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Department of Biochemistry /Molecular and Cellular Biology

Dissertation

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Option: Proteomic Analysis and Health

Effect of Dietary Addition of 2.5% Fenugreek on Serum Lipids and Body Fat in Rats

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DEDICATION

I dedicate my dissertation work to my family and friends.

A special feeling of gratitude to my loving parents, for their continual faithful support during my study.

Thanks and gratitude are also extended, to my brothers, Wakil and Chihab, my sisters, Afifa, Ghada, Hadjer, my tante Houria , my uncles Abd-elhamid and Hichem.

I also pay thankfulness to my best friends; Mamiche, Imane, Mohammed, Amin and Mohammed Larbi.

Finally I pray that Allah accept this effort and make it of real benefit to all who read it.

Kawther

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Finally I pray that Allah accept this effort and make it of real benefit to all who read it.

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ABREVIATIONS:

GAL: Galactomannans.

HDL: High density lipoprotein-cholesterol.

LDL: Low density lipoprotein-cholesterol.

TR: Trigonelline.

4-HI: 4-hydroxyisoleucine.

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INTRODUCTION

INTRODUCTION

Plants are used medicinally in different countries and are a source of many potent and powerful drugs. Medicinal plants are used by 80% of the world population as the only available medicines especially in developing countries (Hashim *et al*., 2010).

Fenugreek (*Trigonella foenum-graecum*) is an annual herb that belongs to the family of leguminosae. It has a long history both as a culinary and as a medicinal herb (Kaviarasan *et al.,* 2006). The seeds of fenugreek are commonly used in Egypt, India and in other oriented countries as a spice in food preparations due to their strong flavour and aroma (Kaviarasan *et al.,* 2007) and as herbal medicine for their carminative, tonic and aphrodisiac effects (Xue *et al.,* 2007). It is well known that Fenugreek seeds consumption lead to hypoglycemia (Ethan *et al.,* 2003; Khalki *et al*., 2010), Hypochlesterolemia, hypotriglyceridemia (Mathern *et al.,* 2009) and reducing the risk factors for coronary heart disease by lowering LDL cholesterol (Nasri and Tinay., 2007).

Obesity is defined as a phenotypic manifestation of abnormal or excessive fat accumulation that alters health and increases mortality, it is the most common cause of dyslipidemia. Lipid oversupply in a state of obesity, hyperinsulinemia, and/or insulin resistance results in increased non-esterified fatty acid availability and, in turn, higher triglyceride stores (Marchesini *et al*., 2001). Indeed, obesity facilitates the development of metabolic disorders such as diabetes, hypertension, and cardiovascular diseases; it increases the risk for heart failure, sudden cardiac death, angina or chest pain, and abnormal heart rhythm. In addition to chronic diseases such as stroke,osteoarthritis, sleep apnea, some cancers, and inflammation-bases pathologies (Singla *et al*., 2010).

The incidence of obesity is rising at an alarming rate and is becoming a major public health concern (Popkin., 2010). Causes of obesity involve genes, metabolism, diet, physical activity, and the socio-cultural environment that characterizes 21st century living. A food field research that has recently aroused considerable interest is the potential of natural products to counteract obesity.

Taking all these previous studies in consideration, both of obesity and fenugreek are well known to be associated with numerous diseases such as: diabetes, dyslipidemia, cardiovascular diseases, and cancer. However, little is known about the effect of fenugreek on body fat. This study was conducted to elucidate the effect of 2.5% fenugreek on the body fat. Here we describe that fenugreek not only provide a new information on the regulation of lipid metabolism, but also suggest a possible role on fat accumulation.

BRIEF REVIEW ON FENUGREEK

1. BRIEF REVIEW ON FENUGREEK

1.1 Fenugreek

1.1.1 Definition

Fenugreek (*Trigonella foenum-graecum*) is a leguminous herb cultivated in India and North African countries **Figure 1**. It belongs to the family Fabaceae **Table 1.**

Table 1: Botanical classification of fenugreek[1] (*Trigonella foenum-graecum Linn*).

Kingdom	*Plantae*
Subkingdom	*Tracheobionta*
Branch	*Magnoliophyta*
Sub-branch	*Magnoliophytina*
Class	*Magnoliopsida*
Subclass	*Rosidae*
Order	*Fabales*
Family	*Fabaceae (Leguminosae)*
Subfamily	*Faboideae*
Tribe	*Trifolieae or Papilionaceae*
Genus	*Trigonella*
Species	***T. foenum-graecum***

[1]http://www.itis.gov/ (Integrated Taxonomic Information System).

[1]http://epic.kew.org/ (Electronic Plant Information Centre, Kew).

[1]http://www.ars-grin.gov/ (Agricultural Research Service, Germplasm Resources Information Network).

Botanical Description

Fenugreek is an annual herb, with a well-developed taproot and a spreading, fibrous, root system. The stem is green to purple, smooth, and erect up to 140 cm (1.5 ft) high. The light-green leaves are alternate and pinnate, consisting of three ovate leaflets. The inflorescence is a terminal, compound umbel. The flowers are white to whitish-yellow. The fruit is light green to yellow brown, ovoid-cylindrical, and slightly curved, with 20–30 small, smooth brownish seeds. The pod shape also gives the name "goat's horn" to the plant. The pods contain about 10-20 yellowish seeds with an appetizing pleasing aroma (Charles., 2013).

The fenugreek seed contains a central hard, yellow embryo surrounded by a corneous and comparatively large layer of white, semi-transparent endosperm (Betty., 2008), a tenacious and dark brown husk surrounds the endosperm. The color of the gum fraction depends upon the amount of outer husk (brown color) and cotyledon (yellow color) present.

1.1.2 Fenugreek composition

Fenugreek seeds contain alkaloids, saponin, tannin, flavonoid, steroid, glycoside and protein (Patel *et al.,* 2013). carotenoids and coumarins (Kulkarni *et al.,* 2012). Its seeds were separated into husk and endosperm. The proximate composition of fenugreek seeds, husk and cotyledons showed that endosperm had the highest saponin (4.63 g/100 g) and protein (43.8 g/100 g) content. In contrast, husk had higher total polyphenols (Naidu *et al.,* 2011) **Table 2.**

The active compounds of fenugreek included diosgenin (Uemura *et al.,* 2010), fiber galactomannans (Kamble *et al.,* 2013), 4-hydroxyisoleucine (Singh *et al.,* 2010), trigonelline (Moorthy *et al.,* 2010) and flavonoids (luteolin, vitexin, isovitexin, quercetin) (Swati *et al.,* 2014).

Table2: Chemical constituents of fenugreek.

classes of chemical constituents	chemical constituents	References
proteins	globulin, albumin and lecithin	(Youssef *et al.,* 2009). (Naidu *et al*., 2011).
Lipids	Fatty acids: linoleic acid, a-linolenic, oleic, palmitic and stearic acids. Sterols: b-Sitosterol Campesterol , cycloartenol. triunsaturated and diunsaturated triacyl glycerides.	(Ciftci *et al.,* 2011).
carbohydrates	Mucilage (gum):galactomannan	(Kamble *et al.,* 2013).
Saponins	Graecunins, fenugrin B, fenugreekine, trigofoenosides A-G.	(Kang *et al.,* 2013)
Steroidal saponins	Diosgenin, yamogenin, gitogenin, tigogenin, neogitogenin, neogitogenin, smilagenin, sarsasapogenin, neogitogenin, yuccagenin.	(Kang *et al*., 2013.)
Flavonoids	Apigenin, luteolin, vitexin, isovitexin, quercetin, Kaempferol-dirhamnoside, Kaempferol rhamnoside. Orientin,biochanin A, formononetin, irilone, tricine, daidzein, calycosin.	(Swati *et al*., 2014).
alkaloids	Trigonelline (yields nicotinic acid with roasting), gentianine, carpaine, choline	(Yoshinari *et al.,* 2010).
Amino acids	4-hydroxyisoleucine, histidine, lysine, arginine and L- tryptophan	(Narender *et al*., 2006)
Others	Coumarin: scopoletin.vitamins : vitamin C, Vitamin B12, Vitamin A,Vitamin D. Minerals : calcium, iron, zinc	(Kulkarni *et al*., 2012).

1.1.2.1 Diosgenin

Diosgenin is a crystalline steroidal sapogenin of the triterpene group, an important raw material for the semi-synthesis of steroid hormones (Lee *et al.,* 2012) such as cortisone and progesterone (Aggarwal *et al.,* 2006) and has a great importance in the pharmaceutical industry (Kohara *et al.,* 2005). Where, saponins are transformed in the gastrointestinal tract into sapogenins.

1.1.2.1.1 Chemical structure of Diosgenin

The typical sugars present in the steroidal saponins of are xylose, rhamnose and glucose (Jing *et al*., 2003; Xu *et al*., 2011). The glycosyl groups are attached to the steroidal aglycones at the C3 and/or C26 hydroxy groups **Figure 2.**

1.1.2.1.2 Mechanism of action

Diosgenin in fenugreek from used for the treatment of diabetes (Gupta *et al.,* 2010), hypercholestrolemia (Pandian *et al.,* 2002). It also prevented cell growth and induced apoptosis in the human colon cancer cell line and fenugreek seed was found to have hepatoprotective properties (Thirunavukkarasu *et al.,* 2003). It may also suppress NF-kappa B activation and antiapoptotic gene products and induce apoptosis in cancer cells (Aggarwal *et al.,* 2006). Recent study on the effect of diosgenin on Vascular smooth muscle cell under TNF-a-induced condition reports that diosgenin abrogated tumor necrosis factor-a (TNF-a). It has also the ability to prevent invasion, suppress proliferation and osteoclastogenesis through inhibition of necrosis factor NF-kappa B-regulated gene expression and enhances apoptosis induced by cytokines and chemotherapeutic agents.

1.2.2.2 Galactomannan

Galactomannans (GAL) are a group of storage polysaccharides from plant seeds that reserve energy for germination in the endosperm. It is mucilaginous fiber (Prajapati *et al.,* 2013).

1.2.2.2.1 Chemical structure of Galactomannan

GAL are hemicellulosic (Wang *et al* ., 2012) heterogeneous polysaccharides composed by a β-(1-4)-D-mannane backbone with a single D-galactose branch linked α-(1-6). They differ from each other by the mannose/galactose (M/G) ratio. Fenugreek GAL is a group of polysaccharides composed of mannose as backbone with galactoseas side groups in the ratio of 1:1. Fenugreek GAL has the highest galactose (∼48%; M/G) in its molecule **Figure 3.**

1.2.2.2.2 Mechanism of action

In 2008, Srichamroen *et al* showed that the fiber galactomannan has the potential to modifying both glycemic and lipidemic status as well as body weight in rats. Also, galactomannans component from many other plants showed ability in inhibiting the pro-inflammatory cytokines (Badia *et al.*, 2012). As well as, low molecular weight galactomannans from fenugreek seeds plays an important role in inhibiting diabetic cachexia by inhibiting pro inflammatory cytokines in diabetic conditions and it was found to protect pancreas from alloxan-induced destructive changes perhaps by preventing these causative factors. So, galactomannan is suggested to be acting by extrapancreatic pathway rather than insulin stimulating effects (Kamble *et al.*, 2013).

1.2.2.3 (4-hydroxyisoleucine)

4-hydroxyisoleucine (4-HI) constitutes about is 80% of the total content of free amino acids in Trigonella foenum-graecum seeds, it is a natural nonproteinogenic amino acid. 4-HI is structurally similar to branched chain amino acid. 4-HI found exclusively in the fenugreek seeds and do not present in mammalian tissues (Broca *et al.*, 2000; 2004) **Figure 4.**

1.2.2.3.1 Chemical structure of 4-hydroxyisoleucine

The molecule has three chiral centres and 90% is found in the form with stereochemistry (2S, 3R, 4S) and 10% with stereochemistry (2R, 3R, 4S). A comparison of the activity of the (2S, 3R, 4S) stereoisomer with the (2R, 3R, 4S) isomer and another 10% congeners revealed the (2S, 3R, 4S) isomer to be the most potent form tried, when measuring insulin release from isolated rat pancreatic islets (Broca *et al.*, 2000).

1.2.2.3.2 Mechanism of action

(4-HI) is a natural nonproteinogenic amino acid possessing insulinotropic biological activity (Broca *et al.*, 2004). This unique property of 4-HI allows the avoidance of undesirable side-effects, such as hypoglycemia, in the therapy of type II diabetes. Thus, 4-HI seems a very promising dietary supplement in

the treatment and prevention of this chronic disease. Because of the high incidence of type II diabetes at present and the unfavorable prognosis of its spread in the future, the development of an industrial 4-HI synthesis process has been highly desired.

1.2.2.4 Trigonelline

Trigonelline is the major alkaloid present in fenugreek, an active component has been reported to possess several biologic activities.

1.2.2.4.1 Chemical structure of Trigonelline

Trigonelline is a pyridine (1-methylpyridinium-3-carboxylate, TR) – a derivative of nicotinamide that also has been found in coffee beans and the western rock lobster. In animal cells **Figure 5.**

1.2.2.4.2 Mechanism of action

In 2006, Shah *et al.,* showed that the onset of action and maximum decrease in serum glucose were similar in glyburide, glibenclamide and trigonelline treatment in diabetic animals. Furthermore, it has been found to have a function as a hormone that controls plant cell cycle (Mehrafarin *et al.*, 2010). As well as, (Panda *et al.,* 2013) for the first time revealed that trigonelline isolated from fenugreek seed protects against isoproterenol-induced myocardial injury through down-regulation of two small stress proteins, heat shock protein (Hsp27) and αB-crystallin. Whereas, TR has been reported to possess several biologic activities including antibacterial and anti-cancerous properties, of trigonelline and its beneficial influence on lipid profile have been proven (Yoshinari *et al.,* 2010).

On the other hand , this alkaloid may taken as a potential neuroprotective agent, especially in Alzheimer's disease, interesting fact about trigonelline demonstrated the association of some pyridine and piperidine alkaloids, including trigonelline, with β-amyloid (Grabowska *et al.,* 2010). By interacting with key residues (His6, Tyr10, His13 and His14) of β-amyloid (1–42) involved in its aggregation (Makowska *et al.,* 2013).

1.2.2.5 Flavonoids

Flavonoids are not synthesized in animal cells, and their presence in tissues strictly depends on the intake of plant products (Kyle *et al.,* 2006). It is noteworthy that flavonoids and their chemical derivatives are often less toxic and reveal lower side effects than derivatives produced from other natural compounds. Nevertheless, similar to any chemical, flavonoids can be harmful at high doses (Ebrahimi *et al*., 2012).

1.2.2.5.1 Chemical structure of Flavonoids

The major flavonoids isolated from fenugreek are vitexin (apigenin-8-*C*-glucoside), isovitexin (apigenin-6-*C*-glucoside), luteolin (3´, 4´, 5, 7-tetrahydroxyflavone) and quercetin (Swati *et al.,* 2014) **Figure 6.**

Flavonoids represent diverse classes of natural polyphenols. By both aglycones (ex: luteolin, quercetin) and various glycosides (ex: vitexin, isovitexin). In which the glycoside part is attached to an oxygen atom. Glycosides usually bear one or several pyranoside or furanoside carbohydrate residues and may be composed not only of glucose and mannose but also of some rare sugars, such as allose, galacturonic acid, and apiose.

The 15-carbon frame of flavonoids consists of two aromatic rings (A and B) connected by three carbon atoms. In general, the distinctive features of this C3 chain are associated with the presence or absence of the double bond, the choice of carbonyl or carboxyl moiety, and the possibility of forming a penta- or hexagonal ring C.

In the intestinal lumen, flavonoid-O-glycosides are attacked by hydrolases exhibiting multiple enzymatic activities, which results in the release of flavonoid aglycones. The aglycones are delivered to the human body through the membranes of the intestinal epithelium, which covers more than 90% of the intestinal surface (Day *et al.,* 2000). The bioavailability of flavonoids is very low. Less than 1% of the consumed flavonoids enter the blood (Manach *et al.,* 2005). The hydrolysis of flavonoid glycosides by β-glucosidase and the subsequent attachment of glucuronic acid occur after the penetration of the glycosides into the cytoplasm of enterocytes, which are the cells of the intestinal epithelium (Gee *et al.,* 2000).

Furthermore, the portal vein transports these substances to the liver, where they are methylated and sulfated with appropriate transferases (Alvarez *et al*., 2010). Thus, in the blood, only 5–10% of the flavonoids are not modified (Clifford., 2004).

1.2.2.5.2 Mechanism of action

Antitumor activity of fenugreek extract might be mediated through scavenging of free radicals and their anti-inflammatory activity, accumulative evidence revealed that the antioxidant activity of fenugreek could be attributed to the presence of flavonoids which act as scavengers of reactive oxygen species (Abou El-Soud *et al.,* 2007; Belguith-Hadriche *et al.,* 2010). Vitexin has been shown inhibit adipogenesis in vitro (Kim *et al.,* 2010). Isovitexin acts an insulin secretagogue in non-diabetic rats (Folador *et al.,* 2010). While, both the compounds showed strong antioxidant ability, which in addition to their known properties could be an added advantage in diabetic. Luteolin possesses a wide range of pharmacological effects, including antioxidant, anti-neoplastic and anti-inflammatory (Wu *et al.,* 2005).

Quercetins are effective inhibitors of a beta protein aggregation and the assembly of amyloid fibers, which are responsible for the development of Alzheimer's disease (Wang *et al.,* 2011; Tarozzi *et al.,* 2012).

1.2 Therapeutic effect of fenugreek

Some of the therapeutic uses of Fenugreek include its use as antioxidant (Kaviarasan *et al.,* 2007), antineoplastic, antiulcerogenic, antipyretic, antitumor and immunomodulatory effects (kumar *et al.,* 2012). Thereby, the role of fenugreek seeds in neurodegenerative diseases and especially against aluminum-induced changes has not so far been considered. Fenugreek was used to ease childbirth and to increase milk flow, for instance it is taken by Egyptian women for menstrual pain and as hilba tea to ease stomach problems of tourists; Fresh fenugreek leaves for the treatment of indigestion, flatulence and a sluggish liver (Basch *et al.,* 2003). An infusion of the leaves is used as a gargle for recurrent mouth ulcers. A gargle made from the seeds is best for ordinary sore throat. Fresh Fenugreek leaves paste applied over the scalp regularly before bath helps hair grow, preserves natural color, keeps hair silky and also cures dandruff, the gelatinous texture of fenugreek seed may have some benefit for soothing skin that is irritated by eczema or other conditions.

1.2.1 Antioxidant activity

Oxidative damage at the cellular or subcellular level is now considered to be a major event in disease processes like coronary vascular disease, inflammatory disease, diabetes, carcinogenesis,and aging. Reactive oxygen radicals are detrimental to cells at both membrane and genetic levels. They induce lipid peroxidation in cellular membranes, generating lipid peroxides that cause extensive damage to membranes and membranemediated chromosomal damage. Fenugreek contains phenolic and flavonoid compounds which help to enhance its antioxidant capacity (Dixit et al., 2005). It has been shown to counter the increased lipid peroxidation and alterations in the content of circulating antioxidant molecules, such as glutathione, β-carotene and α-tocopherol, and decreasing liver and muscle glycogen, and activities of antioxidant enzymes i.e. superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) ((Baquer et al., 2011). The enhanced lipid peroxidation and increased susceptibility to oxidative stress associated with depletion of antioxidants in liver, kidney and pancreas observed in diabetic rats were observed to be normalised with fenugreek seed powder treatment.

1.2.2 Anticarcinogenic activity

Fenugreek is a promising protective medicinal herb for complementary therapy in cancer patients under chemotherapeutic interventions because fenugreek extract shows a protective effect by modifying the cyclophosphamide induced apoptosis and free radical-mediated lipid peroxidation in the urinary bladder of mice (Bhatia et al., 2006). The seed powder in the diet due to the presence of fiber, flavonoids and saponins decreased the activity of β-glucuronidase significantly and prevented the free carcinogens from acting on colonocytes.

1.2.3 Care, Concern and Safety in Fenugreek Use

In 1999, Muralidhara et al investigated toxic effects of fenugreek powder on acute and subchronic regimes in mice and rats, and could not find any sign of toxicity, mortality, Food quality and safety of fenugreek is determined by its production practices, handling, preparation and storage. Diabetic patients should avoid its use along with therapeutic medication because fenugreek could interfere with the absorption of those therapies that control blood sugar. It should not be consumed in excess amount because it has high content of fibre which may cause problem with digestion. There is a problem of odd smelling in sweet after consuming fenugreek, hence it should be used in limited quantity. The application of vanadium alone in rats created toxicity but was nullified when fenugreek powder was given. But it might cause several adverse reactions including hypoglycemia, diarrhea and galactorrhea, when an excessive amount of fenugreek was ingested (Zuppa *et al.,* 2010).

MATERIALS AND METHODS

2. Materials and Methods

2.1 Animals and diets

Male Albino Wistar rats weighing 87.0 ~ 222 g (starting weight) were used. Rats were individually housed in plastic cages. Room temperature was kept at 20~24 C° on a 12 h light-dark cycle (lights on, 08:00~ 20:00 h). All rats were given free access to water. Composition of the basal diet is shown in **Table 3**. Fenugreek (from north-east of Algeria) was added to fenugreek group at the level of 2.5%. Supplementation of fenugreek was at the expense of corn starch. All animals were fed the same amount of experimental diets (8g for d1, 10g for d2, 12g for d 3, 14g for d 4-5, 18g for d 6-7, 20g for d 8-19 and 22 for d 20-35). All the diets were daily incorporated into food cups in the cages at 10:00h; and all rats completely consumed the diets until the next morning. After 35days of consuming diets, food was removed from the cages at 08:00 h and the rats were lightly anesthetized with chloroform and killed between 11:00 and 14:00 h. Blood was collected and samples were allowed to clot on ice. Serum samples were obtained by centrifugation (2500 rpm for 15 min). Liver and abdominal adipose tissues (epididymal and perirenal adipose tissues) were immediately removed, weighed and stored at -80 C° until use

2.2 Experiment

Rats were divided into two groups of 6 rats each, control group (C) and fenugreek diet group (F).

Table 3: Composition of basal diets[1]

Ingredients	Control	Fenugreek
	% (W/W)	
DL-Methionine	0.3	0.3
Vitamin mixture	1.0	1.0
Salt mixture	3.5	3.5
Casein	3.33	3.33
Corn oil	5.0	5.0
Sucrose	6.66	6.66
Cellulose powder	20.03	20.03
α-Corn starch	60.16	57.66
Fenugreek	0	2.5

[1]Diets are modified version of AIN J. Nutr. 107: 1340-1348 (1977).

2.3 Analytical procedure

Serum concentrations of glucose, total cholesterol, triglycerides and albumin were measured by kits (SPINREACT; SPAIN). HDL-Cholestrol and Creatinine levels were measured using automate (ADVIA 1650, laboratory of biochemistry, CHU, Constantine).

2.3.1 Quantitative determination of glucose

b. Test principe of glucose

Glucose oxidase (GOD) catalyses the oxidation of glucose to gluconic acid. The formed hydrogen peroxide (H2O2) is detected by a chromogenic oxygen acceptor, phenol, 4 –aminophenazone (4-AP) in the presence of peroxidase (POD):

$$\beta\text{-D-Glucose} + O_2 + H_2O \xrightarrow{GOD} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{Phenol} + \text{4-AP} \xrightarrow{POD} \text{Quinone} + H_2O$$

The intensity of the color formed is proportional to the glucose concentration in the sample.

a. Procedure:

One milliliter of the reaction mixture containing 92 mM TRIS buffer (pH 7.4), 0.3 mM phenol, 15000 U/L glucose oxidase (GOD), 1000U/L peroxydase (POD) and 2.6 mM 4-aminophenazone (4-AP) was incubated with10 µl sample. After incubation at 37ºC for 10 min, optical density of sample and standard (Glucose aqueous primary standard 100 mg/dL) were recorded against blank. At the wavelength 505nm using Spectrophotometer (JENWAY, 6715 UV/Vis, CHINA).

The concentration of serum glucose was calculated by the difference in absorbance between the standard and the sample (Eq. 1) and (Eq.2)

(A) Sample / (A) Standard X 100 (Standard conc.) = mg/dL glucose in the sample (Eq.1).

Then mg/dL X 0.0555 (conversion factor) = mmol/L (Eq.2)

2.3.2 Quantitative determination of Triglycerides

a. Test principe of triglycerides

Sample triglycerides incubated with lipoproteinlipase (LPL), liberate glycerol and free fatty acids. Glycerol is converted to glycerol-3-phosphate (G3P) and adenosine-5-diphosphate (ADP) by glycerol kinase (GK) and ATP. Glycerol-3-phosphate (G3P) is then converted by glycerol phosphate dehydrogenase (GPO) to dihydroxyacetone phosphate (DAP) and hydrogen peroxide (H2O2). In the last reaction, hydrogen peroxide (H2O2) reacts with 4-aminophenazone (4-AP) and p-chlorophenol in presence of peroxidase (POD) to give a red colored dye:

$$\text{Triglycerides} + HO_2 \xrightarrow{\text{LPL}} \text{Glycerol} + \text{free fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow{\text{GPO}} \text{dihydroxyacetone} + \text{phosphate} + H_2O_2$$

$$H_2O_2 + \text{4-AP} + \text{p-chlorophenol} \xrightarrow{\text{POD}} \text{Quinone} + 4H_2O$$

The intensity of the color formed is proportional to the triglycerides concentration in the sample.

b. Procedure

One milliliter of working reagent contain 50 mM GOOD buffer (pH 7.5), 2 mM 4-chlorophenol, LPL 150000 U/L, GK 500 U/L, GPO 2500 U/L, POD 440 U/L, 4-AP 0.1 Mm and 0.1 mM ATP was incubated with10 µl sample. After incubation at 37ºC for 10 min, optical density of sample and standard (Glucose aqueous primary standard 100 mg/dL) were recorded against blank. At the wavelength 505nm using Spectrophotometer (JENWAY, 6715 UV/Vis, CHINA).

The concentration of serum triglycerides was calculated by the difference in absorbance between the standard and the sample (Eq. 3) and (Eq.4)

(A) Sample / (A) Standard X 200 (Standard conc.) = mg/dL triglycerides in the sample (Eq.3).

Then mg/dL X 0.0113 (conversion factor) = mmol/L (Eq.4)

2.3.3 Quantitative determination of cholesterol

a. Test principe of cholesterol

The cholesterol present in the sample originates a colored complex, according to the following reactions:

$$\text{Cholesterol esters} + H_2O \xrightarrow{\text{CHE}} \text{Cholesterol} + \text{fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{CHOD}} \text{4-Cholestenona} + H_2O_2$$

$$2\,H_2O_2 + \text{Phenol} + \text{4-AP} \xrightarrow{\text{POD}} \text{Quinonimine} + 4H_2O$$

The intensity of the color formed is proportional to the cholesterol concentration in the sample.

b. Procedure:

One milliliter of the reaction mixture containing 90 mM PIPES buffer (pH 6.9), 26 mM phenol, 1000 U/L Cholesterol esterase (CHE), 300U/L Cholesterol oxidase (CHOD) , 650 U/L Peroxidase (POD) and 0.4 mM 4 – Aminophenazone (4-AP) was incubated with10 µl sample. Each sample had both blank and standard (Cholesterol aqueous primary standard 200 mg/dL). After incubation at 37ºC for 10 min, optical density at the wavelength 505 nm was recorded using Spectrophotometer (JENWAY, 6715 UV/Vis, CHINA). The concentration of serum cholesterol was calculated from the difference in absorbance between the standard and the sample (Eq. 5) and (Eq. 6).

(A) Sample / (A) Standard X 200 (Standard conc.) = mg/dL cholesterol in the sample (Eq. 5).

Then mg/dL X 0.0258 (conversion factor) = mmol/L (Eq. 6).

2.3.4 Quantitative determination of HDL-Cholesterol

a. Test principe of HDL-Cholesterol

Directly determination of serum HDLc (high-density lipoprotein cholesterol) levels without the need for any pre-treatment or centrifugation of the sample.

The assay takes place in two steps.

1º Elimination of lipoprotein no-HDL

$$\text{Cholesterol esters} + H_2O \xrightarrow{\text{CHE}} \text{Cholesterol} + \text{fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{CHOD}} \text{4-Cholestenona} + H_2O_2$$

$$2\ H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

2º Measurement of HDL-c

$$\text{Cholesterol esters} + H_2O \xrightarrow{\text{CHE}} \text{Cholesterol} + \text{fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{CHOD}} \text{4-Cholestenona} + H_2O_2$$

$$2\ H_2O_2 + \text{HDAOS} + \text{4-AP} \xrightarrow{\text{POD}} \text{Quinonimine} + 4H_2O$$

The intensity of the color formed is proportional to the HDLc concentration in the sample**.**

b. Procedure

Two reagents the first one is R1 for elimination of lipoprotein no-HDL , 300 microlitter contain N,N-bis(2-hydroxyethyl)-2- aminoethanesulphonic acid (pH 6.6) 100 mM , N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline (HDAOS) 0.7 mM, Cholesterol Esterase ≥ 800 U/L, Cholesterol oxidase≥ 500 U/L, Catalase ≥ 300 U/L Ascorbic oxidase ≥ 3000 U/L was incubated with 3 µl sample for 5 min at 37ºC. The absorbances (A1) of the samples and standard (Lyophilized human serum) were recorded against blank using automate (ADVIA 1650, laboratory of biochemistry, CHU, Constantine) at the wavelength 600 nm.

Then adding 100 µl of the second reagent R2 contain N,N-bis(2-hydroxyethyl)-2-aminoethanesulphonic acid pH 7.0 100 mM , 4 – Aminoantipyrine (4-AP) 4 mM, Peroxidase ≥ 3500 U/L and incubate for 5 min at 37ºC again. After that reading the absorbance (A2) of the samples and calibrator, against the blank and calculate the increase of the absorbance ΔA= A2 – A1.

The concentration of serum HDL-Cholesterol was calculated from the difference in absorbance between the standard and the sample (Eq. 7) and (Eq. 8).

(ΔA) Sample / (ΔA) Standard X (Standard conc.) = mg/dL HDL-Cholesterol in the sample (Eq. 7). Then mg/dL X 0.0259 (conversion factor) = mmol/L (Eq. 8).

2.3.5 Quantitative determination of albumin

a. Test principe of albumin

Albumin in the presence of bromcresol green at a slightly acid pH, produces a colour change of the indicator from yellow-green to green-blue. The intensity of the color formed is proportional to the albumin concentration in the sample.

b. Procedure

One milliliter of the reaction mixture containing 0.12 mM Bromcresol green buffer (pH 4.2) was incubated with 5 µl sample. Each sample had both blank and standard (Albumin aqueous primary standard 5 g/dL). After incubation for 10 min at room temperature (15-25 °C) optical density at the wavelength 630 nm was recorded using Spectrophotometer (JENWAY, 6715 UV/Vis, CHINA). The concentration of serum albumin was calculated from the difference in absorbance between the standard and the sample (Eq. 9) and (Eq. 10).

(A)Sample / (A) Standard X 5 (Standard conc.) = g/dL albumin in the sample (Eq. 9).

Then g/dL X 144.9 = µmol/L (Eq. 10).

2.3.6 Quantitative determination of Creatinine

a. Test principe of creatinine

At an alkaline pH, creatinine reacts with picrate to form a Janovsky complex.The rate of increase in absorbance at 492 nm due to the formation of the creatinine-picrate complex is directly proportional to the concentration of creatinine in the sample.

b. Procedure

A Creatinine Base Reagent containing 0.29 mol/L sodium hydroxide and surfactants and a Creatinine Picrate Reagent containing 17.5 mM picric acid can be used separately on two reagent systems. A working reagent may be prepared by combining one volume of creatinine picrate reagent and four volumes of creatinine base reagent. Mix well before using.

Studies using this reagent were performed on an automated analyzer using a kinetic test mode, with a sample to reagent ratio of 1:24, and a wavelength reading of 505 nm. The analyzer automatically calculates the creatinine concentration of each sample.

2.4 Statistical analysis

All results were tested for statistical significance by Student's t-test.

RESULTS AND DISCUSSION

Dietary addition of 2.5% fenugreek slightly decreased the final body weight. However, relative liver and kidney weight were unaffected (P ‹ 0.05, Fig. 7, 8 and 9).

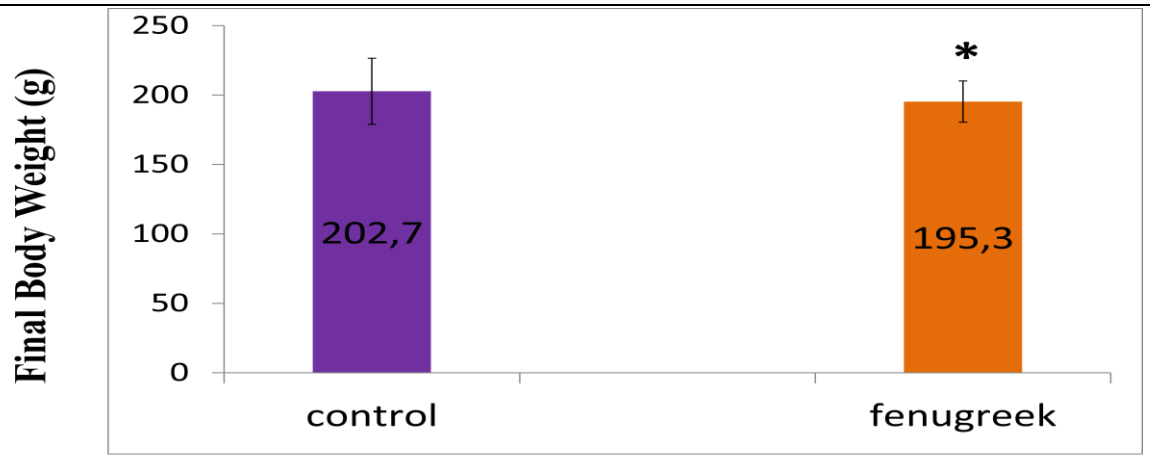

Figure 7: Effect of 2.5% fenugreek on Final Body Weight[1]

[1]Values are means ± SE (n=6) for each group.

*Significantly different from control by student's t-test (P ‹ 0.05).

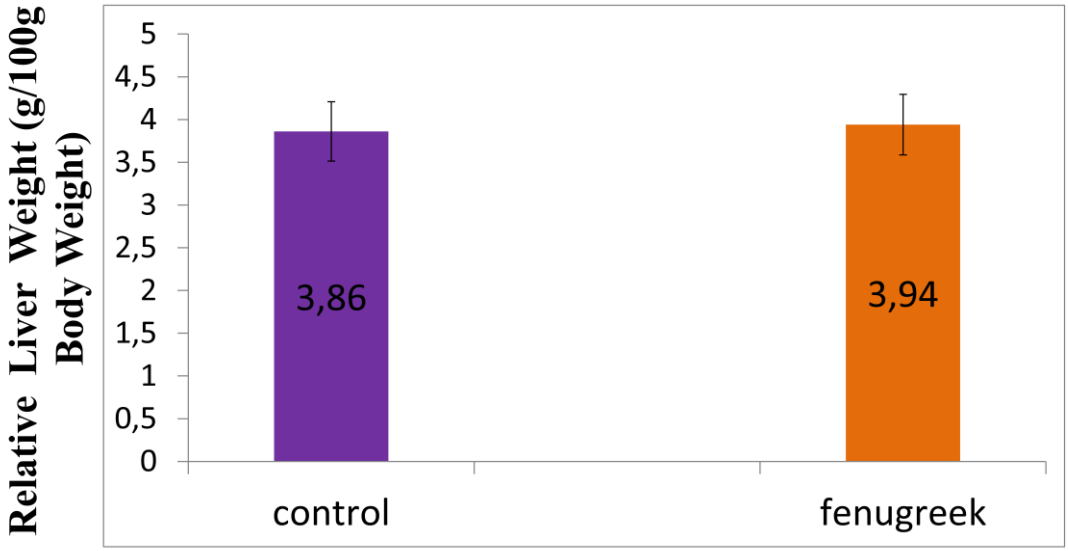

Figure 8: Effect of 2.5% fenugreek on relative Liver Weight[1]

[1]Values are means ± SE (n=6) for each group.

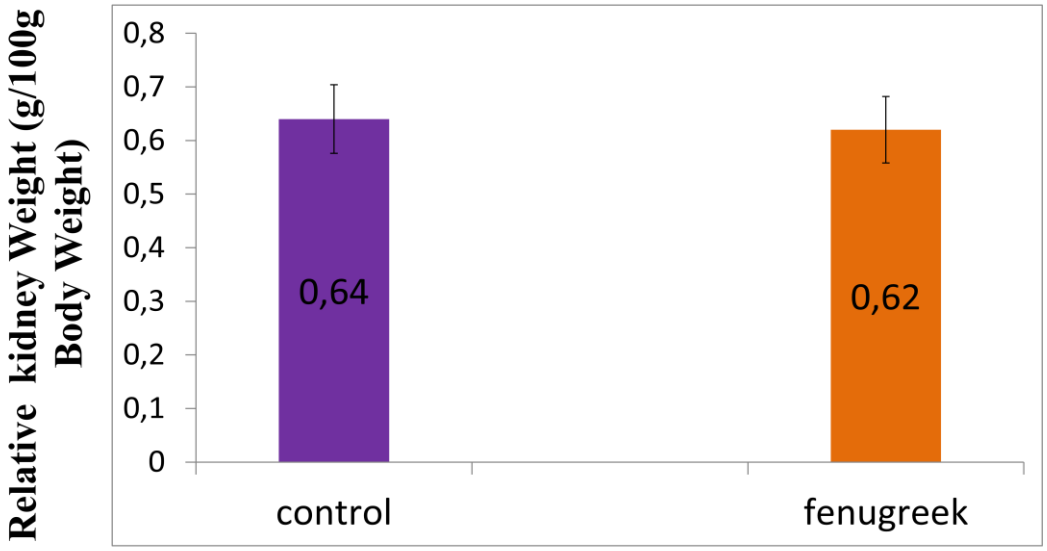

Figure 9: Effect of 2.5% fenugreek on relative kidney Weight[1]

[1]Values are means ± SE (n=6) for each group.

The results showed that 2.5% fenugreek feeding significantly caused 30% and 37% reduction in relative epididymal and perirenal adipose tissue weight respectively (P ‹ 0.05, Fig.10 and11). Previous studies showed that diosgenin, promote both the adipocyte differentiation and the size reduction (Sauvaire *et al.*, 1998; Hirai *et al.*, 2010). Furthermore, trigonelline also attenuates the adipocyte differentiation and lipid accumulation (Ilavenil *et al.*, 2014). Moreover, Fenugreek limits the adipogenesis process, the adipocyte differentiation, lipid accumulation, lipogenesis and adipogenesis by inhibition of adiponectin, adipogenin, and leptin expression. In view of these facts, our findings suggest that the anti-obesity effect of fenugreek is mediated through these active compounds such as diosgenin and trigonelline by inhibiting adipocyte differentiation.

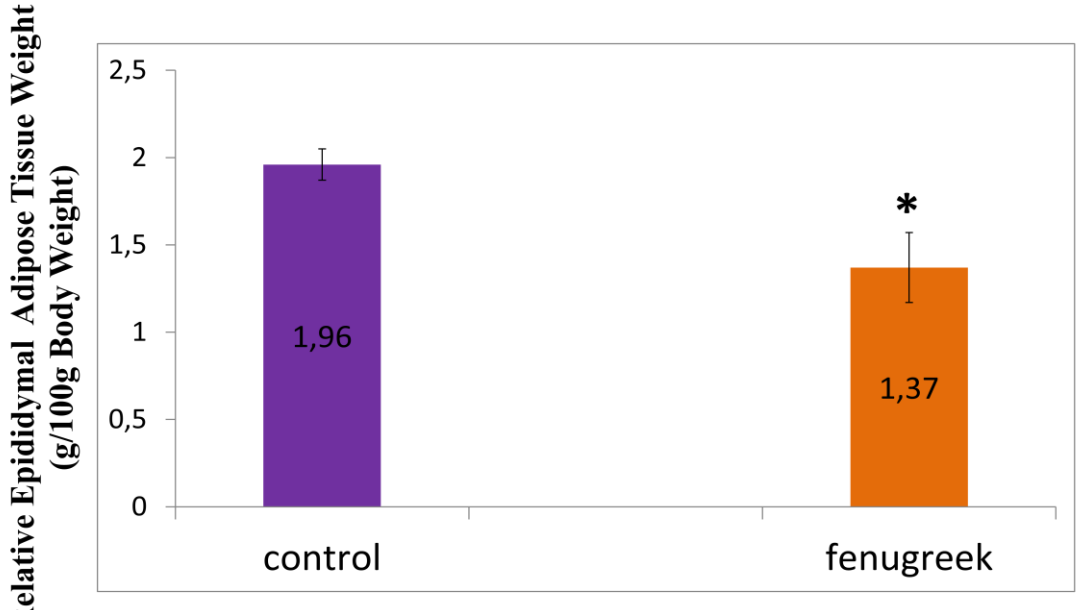

Figure 10: Effect of 2.5% fenugreek on relative Epididymal Adipose Tissue Weight[1]

[1]Values are means ± SE (n=6) for each group.

*Significantly different from control by student's t-test (P ‹ 0.05).

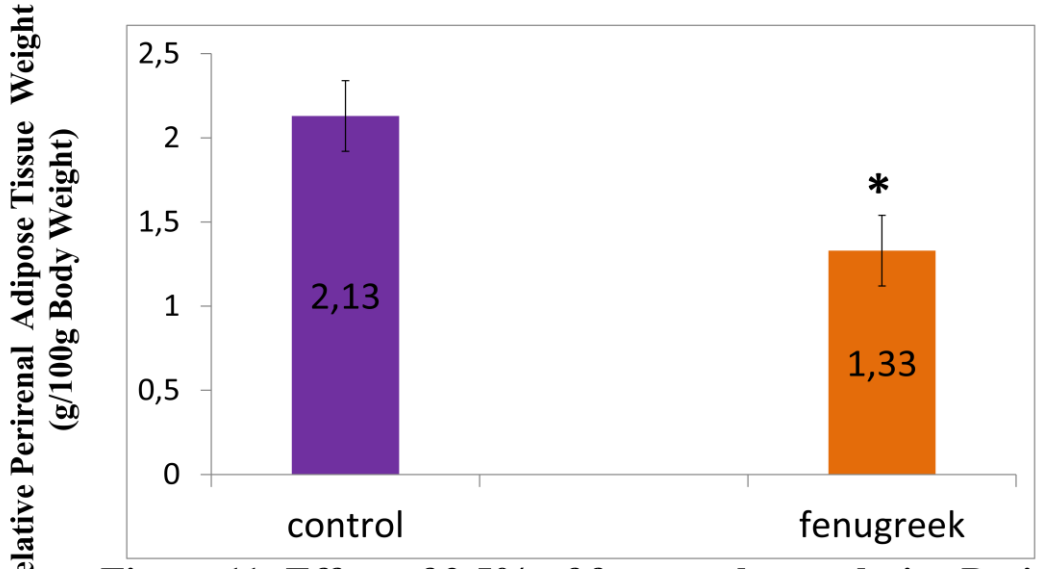

Figure 11: Effect of 2.5% of fenugreek on relative Perirenal Adipose Tissue Weight[1]

[1]Values are means ± SE (n=6) for each group.

*Significantly different from control by student's t-test (P ‹ 0.05).

The results showed that 2.5% fenugreek feeding significantly caused 27% decreasing in serum glucose concentration (P ‹ 0.05, Fig.12.). Fenugreek has been well known to be used as antidiabetic remedy for both type I and II diabetes and has been extensively used as a source of antidiabetic compounds,from its seeds, leaves and extracts in different model systems (Analava and Debaprasad., 2004; Khalki *et al.*, 2010). Galactomannan (GAL) has the potential to modifying the glycemic index by reducing intestinal absorption of low or high concentrations of glucose and hence for the benefit of blood glucose management (Srichamroen *et al.*, 2008, 2009). Both of Quercetin and of 4-hydroxyisoleucine (4-HI) have been shown to stimulate glucose-induced insulin secretion (Kodera *et al.*, 2009, Bardy *et al*., 2013). These previous results agree with our results and suggest that the hypoglycemic effect of fenugreek is mediated through these active compounds of fenugreek such as Galactomannan, 4-hydroxyisoleucine and Quercetins.

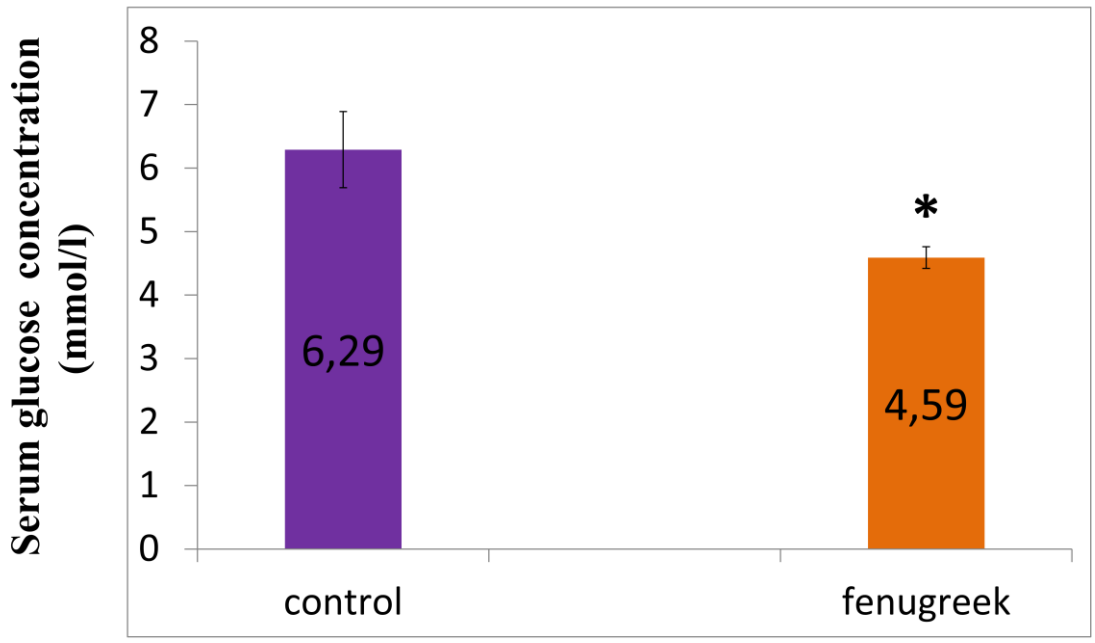

Figure 12: Effect of 2.5% fenugreek on Serum Glucose[1]

[1]Values are means ± SE (n=6) for each group.

*Significantly different from control by student's t-test (P ‹ 0.05).

Dietary addition of 2.5% Fenugreek significantly caused 59%, 35% decreasing in serum triglyceride, serum cholesterol respectively (Fig. 13, 14). Our results are in agreement with the previous studies. It has been shown that fenugreek exhibit hypocholesterolemic effects, lowered serum cholesterol, triglyceride, LDL in hypercholesterolemia suffering patients and experimental models (Mathern *et al*., 2009; Ramadan *et al*,. 2010; Rashmi *et al.*, 2011; Reddy and Srinivasan ., 2011).

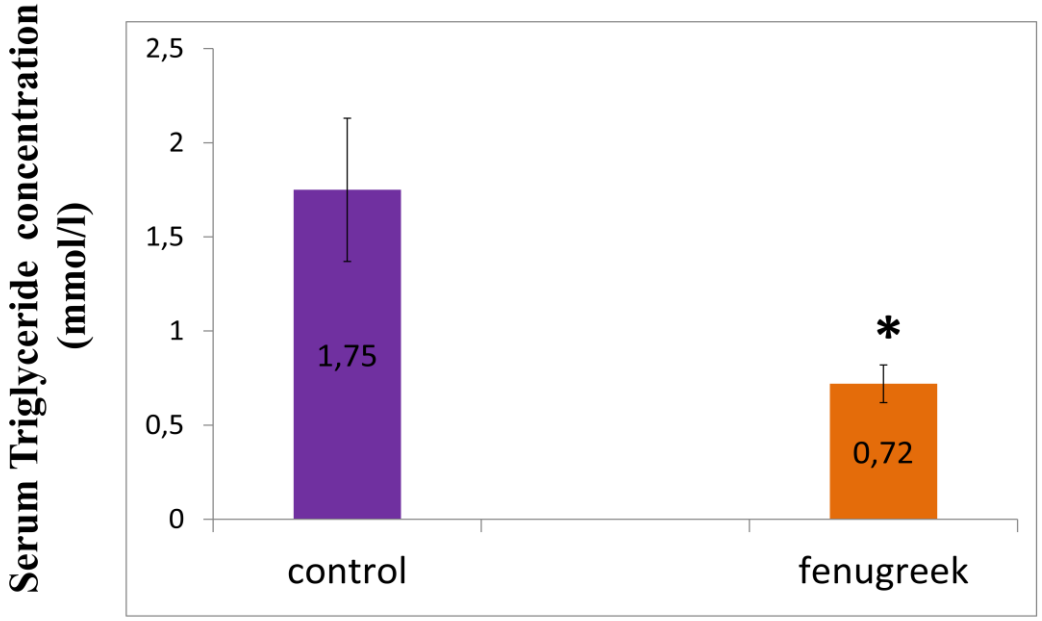

Figure 13: Effect of 2.5% Fenugreek on Serum Triglyceride[1]

[1]Values are means ± SE (n=6) for each group.

*Significantly different from control by student's t-test (P ‹ 0.05).

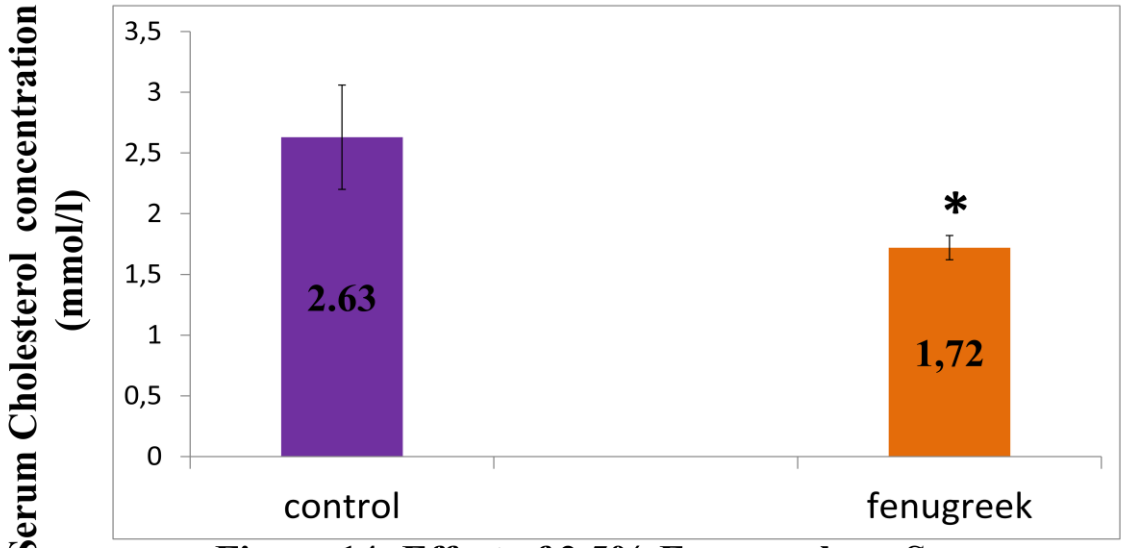

Figure 14: Effect of 2.5% Fenugreek on Serum Cholesterol[1]

[1]Values are means ± SE (n=6) for each group.

*Significantly different from control by student's t-test (P ‹ 0.05).

Serum HDL-Cholestrol was not affected by 2.5% fenugreek treated group. However, the ratio of total cholesterol/HDL cholesterol significantly decreased. (Fig 15, 16). It has been reported that Diosgenin lower incidence for coronary artery diseases and disorders related to estrogen deficiencies in humans who have a high consumption of diet rich in phytoestrogens (Au AL *et al*., 2004). 4-hydroxyisoleucine also significantly decreased the plasma triglyceride levels, total cholesterol and free fatty acids accompanied by an increase in HDL–Chol/ Chol ratio by 39% in the dyslipidemic hamster model (Narender *et al*., 2006). Feeding galactommanan has been also shown to reduce both total and LDL cholesterol levels in healthy animals (Boban *et al*., 2006; Naidu *et al*., 2011). Our results support these findings and suggest a possible role of fenugreek in preventing atherosclerosis and heart attacks through the active compounds such as diosgenin, 4-hydroxyisoleucine and galactommanan.

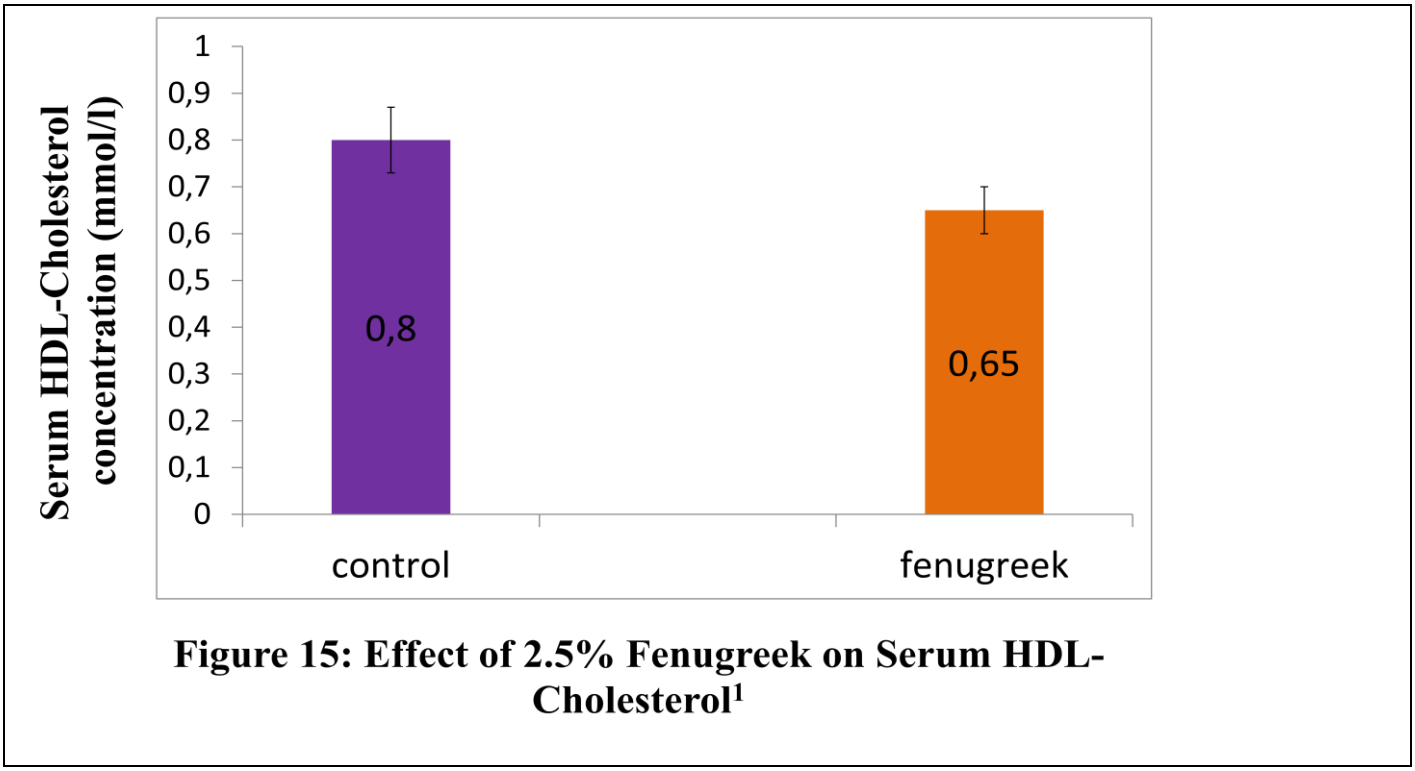

Figure 15: Effect of 2.5% Fenugreek on Serum HDL-Cholesterol[1]

[1]Values are means ± SE (n=6) for each group.

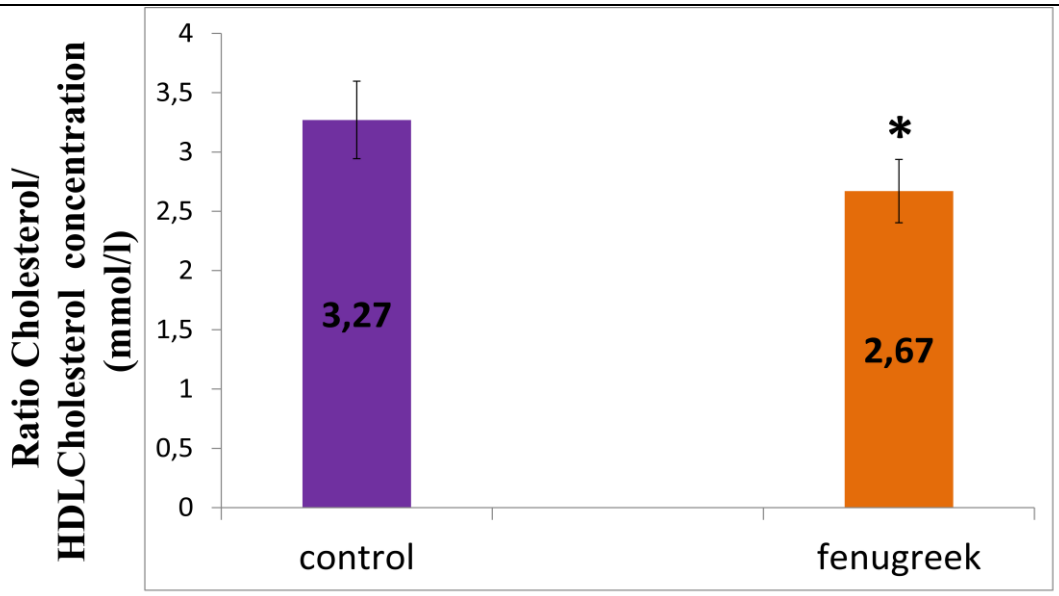

Figure 16: Effect of 2.5% Fenugreek on Ratio Cholesterol/ HDLCholesterol

[1]Values are means ± SE (n=6) for each group.

*Significantly different from control by student's t-test (P ‹ 0.05).

Serum concentrations of albumin and creatinine (indicators of renal function) were unaffected by 2.5% fenugreek treatement (Fig 17 and 18). Nephrotic syndrome includes hyperlipidemia, proteinuria and hypoalbuminemia (Fujisawa *et al.* 1994). Hyperlipidemia associated with nephrosis is generally thought to occur as a result of an increased synthesis of lipids and apoproteins in liver (Marsh., 1984; Fujsawa *et al.*, 1994). Thus, in the present study, the unaffected serum albumin and creatinine by 2.5% fenugreek treated animals, suggesting that hypolipidemia induced by 2.5% fenugreek is not mediated through a mechanism involving renal dysfunction.

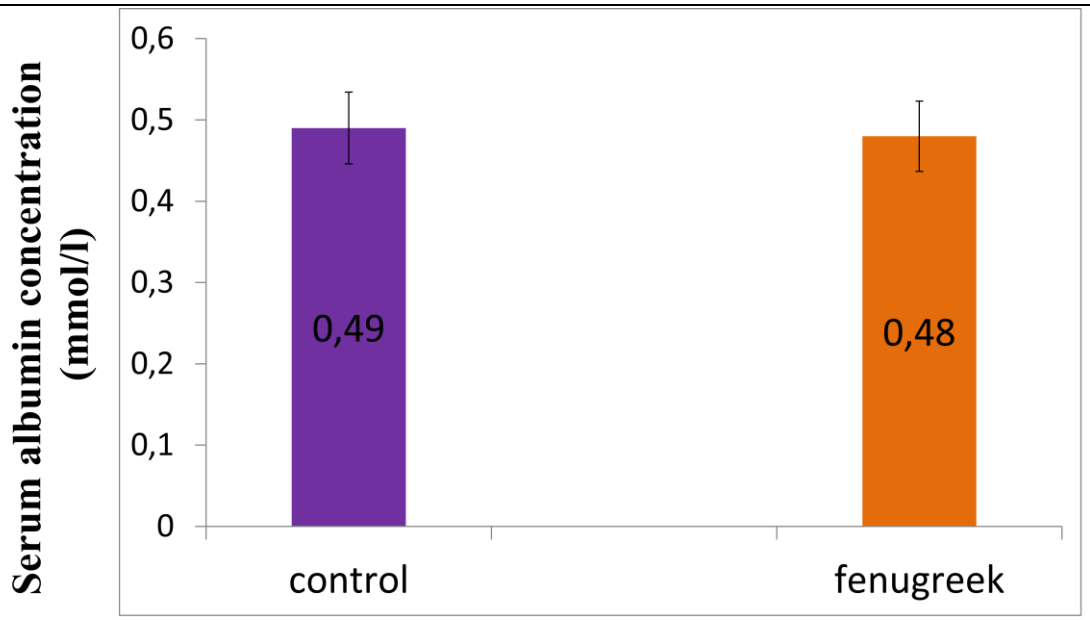

Figure 17: Effect of 2.5% Fenugreek on Serum albumin[1]

[1]Values are means ± SE (n=6) for each group.

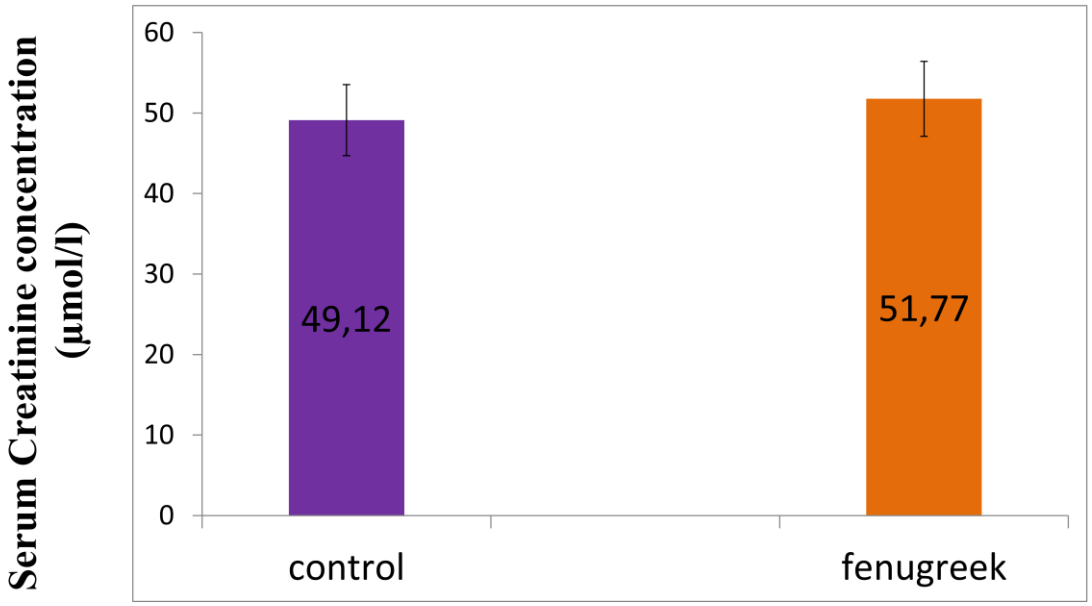

Figure 18: Effect of 2.5% Fenugreek on Serum Creatinine[1]

[1]Values are means ± SE (n=6) for each group.

CONCLUSION AND PERSPECTIVE

CONCLUSION

This study provided that feeding rats 2.5% fenugreek caused not only hypoglycemia and hypolipidemia but also lower body fat.

Obesity, defined as an unhealthy amount of body fat, it is a very serious health problem in worldwide. Obesity is closely associated with the prevalence of diabetes, severe cardiovascular disease (Hursting and Dunlap., 2012), Adipocytes differentiations and lipid accumulations (Camp *et al.*, 2002).

The active compounds such as the steroidal saponin diosgenin inhibit adipogenesis and lipogenesis process, lowered incidence for coronary artery diseases (Sauvaire *et al.*, 1998; Hirai *et al.*, 2010) and also the alkaloid trigonelline (Ilavenil *et al*., 2014). Galactomannan has hypoglycemic effect (Srichamroen *et al*., 2008, 2009). Both of Quercetin and of 4-hydroxyisoleucine have been shown to stimulate glucose-induced insulin secretion (Kodera *et al.*, 2009; Bardy *et al.*, 2013).

In summary, this study demonstrated lower body fat and hypolipidemia caused by 2.5% fenugreek and suggest that this reduction is mediated by the active compounds of fenugreek. This study further raises an important interaction between these active compounds and oxidative stress. Further study is needed to elucidate this association.

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Summary

Fenugreek is an annual herb that belongs to the family of leguminosae. It contains saponin such as diosgenin, alkaloids as trigonelline, galactomannan, 4-hydroxyisoleucine and some flavonoids, these compounds make it a potent medicinal herb. Fenugreek is used for treatment of many diseases such as dyslipedemia, diabetes, cardiovascular diseases, cancer and for regulation of lipid metabolism. Obesity is abnormal or excessive fat accumulation and it facilitates the development of metabolic disorders such as diabetes, dyslipidemia, lipid oversupply, hypertension, cardiovascular diseases and cancer. These associations between fenugreek and lipid metabolism, obesity and lipid accumulation, suggest a possible effect of fenugreek on obesity.

This study was conducted to define the effect of 2.5% fenugreek on serum lipid and body fat accumulation. Rats were fed for 35 days diets with or without 2.5% fenugreek. Feeding a diet containing 2.5% fenugreek caused 30% and 37% reduction in relative epididymal and perirenal adipose tissue weight respectively, 27% decreasing in serum glucose concentration, 59%, 35% decreasing in serum triglyceride, serum cholesterol concentrations respectively, decreasing the ratio cholesterol/HDL-cholesterol and slightly decreasing in final body weight.

This study provided the first evidence suggesting that 2.5% fenugreek lower body fat accumulation. Fenugreek appears to have anti-obesity effect by inhibiting adipocyte differentiation through a mechanism involving its active compounds such as the saponin diosgenin, the alkaloid trigonelline, galactomannan and 4-Hydroxyisoleucine.

***Keywords*: Fenugreek, Hypocholesterolemia,Hypotriglyceridemia,Hypoglycemia,Obesity**

Résumé

Le fenugrec est une plante herbacée annuelle qui appartient à la famille des légumineuses. Il contient de la saponine telle que la diosgénine, alcaloïdes que trigonelline, galactomannane, 4-hydroxy-isoleucine et certains flavonoïdes, ces composés font une plante médicinale efficace. Le fenugrec est utilisé pour le traitement de nombreuses maladies telles que la dyslipidémie, le diabète, les maladies cardiovasculaires, le cancer et pour la régulation du métabolisme des lipides. L'obésité est l'accumulation excessive ou anormale des graisses et il facilite le développement de troubles métaboliques tels que le diabète, la dyslipidémie, l'hypertension, les maladies cardiovasculaires et le cancer. Ces associations entre le fenugrec et le métabolisme des lipides, de l'obésité et de l'accumulation des lipides, suggèrent un effet possible de fenugrec sur l'obésité.

Cette étude a été menée pour définir l'effet de 2,5% fenugrec sur lipides sériques et accumulation de graisse corporelle. Un groupe des rats ont été nourris du régime avec 2.5% du fenugrec et d'autre groupe a été nourri régime sans fenugrec. Le régime qui contient 2.5%Fenugrec a provoqué une réduction de 30% et 37% des poids de tissue adipeux épididymites et de tissu adipeux péri-rénal respectivement, 27% diminution de la glycémie, 59%, 35% diminution de triglycéridémie et cholestérolémie respectivement avec réduction le rapport cholestérol / HDL-cholestérol et de diminuer légèrement en poids final.

Cette étude est la première qui suggérant que 2.5% fenugrec diminue l'accumulation des graisse corporelle. Le fenugrec semble avoir un effet anti-obésité en inhibant la différenciation des adipocytes par un mécanisme impliquant ses composés actifs, tels que la diosgénine de saponine, l'alcaloïde trigonelline, le galactomannane et de 4-hydroxyisoleucine.

Mots-Clés **: Fenugrec, Hypocholestérolémie, Hypotriglycéridémie, Hypoglycémie, L'obésité.**

ملخص

الحلبة هي عشبة حولية تنتمي إلى عائلة القرنيات تحتوي على الصابونيين مثل ديوسجينين، قلويدات كالترايجونيلين ،جلاغتومنان ، 4-هيدروكيسي ازولوسين وبعض الفلافونويدات، هذه المركبات تجعلها من الأعشاب الطبية الفعالة. الحلبة تستعمل في معالجة الكثير من الامراض مثل دسليبيدميا، مرض السكري ، أمراض القلب والأوعية الدموية ، السرطان و تنظيم ايض الدهون. السمنة هي زيادة غير طبيعية أو مفرطة للدهون تسهل حدوث الإضطرابات الأيضية مثل مرض السكري، دسليبيدميا، أمراض القلب والأوعية الدموية، ارتفاع ضغط الدم والسرطان. هذه العلاقات بين الحلبة وأيض الدهون، السمنة و تراكم الدهون، تشير إلى تأثير ممكن للحلبة على السمنة.

صُممت هذه الدراسة لتحديد مدى تأثير 2.5 % من الحلبة على نسبة الدهون في الدم وتراكم الدهون في الجسم. تمت هندسة التجربة بتغذية الفئران إما بغذاء يحتوي على 2.5% من الحلبة او بدونها لمدة 35 يوما . نتائج التجربة تتمثل في انخفاض بمقدار 30% و 37% في النسيج الدهني البطني و النسيج الدهني المحيط بالكلى على التوالي. وكذلك انخفاض بمقدار 27% ، 59% ، 35% في نسبة السكر،الجليسريدات الثلاثية و نسبة الكولسترول في الدم على التوالي. كما لوحظ انخفاض في معدل الكوليسترول / البروتين الدهني مرتفع الكثافة و انخفاض طفيف في الوزن النهائي للجسم بالنسبة للمجموعة التي تغذت على 2.5% من الحلبة مقارنة بالمجموعة الشاهد.

استنادا إلى كل ما ذكر هذه الدراسة تبين أن 2.5% من الحلبة لها تأثير على خفض الدهون في الجسم و ربما للحلبة تأثير ضد السمنة بواسطة تثبيط تمايز الخلية الدهنية وذلك راجع لمكوناتها النشطة مثل الصابونيين ديوسجينين ، القلويد ترايجونيلين ،جلاغتو منان و 4-هيدروكسي ازولوسين

الكلمات المفتاحية: الحلبة، الكولسترول، الجليسريدات الثلاثية، السكر، السمنة.

BOUCHAREF Kawther and ABBAD Sarra

Presented in 25th June 2014

Dissertation to Get a Diploma of Master in Biochemistry

Option: Proteomic Analyze and Health

Theme : Effect of Dietary Addition of 2.5% Fenugreek on Serum Lipids and Body Fat in rats

Summary

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