Research programme

**Title of research proposal:** Effect of fenugreek sprout extracts on diabetic and hyperlipidemic markers in Streptozotocin (STZ) induced diabetic rats.

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Introduction

Medicinal plants have been known for millennia and are highly esteemed all over the world as a rich source of therapeutic agents for the prevention of diseases and ailment. The demand for plant based medicines, health products, pharmaceuticals, food supplement and cosmetics are increasing in both developing and developed countries, due to the growing recognition that the natural products are non-toxic, have less side effects and easily available at affordable prices. To this end, research has begun to embrace traditional medicines from various cultures, as scientists search for clues to discover new therapeutic drugs for diabetes. Traditional Indian and Chinese medicines have long used plant and herbal extracts as anti-diabetic agents. Therefore, investigation on such agents from traditional medicinal plants has become more important and researches are competing to find the new effective and safe therapeutic agent for the treatment of diabetes.

Diabetes mellitus (DM) is a complex metabolic disorder that is increasing tremendously all over the world. By the year 2010, the total number of people worldwide with DM is projected to reach 239 millions [1]. Plants have been used to treat Diabetes since the ancient times as reported in the Ebers papyrus in Egypt in 1550 BC [2]. Among the anti-diabetic plants used in Egyptian folk medicine are fenugreek (Trigonella foenum graecum, family Leguminosae) and Balanites (Balanites aegyptiaca, family Balanitaceae). The hypoglycemic effect of fenugreek seeds has been studied in many...
animal model systems [3-6], as well as in humans in IDDM patients [7] and NIDDM patients [8]. In addition, Fenugreek seeds have been shown to possess hypocholesterolemic effect [9, 10], an encouraging antioxidant property [11, 12] and can be a valuable candidate in the treatment and/or prevention of diabetes complications. Recently, fenugreek seeds have been reported to protect against histopathological abnormalities in diabetic rat kidney [13]. The hypoglycemic effect of Fenugreek seeds has been granted to the presence of coumarins and the alkaloid trigonelline [14] as well as to the steroid saponins and fibre content in the seeds [15, 16].

Fenugreek, or Trigonella foenum-graecum, is a plant indigenous to the Mediterranean region, Ukraine, India, and China. Fenugreek seeds have long been prized for their medicinal properties. The ripe, dry seeds have been used for thousands of years in Arabian, Greek, Indian, and Chinese medicines. Crushed or powdered, Fenugreek seeds can be used externally and applied as poultices for boils, hives, ulcers, and eczema. Internally, Fenugreek seeds have been used to reduce blood sugar, increase lactation, and to treat pellagra, appetite loss, indigestion, dyspepsia, bronchitis, fever, hernia, impotence, vomiting, and stomach ulcers.

Fenugreek is much used in herbal medicine in many areas of the world. The seeds are very nourishing and are given to convalescents. Research has shown that the seeds can inhibit cancer of the liver, lower cholesterol levels, and have an anti-diabetic effect. Fenugreek seed and leaves also have anti-inflammatory, anti-tumor, carminative, demulcent, deobstruent, emollient, expectorant, febrifuge, laxative, parasiticide, and restorative properties. Fenugreek seeds yield strong mucilage and are therefore useful in
the treatment of stomach ulcers. For external use, fenugreek seeds are ground into a powder and used as a poultice for abscesses, boils, ulcers, and burns.

Usually, the defatted portion of Trigonella foenum graecum seed has been used as a remedy for diabetes. Alkaloid trigonelline, nicotinic acid, and coumarin are contained in this fraction. Consuming 1.5-2.0 g daily of the defatted seed was shown to reduce fasting and postprandial blood glucose levels, glucagon, somatostatin, insulin, total cholesterol, and triglycerides, and increased HDL-cholesterol levels in diabetic dogs. The fact that seeds are comprised of 50 percent fiber alludes to the possibility of glycemic influences related to total dietary fiber.

As much as Type 2 diabetes mellitus is concerned it is affecting more than 150 million people worldwide. It is characterized by peripheral insulin resistance (reduced uptake of glucose from blood into the skeletal muscle), dysfunction of the pancreatic b-cells, and increased endogenous glucose production (liver). The causes for the development of diabetes are environmental (nutrition, physical activity) and genetic, since type 2 diabetes is highly hereditary. Besides a few percent of all cases with monogenetic disease, several genetic polymorphisms contribute to the development of diabetes. This renders all genes that are involved in the transmission of the cellular insulin response to candidate genes the malfunction of which may be causally involved in the development of diabetes.

Thus the worldwide epidemic of type 2 diabetes (NIDDM) has been stimulating the quest for new concepts and targets for the treatment of this incurable disease. Most current therapies were developed in the absence of defined molecular targets. Increasing knowledge on the biochemical and cellular alterations occurring in NIDDM has led to the
development of novel and potentially more effective therapeutic approaches to treat the disease. In Akt pathway two targets, protein tyrosine phosphatase 1B (PTP-1B) and glycogen synthase kinase-3 (GSK-3), have emerged as validated targets for treating this disease. The activity of various nonpeptidic small molecules as well as small peptides like PTP-1B inhibitors has been studied. Likewise, GSK-3, which plays a key role in the insulin signaling pathway, has been intensely studied by various companies as a potential target for the development of antidiabetic therapies. PTP-1B knockout mice have shown increased insulin sensitivity and decreased weight gain after a high-fat diet. All these evidences help to validate PTP-1B as a key negative regulator of insulin signal transduction and a potential therapeutic target in the treatment of NIDDM and obesity13. A study has demonstrated that supplementation of fenugreek leaves lowered the lipid profile in STZ-induced diabetic rats [17].

Similarly, hyperlipidemia is the current medical as well social problem, specially associated with diabetes mellitus leading to increasing morbidity and mortality. The major risk factors of hyperlipidemia are associated with atherosclerosis which predisposes ischemic heart disease and cerebro-vascular disease. In type 2 diabetic patients there is mild to moderate hyper triglyceridemia, low level of high density lipoprotein (HDL) and over pro-duction of very low density lipoprotein (VLDL). Serum total cholesterol is also increased [18].

As far as sugar metabolism is concerned Akt is regarded as a key signal molecule mediating the metabolic actions of insulin in muscle, fat, and the liver. Akt is critical in insulin-induced metabolism of glucose and lipids. Akt has been shown to play a critical role in insulin-induced metabolic actions. In fat and muscle, the role of Akt has been
widely recognized as mediation of insulin-stimulated glucose uptake. Indeed, overexpression of constitutively active forms of Akt is sufficient to induce glucose transport in 3T3-L1 adipocytes and L6 muscle cells, whereas Akt2-deficient mice showed impaired glucose tolerance due to a decrease in insulin-induced glucose uptake in skeletal muscle and increased hepatic glucose production. In Akt pathway there are two emerging targets which are insulin sensitizers: protein tyrosine phosphatase-1B (PTP-1B) and glycogen synthase kinase-3 (GSK-3). Development of specific inhibitors for GSK-3 could have therapeutic implications for type 2 diabetes. Normally insulin induced inactivation of GSK-3 contributes to glucose uptake and glycogen synthesis. The mechanisms contributory to insulin resistance and type 2 diabetes are multifactorial, but one factor is certainly due to inadequate inhibitory control of GSK-3. Interesting reports have shown that elevated levels of GSK-3 have been observed in diabetic and obese mouse strain and in skeletal muscles from patients with type 2 diabetes [19].

Current studies have investigated the blood cholesterol-lowering and blood glucose-lowering properties of fenugreek seeds, both in normal subjects and in those with diabetes. Significant reductions in total cholesterol, LDL cholesterol and triglyceride levels, but not HDL cholesterol levels, have been observed in non-insulin-dependent diabetics consuming 25 grams of fenugreek per day. The beneficial effects were sustained over five to six months. With only five grams of fenugreek a day, fasting and postprandial blood glucose levels were significantly reduced in those persons with diabetes. Today fenugreek is recognized as a useful botanical aid in the treatment of persons with diabetes. Some recent studies have shown that dry fenugreek seeds are low in phenolics with poor antioxidant activity. Seed sprouting is gaining importance
commercially because it not only improves the nutritional and antioxidant value of seed but also known to remove some antinutrients like enzyme inhibitors in seeds that make sprouts safe for diet. Fenugreek sprouts are native to arid countries and are commonly used to eat, or in seed form to season food. Women who are lactating may benefit from the addition of fenugreek in the diet to stimulate milk production. Prepared sprouts provide a good source of many vitamins, minerals and protein.

Nowadays there is much work underway to develop treatments for common oxidative stress linked diseases such as diabetes, cardiovascular disease and certain cancers. Because of the oxidative nature of these diseases a significant role for the use of dietary antioxidant photochemicals in their prevention and treatment is emerging. Such dietary constituent showing functionality against above mentioned problem will be preferred over drugs without any possible side effect even though consuming it for long time. Phenolic enriched pea sprouts have been reported recently to possess much higher hypoglycemic activity than its seeds in relation to diabetes management. Thus prime contribution of the proposed work will be the first step of understanding the role of bioactive compounds in fenugreek sprouts. At the same determination of the biochemical and molecular targets of such phytochemical rich sprouts in response to its anti diabetic effects under in vitro and in vivo conditions will be of invaluable importance for management of diabetes and hyperlipidemia with such nontoxic functional food.
OBJECTIVES

1. Phytochemical analysis of important bioactive compounds (e.g. total phenols, quercetin, diosgenin, fenugreekine and trigonelline) at different stages of growth in sprouts obtained from different cultivars of fenugreek.

2. Analysis antidiabetic activity of fenugreek sprouts under in vitro conditions.

3. Evaluation of best fenugreek sprouts on the basis of in vitro studies and determination of their antidiabetic and lipid lowering activity under in vivo conditions.

4. Determination of effect of sprout extracts on insulin signaling pathway in streptozotocin induced rats

Methodology

1. **Seed germination:** Dry seeds of fenugreek will be soaked in distilled water distilled water on orbital shaker at a speed of 120 rpm at room temperature for 12-48 hours and then germinated in flats lined with moist paper towels. The flats will be covered with aluminum foil and the seeds germinated in dark. The germinating seeds will be kept moist with distilled water and the assays performed daily for
next 10 days. Each experiment will have three replications and each experiment will be repeated three times. Sprouts can also be produced in glass jars.

2. **Estimation of fenugreekine, quercetin, Trigonelline and diosgenin:** The concentration of all the four rare components of fenugreek viz quercetin, fenugreekine, Trigonelline and diogenin associated with hypoglycemic and lipid lowering effects will be simultaneously determined in fenugreek seeds and sprouts by HPLC throughout all the ten days of germination.

3. **In vitro assay for determination of fenugreek sprout extract as a Amylase inhibitor, a Glucosidase inhibitor and sucrase inhibitor:** The a Amylase and a Glucosidase inhibitor assay will be carried out as per methods given by Burguieres et al (J Food Biochem, 2008).

4. **Preparation of extracts for in vivo study:** In brief, 100 g of Fenugreek sprouts will be defatted with 500 ml petroleum ether for 8 h. The defatted seedlings will be boiled under a reflux for 2 h with 30% methanol (2 x 500 ml), the extract will be evaporated under vacuum to yield yellow residue, which will be standardized and used for the study.

5. **Effect of extracts on starch digestion and absorption in Normal rats:** This experiment is based on measuring blood glucose level in rats after 18 h fasting and at different time intervals following starch ingestion either alone or in combination with one of the plant extracts. Rats (n = 24) will be fasted for 18 h, blood samples will be taken from the tail vein, and the animals divided into normal (n = 8) and two treated groups (n = 8 for each). The rats in the normal
group will be given (3 g/kg) oral starch load and blood samples withdrawn at 15, 30, 60, 90, 120 and 150 min after starch ingestion. Blood glucose levels will be measured. In the treated groups, the same previous regimen will followed except that (1.5 g/kg) of Fenugreek extract given orally just before the starch load.

6. **Effects of extracts on Diabetic and hyperlipidimic markers in Streptozotocin (STZ)-induced diabetic rats:** The male albino rats weighing 150-200 g, would be taken from concerning institution. Rats will be divided into normal (n = 8) and diabetic groups (n = 8), the latter will be made diabetic by i.p. injection of STZ (50 mg/kg) freshly prepared in 0.1 Mcitrate buffer at pH 4.5. Forty-eight hours after STZ-administration, diabetic rats with non-fasting plasma glucose >300 mg/dl will be considered diabetic and randomly divided into three groups. The first group will be left without treatment and served as a control diabetic group. The second and the third groups will receive oral daily dose of (1.5 g/kg) of Fenugreek sprout extracts, respectively for 21 days. At the end of the experimental period, animals in the normal and the three diabetic groups will be deprived from food for 3 h, and then sacrificed. Blood will be collected for estimation of blood glucose, serum insulin levels and complete lipid profile determined.

7. **Determination of Akt expression and related proteins:** Liver tissue lysates from extract treated and untreated mice will be prepared in cold lysis buffer [0.05 mmol/L Tris-HCl, 0.15 mmol/L NaCl, 1 mol/L EGTA, 1 mol/L EDTA, 20 mmol/L NaF, 1Q0 mmol/L Na3V04, 0.5% NP40, 1% Triton X-100, 1 mol/L
phenyl methylsulfonyl fluoride (pH 7.4) with freshly added Protease Inhibitor Cocktail Set III (Calbiochem). The lysate will be collected and cleared by centrifugation, and the supernatant was aliquot and stored at 80°C. The protein content in the lysates will be measured by BCA protein assay (Pierce) as per vendor's protocol. For Western blot analysis, 25 to 40 μg of protein will be resolved over 12% Tris-glycine polyacrylamide gels (Novex) under nonreduced conditions, transferred onto nitrocellulose membranes, and subsequently incubated in blocking buffer (5% nonfat dry milk/1% Tween 20, in 20 mmol/L TBS, pH 7.6) for 2 h. The blots will be incubated with appropriate primary antibody, washed, and incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Amersham Biosciences). The blots will be detected with chemiluminescence (ECL kit, Amersham Biosciences) followed by autoradiography.

References:


