HOMA-IR score was counteracted to significant extent by dietary incorporation of FGM/LGM/diosgenin, when administered alone or in combination. The HOMA-IR score in HFD-FGM, HFD-LGM, HFD-D, HFD-FGMD and HFD-LGMD groups were calculated to be 6.32 ± 1.15, 5.45 ± 0.63, 8.34 ± 2.13, 6.73 ± 2.08 and 6.12 ± 0.86, respectively. The administration of LGM seems to be more effective in comparison to FGM and also diosgenin. However, the HOMA-IR scores were not found to differ significantly from one another. Furthermore, no additive effect could be seen on co-administration of galactomannan and diosgenin (HFD-FGMD/HFD-LGMD).

4.1.5 Serum lipid profile

The serum lipid profile was determined in different treatment groups to evaluate the effects of dietary incorporation of galactomannan (FGM/LGM) and a sapogenin (diosgenin) after 6 &18 weeks feeding. The mean values of serum triglycerides (TG), total cholesterol (TC), HDL-cholesterol (HDL-C), LDL-cholesterol (LDL-C) and VLDL-cholesterol (VLDL-C) are presented in Fig. 4.7A-E. The results indicate the development of dyslipidemia as a consequence of feeding high fat diet which was more pronounced after 18 weeks.

At 6 week, the serum TG level was found to be higher in HFD group (120.2 ± 10.78 mg/dl) as compared to the Ctrl groups 89.58 ± 12.52 mg/dl. However, the values were not found to differ significantly ($p>0.05$). Dietary incorporation of FGM/LGM/diosgenin in high fat diet (alone or in combination) exhibited a tendency to resist the rise in serum TG level, but not to the significant extent ($p>0.05$). Feeding of high fat diet for a long duration of 18 weeks, serum TG level was found to increase significantly ($p<0.05$) and reached to the level of 145.2 ± 14.56 mg/dl compared to a low level of 97.52 ± 8.60 mg/dl in Ctrl group. Both types of galactomannans (FGM and LGM) were found to be equally and significantly effective in resisting the rise in serum TG resulting in normalization of TG level. The serum TG levels were measured to be 96.41 ± 8.38 and 102.6 ± 6.75 mg/dl (mean ± SEM) in HFD-FGM and HFD-LGM group, respectively. However, these were not found to differ significantly from each other. Diosgenin feeding also exhibited a tendency to decrease serum TG level (118.2 ± 8.22 mg/dl), but not to the significant extent when compared with HFD group. The data also revealed the efficacy of diosgenin to be lower than that exhibited by either type of galactomannan. Dietary

incorporation of both the functional fibers (FGM/LGM) in combination with diosgenin also showed protective effects, however, no additive effects were visible and the serum TG levels were almost comparable to the Ctrl group level.

The serum total cholesterol (TC) level was found to increase as a consequence of feeding high fat diet. However, the serum TC level in HFD group (107.7 ± 8.45 mg/dl) did not reached to a significantly higher level ($p>0.05$) in comparison to Ctrl group (89.65 ± 5.45 mg/dl), after 6 weeks. The dietary incorporation of either type of galactomannan (FGM/LGM) and diosgenin (alone or in combination) exhibited a trend towards resisting the rise in serum TC level when compared with HFD group. However, the mean serum TC levels among different treatment groups were not found to differ significantly ($p>0.05$). High fat diet feeding for longer duration (18 weeks) resulted in a significant ($p<0.05$) increase in serum total cholesterol level in HFD group (137.6 ± 12.97 mg/dl) compared to the Ctrl group (95.70 ± 9.84 mg/dl). The results indicate that the dietary incorporation of galactomannan (FGM/LGM) and diosgenin were equally and significantly ($p<0.05$) effective in counteracting the rise in serum TC level due to high fat diet feeding, and the TC levels were normalized. The protective effect to the significant extent could also be seen on co-administration of galactomannan and diosgenin (HFD-FGMD/HFD-LGMD groups). Importantly, the mean serum values in HFD-FGM (88.94 ± 8.76 mg/dl), HFD-LGM (94.53 ± 7.89 mg/dl), HFD-D (96.15 ± 9.07 mg/dl), HFD-FGMD (95.16 ± 7.72 mg/dl) and HFD-LGMD (95.03 ± 6.84 mg/dl) were comparable to the serum TC level measured in control group. The data on HDL-C levels measured in different treatment groups after 6 as well as 18 week revealed that HDL-C level remained almost unaffected on feeding functional ingredients viz., fenugreek galactomannan, locust been galactomannan and diosgenin, whether administered alone or in combination.

High fat diet feeding was found to increase the serum LDL-C level, however, it could not reach to a significantly higher level in HFD group after 6 weeks when compared with the Ctrl group. Dietary incorporation of FGM/LGM seemed to resist the increase in LDL-C level, however, the mean values in HFD-FGM (27.33 ± 4.61 mg/dl) and HFD-LGM (26.53 ± 5.92 mg/dl) were not significantly lower ($p>0.05$) in comparison to the HFD group (40.06 ± 6.74 mg/dl). The protective effects was also visible on feeding diosgenin (alone or in combination with galactomannan), but the levels were again not found to be significantly lower than HFD

group. Feeding of high fat diet for 18 weeks led to an increase in serum LDL-C to a significantly higher ($p<0.05$) level in HFD group (64.93 ± 9.65 mg/dl) compared to a low level of 31.36 ± 4.60 mg/dl in Ctrl group. The dietary incorporation of galactomannans were observed to exhibit protective effect as evinced by the mean LDL-C levels in HFD-FGM (26.95 ± 6.14 mg/dl) and HFD-LGM (32.45 ± 4.93 mg/dl), which were significantly lower than the HFD group. Administration of diosgenin also showed protective effect when administered alone or in combination with galactomannan (FGM/LGM). The mean serum LDL-C level in HFD-D, HFD-FGMD and HFD-LGMD were measured to be 31.30 ± 5.42, 32.51 ± 5.50 and 31.02 ± 6.00 mg/dl, respectively. However, these values did not differ significantly then the HFD-FGM and HFD-LGM groups which showed no additive effect of co-administration of functional ingredients.

As shown in Fig. 4.6D, the VLDL-C level was increased as a consequence of high fat diet feeding. Though the levels in HFD (24.04 ± 1.68 mg/dl) and Ctrl (17.91 ± 1.94 mg/dl) did not differ significantly at 6 week, the serum VLDL-C level reached to a significantly higher level in HFD group (29.04 ± 2.91 mg/dl) as compared to the control group (19.51 ± 1.73 mg/dl) after the experimental period of 18 weeks. Different dietary interventions involving FGM, LGM and diosgenin exhibited a tendency of resisting the rise in serum VLDL-C, but not to the significant extent at 6 weeks. Significant protective effects of administration of galactomannans could be seen after 18 weeks. The mean serum VLDL-C levels in HFD-FGM and HFD-LGM were measure to be 19.27 ± 1.68 and 20.52 ± 1.35 mg/dl, respectively, however, these were not significantly different from each other. Though, dietary incorporation of diosgenin did show the resistance to raise in serum VLDL-C level but the mean value was not significantly different than the HFD group. The co-administration o f diosgenin and galactomannan (FGM/LGM) exhibited a significant protection from rise in serum VLDL-C levels due to HFD. However, no additive effects were visible as the mean values of serum VLDL-C in HFD-FGMD (19.71 ± 1.62 mg/dl) and HFD-LGMD (20.53 ± 1.62 mg/dl) were comparable and did not differ significantly from the levels measured in HFD-FGM and HFD-LGM groups.

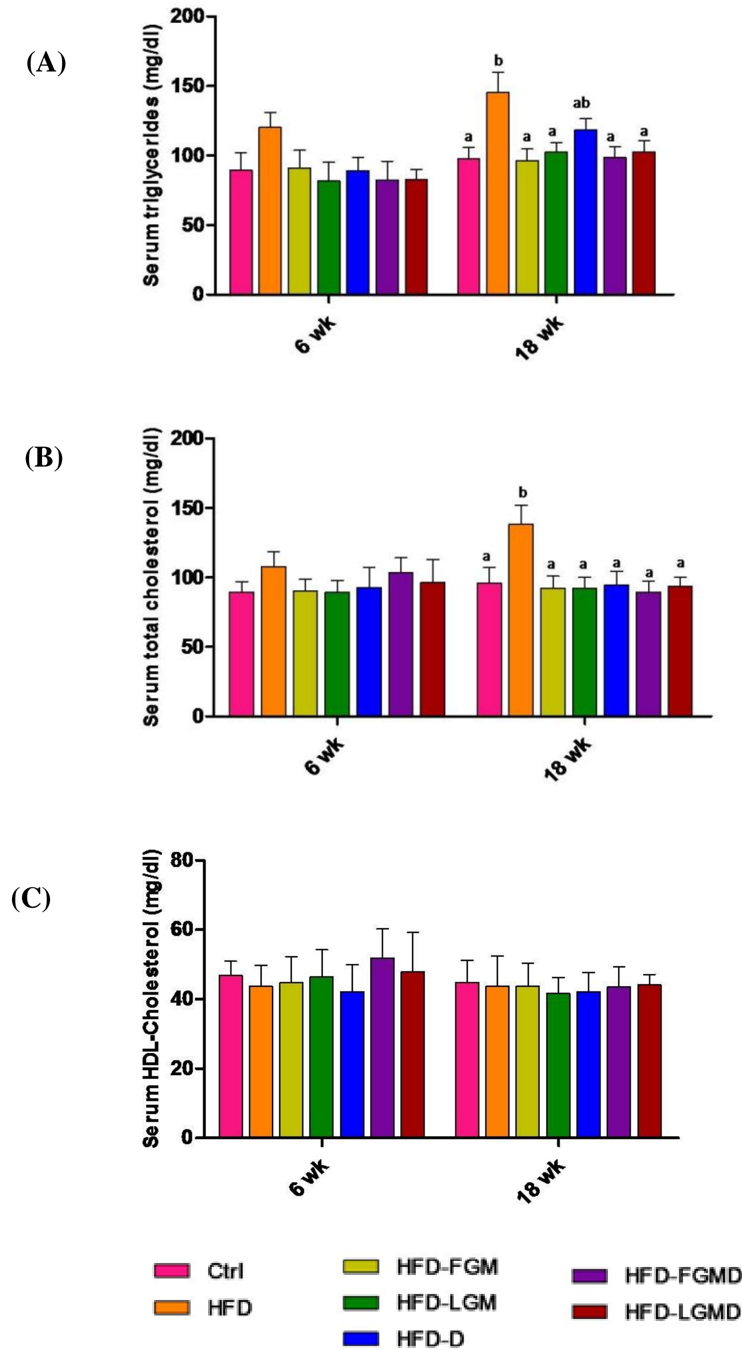


Fig. 4.7A-E (contd.) Effect of dietary incorporation of galactomannan (FGM/LGM) and diosgenin alone or in combination on serum lipid profile in mice fed high fat diet. Mean values with different superscript letters differ significantly ($p<0.05$). Values are expressed as mean ± SEM (n = 4 at 6 wk; 5 at 18 wk.

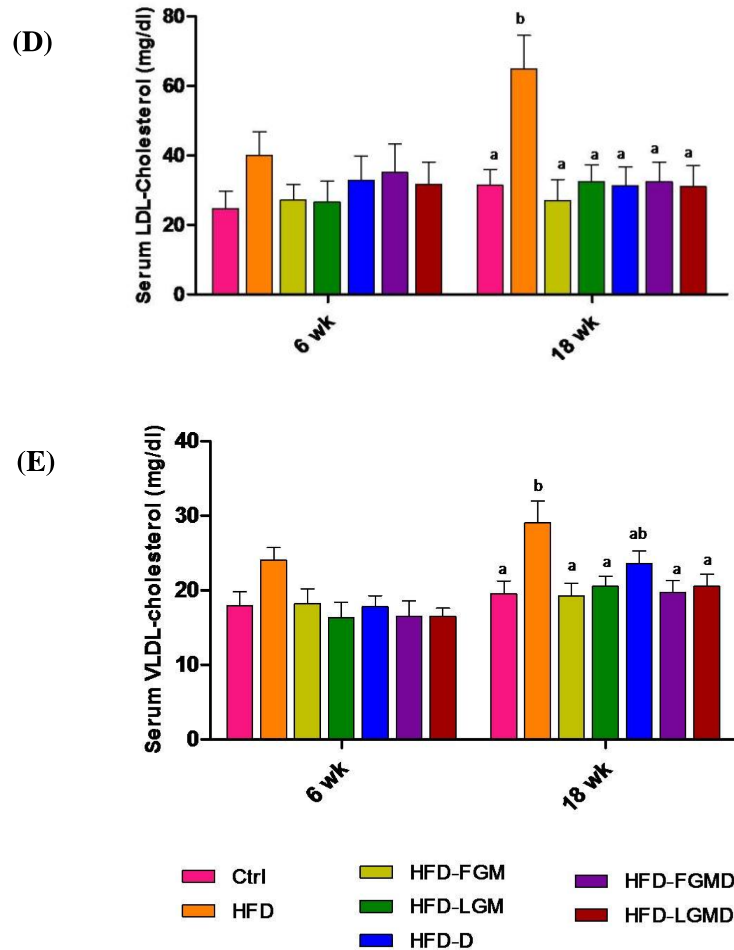


Fig. 4.7A-E Effect of dietary incorporation of galactomannan (FGM/LGM) and diosgenin alone or in combination on serum lipid profile in mice fed high fat diet. Mean values with different superscript letters differ significantly ($p<0.05$). Values are expressed as mean ± SEM (n = 4 at 6 wk; 5 at 18 wk).

4.1.6 Atherogenic Index (AI)

Results on atherogenic index calculated for different treatment groups (6 & 18) weeks are depicted in Fig. 4.8. High fat diet feeding resulted in an increase atherogenic index (AI) at 6 as well as 18 week. Though, the AI was not to a significantly higher in HFD group compared to Ctrl at 6 weeks, it was found to reached to a significantly higher ($p<0.05$) level of 2.60 ± 0.56 (mean± SEM) in HFD group when compared with the control group (1.18 ± 0.10). The increase in AI by high fat diet feeding was significantly resisted by FGM/LGM feeding. The mean AI in HFD-FGM group (1.12 ± 0.24) was slightly lower than HFD-LGM (1.30 ± 0.15), but these

values were not found to differ significantly. Diosgenin feeding also exhibited a protective effect, however, the AI was not found to be significantly lower than the HFD group. Resistance to rise in AI to the significant extent could be seen in HFD-FGMD and HFD-LGMD groups where the values were found to be 1.28 ± 0.21 & 1.20 ± 0.18, respectively.

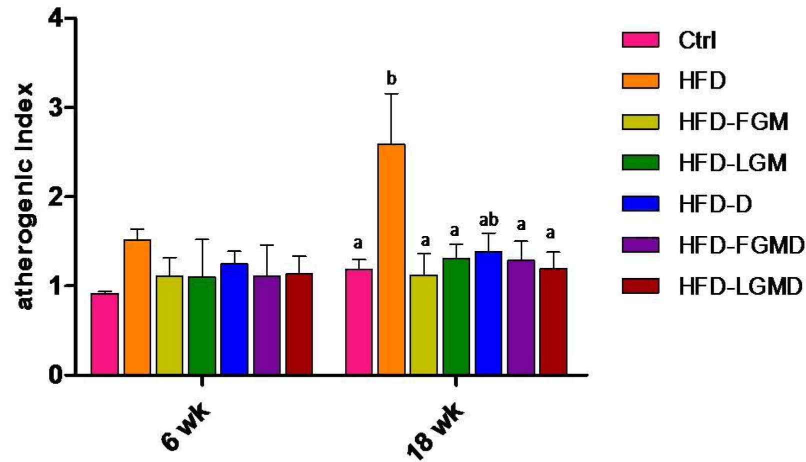


Fig. 4.8 Effect of dietary incorporation of galactomannan (FGM/LGM) and diosgenin alone or in combination on atherogenic index in mice fed high fat diet. Mean values with different superscript letters differ significantly (p<0.05). Values are expressed as mean ± SEM (n = 4 at 6 wk; 5 at 18 wk).

4.1.7 Liver lipids

Effects of administering galactomannans and a sapogenin viz., FGM, LGM and diosgenin in C57BL/6 mice on total TG and TC in liver tissue extracts are presented in Fig. 4.9A-B. The liver TG level exhibited an increase of 54.8%, in HFD group, however, not significantly (p>0.05) different compared to Ctrl group level (15.28 ± 2.04 mg/g tissue) after 18 weeks of feeding. Dietary incorporation of FGM/LGM and diosgenin (alone or in combination) showed protective effects, as indicated by the resistance to the rise in liver TG level due to high fat diet feeding. Mean liver TG levels in HFD-FGM, HFD-LGM, HFD-D, HFD-FGMD and HFD-LGMD were measured to be 13.37 ± 2.04, 13.79 ± 1.48, 14.66 ± 1.72, 13.11 ± 1.43 and 12.71 ± 1.61 mg/g tissue, respectively, which were significantly lower in HFD-FGM, HFD-LGM & HFD-D (p<0.05) and HFD-FGMD & HFD-LGMD (p<0.01) as compared to the HFD group value (23.65 ± 2.68 mg/g tissue). Importantly, the mean values in different groups fed functional ingredients did not differ significantly from one another. However, these values were somewhat

lower than control group value.

The high fat diet feeding elevated the liver total cholesterol level also in HFD group but not to the significant extent as compared with Ctrl (6.05 ± 0.58 vs. 3.68 ± 0.86 mg/g tissue). Dietary incorporation of galactomannans (FGM/LGM) showed protective effect but the liver TC levels in HFD-FGM (3.59 ± 0.46 mg/g tissue) as well as HFD-LGM (4.04 ± 0.62 mg/g tissue) though lower than the HFD group value, but not found to be different statistically. On the contrary, administration of diosgenin was observed to counteract the rise in TC level significantly ($p<0.05$), the value being 3.19 ± 0.55 mg/g tissue (mean ± SEM). The co-administration of diosgenin and galactomannan (FGM/LGM) also exhibited significant protective effect, however, the mean TC levels in HFD-FGMD (3.25 ± 0.55 mg/g tissue) as well as HFD-LGMD (2.97 ± 0.50 mg/g tissue) did not differ significantly compared to HFD-D group value.

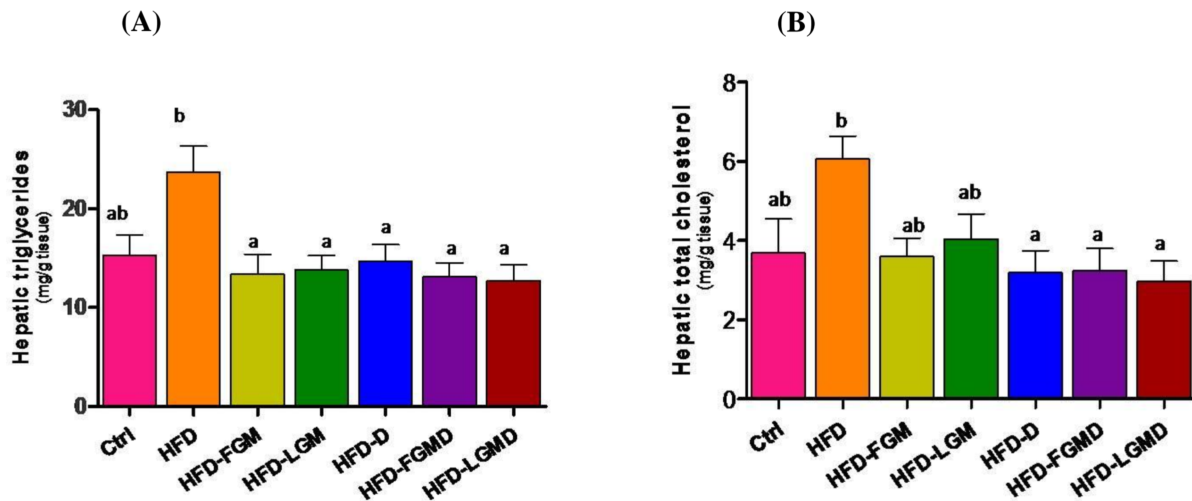


Fig. 4.9A-B Effect of dietary incorporation of galactomannan (FGM/LGM) and diosgenin alone or in combination on hepatic TG and TC in mice fed high fat diet. Mean values with different superscript letters differ significantly ($p<0.05$). Values are expressed as mean ± SEM (n =5).

4.1.8 Adipocyte size and number

Histological analysis of epididymal fat tissue was carried out to examine the influence of dietary incorporation of two types of galactomannans and diosgenin in high fat diet on adipocyte size and number. After H & E staining, viewed at 200x magnification (Fig. 4.10A-G). The adipocyte size measured and the number determined using Image J software. Comparison of

mean adipocyte size and number among different treatment groups was done and the data are presented in Fig. 4.11A-B.

The mean adipocyte size in high fat diet fed group was found to increase to the significant extent ($p<0.001$) as compared to Ctrl group, the mean values being 3782 ± 115.6 and 4721 ± 228.7 μm^2 in Ctrl and HFD group, respectively. Similar to fat mass accumulation, the increased mean adipocyte area showed increased adiposity. The dietary incorporation of FGM/LGM/diosgenin, alone or in combination, exhibited significant protective effect ($p<0.001$) when compared with HFD group. The mean adipocyte size in different groups viz., HFD-FGM, HFD-LGM, HFD-D, HFD-FGMD and HFD-LGMD were measured to be 3479 ± 94.29, 3605 ±124.8, 3389 ± 99.72, 2891 ± 69.09 and 2789± 116.8 μm^2, respectively. Both types of galactomannans and diosgenin were equally effective in resisting the increase in adipocyte size due to HFD feeding. Moreover, feeding of diosgenin with either of the galactomannan seemed to have additive effect and mean values in HFD-FGMD and HFD-LGMD groups were even lower than Ctrl group value. Importantly, the HFD- LGMD showed maximum effect among all type of dietary interventions and the mean adipocyte size was significantly lower ($p<0.05$) than the group fed diosgenin only (HFD-D).

As expected, significantly less ($p<0.05$) mean adipocyte number in epididymal fat tissue of HFD group could be found compared with Ctrl group (78.56 ± 6.69 vs. 123.1 ± 10.45). The Fig. 4.11B shows that administration of either type of galactomannan (FGM/LGM) and diosgenin in high fat diet were found to be equally and significantly ($p<0.05$) effective in counteracting the decrease in adpocyte number under high fat diet fed conditions. A little additive effect could be seen on feeding of both the functional components (galactomannan and diosgenin) in combination, however, the mean adipocyte number in HFD-FGMD (135.0 ± 11.6) and HFD-LGMD (131.6 ± 10.44) were not significantly lower in comparison to HFD-FGM, HFD-LGM and HFD-D groups.

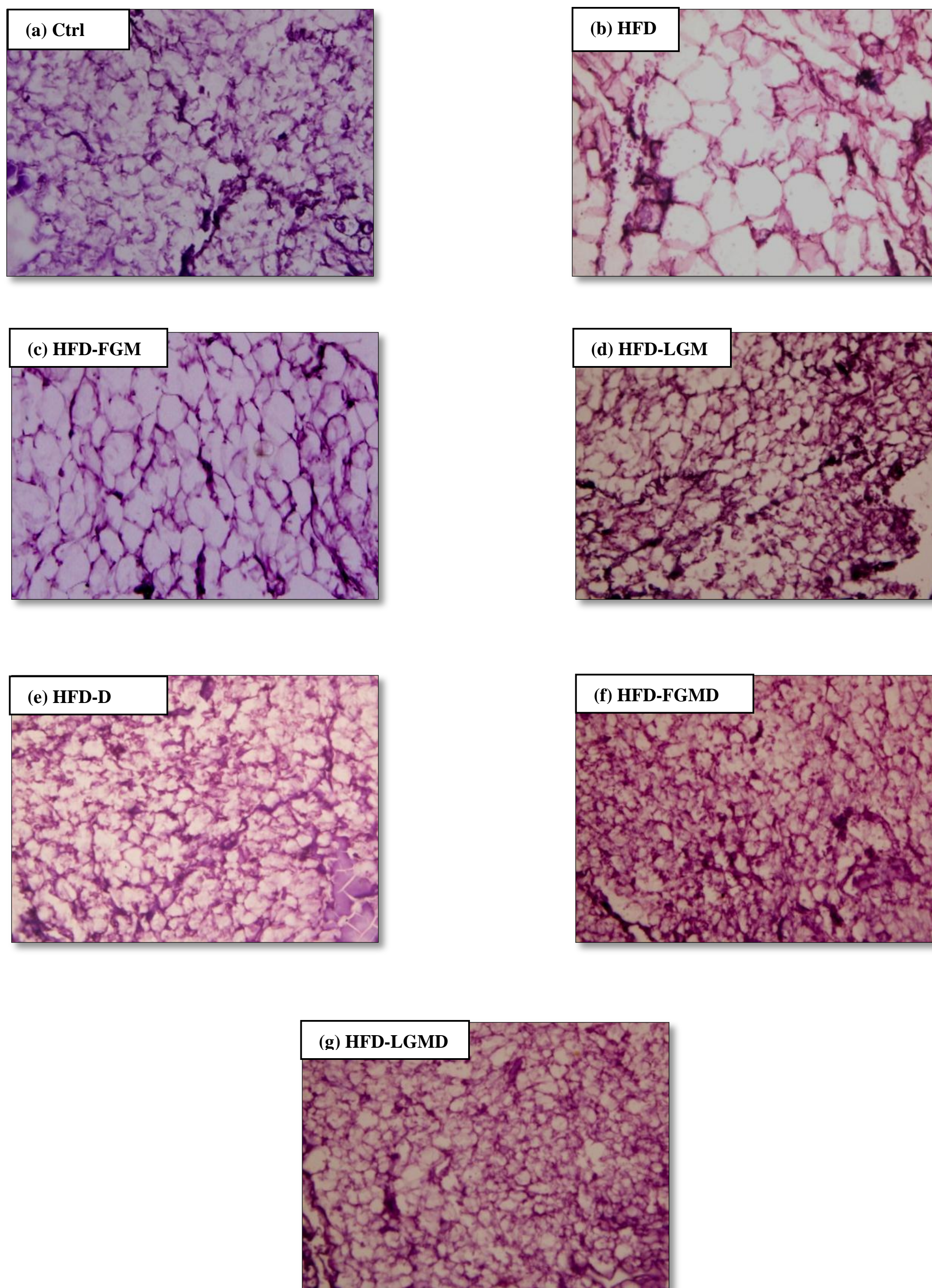


Fig. 4.10 (a-g) Effect of dietary incorporation of galactomannan (FGM/LGM) and diosgenin alone or in combination on adipocyte size (μm^2) in mice fed high fat diet for 18 weeks (Representative photomicrographs of hematoxylin and eosin staining of adipocytes, 200X magnification).

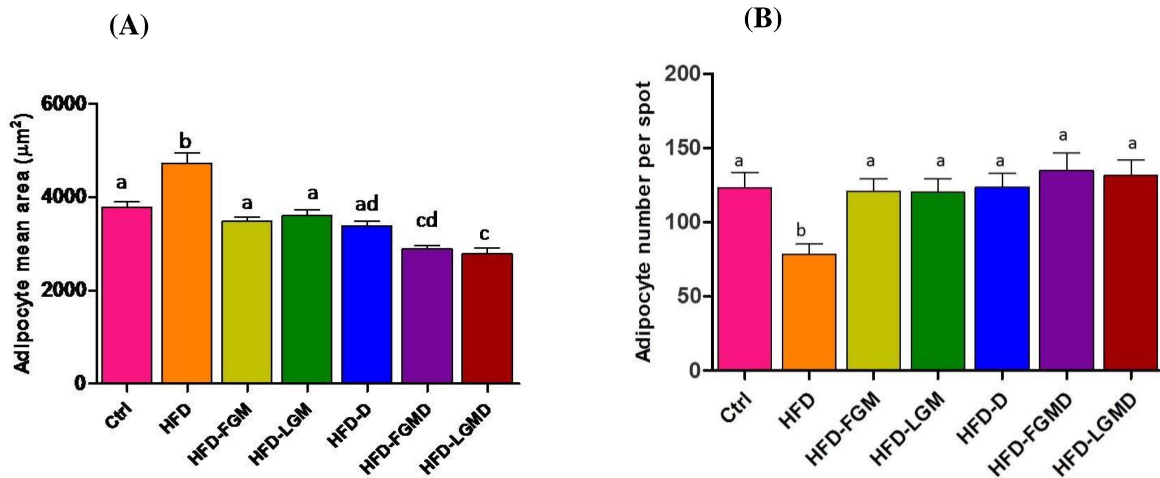


Fig. 4.11A-B Effect of dietary incorporation of galactomannan (FGM/LGM) and diosgenin alone or in combination on (A) mean adipocyte area (μm^2) and (B) adipocyte number per spot in mice fed high fat diet. Mean values with different superscript letters differ significantly ($p<0.05$). Values are expressed as mean ± SEM (n =3).

4.1.9 Hepatic enzymes activities

Liver plays a pivotal role in maintenance of glucose homeostasis and the effect of administration of fenugreek & locust bean galactomannan, and diosgenin on activities of key regulatory enzyme of glycolysis (PFK, PK and GK) and gluconeogenesis (PEPCK and FBPase1; were determined by spectrophotometric-coupled enzyme assays.

4.1.9.1 Effect on key enzymes of glycolysis

To get an insight into the status of glycolytic flux, the activities of key regulatory enzymes were assayed in different experimental groups (Fig. 4.12A-C). The activities of phosphofructokinase1 (PFK1) and pyruvate kinase (PK) were decreased as a consequence of feeding high fat diet in HFD group, but not to the statistically significant level ($p>0.05$) compared to their corresponding controls (Ctrl groups). The mean enzyme activity values of PFK were measured to be 2.32 ± 0.28 vs. 2.67 ± 0.37 mU/mg protein (HFD vs. Ctrl), while in case of PK the activities were 18.69 ± 2.63 vs. 29.18 ± 2.42 mU/mg protein (HFD vs. Ctrl) in liver tissue. The incorporation of FGM/LGM/diosgenin in high fat diet tends to resist the decrease in PFK1 activity, though the PFK activity levels in HFD-FGM (3.03 ± 0.37 mU/mg protein), HFD-LGM (2.76 ± 0.46 mU/mg protein) and HFD-D (2.86 ± 0.41 mU/mg protein) groups were higher than HFD group and the Ctrl group also, the differences among different treatment groups were

not found to be statistically significant. Almost a similar trend was visible in HFD-FGMD and HFD-LGMD groups when compared with HFD group and exhibited no additive effect of co-administration of functional ingredients.

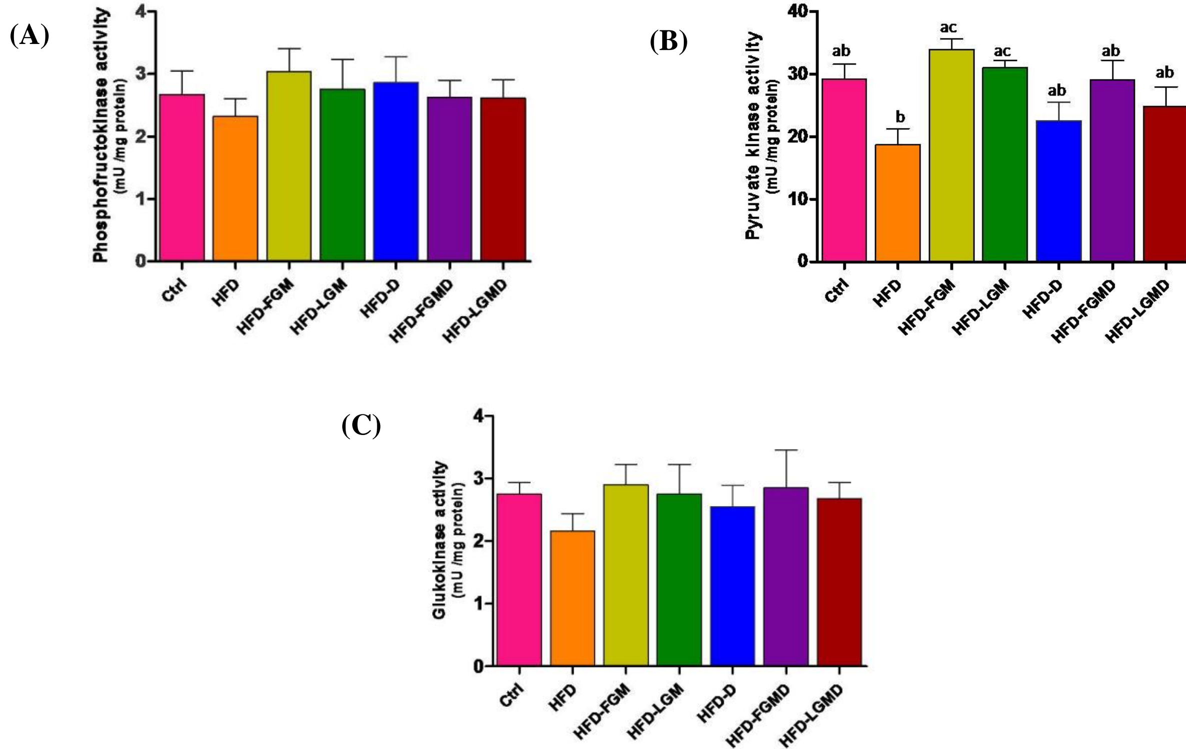


Fig. 4.12A-C Effect of dietary incorporation of galactomannan (FGM/LGM) and diosgenin alone or in combination on key glycolytic enzymes in liver (A) phosphofuctokinase (B) pyruvate kinase (C) glucokinase in mice fed high fat diet. Mean values with different superscript letters differ significantly ($p<0.05$). Values are expressed as mean ± SEM (n =4).

In case of PK, significant resistance to decrease in activity on feeding FGM/LGM could be observed as evinced by higher activity levels 33.98 ± 1.70, 31.06 ± 1.14 mU/mg protein in HFD-FGM and HFD-LGM groups, respectively, as compared to a low level of enzyme activity (18.69 ± 2.63 mU/mg protein) in HFD group. On the contrary, feeding of diosgenin did not show any protective effect. The co-administration of galactomannans and diosgenin did show resistance to decrease in PK activity due to high fat diet feeding, but no additive effect was visible. The results indicate the maximum efficacy on administration of FGM or LGM individually.

The glucokinase (GK) plays a pivotal role in maintenance of blood glucose level. Similar to PFK and PK, the GK activity in liver tissue also tends to decrease in HFD group compared to Ctrl group (2.15 ± 0.28 vs. 2.75 ± 0.18 mU/mg protein, respectively). The different dietary interventions involving the galactomannan (FGM/LGM) and diosgenin, whether administered alone or in combination, appeared to resist the decrease in GK activity due to high fat diet feeding but not to the significant extent. The mean values of GK activity in HFD-FGM, HFD-LGM, HFD-D, HFD-FGMD and HFD-LGMD were measured to be 2.90 ± 0.32, 2.75 ± 0.47, 2.54 ± 0.35, 2.85 ± 0.60, and 2.67 ± 0.26 mU/mg protein, respectively, which were higher than HFD group value and comparable with the GK activity in Ctrl group. The different dietary treatments seem to normalize the GK activity levels.

4.1.9.2 Effect on key enzymes of gluconeogenesis

The activities of phosphoenolpyruvate carboxykinase (PEPCK) and fructose1,6-bisphospghatase (FBPase1) the important regulatory enzymes of gluconeogenesis, measured in liver tissue of mice from different experimental groups, and the results are illustrated in Fig. 4.13A-B.

The PEPCK activity in HFD group (34.68 ± 3.38 mU/mg protein) was significantly higher ($p<0.001$) as compared to the Ctrl group (17.93 ± 1.16 mU/mg protein). Administration of all types of functional ingredients viz., FGM, LGM and diosgenin exhibited potential to resist the rise in PEPCK activity under high fat diet fed conditions. The mean values of PEPCK activity in HFD-FGM, HFD-LGM and HFD-D were significantly lowered as compared to HFD group (FGM, $p<0.05$; LGM & diosgenin, $p<0.01$). Importantly, the administration of FGM/LGM in combination with diosgenin seemed to exhibit additive effect as indicated by the mean PEPCK activity in HFD-FGMD (19.25 ± 1.46 mU/mg protein) and HFD-LGMD (17.89 ± 1.83 mU/mg protein) groups which were lower than those of HFD-FGM (23.54 ± 2.14 mU/mg protein), HFD-LGM (21.87±1.21 mU/mg protein) and HFD-D (22.10 ± 1.89 mU/mg protein) groups. However, the differences were not statistically significant among these groups.

Similar to the trend observed in case of PEPCK, FBPase1 activity was also found to increase under high fat diet fed conditions, and reached to a significantly higher ($p<0.01$) level in HFD group (152.2 ± 13.20 mU/mg protein) compared to the Ctrl group (84.66 ± 13.72 mU/mg protein). Moreover, all types of functional ingredients seemed to counteract the increase in

FBPase1 activity. However, the mean values of enzyme activities in HFD-FGM, HFD-LGM and HFD-D were not significantly lower when compared with HFD group. Importantly, the FBPase 1 activities in HFD-FGMD (88.77 ± 5.15 mU/mg protein) and HFD-LGMD (92.18 ± 6.63 mU/mg protein) were significantly lower ($p<0.01$) than that in HFD group. The data also reveal the additive effect and maximum effectiveness of co-administration of galactomannan and diosgenin among all dietary interventions.

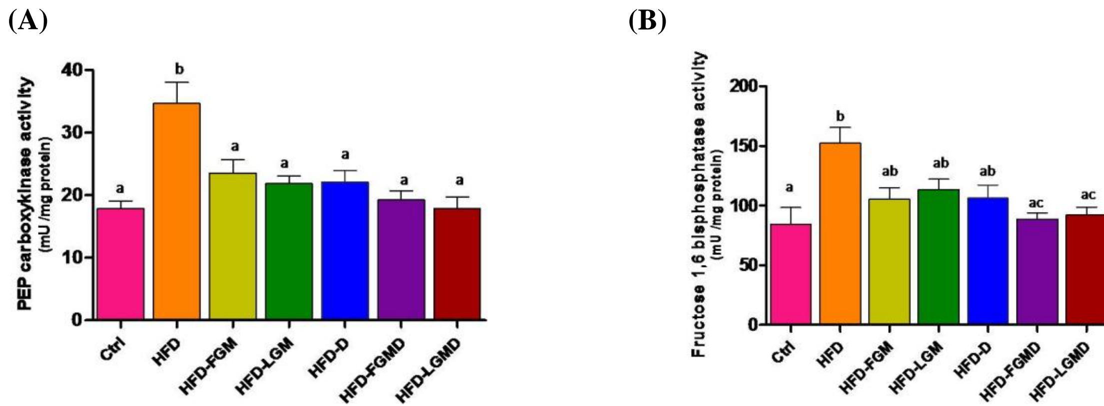


Fig. 4.13A-B Effect of dietary incorporation of galactomannan (FGM/LGM) and diosgenin alone or in combination on key gluconeogenic enzymes in liver (A) PEPCK and (B) FBPase in mice fed high fat diet. Mean values with different superscript letters differ significantly ($p<0.05$). Values are expressed as mean ± SEM (n =4).

4.2 ANALYSIS OF EXPRESSION PATTERN OF GENES RELATED TO LIPID METABOLISM AS AFFECTED BY GALACTOMANNAN AND DIOSGENIN FEEDING

Quantitative mRNA analysis of different genes viz., adiponectin, leptin, PPARγ, GPCR43, GLUT4, MCP-1 & TNF-α in epididymal fat tissue; CPT1, PPARα & SREBP-1c in liver tissue, and FIAF, ZO-1, occludin, PEPCK and glucose 6-phosphatase in distal small intestine as affected by fenugreek and locust bean galactomannan (FGM & LGM) and diosgenin feeding was also performed.

4.2.1 Gene expression analysis in epididymal adipose tissue

4.2.1.1 Adiponectin

Adiponectin is exclusively produced by adipocytes and plays a significant role in energy homeostasis. The mRNA expression levels of adiponectin in epididymal fat tissues of mice from

different experimental groups were determined, and the data are presented in Fig. 4.14A. The expression of adiponectin in HFD group was observed to decrease (49 %) as compared to the mRNA expression level in Ctrl group, but the difference between two groups was not statistically significant ($p>0.05$). Dietary incorporation of FGM/LGM/diosgenin in high fat diet significantly resisted ($p<0.001$) down regulation of adiponectin due to HFD feeding. The expression levels in HFD- FGM, HFD-LGM, HFD-D groups reached to the level of 2.55 ± 0.11, 2.60 ± 0.13, 2.29 ± 0.14 fold, respectively, which were significantly higher in comparison to Ctrl group also ($p<0.001$). The administration of galactomannan (FGM or LGM) with diosgenin appeared to be even more effective, however, the expression levels in HFD-FGMD and HFD-LGMD groups were not significantly higher as compared to HFD- FGM, HFD-LGM, HFD-D.

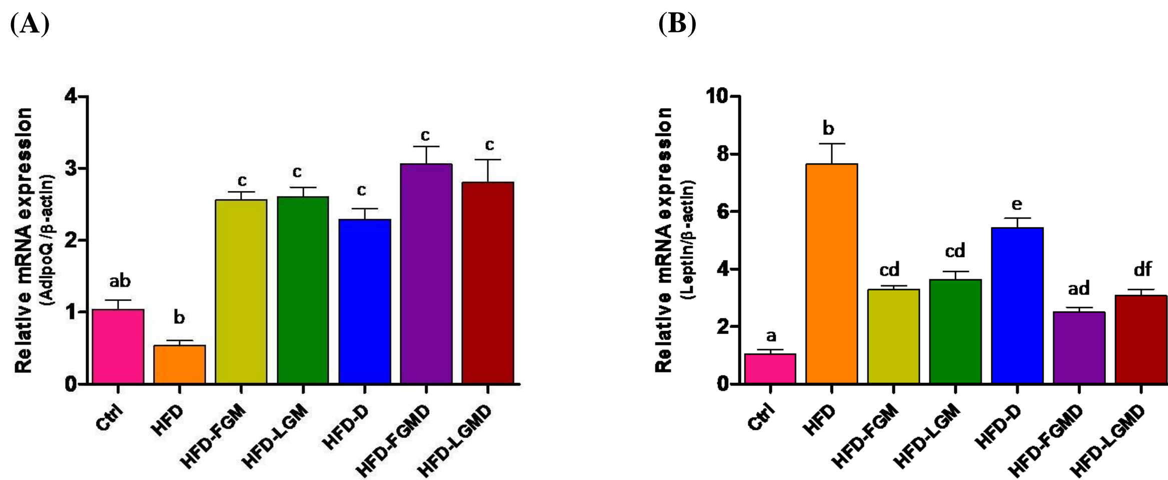


Fig. 4.14A-B Effect of dietary incorporation of galactomannans (FGM/LGM) and diosgenin alone or in combination on (A) adiponectin and (B) leptin (involved in energy homeostasis) gene expression in epididymal adipose tissue of mice fed high fat diet. Mean values with different superscript letters differ significantly ($p<0.05$). Values are expressed as mean ± SEM (n =3).

4.2.1.2 Leptin

The results on relative mRNA expression of leptin (linked with adiposity) in different treatments groups are presented in Fig. 4.14B. Relative mRNA expression of leptin in HFD group was found to increase significantly (>6.5 fold) compared with the Ctrl group, which is well correlate with the significant increase in fat mass accumulation under high fat diet fed

conditions. Dietary incorporation of functional components (galactomannan/diosgenin) significantly resisted the up-regulation of leptin, compared with HFD group. The relative mRNA expression levels in HFD-FGM, HFD-LGM and HFD-D were calculated to be 3.26 ± 0.12 ($p<0.001$), 3.63 ± 0.28 ($p<0.001$) and 5.43 ± 0.33 ($p<0.01$) fold, respectively. These expression levels also show that the efficacy of FGM and LGM were significantly higher compared to diosgenin (HFD-FGM vs. HFD-D, $p<0.01$; HFD-LGM vs. HFD-D, $p<0.05$). Furthermore, the expression levels in HFD-FGMD (2.49 ± 0.17 fold) and HFD-LGMD (3.07 ± 0.22, fold) were lower than those in HFD-FGM, HFD-LGM and HFD-D groups, but these were found to be significantly lower ($p<0.001$) compared to HFD-D group only. The trend observed also suggests the additive effect of co-administration of galactomannan and diosgenin

4.2.1.3 Peroxisome proliferator-activated receptor gamma (PPARγ)

Expression of PPARγ (regulator of adipogenesis) in epididymal fat tissue was analyzed in different treatment groups after 18 weeks of feeding and the results are depicted in Fig. 4.15A. Feeding of high fat diet resulted in increase of PPARγ relative mRNA expression reaching to the level to the level of 2.67 ± 0.14 fold which was significantly higher ($p<0.001$) compared to Ctrl group. Dietary incorporation of soluble fibers (FGM/LGM) in high fat diet exhibited protective effect. The relative mRNA expression levels in HFD-FGM group (1.28 ± 0.12 fold) and HFD-LGM (1.73 ± 0.16 fold) were significantly lower compared to the HFD group. Though the efficacy of FGM seem to be better than that of LGM, however, the different between the expression levels was not statistically significant. Feeding of diosgenin tends to resists the increase in PPARγ expression but not to significant extent ($p>0.05$ vs. HFD). The efficacy of co-administration of galactomannans (FGM/LGM) with diosgenin was not better than that exhibited by the galactomannas when administered individually.

4.2.1.4 G-protein coupled receptor 43 (GPCR43)

Among the varied type of functions, GPCR43 has been linked with promotion of leptin secretion and adipogenesis. As shown in Fig. 4.15B the relative mRNA expression of GPCR43 was found to be significantly increased and reached to the level of 3.30 ± 0.18 fold (>2.3 fold change) in HFD group as compared to that in case of Ctrl group. It could be seen that all types of dietary interventions involving the administration of galactomannan and diosgenin (HFD-FGM, HFD-LGM, HFD-D, HFD-FGMD and HFD-LGMD)) exhibited significant resistance ($p<0.001$

vs. HFD) to the up-regulation of GPCR43 by high fat diet (reduction 74%-83%). Interestingly, the expression levels of GPCR43 in HFD-FGM, were either comparable or even lower than the expression level in Ctrl group.

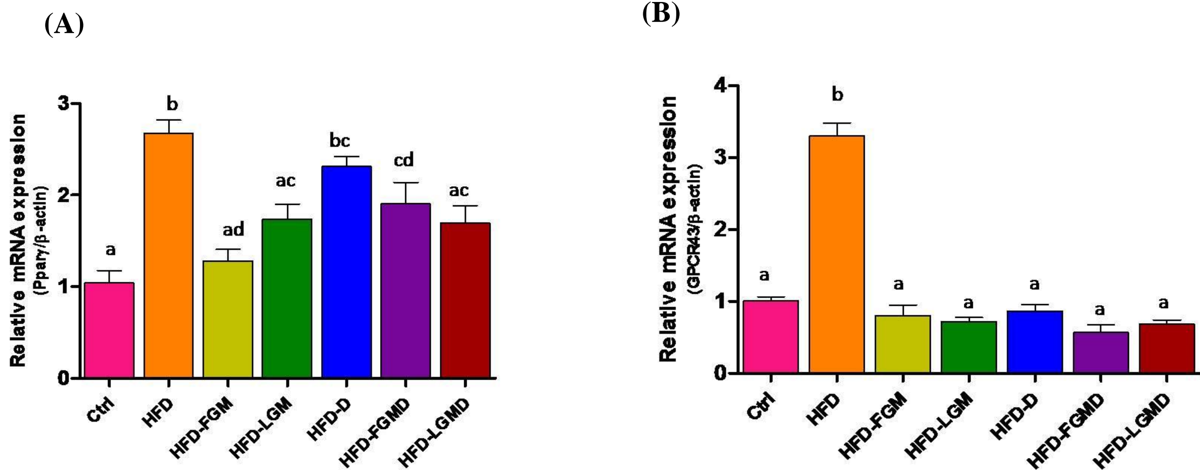


Fig. 4.15A-B Effect of dietary incorporation of galactomannans (FGM/LGM) and diosgenin alone or in combination on (A) PPARγ and (B) GPCR43 (linked with adipogenesis) gene expression in epididymal adipose tissue of mice fed high fat diet. Mean values with different superscript letters differ significantly (p<0.05). Values are expressed as mean ± SEM (n =3).

4.2.1.5 Glucose transporter 4 (GLUT4)

GLUT4 is the important insulin responsive glucose transporter in adipose tissue. The data on relative mRNA expression of GLUT4 are presented in Fig. 4.16. High fat diet feeding resulted significant reduction (66%) in HFD group as compared to that in Ctrl group (p<0.01). Enhanced GLUT4 expression could be seen as a consequence of dietary incorporation of FGM/LGM/diosgenin and the expression levels in HFD-FGM (0.13 ± 0.11, fold), HFD-LGM (1.25 ± 0.10, fold), and HFD-D (1.36 ± 0.14, fold) were significantly higher when compared with the HFD group (p<0.001 vs. HFD). The co-administration of FGM/LGM in combination with diosgenin seemed to be more effective than the individual functional ingredients. However, the expression levels in HFD-FGMD (1.80 ± 0.15 fold) and HFD-LGMD (1.66 ± 0.13 fold) group were not significantly higher compared to HFD-FGM, HFD-LGM and also HFD-D group.

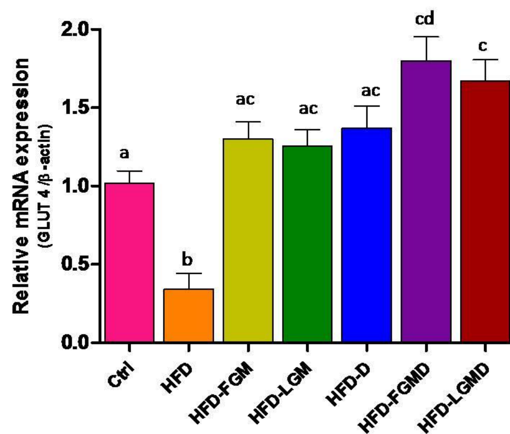


Fig. 4.16 Effect of dietary incorporation of galactomannans (FGM/LGM) and diosgenin alone or in combination on glut-4 (glucose transporter) expression in epididymal adipose tissue of mice fed high fat diet. Mean values with different superscript letters differ significantly ($p<0.05$). Values are expressed as mean ± SEM (n =3).

4.2.1.6 Monocyte Chemoattractant Protein-1 (MCP-1)

In view of the fact that increased adiposity is associated with inflammatory responses and insulin resistance, the mRNA expression of pro-inflammatory markers in epididymal fat mass was analyzed. The results on relative mRNA expression of MCP-1 are illustrated in Fig.4.17A. Expression of MCP-1 due to high fat diet feeding were found to be significantly ($p<0.001$) up-regulated, the level being 1.73 ± 0.03 fold in HFD group as compared with Ctrl. Feeding of soluble fiber (galactomannan) and diosgenin, alone or in combination showed significant resistance to the increase in MCP-1 expression. The mRNA expression levels were found to be 1.18 ± 0.11, 1.21 ± 0.09, 1.08 ± 0.11, 0.87 ± 0.08 and 0.93 ± 0.08 fold in HFD-FGM, HFD-LGM, HFD-D, HFD-FGMD and HFD-LGMD, respectively. The data reveal the equal effectiveness of two types of galactomannans. Diosgenin effect appeared to be more, however, the expression level was not significantly different than those in case of HFD-FGM and HFD-LGM. The expression levels in HFD-FGMD and HFD-LGMD groups also indicate maximum efficacy of co-administration of galactomannans and diosgenin. Though, the expression levels in this two groups were somewhat lower than the Ctrl group, these did not differ significantly when compared with HFD-FGM, HFD-LGM and HFD-D group.

4.2.1.7 Tumor necrosis factor alpha (TNFα)

TNFα is another important pro-inflammatory marker. Similar to the trend observed in case of MCP-1, the TNFα expression was also found to increase significantly ($p<0.001$) under high fat diet fed conditions and the mRNA expression reached to the level of 1.90 ± 0.09 fold (Fig. 4.17B). The two types of galactomannans (FGM & LGM) and diosgenin were observed to be equally effective in resisting the increase in TNFα expression. The relative mRNA expression levels in HFD-FGM (1.24 ± 0.09 fold), HFD-LGM (1.18 ± 0.12 fold) and HFD-D (1.25 ± 0.13 fold) groups were significantly lower as compared to that in HFD group. Additive effect of co-administration of galactomannans with diosgenin were also visible as the mean values of TNFα expression in HFD-FGMD (0.95 ± 0.11 fold) and HFD-LGMD (1.02 ± 0.06 fold) were lower than those calculated for HFD-FGM, HFD-LGM and HFD-D groups. However, the differences were not statistically significant.

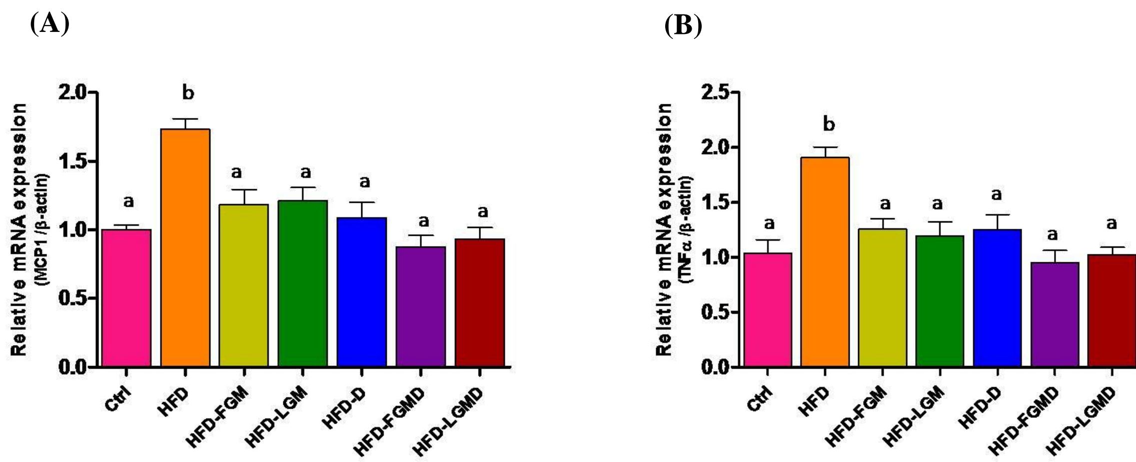


Fig. 4.17A-B Effect of dietary incorporation of galactomannans (FGM/LGM) and diosgenin alone or in combination on (A) MCP-1 and (B) TNFα (inflammatory markers) gene expression in epididymal adipose tissue of mice fed high fat diet. Mean values with different superscript letters differ significantly ($p<0.05$). Values are expressed as mean ± SEM (n =3).

4.2.2 Gene expression analysis in liver

Carnitine palmitoyltransferase1 (CPT1) is involved in regulating the transport of long chain fatty acids into the mitochondria for their β-oxidation while PPAR α is a important transcription factor controlling the expression of genes related to enzymes involved β-oxidation.

Sterol regulatory element binding protein-1c (SREBP-1c) known to be the key regulator of lipogenesis in liver. In order to assess the effects of dietary incorporation of galactomannans (FGM & LGM) and diosgenin on genes related to lipid metabolism, expression patterns of above mentioned genes in liver tissue of mice from different experimental groups was analyzed.

4.2.2.1 Carnitine palmitoyltransferase1 (CPT-1)

The relative mRNA expression levels of CPT-1 in different treatment groups were determined and the data are illustrated in Fig 4.18A. The expression of CPT-1 was significantly reduced (50%, $p<0.05$) in HFD group as compared to Ctrl group. All types of dietary interventions involving administration of FGM, LGM and diosgenin (alone and in combination) were effective in resisting the decrease in CPT-1 expression to the significant extent. The expression levels in HFD-FGM (1.56 ± 0.10), HFD-LGM (1.29 ± 0.12) and HFD-D (1.03 ± 0.09) were found to be significantly higher compared that in HFD group ($p<0.001$, $p<0.001$ and $p<0.05$, respectively). The data reveal the maximum effectiveness of FGM in resisting the reduction in CPT-1 expression among different functional ingredients. A significant protective effect could also be seen when galactomannan (FGM/LGM) and diosgenin were administered in combination, but no additive effect was visible.

4.2.2.2 Peroxisome proliferator-activated receptor alpha (PPARα)

As shown in Fig. 4.18B, PPARα expression was also reduced to the significant extent (56 %) in HFD group. The mRNA expression levels in HFD-FGM, HFD-LGM and HFD-D (1.58 ± 0.13 fold) exhibited a trend similar to that followed by CPT-1 which showed the maximum protective effect of FGM. The expression levels in HFD-FGM, HFD-LGM and HFD-D were significantly higher ($p<0.001$) compared to HFD and the values being 1.93 ± 0.15, 1.75 ± 0.18 and 1.58 ± 0.13 fold respectively. Importantly, the additive effects of co-administration of galactomannan (FGM/LGM) and diosgenin were distinctly visible. The expression levels in HFD-FGMD (2.61 ± 0.06) and HFD-LGMD (2.40 ± 0.11 fold) reached to the significantly higher levels ($p<0.001$) compared to that of HFD group (0.45 ± 0.04).

4.2.2.3 Sterol regulatory element binding protein-1c (SREBP-1c)

The results on expression of SREBP-1c in liver tissue are presented in Fig. 4.18C. The high fat diet feeding led to increase in SREBP-1c expression to the level 2.73 ± 0.10 fold, being

significantly higher ($p<0.001$) as compared to the Ctrl group. Dietary incorporation of galactomannans and diosgenin tend to resist the up-regulation of SREBP-1c. Among HFD-FGM, HFD-LGM and HFD-D, diosgenin appeared to be more effective as compare to FGM and LGM, however, the differences in expression levels among three groups did not differ significantly. As evinced by the expression levels in HFD-FGM, HFD-LGM and HFD-D groups which were found to be 1.83 ± 0.21, 1.72 ± 0.18 and 1.53 ± 0.20 fold, respectively. The relative mRNA expression levels in HFD-FGMD and HFD-LGMD were apparently lower than those in case of HFD- FGM, HFD-LGM and HFD-D groups, which suggest some additive effect (but not to the significant extent) of co-administration of functional ingredients.

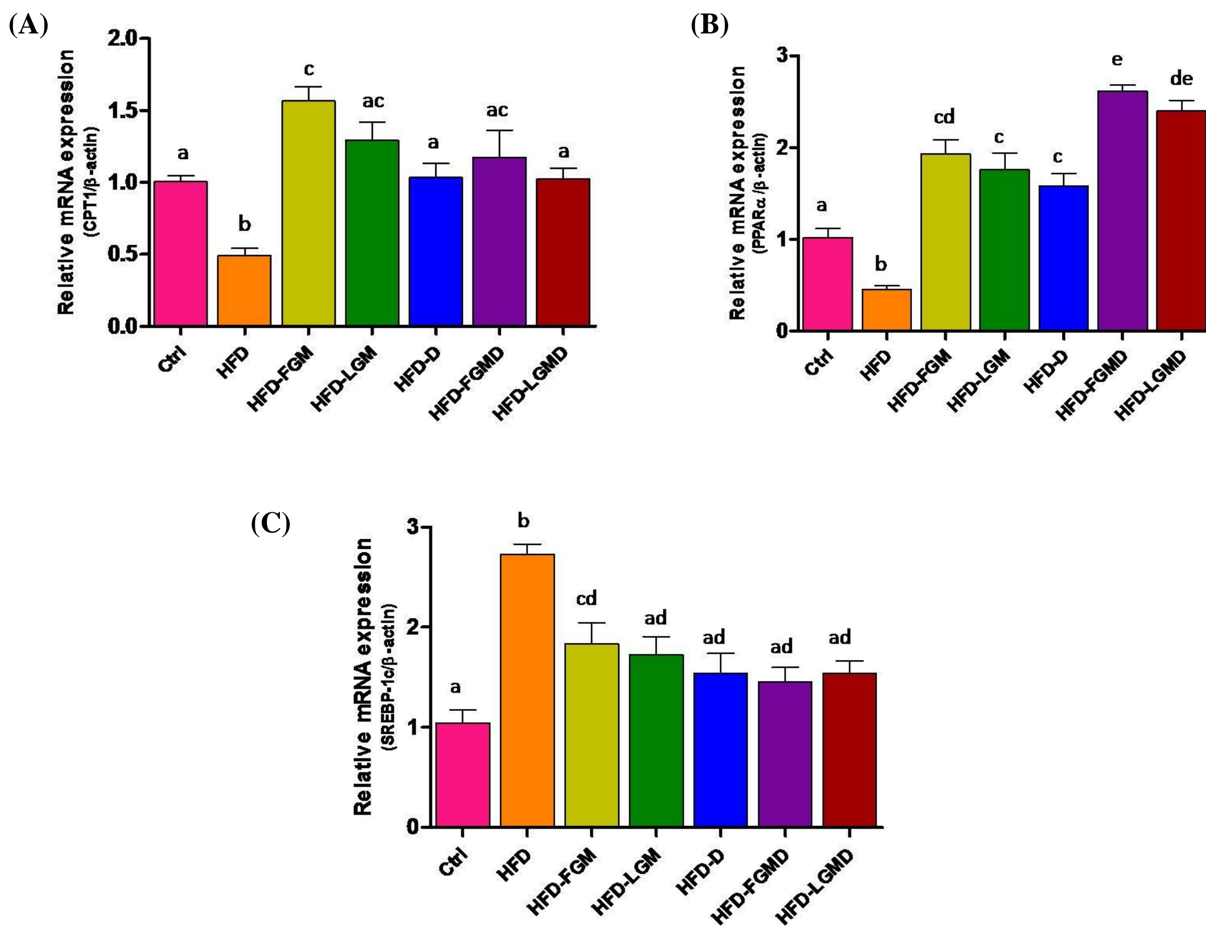


Fig. 4.18A-C Effect of dietary incorporation of galactomannans (FGM/LGM) and diosgenin alone, and in combination on (A) CPT-1 (B) PPARα and (C) SREBP-1c (involved in fatty acid oxidation & synthesis) gene expression in liver tissue of mice fed high fat diet. Mean values with different superscript letters differ significantly ($p<0.05$). Values are expressed as mean ± SEM (n =3).

4.2.3 Gene expression analysis in small intestine

4.2.3.1 Fasting induced adipose factor (FIAF)

FIAF is mainly produced by intestine and is recognized to be a circulating lipoprotein lipase (LPL) inhibitor which controls fat storage in adipose tissue. Relative mRNA expression levels of FIAF calculated for different groups are presented in Fig 4.19. Its expression was found to be significantly reduced (57%) on feeding high fat diet as compared to the level in Ctrl group ($p<0.05$). Among FGM, LGM and diosgenin, feeding of FGM exhibited maximum potential to resist the decrease in FIAF expression and the expression level in HFD-FGM reached to the level 1.50 ± 0.16 fold. Diosgenin feeding also significantly resisted the decrease in FIAF expression but it was lower than that exhibited by LGM. However, on comparison the expression levels in HFD-FGM, HFD-LGM and HFD-D were not found to be statistically different. Though the expression levels in HFD-FGMD and HFD-LGMD groups were also significantly higher than HFD group but did not exhibit any additive effect of co-administration of functional ingredients.

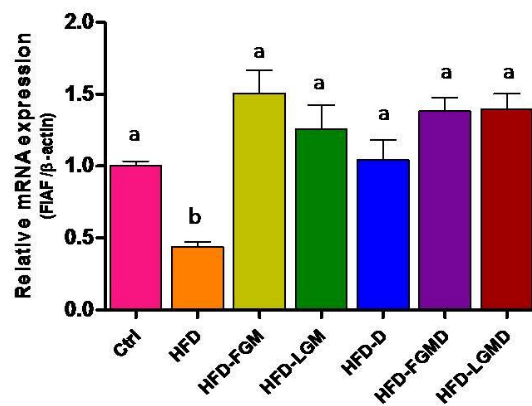


Fig. 4.19 Effect of dietary incorporation of galactomannan (FGM/LGM) and diosgenin alone, and in combination on FIAF expression in intestine of mice fed high fat diet. Mean values with different superscript letters differ significantly ($p<0.05$). Values are expressed as mean ± SEM (n =3).

4.2.3.2 Zonula Occludens (ZO-1)

As intestinal integrity is generally affected under high fat diet fed conditions, the relative mRNA expression of tight junction proteins was analyzed. As expected, ZO-1 expression in intestine of mice fed high fat diet was found to decrease significantly ($p<0.01$) and reached to the level of 0.41 ± 0.04 fold (mean ± SEM) as compared to the Ctrl group (Fig 4.20A). In groups fed

galactomannan and diosgenin alone, the ZO-1 expression levels were 0.97 ±0.10, 0.89 ± 0.09 and 0.98 ± 0.15 fold in HFD-FGM , HFD-LGM & HFD-D groups, respectively, which were significantly higher than HFD fed group ($p<0.01$, $p<0.05$ and $p<0.01$, respectively). The data reveal that all three treatments were equally effective in maintaining the ZO-1 expression near to Ctrl level. The mRNA expression levels in HFD-FGMD (1.27 ± 0.12 fold) & HFD-LGMD (1.15 ± 0.09 fold) groups suggested additive effect as a result of co-administration of functional ingredients, however the expression levels did not reach to the significantly higher level ($p>0.05$) as compared to the groups fed HFD-FGM, HFD-LGM and HFD-D.

4.2.3.3 Occludin

As shown in Fig. 4.20B, the mRNA expression of occludin was decreased significantly by high fat diet feeding to the level of 0.45 ±0.05 fold (HFD), indicating reduction almost to the same extent as observed in case of ZO-1. Also, the administration of FGM/LGM/diosgenin exhibited the protective effect similar to the trend observed with expression levels of ZO-1 in different treatment groups. Conversely, no additive effects were visible in groups fed galactomannan (FGM/LGM) with diosgenin.

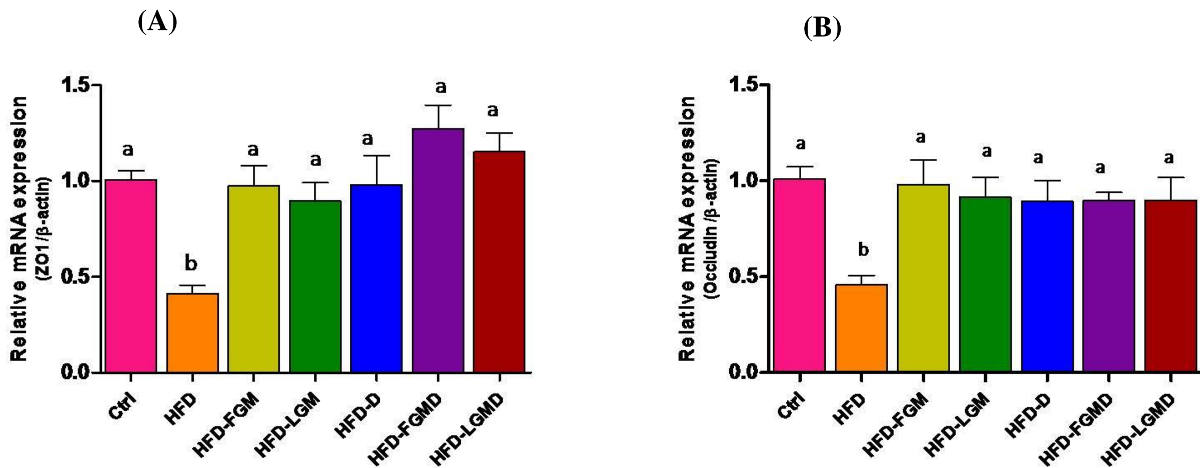


Fig. 4.20A-B Effect of dietary incorporation of galactomannans (FGM/LGM) and diosgenin alone, and in combination on (A) Occludin and (B) ZO-1 (tight junction proteins) gene expression in intestine of mice fed high fat diet. Mean values with different superscript letters differ significantly ($p<0.05$). Values are expressed as mean ± SEM (n =3).

4.2.3.4 Phosphoenolpyruvate carboxykinase (PEPCK)

In view of the fact that intestinal gluconeogenesis is also becoming the focus of attention, attempt was also made to examine the effects of galactomannan and diosgenin on relative mRNA expression of PEPCK in intestinal (distal) tissue of mice from different experimental groups (Fig 4.21A). High fat diet feeding seemed to enhance the expression of PEPCK in HFD group, but the mRNA expression level (1.83 ± 0.18) was not significantly higher compared to that in animals fed control diet (Ctrl). Dietary interventions involving FGM/LGM/diosgenin (alone or in combination) tend to counteract the up-regulation of PEPCK but the expression levels in HFD-FGM (1.47 ± 0.15), HFD-LGM (1.51 ± 0.13), HFD-D (1.56 ± 0.25), HFD-FGMD (1.56 ± 0.14) and HFD-LGMD (1.54 ± 0.19) groups were not significantly lower than that in HFD group.

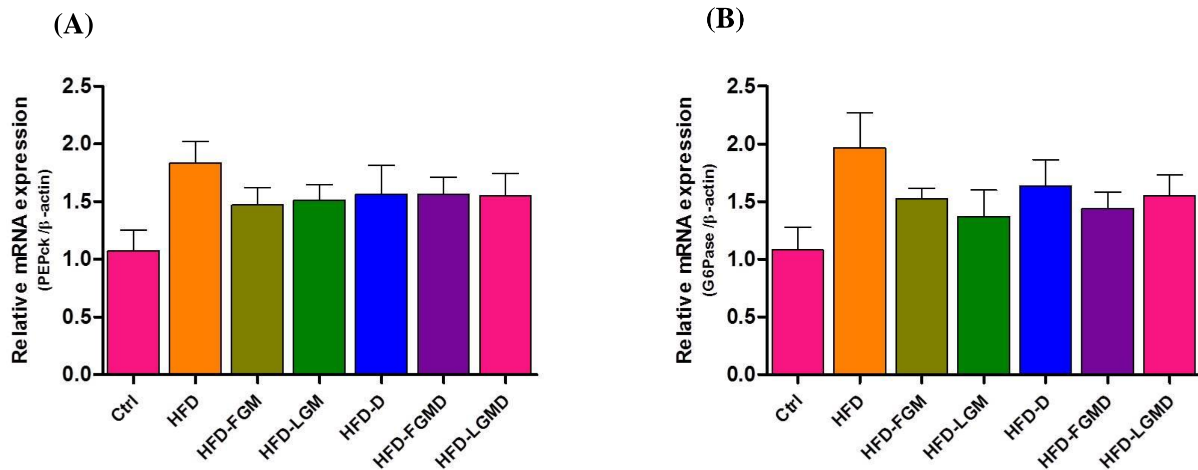


Fig. 4.21A-B Effect of dietary incorporation of galactomannans (FGM/LGM) and diosgenin alone, and in combination on (A) PEPCK and (B) glucose 6-phosphatase (gluconeogenic enzymes) gene expression in intestine of mice fed high fat diet (p>0.05). Values are expressed as mean ± SEM (n =3).

4.2.3.5 Glucose 6-phosphatase (G6pase)

The results on G6pase expression in intestine tissue are illustrated in Fig 4.21B. Similar to the trend observed in case of PEPCK, the G6pase expression appeared to be up-regulated as a consequence of high fat diet feeding reaching to the level of 1.96 ± 0.30 fold. However, the difference in expression levels between HFD and Ctrl groups was not found to be statistically significant. Administration of FGM/LGM/diosgenin (alone or in combination) exhibited

tendencies to resist the increase in g6pase expression caused by high fat diet. However, the expression levels determined for HFD-FGM, HFD-LGM, HFD-D, HFD-FGMD and HFD-LGMD were neither significantly lower compared to HFD group nor these were statistically different from one another.

CHAPTER - 5

Discussion

5.0 DISCUSSION

Obesity is defined as a phenotypic manifestation of abnormal or excessive fat accumulation that alters health and increases mortality. It is considered as an epidemic by the World Health Organization (Pimentel et al., 2010; Giskes et al., 2011). In fact, obesity is more frequent in populations living in environments charecterized by long-term energy imbalance due to sedentary life style, low resting metabolic rate, or both (International Obesity Task Force-IOTF). In the recent past, there has been increased interest in finding nutritional strategies for management of obesity and associated metabolic disorders. Accordingly, in the field of food research, the potential of various natural products is being explored to counteract obesity. The benefits of consuming foods rich in fiber are numerous, ranging from improved large bowl function to slow digestion and absorption of carbohydrate and fat, and a reduced risk of diseases. Increased consumption of dietary fiber has been associated with management of metabolic syndrome and other obesity associated diseases (Galisteo et al., 2008; Anderson et al., 2009; Threapleton et al., 2013). However, it is clear that not all fibers are equal in terms of the types and extent of health benefits they provide. Different characteristics such as bulking, solubility, water holding capacity, adsorption/binding, fermentability, and viscosity are important determinants of the effects of the fibers will have in the body. Due to the variability of effects in the body, it is important to consume fiber from a variety of sources (Slavin, 2013).

There are many naturally occurring fibers among which gums form an important class e.g. fenugreek galactomannnan, guar gum, locust bean gum, tara gum, gellan gum, and gum acacia. Gums have many culinary and medicinal uses. These are widely used as additives in various industries such as food, pharmaceuticals, paper, textile, oil well drilling and cosmetics. These are also beneficial in the control of many health problems like diabetes, bowel movements, heart disease and colon cancer due to the dietary fiber action (Barak and Mudgil., 2014). Galactomannans generally consists of a β,1-4 linked linear mannan backbone, to which single galactose grafts are linked randomly by α,1-6 glycosidic bond. Galactomannans from different legume seeds differ in M:G ratio, molecular weights, and mode of placement of the galactose grafts which are expected to determine their functional properties (Andrews et al., 1952).

Phytosterols are naturally occurring plant sterols that are structurally similar to

cholesterol. Although, phytosterol have been used to treat hypercholesterolemia since the 1950's (Miettinen, 2001), it is only in the last few decades that they have gained wide acceptance and have become available to consumers as ingredients in functional foods. Phytosterols are now being added to commercially available foods e.g., fat-based spreads, yoghurt, and cheese to facilitate reduction of serum cholesterol levels (Law, 2000; Kwak et al., 2005). As in the case with several phytochemicals, phytosterols also exhibit antioxidative activity. Recently, the interest in phytosterols in human diet has been related not only to their property of lowering blood cholesterol levels, but also to their hypoglycemic effects. Different types of phytosterols may cause different biological effects due to the difference in their chemical structures. Diosgenin, a naturally-occurring steroid sapogenin, is found abundantly in legumes (*Trigonella* sp.) and yams (*Dioscorea* sp.). It is a precursor of various synthetic steroidal drugs that are extensively used in the pharmaceutical industry. Diosgenin has been proposed to be effective against variety of pathologies, including diabetes, hyperlipidemeia, cancer, cardiovascular disease, oxidative stress and inflammation (Yamada et al., 1997; Son et al., 2007; Hirai et al., 2010; Das et al., 2012; Pari et al., 2012; Kalailingam et al., 2014). Recently, Huang et al. (2012) reported that other than carbohydrates, a phytosterol diosgenin also has prebiotic effects. The presence of diosgenin was reported to significantly enhance the growth of *Lactobacillus murinus* and *Lactobacillus reuteri*, but not enterococci.

To the best of our knowledge, no information is available with reference to the role of pure form of galactomannans and sapogenins like diosgenin in reduction of obesity under high fat/energy dense diet fed conditions. Fenugreek galactomannan (FGM) and locust bean galactomannan (LGM) differ in their M:G ratios as well as molecular weights (Brummer et al., 2003). We hypothesized that these two types of gums, differing in chemical architecture, might affect the host metabolism differently. Also, the administration of galactomannans and diosgenin in combination might result in synergistic effects due to their different modes of action. The present study was, therefore, conducted to examine the effects of two types of functional fibers viz., fenugreek galactomannan (FGM) & locust bean galactomannan (LGM), and a sapogenin (diosgenin), alone or in combination on biomarkers of obesity and expression of genes related to lipid metabolism in C57BL/6 mice fed high fat diet (60% energy from fat) for 18 weeks. The results of the study are discussed in the following sections.

5.1 TO STUDY THE EFFECTS OF GALACTOMANNAN AND DIOSGENIN ON PROGRESSION OF OBESITY IN HIGH FAT DIET FED MICE

The effects of dietary incorporation of two types of functional fibers viz., fenugreek galactomannan (FGM) & locust bean galactomannan (LGM) @ 5% (w/w) and a sapogenin (diosgenin) @ 0.5%, (w/w), alone or in combination, in high fat diet on different parameters were examined in 18 weeks of feeding schedule. As a consequence of feeding of high fat diet (60% kcal from fat), the body weight was found to remain higher in HFD group compared to the animals fed Ctrl diet throughout the feeding schedule of 18 weeks. The animals fed high fat diet seemed to gain more weight despite the fact that the cumulative feed intake in HFD group was lower than the animals fed control diet. Dietary incorporation of galactomannans and diosgenin significantly resisted the gain in body weight. However the results did not show any additive effect of co-administration of galactomannans and diosgenin. Handa and co-workers (2005) extracted fenugreek seeds with ethanol and evaporated the solution. Female ddY mice were fed high fat diet (40% beef tallow) containing 0.3% and 1% fenugreek seed extract for a short duration of 22 days. Similar to our observations, the fenugreek seed extract did not affect the food intake. They reported significant reduction in body weight gain to the same extent in animals fed 0.3% or 1% fenugreek extract. On the contrary, Muktamba and Srinivasan (2016) could not observe significant reduction in body weight of Wistar rats on incorporation of 10% fenugreek seed powder in high fat diets (59% fat) despite following a feeding schedule of longer duration of 8 weeks. The differences in anti-obesity effects might be due to the different types of preparations as Handa and co-workers (2005) administered the ethanolic extract of fenugreek seeds which is expected to contain the higher concentration of galactomannans as compared to the seed powder. Significant reduction in weight gain in high fat diet induced obese female Wistar rats has also been reported on oral administration of aqueous extracts of *Trigonella foenum-graecum* (AqE-TFG) for 21 days (Kumar et al., 2014). To the best of our knowledge, the present investigation is the first report demonstrating the reduction in body weight gain on dietary incorporation of LGM in high fat diet fed mice. We could also demonstrate the effectiveness of diosgenin to resist the gain in body weight.

Very little information is available on effects of administration of diosgenin with diet on body weight. In one study, male SD rats were fed high fat diets with or without *Dioscorea nipponica* (DN) root powder (mixed at a concentration of 2% or 5%) for 8 weeks (Kwon et al.,

2003). Feeding of high fat diet plus 5% DN powder was reported to significantly suppress the body weight gain compared to the control group fed on high fat diet alone during the experimental period. They also showed the fecal fat excretion of the experimental groups fed with the 2 and 5% DN powder to be significantly higher than the control group and suggested that the DN powder prevented dietary fat absorption in rats, which may explain one of the mechanisms for decrease body weight gain and fat mass accumulation.

In the present study, feeding of high fat/energy dense diet resulted in significantly higher accumulation of epididymal fat after 18 weeks, indicating the induction of adiposity by high fat diet, which was well correlated with the significant increase in mean adipocyte size and decrease in adipocyte number (determined by histological analysis) in HFD group. Dietary interventions involving incorporation of galactomannans/diosgenin exhibited anti-adiposity effect as revealed by the reduction in fat mass accumulation & adipocyte size, and also correspondingly the increased adipocyte number. Only a few reports are available related to efficacy of galactomannans and diosgenin in regulating fat mass accumulation. Uemura and co-workers (2010) investigated the effects of fenugreek supplementation (0.5 or 2% w/v) in high fat diet (60% kcal from fat) in KK-Ay mice and reported that fenugreek affected neither the body weight nor total WAT weight of mice which might be due to the fact that the feeding period was of short duration of 4 weeks only. Interestingly, the increased adipocyte size induced by HFD was less pronounced in 2% fenugreek fed mice, however, this interpretation was based on histological analysis of epididymal WAT without doing any quantification of adipocyte size and number. Significantly lowered WAT has been reported on oral administration of aqueous extract of *Trigonella feonum graecium* @ 0.5 and 1.0 g/ kg with HFD for 21 days in Wistar rats (Kumar et al., 2014). Like fenugreek and locust bean gums, the guar gum is also an important soluble fiber rich in galactomannans. David et al. (2014) determined the effects of one fermentable (guar gum) and one non-fermentable fiber (cellulose) on development of adiposity in diet induced obesity model. They demonstrated that epididymal fat pads of Wistar rats, fed 5% guar gum for 10 weeks, weighed less than the cellulose fed group.

Different types of dietary fibers may differ in their chemical architecture, viscosity, gel forming ability, fermentability etc. Evidently, several factors might be contributing to varying extent in regulating the body mass and fat mass accumulation. Viscous fibers might be fermentable or non fermentable. Viscosity is a key factor in influencing gastric emptying of food

and increased viscosity is associated with more prolonged satiety (Marciani et al., 2000). On the basis of experiment involving non-fermentable (HPMC, hydroxypropyl methylcellulose) and fermentable (GG, guar gum), David et al. (2014) concluded that addition of viscous fibers to the diet, regardless of fermentability, may have utility for decreasing obesity and fatty liver and improving metabolic flexibility. In a recent report, guar gum (rich in galactomannans) has been shown to possess distinct bifidogenic effect independent of viscosity, increasing the cecal abundance of bifidobacteria, which was also associated with butyrate levels, both in cecum and in the systemic circulation (Fak et al., 2015). Increase in bifidobacteria has been reported to be inversely correlated with body weight gain in Fa/Fa obese rats (Waldran et al., 2009). In another study, Kondo et al. (2010) found that mice fed a high fat diet supplemented with *Bifidobacterium brevi* B-3 for 8 weeks resulted in suppression of the accumulation of body weight and epididymal fat. It can be surmised that both types of galactomannan preparations (FGM and LGM) might be acting through stimulating the proliferation of bifidobacteria in gut. Other researchers have attributed the weight loss on consumption of fibers to the reduction of bile acid pool in the body (Babiker et al., 2012); decrease in metabolizable energy of diet (Baer et al., 1997).

The detailed mechanisms by which phytosterols effect a reduction of adipose tissue and body weight have not been fully elucidated. In the present study, we could demonstrate the efficacy of pure form of diosgenin in decreasing the gain in body weight together with reduction in epididymal fat mass. Kwon and co-workers (2003) reported the lipase inhibitory activity of saponins isolated from *Dioscorea nipponia* (DN). They ascribed the weight reduction by DN powder to the decreased fat absorption and it did not depend upon a decreased food and energy intake. Huang and co-workers (2012) for the first time demonstrated the prebiotic potential of diosgenin. Prebiotics are well recognized to improve the gut microbial balance in favor of health promoting bacteria. Obesity is known to be a chronic low grade inflammatory condition induced by lipopolysaccharides produced from Gram -ve bacteria. Therefore, to investigate the positive effects of sapogenins like diosgenin on gut environment can be an exciting area of research.

It is well recognized that the progression of diet induced obesity is linked with development of insulin resistance, type 2 diabetes and other associated complications. In the present investigation consumption of high fat diet (60% kcal from fat) caused overweight, and resulted in induction of hyperglycemia and hyperinsulinemia. High fat diet feeding elevated the

fasting blood glucose level after 6 weeks, however, feeding of high fat diet for a long duration of 18 weeks resulted in induction of hyperglycemic conditions as evinced by the significant rise in fasting blood glucose level. The serum insulin levels were also increased under high fat diet fed conditions corresponding to the rise in blood glucose levels. HOMA-IR score, an indicator of insulin resistance, was found to be significantly higher in HFD group as compared to the score in Ctrl group. Our results are in agreement with the findings of other researchers where consumption of high fat diet has been reported to induce hyperglycemia along with hyperinsulinemia and insulin resistance (Dewulf et al., 2011; Neyrinck et al., 2011; den Besten et al., 2014; Heijden et al., 2015). In the present study, it could be demonstrated that both types of galactomannans and the diosgenin were significantly effective in normalizing the fasting blood glucose levels. The positive effects of dietary incorporation of different functional ingredients were also revealed by the protection from high fat diet induced hyperinsulinemia and the insulin resistance (improvement of HOMA-IR). A trend was visible where administration of locust bean galactomannan (LGM) seems to be more effective than fenugreek galactomannan, and the efficacy of both types of soluble fibers (FGM and LGM) was more than that of sapogenin (diosgenin) in counteracting the rise in HOMA-IR score under high fat diet fed conditions.

The available literature related to both rodents and humans indicates that insulin sensitivity improves as visceral fat reduces (Fantuzzi and Mazzone., 2007; Uemura et al., 2010). Uemura and co-workers (2010) reported lowering of fasting plasma glucose & insulin levels and also the improvement in insulin sensitivity in obese diabetic model mice (KK-Ay) fed HFD supplemented with 2% fenugreek seed powder for 4 weeks. As far as locust bean galactomannan is concerned, ethanolic extracts of LBG have been reported to decrease the blood glucose level in STZ induced diabetic Wistar rats (Mokhari et al., 2011). The efficacy of fenugreek extracts to improve the hyperglycemic & hyperinsulinemic condition, and insulin sensitivity in high fat diet fed rodents has also been demonstrated by orally administering the different types of extracts: *Trigonella foenum graecum* (TFG) extract administered (2 g/kg, daily) in HFD fed C57BL/6J mice model, in both the preventive as well as curative study (Hamza et al., 2012); treatments with Aq-TFG (0.5 and 1g/kg) for 21 days in Wistar rats (Kumar et al., 2014). Galactomannans are the non-digestible viscous fibers and this property is expected to decrease the intestinal glucose uptake. Galactomannan from Canadian fenugreek seed (*Trigonella foenum graecum* L) has been reported to inhibit the intestinal glucose uptake in jejunum and ileum segments of JCR

rats due to viscosity of galactomannan (Srichamroen et al., 2009). The other possible mechanism for the hypoglycemic effect of galactomannans might be attributed to their inhibitory effects on intestinal disaccharidases. This is supported by the experiments conducted by Hamden and co-workers (2010) where administration of galactomannan to diabetic rats significantly decreased the maltase, lactase and sucrose activities. The results of present study on blood glucose level suggested that the hypoglycemic effects observed in different treatment groups might be due to the improvement in insulin sensitivity. Vijayakumar and coworkers (2005) demonstrated the *in vivo* hypoglycemic activity of i.p. administration of a dialyzed fenugreek seed extract (FSE) in alloxan (AXN) induced diabetic mice. They investigated the mechanism by which FSE attenuated hyperglycemia in an *in vitro* experiment, and concluded that FSE induces a rapid, dose-dependent stimulatory effect on cellular glucose uptake by activating cellular responses that lead to GLUT4 translocation to the cell surface.

No information is available on role of diosgenin in amelioration of hyperglycemia induced by high fat diet. Mc Anuff et al. (2003) studied the effect of 1% bitter yam steroidal sapogenin extract and commercial diosgenin on intestinal morphology in streptozotocin (STZ) induced diabetic rats. They reported that induction of diabetes significantly increased villus and crypt depth and supplementation of the diet with sapogenin extract or commercial diosgenin significantly increased villus width and significant decreased the crypt depth. They linked the decreased crypt depth is indicative of decreased surface area for absorption, to the observed decrease in level blood glucose. In another study (Mc Anuff et al., 2005) significant reduction in Na^+-K^+ ATPase activity on supplementation of diet with bitter yam steroidal sapogenin extract or commercial diosgenin was given an explanation to account for their hypoglycemic properties. Amelioration of hyperglycemia in diabetic rat models by oral administration diosgenin has also been reported (Sangeeta et al., 2013; Kalailingam et al., 2014). Whereas on plausible mechanism suggested for diosgenin reducing hyperglycemia is by promoting insulin level in circulation which is due to the protection and regeneration of β-cells (Sangeeta et al., 2013). Kalailingam and co-workers (2014) have further reported that glucokinase activity was decreased significantly in the liver of STZ induced diabetic rats and the treatment with diosgenin decrease blood sugar levels, possible by increasing insulin secretion, which would activate the glucokinase and increased the utilization of glucose.

High fat diet consumption is known to cause dyslipidemia (Choi et al., 2014; Wu et al.,

2015). In the present investigation, lipid profile was observed to be disturbed as indicated by significant increases in serum TG (48.9%), TC (43.8%), LDL-C (107%), VLDL-C (48.8%) and AI (120.3%) in HFD group compared to Ctrl group after 18 weeks. However, the HDL-C level remained unaffected due to high fat diet feeding. Both the galactomannans (FGM and LGM) were found to resist the increase in serum TG, TC, LDL-C, VLDL-C & AI levels significantly and appeared to exhibit the normalizing effects on these parameters. Furthermore, from the serum TG and LDL-C levels, efficacy of FGM seems to be slightly better than that of LGM. Similar to FGM and LGM, administration of diosgenin did show maximum protective effect against rise in serum TC level under high fat diet fed conditions. However, such trend was not visible in case of serum TG levels, where the ~19% reduction could be seen due to diosgenin (HFD-D group). The data also reveal that generally maximum protective effects could be achieved by individual functional components and no additive effects were visible. Importantly, the feeding of high fat diet for a long duration is accompanied with corresponding increases in hepatic TG and TC stores. However, in the present investigation, hepatic TG and TC were not found to increase significantly due to high fat diet. Of Course, maximum protective effect toward normalizing the hepatic TG content was clearly visible by feeding galactomannans or diosgenin. As for as hepatic TC levels are concerned, diosgenin seem to be more effective in comparison to galactomannans.

The hypolipidemic effects observed in our study are in agreement with the finding of Muktamba and Srinivasan (2016) who reported the serum lipid lowering (TG,TC,LDL-C & AI) and also hepatic TG & TC levels due to dietary incorporation of fenugreek seed powder (2% w/w) in high fat diet fed to Wistar rats for 8wk. However, contrary to our results, they could observe increase in serum HDL-C. In another study, Kumar et al., (2014) could observe significant decrease in level of serum TC, TG, LDL-C and VLDL-C and a significant increase in level of HDL-C after oral administration of Aq-TFG (0.5 and 1.0 g/kg) 21 days in HFD fed rats. Hamza et al. (2012) administered ethanolic extract of *Trigonella foenum* (2 g/kg, daily) orally by gavage in C57BL/6J fed high fat diet for 20 wks and demonstrated significant reduction in serum TG levels. The beneficial effects of dietary fenugreek in reduction of hepatic and plasma TG & TC in rats fed a high fat high sucrose diet has been demonstrated by other researchers also Muraki et al. (2011) reported that fenugreek seeds in reduction of hepatic and plasma TG & TC in rats fed a high fat high sucrose diet. Srinivasan (2013) has also reported that fiber and saponin

components of fenugreek seeds are responsible for cholesterol lowering or lipid lowering activity of fenugreek seeds. Guar gum, a viscous fiber is known to be rich in galactomannans. Hypocholesterolemic and hypolipidemic effect of guar gum have also been documented in rats Shahzadi et al., 2007; Fak et al., 2015). The beneficial effect of *Trigonella foenum* soluble dietary fiber (Tf-sdf) has been attributed to retardation of carbohydrate and fat absorption due to the presents of bioactive fiber (Hannan et al., 2003); excretion of bile acids and neutral sterols in feces due to dietary fenugreek galactomannan in WNIN(GR-Ob) obese rats (Ramulu et al., 2011). Inhibitory potential of dioscin and its aglycone, diosgenin against fat absorption in SD rats fed high fat diets containing 5% DN powder has also been demonstrated (Kwon et al ., 2003). These researchers also demonstrate the inhibition of pancreatic lipase activity by diosgenin. The hypocholesterolemic effect of ethanolic extract derived from fenugreek in high cholesterol diet fed rats has been attributing to saponins (Stark and Madar, 1993). More studies are needed related to hypolipidemic effects of galactomannans and diosgenin as for example how the HDL-C is increased as reported in certain studies; then contribution of endogenous cholesterol in experimental animals fed galactomannan and sapogenin (diosgenin).

Dysregulation of energy expenditure in obesity can be attributed to impaired glucose and lipid metabolism. Liver plays a critical role in blood glucose homeostasis. In the present investigation, we could demonstrate a decreasing trend in the activities of key regulatory enzymes of glycolysis viz., phosphofructokinase (PFK), pyruvate kinase (PK) and glucokinase (GK) in liver tissue in high fat diet fed group (HFD). Furthermore, the activities of key regulatory enzymes of gluconeogenesis viz., PEP carboxykinase (PEPCK) and fructose 1,6-bisphosphatase (FBPase1) were found to increase significantly due to high fat diet feeding. Protection against the decrease in activities of PFK, PK and GK could be observed on feeding galactomannans (FGM & LGM) and diosgenin, however, it was more conspicuous in case of PK on administration of galactomannans. The positive effects of galactomannans and diosgenin on gluconeogenesis were also visible as revealed by the reduction in activity of most important regulatory enzyme, PEPCK, under high fat diet fed conditions. Vats and co-workers (2003) administered fenugreek orally to STZ induced diabetic rats for 30 days and reported significant increase in PFK, GK and HK activities. They suggested that the cellular entry of glucose was facilitated by fenugreek, which in turn stimulated the activity of GK and PFK enzymes. However, this was not supported by the mechanistic details. Reports are also available where

feeding *Trigonella foenum graecum* seed powder (TSP) to diabetic rats has been reported to result in restoration PK and PEPCK activities (Mohamad et al., 2004); hexokinase (I, II, and IV) PFK, PK, glucose 6-phosphatase and fructose1, 6-bisphophatase activities (Raju et al., 2001). In one study, feeding high fat diet supplemented with guar gum (GG) to Wistar rats for 10 wk has been reported to result in lowered hepatic glucose 6-phosphatase and phosphoenolpyruvate carboxykinase gene expression compared with the high fat diet control (cellulose) group (David et al., 2014).

Molecular mechanisms of disturbed hepatic metabolism under high fat diet conditions are still to be clearly elucidated. In obese or type 2 diabetic patients, insulin fails to suppress hepatic glucose output, which leads to hyperglycemia via an up regulation of the gluconeogenic enzymes, glucose 6-phosphatase and phosphoenolpyruvate carboxykinase (Magnusson et al., 1992). Jiang and co-workers (2011) reported that mRNA expression of hepatic glucokinase (Gck) and L-type pyruvate kinase (LPK) was reduced dramatically in HFD rats. Furthermore, they observed the decreasing trend of Gck and LPK activities very similar to that of their mRNA expression. DNA methylation was correlated with the decline in hepatic Gck and LPK expression in high fat diet induced obese rats as compared with the control group. In view of above, it appears to be of great significance to investigate the mechanistic details at the molecular level as to how the different functional components varying in their chemical architecture like fermentable fibers and a range of phytosterols affect the key regulators of energy homeostasis, both at the activities and expression levels.

5.2 ANALYSIS OF EXPRESSION PATTERN OF GENES RELATED TO LIPID METABOLISM AS AFFECTED BY GALACTOMANNAN AND DIOSGENIN FEEDING

A wide variety of changes in nutritional conditions can lead to adjustments/imbalance in carbohydrate and fat metabolism. Dysregulation of energy expenditure is one if the important causes of development of obesity and related metabolic disorders. Gene expression can play a critical role in the adaptive responses. Alterations in activities of different enzymes involved in energy metabolisms can also be a significant consequence during altered metabolism. The relative mRNA expression of genes were measured in different tissues viz., epididymal fat, liver and distal small intestine of C57BL/6 mice fed high fat diet and administered soluble fibers (FGM/LGM) and diosgenin alone or in combination to evaluate the effects if different functional

components. Earlier studies on galactomannans and diosgenin have mostly dealt with their effects on body weight, fat mass, and hypoglycemic & hypolipidemic properties (Vijaya kumar et al., 2005; Uemura et al., 2010; Sangeeta et al., 2013; Kalailingam et al., 2014; Sato et al., 2014; Muktamba and Srinivasan., 2016; Sharma and Choudary., 2016). However, not much work has been done regarding the exact mechanism by which of soluble fiber (galactomannan) and diosgenin regulate the host metabolism and this still needs to be fully understood.

Adipose tissue used to be considered simply a fat storage depot. The increasing information related to this organ recognizes it a dynamic organ which plays an important role in energy homeostasis via some secretary proteins especially adiponectin and leptin. High fat diet causes decreased adiponectin (inversely correlated with body mass) and increased leptin levels (positively correlated with adiposity). In the present investigation, high fat diet feeding resulted in decreased mRNA level expression of adiponectin while the leptin expression was significantly up-regulated (>6.5 fold). These data are very well correlated with the significantly higher accumulation of epididymal fat, increased mean adipocyte size, and the decreased adipocyte number in HFD group. High fat diet feeding also resulted in significant down-regulation of GLUT4, the important transporter in adipose tissue. This finding is well correlated with the hyperglycemia and increase in HOMA-IR score due to high fat diet feeding. Both types of galactomannans and the sapogenin (diosgenin) exhibited significant potential to counteract the effects of high fat diet. Both, FGM as well as LGM, were more effective than diosgenin in resisting the down regulation of adiponectin and GLUT4. The higher efficacy of galactomannans was further corroborated by leptin expression data where FGM as well as LGM could significantly resist the up-regulation of leptin and down-regulation of GLUT4. Furthermore, FGM tends to be relatively more effective than LGM. Diosgenin also exhibited similar effect in resisting leptin expression, but the protective effects of galactomannans were significantly higher than that due to diosgenin. David and coworkers (2014) found significantly increased adiponectin and decreased leptin levels on administration of highly viscous fibers - (5%, w/w) hydroxypropyl methylcellulose (HPMC; nonfermentable) or guar gum (GG; fermentable) than the cellulose in high fat diet fed group and they also suggested that increased plasma adiponectin can increase activation of hepatic AMP kinsae, which in turn increases fatty acid oxidation by increasing the activity of CPT1. In our study, the significantly increased mRNA expression of CPT1could be observed due to FGM.LGM, and also diosgenin.

Uemura et al. (2010) fed KK-Ay mice HFD diet supplemented with fenugreek @ 0.5 and 2 % for 4 weeks and reported higher adiponectin levels than the HFD fed group, and they also suggested diosgenin to be the bioactive component in fenugreek is responsible for its positive effects. However, they did not examine the effects of other components like galactomannans. Contrary to the findings of present study as well as the study conducted by Uemura et al., (2010), Furlan and coworkers (2013) demonstrated no improvement of adiponectin level on supplementation of @ 2% phytosterol in high fat diet.

PPARγ is the marker gene responsible for adipocyte differentiation (Siersbaek et al., 2010). GPCR43 has been reported to be expressed in adipose tissue, intestine and immune tissues (Hong et al., 2005; Maslowski et al., 2009). Certain reports have indicated that GPCR43 promotes leptin secretion, adipogenesis and inhibition of lipolysis in adipose tissue and adipocytes, thus regulating energy metabolism (Hong et al., 2005; Ge et al., 2008; Zaibi et al., 2010). Though, in immune system regulation of inflammatory responses by GPCR43 is well studied process, GPCR43 has not been as well studied in the adipose tissue (Kimura et al., 2013). In the present investigation, the dietary supplementation of FGM/LGM as well as diosgenin (alone, or in combination) was observed to resist the rise in mRNA expression of PPARγ and GPCR43 in epididymal fat. Though, both galactomannan exhibited higher effectiveness compared to diosgenin, dietary incorporation of fenugreek galactomannan appeared to be more effective in resisting PPARγ expression. To the best of our knowledge, no animal studies are available dealing with the effects of pure galactomannans and diosgenin on differentiation of adipocytes, and PPARγ & GPCR43 expression. Recently, den Besten et al. (2015) demonstrated that supplementation of guar gum seed powder (rich in galactomannans) was effective against high fat diet induced obesity and insulin resistance, by repressing PPARγ expression, subsequently increasing mitochondrial UCP2 expression and AMP/ATP ratio, leading to the activation of AMPK and culminating in enhanced fatty acid oxidation in both liver and adipose tissue of mice. Vijayakumar et al. (2010) treated 3T3-L1 cells with novel thermostable extract of fenugreek seeds (TEFS) and reported that TEFS inhibited fat accumulation in differentiating as well as differentiated 3T3L1 cells via, decreased expression of adipogenic transcription factors viz., PPARγ, c/EBP-α, SREBP-1 in a dose depended manner. No difference in GPCR43 gene expression was found on feeding guar gum compared with cellulose in high fat diet and normal

fat diet (David et al., 2014). This seems to be in contradiction with the results in present study on GPCR43 as affected by pure galactomannans.

GLUT4 is a very important insulin-responsive glucose transporter in adipose tissue, and the development of insulin resistance is the result of impaired GLUT4 signaling pathway. The improvement in insulin sensitivity (decreased HOMA-IR score), observed in the present investigation, especially in FGM and LGM fed groups, could be linked to the relatively higher expression of GLUT4 under the influence of galactomannans. Though, diosgenin also exhibited similar effect, but it was lower than that exhibited by galactomannan. Significantly higher GLUT4 expression has been reported in 2% fenugreek powder fed mice (Uemura et al., 2010). Furthermore, these researchers could also observe higher expression levels in 3T3-L1 cells treated with diosgenin. On the contrary, Vijayakumar et al. (2010) did not observe any alteration in GLUT4 expression on treatment of 3T3-L1 cells with thermostable extract of fenugreek. Probably, they could not observe the effect on GLUT4 as the fenugreek extract was administered, rather than a pure component.

The results of present investigation indicate the significant down regulation of genes related to regulation of fatty acid oxidation (CPT-1 and PPARα) due to high fat diet. We were able demonstrate the significant resistance to the decrease in expression of CPT1 as a consequence of feeding galactomannans/diosgenin. Furthermore, efficacy of both the fibers (FGM and LGM) were higher than that of diosgenin, and between two types of galactomannans, FGM exhibited better protective effect as compared to LGM. Same trend was visible in case of PPARα. Importantly, additive effects could also be observed on administration of either type of galactomannan in combination with diosgenin (HFD-FGMD & HFD-LGMD). mRNA level expression of SREBP-1c (related with lipogenesis) was found to be up regulated due to high fat diet feeding. However, the resistance to its up regulation was maximum due to diosgenin followed by LGM and FGM. These results clearly suggest that all the functional components were significantly effective in modulating the expression of genes related to lipid metabolism towards increased energy expenditure and decrease in lipogenesis. Furthermore, these findings are in consonance with the decreased epididymal fat mass accumulation and also mean adipocyte size. Adiponectin is the key adipokine responsible for maintenance of energy homeostasis. It work through AMPK and enhances the fatty acid oxidation. Evidently, the effects of galactomannans as well as diosgenin on mRNA expression of CPT1, PPARα and SREBP-1c

in liver are in conformity with the significant upregulation of adiponectin and down regulation of leptin observed in adipose tissue. FIAF is a circulating lipoprotein lipase (LPL) inhibitor that controls triglyceride deposition into adipose tissue (Yoshida et al., 2002; Sukonina et al., 2006). It is also a downstream target gene of PPAR's. Similar to the observations in case of CPT1 and PPARα, the mRNA expression of FIAF was found to decrease significantly as a consequence of high fat diet feeding which can be easily correlated with the increased fat mass accumulation in HFD group. Furthermore, all dietary interventions involving galactomannans and diosgenin were found to be significantly effective in counteracting the effects of high fat diet and their efficacy was in the order: FGM>LGM>D, this being in harmony with that seen in case of CPT1 as well as PPARα.

There is paucity of information related to effects of galactomannans and diosgenin on expression of genes related to regulation of lipid metabolism linking with adiposity and energy homeostasis under the high fat/energy dense fed conditions. Uemura et al. (2011) conducted studies to examine the effect of 2% fenugreek in HFD fed mice, and due to diosgenin treatment in T0901317 induced HepG2 cells. They reported decreased mRNA expression of SREBP-1c and FAS, SCD1and ACC in liver of 2% fenugreek fed group. In addition, they also found that diosgenin dose dependently suppressed FAS, SCD1 and ACC mRNA expression in HepG2 cells, and the expressions of SREBP-1c, both at mRNA and protein level. David et al. (2014) fed HPMC (nonfermentable) and guar gum (fermentable fiber, rich in galactomannans) in high fat diet and observed lowered SREBP-1c mRNA expression. Oral administration of Aloe vera phytosterols in HFD has also been reported to significantly increase the expression levels of liver genes involved in fatty acid uptake (FATP1 and FAT/CD36) and fatty acid oxidation (ACOX-1, CPT 1a, CYP4a10 and CYP4a14) in C57BL/6J mice (Nomaguchi et al., 2011). Contradictory report is also available where supplementation of 2% phytosterol with control diet did not affect PPARα protein expression in liver of C57Bl/6J mice (Rideout et al., 2010).

Obesity is considered a systemic chronic low-grade inflammation, and the low grade inflammation can play a causal role in obesity associated insulin resistance. Adipose tissue is an important site of inflammatory events in obesity. In addition to regulating fat mass and nutrient homeostasis, adipocytes mediate the inflammatory response through the secretion of adipokines, cytokines, and chemokines that enhance the recruitment of immune cells, especially macrophages, to adipose tissue (Cinti et al., 2005). High fat diet is known to disturb the gut

environment, creates dysbiosis and affects the intestinal functionality also by affecting the intigrety/permeability. In the present study, high fat diet feeding significantly enhanced the Monocyte Chemoattractant Protein-1 (MCP-1) and TNFα in epididymal fat tissue indicating the pro-inflammatory response which can be linked with the increased insulin resistance (HOMA-IR score) observed due to high fat diet feeding for a long duration. Though, FGM/LGM and diosgenin tend to resist the up regulation of TNFα to the same extent, MCP-1 expression was maximally resisted by diosgenin. Uemura and co-workers (2010) fed 2% fenugreek in HFD to KK-Ay mice and observed decreased mRNA expression of F4/80, MCP-1 and TNFα in adipose tissue and they also found decreased mRNA expression of MCP-1 in diosgenin treated 3T3-L1 cells in dose depended manner. In another study, arabinoxylan oligosaccharide (AXOS) supplementation counteracted the HF-induced F4/80 expression in visceral adipose tissue suggesting lower macrophage infiltration (Neyrinck et al., 2012). In the present study, the beneficial effects of galactomannans and diosgenin in counteracting the high fat diet induced pro-inflammatory response is in consonance with the positive effects of these functional components on mRNA level expressions of two important tight junction proteins responsible for maintaining the intestinal integrity. Whereas diosgenin and FGM were effective to the same extent in counteracting the up-regulation of ZO-1 under high fat diet fed conditions, occludin expression was up-regulated equally by FGM, LGM and diosgenin. Very scanty information is available on effects of fermentable fiber and sapogenins on expression of genes related to intestinal integrity/permeability. However, Neyrinck and co-workers (2012) observed altered tight junction proteins (Zonula occludens-1 and claudin-3) upon high fat feeding and the up-regulation by supplementation of arabinoxylan oligosaccharides (AXOS), a fermentable carbohydrate.

CHAPTER - 6

Summary and conclusion

6. SUMMARY AND CONCLUSION

Obesity is a complex metabolic disorder which involves storage of abnormally large amount of fat in the body and has become a major public health concern in developed as well as developing countries. Different approaches, such as dietary control and physical activity have been generally unsuccessful in controlling obesity as these rely on an individual's behavior. Moreover, pharmacological and surgical treatments are associated with certain side effects. In the recent past, there has been increased interest in the use of functional ingredients to promote energy expenditure or satiety or dilute energy density which may offer a more physiological solution in obesity management, and functional fibers can be an attractive option in this regard. Although, different studies are available in literature suggesting the various health benefits of consumption of insoluble as well as soluble fibers, the details around mechanism of action of fibers differing in their chemical architecture have not been completely understood. No comparative studies are available on antiobesity potential of galactomannan from fenugreek and locust bean. Furthermore, no studies are available at gene expression level to explain effect of pure form of galactomannans under high fat/energy dense conditions.

Diosgenin, a steroidal saponin (phytosterol) found in many plants such as fenugreek (*Trigonella foenum graecum*), *Costus speciosus*, and different *Dioscorea* species is suggested to exhibit many biological functions. A few reports are available on hypoglycemic and hypolipidemic effects of steroidal sapogenin extract from Jamaican bitter yam (*Dioscorea polygonoides*) or commercial diosgenin. Evidently, it was thought exciting to investigate the effects of pure form of diosgenin under high fat/energy dense conditions.

We hypothesize that the two types of functional ingredients viz., galactomannan (fiber) & diosgenin (a sapogenin) are expected to work through different mechanisms. Galatomannans from two different sources i.e, fenugreek & locust bean differ in M:G ratio which determines the physicochemical properties of the gum (solubility, gel forming capacity and viscosity). In view of above, the present study was conducted to examine the effects of two types of functional fibers viz., fenugreek galactomannan (FGM) & locust bean galacomannan (LGM), and a sapogenin (diosgenin), alone or in combination on biomarkers of obesity and expression of genes related to lipid metabolism in C57BL/6 mice fed high fat diet for 18 weeks. The findings of the study are summarized as below.

1. The final body weight after 18 weeks in HFD fed group was higher as compared to control group. No significant differences were observed in feed intake among different treatment groups fed isocaloric high fat diets. Dietary incorporation of galactomannans (FGM/LGM) alone and in combination with diosgenin in high fat diet resulted in decreased body weight 9 week onwards, but reached statistically significant level after 15th week onwards. Animals fed HFD containing diosgenin alone reduced body weight significantly after 18 weeks of feeding. Further, an additive effect could be seen in FGMD group.
2. A significant increase in epididymal fat (E.fat) mass accumulation (62.2%, $p<0.05$) could be observed in HFD group as compared to control group (1.72 ± 0.15 vs. 1.06 ± 0.09 g). E. fat mass accumulation was found to be significantly lower in either of the galactomannan (5%, w/w) and diosgenin (0.5%, w/w) fed groups, when administered alone or in combination. E.fat mass accumulation was found to be 39.5%, 38.4%, 33.7%, 34.3% and 37.7% lower in HFD-FGM, HFD-LGM, HFD-D, HFD-FGMD and HFD LGMD, respectively, as compared with HFD group. Galactomannans seemed to be more effective than diosgenin in resisting the fat mass accumulation.
3. The dietary incorporation of galactomannans or diosgenin showed a trend in decreasing the liver weight, but not to the statistically significant extent. Further, kidney and spleen weights remained unaffected.
4. The fasting blood glucose level was found to increase significantly due to HFD (178.4 mg/dl) after 18 weeks. The rise in fasting blood glucose due to high fat feeding was significantly ($p<0.05$) resisted by dietary incorporation of FGM/LGM and diosgenin alone or in combination, exhibiting a normalizing effect.
5. A significant rise in insulin levels due to high fat feeding was in conformity with the increase in blood glucose levels. The serum insulin levels & HOMA-IR score in HFD group were measured to be 1.68 ± 0.16 ng/ml & 18.12 ± 1.35, respectively. Both FGM & LGM were equally effective in resisting the hyperinsulinemia as well as increase in HOMA-IR score, and the effect was more than that of diosgenin. However, no additive effects of co-administration of galactomannan and diosgenin could be observed.
6. The HFD feeding resulted in dyslipidemia and significantly increased atherogenic index. The dietary incorporation of FGM/LGM seemed to be more effective than

diosgenin in normalizing the serum TG levels. However, no difference could be observed in efficacy of galactomannans and diosgenin, whether administered alone, or in combination. The LDL-C and VLDL-C levels were also positively affected by both types of galactomannan and diosgenin. HDL-C levels did not differ significantly among all different treatment groups.

7. The increase in hepatic TG was resisted to the same extent by FGM/LGM/diosgenin. Increase in hepatic TC due to HFD was resisted to the significant extent by diosgenin alone and also in combination with either of the functional fiber.
8. High fat diet feeding resulted in significant increase in adipocyte size compared to the Ctrl group. Mean adipocyte size and number indicated the efficacy of FGM/LGM/diosgenin in resisting the adiposity. Moreover, synergistic effects of co-administration of fibers and diosgenin were also visible.
9. Consumption of HFD seemed to affect the glycolytic flux and also the opposing pathway i,e. gluconeogenesis. Reduction in activities of key enzymes of glycolysis in liver viz., GK, PFK & PK were observed in HFD group as compared to control. Significantly higher pyruvate kinase activities were measured in FGM as well as LGM fed groups compared to animals fed HFD only.
10. The gluconeogenesis process seemed to be enhanced under high fat diet fed conditions as evinced by the increased activities of two important regulatory enzymes, PEPCK & fructose1, 6-bisphosphatase. PEPCK activity revealed the positive effects of all dietary treatments, the maximum effects exhibited were on co-administration of functional fiber and diosgenin.
11. The mRNA expression of adiponectin (responsible for regulating energy homeostasis) in E. fat appeared to decrease due to HFD feeding (less energy expenditure). Both the galactomannans (FGM/LGM) were equally effective in resisting the down-regulation of adiponectin and higher than the diosgenin. Further, synergistic effects could be found on co-administration.
12. The highly significant ($p<0001$) increase in leptin expression (adiposity signal) was in agreement with increase epididymal fat mass accumulation and adipocyte size on HFD feeding. A resistance to the up-regulation due to HFD could be observed in all treatment groups. Groups fed galactomannans with diosgenin were more effective when

compare to groups fed galactomannan and diosgenin alone. Galactomannans were seemed to be more effective than diosgenin in resisting the rise in leptin expression.

13. The relative mRNA expression of PPARγ and GPCR43 were up regulated due to high fat diet feeding. Dietary incorporation of both the galactomannans and diosgenin alone or in combination found to be resisted the increase in PPARγ and GPCR43 expression.
14. GLUT4 (glucose transporter in adipose tissue) expression was significantly decreased (66.3%) in HFD feeding and also the protective effect was observed in all treatment groups. Synergistic effect could be seen when administered galactomannan and diosgenin in combination.
15. Expression of pro-inflammatory markers MCP-1 & TNFα in adipose tissue also showed the protective effects of functional fibers and sapogenin (diosgenin). In addition, synergistic effects were also visible.
16. High fat diet feeding significantly increased SREBP 1c (regulates fatty acid synthesis) expression when compare to Ctrl (mean). This shows more fatty acid synthesis/more accumulation in HFD. The expression levels were down-regulated on administration of FGM/LGM/D also in combination. Diosgenin seemed to be more effective than galactomannans in decreasing the SREBP-1c expression. In addition, additive effect could also visible,
17. The CPT-1 (regulator of fatty acid beta oxidation) and PPARα (linked with induction of genes involved in beta oxidation/fatty acid uptake) in liver were found to be down-regulated significantly in HFD group. The dietary incorporation of FGM/LGM/ diosgenin were observed to resist the effect of HFD feeding, which can be surmised as an effect resulting in increased
18. energy expenditure.
19. FIAF is an unfolding chaperon for lipoprotein lipase. The resistance to decrease in FIAF expression to the significant extent and also normalizing effect could be seen in all the treatment groups. FGM/LGM were more effective than diosgenin in resisting the decrease in FIAF expression.
20. Expression of ZO-1 and occludin (tight junction proteins) in intestine indicated the positive effects of FGM/LGM and diosgenin on intestinal integrity under high fat diet fed conditions.

21. The expression level of glucose 6-phosphatase & PEPCK enzymes in intestine were higher in HFD feeding in comparison to Ctrl. All treatment groups are protective in decreasing the rise in expression. However, did not reach to significant level.

The present study provides the first evidence of anti-obesity effects of dietary incorporation of pure forms of fenugreek/locust bean galactomannans and diosgenin in high fat diet fed C57BL/6 mice model. The efficacy of galactomannans in regulating epididymal fat mass as well as improving the insulin sensitivity (HOMA-IR score) was better than that exhibited by diosgenin. However, both types of galactomannans and diosgenin seemed to be equally effective in resisting the rise in blood glucose level due to high fat diet. Analysis of mRNA level expression of selected genes related to energy homeostasis, fatty acid oxidation & synthesis, pro-inflammatory markers and intestinal integrity also suggested the higher efficacy of galactomannans as compared to diosgenin. The additive effects of co-administration of fiber and diosgenin were also visible especially with reference to regulatory enzymes of gluconeogenesis in liver, and expression of genes linked with lipid metabolism & inflammation. Further studies to target the interactions in gut environment in the presence of functional fibers, phytosterols and metabolites produced by gut microbiota under high fat fed conditions can be of great relevance. To investigate as to how the inflammatory responses in adipose tissue affect the energy metabolism in other organs can also be an interesting area of research.

CHAPTER - 7

Bibliography

7. Bibliography

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