Fenugreek (*Trigonellafoenum-graecum* L.) Seed Phytochemicals Protect DNA from Free Radical Induced Oxidative Damage and Inhibit Dipeptidyl Peptidase-IV an Enzyme Associated with Hyperglycemia

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Abstract

Fenugreek (*Trigonellafoenum-graecum* L.; methi) seeds and leaves are used in Asia, Africa, and Mediterranean countries for their nutritional and therapeutic value. It is an ingredient in many traditional Unani and Ayurvedic medicinal systems in India and proven to be very effective in managing blood glucose homeostasis. However, there is a dearth of information on the biochemical rationale for its use in the management of diabetes. In this study, protective effects of fenugreek seed phytochemicals on oxidative stress and inhibition of dipeptidyl peptidase-IV (DPP-IV), a key enzyme associated with diabetes were evaluated. Compositional analysis of phenolics by HPLC revealed that gallic and ferulic acids were the major phenolic acids, and myricetin and rutin were predominant flavonoids in phenolic extracts of fenugreek seeds. Trigonelline content in alkaloid extract was quantitatively determined by reverse phase HPLC and found to be 8.6 mg/g. Alkaloid extract showed superior radical scavenging and metal chelating activities. Besides, these phytochemical extracts also prevented OH• radical-induced DNA damage. Michaelis-Menton and Line weaver-Burk derivations were applied to establish modes of inhibition of DPP-IV activity. Phenolic and alkaloid extracts inhibited DPP-IV activity by mixed non-competitive and non-competitive inhibition mechanisms, respectively. Inhibitory constants of enzyme-inhibitor complexes indicate strong affinity of phenolic (K<sub>i</sub>, 34.93 ± 0.14 μg) and alkaloid extracts (K<sub>i</sub>, 32.3 ± 4.8 μg) for DPP-IV. These results suggest that isolated phytochemicals have the potential as lead compounds for the development of DPP-IV inhibitors to control hyperglycemia and manage diabetes.

Keywords: Fenugreek seeds, Dipeptidyl peptidase-IV, Phenolics, Trigonelline, Antioxidant activity, Mode of inhibition

Introduction

Diabetes mellitus is the world's greatest growing metabolic disorder of the endocrine system. According to WHO, about 422 million adults had diabetes in 2014. Type 2 diabetes is marked by high blood glucose levels (hyperglycemia) and increased insulin resistance along with insufficient insulin secretion. Hyperglycemia has been linked to the onset of the diabetic vascular complications and triggers the generation of free radicals and oxidation-related damage to various organs by producing oxidative stress [1]. Oxidative stress has been forever shown to be a manifestation of many diseases associated with metabolic or vascular disorders including diabetes [2]. Therefore, it is necessary to regulate both blood glucose level and cellular redox status for...
controlling diabetic complications affecting several organs including eyes, kidneys and neurons. Eleven classes of glucose-lowering agents are currently available for the management of diabetes. Of these, dipeptidyl peptidase-IV (DPP-IV; EC 3.4.14.5) inhibitors are among the newest medications that have been introduced to the type 2 diabetes pharmacopeia [3]. DPP-IV is a prolyl peptidase that cleaves proteins and peptides with proline or alanine as the penultimate residue and is widely distributed in almost all human tissues and fluids. Incretin hormones such as a glucose-dependent insulinotropic peptide (GIP) and glucagon-like polypeptide-1 (GLP-1) are endogenous physiological substrates of DPP-IV and are rapidly inactivated by the enzyme [4]. These incretins are gut-derived hormones that stimulate pancreatic glucose-dependent insulin secretion, suppress pancreatic glucagon release, delay gastric emptying, and modulate appetite [5,6].

Synthetic inhibitors of DPP-IV like sitagliptin (Merck & Co.), vildagliptin (Novartis), saxagliptin (Bristol-Meyers Squibb), alogliptin (Takeda) and linagliptin (Boehringer Ingelheim) are being used clinically for effective control of hyperglycemia in diabetic patients. In addition, long-acting (once-a-week use) oral antidiabetic drugs of the DPP-IV inhibitor class such as omarigliptin (Merck & Co.) and trelagliptin (Takeda) are approved for use only in Japan. Albeit their established effectiveness, the inherently associated adverse side effects of these synthetic inhibitors necessitate the need to find natural and safer alternatives. Natural inhibitors of DPP-IV from plant-based foods could be a more effective strategy to control hyperglycemia and provide benefits without the side effects associated with synthetic drugs.

Fenugreek (Trigonella foenum-graecum L) is a leguminous herb cultivated in India and North African countries. The seeds are used as spices worldwide, whereas the leaves are used as green leafy vegetables in the diet. Seeds are bitter to taste and are known for a long time for their medicinal qualities. It is being used as a traditional cure for various disease conditions such as gastrointestinal disorders, gout, wound healing, inflammation, hyperlipidemia, and diabetes [7]. It is also well recognized that fenugreek seeds possess various phytochemicals including flavonoids, saponins, amino acids and soluble dietary fibers with beneficial health effects. The hypoglycemic effects of fenugreek seeds have been attributed to several mechanisms and highlighted in some clinical as well as animal studies. An amino acid 4-hydroxyisoleucine present in fenugreek seeds increased glucose-induced insulin release in human and rat pancreatic islet cells [8]. In humans, fenugreek seeds also exert hypoglycemic effects by stimulating glucose-dependent insulin secretion from pancreatic beta cells [9] and inhibiting the activities of alpha-amylase and sucrase, two intestinal enzymes involved in carbohydrate digestion [10]. Animal studies have shown that fenugreek seed extracts have the potential to reduce post-prandial glucose levels by slowing down the enzymatic digestion of carbohydrates and reduce gastrointestinal absorption of glucose [11]. It also stimulated glucose uptake in peripheral tissues [12] and showed insulin tropic properties in isolated rat pancreatic cells [13]. Furthermore, in vitro inhibition of α-amylase by solid state fermented fenugreek seeds and another processed product from fenugreek seeds were also reported [14]. However, there is a dearth of information on the modulatory effects of active fenugreek components on DPP-IV activity. Therefore, the objective of the present study is to determine the role of fenugreek phytochemicals in the prevention of oxidative stress and to evaluate the inhibitory kinetics and the mode of binding of phenolic and alkaloid fractions in the active site of DPP-IV.

Materials and Methods
Reagents and solutions

Ferrous chloride, trizma base, 2, 2′- diphenyl -1- picrylhydrazyl (DPPH), dipeptidyl peptidase – IV from porcine kidney, Gly-Pro-p-nitroanilide, trigonelline hydrochloride, folin-cioalcalteus reagent, ferrozine, gallic acid, 3,4-dihydroxy benzoic acid, chlorogenic acid, caffeic acid, p-coumaric acid, sinapic acid, ferulic acid, quercetin, kaempferol, myricetin, rutin, luteolin, and apigenin were purchased from Sigma Chemical Co. (St. Louis, MO., U.S.A.). Triethylamine, hydrogen peroxide, calcium chloride, acetonitrile and methanol were purchased from Merck Specialties Pvt Ltd., Germany. Lambda DNA was obtained from Thermo Fisher Scientific, Waltham, USA. All other reagents were of analytical grade.

Sample preparation

Fenugreek seeds were purchased from a local market in Mysore India. Fenugreek seeds were dried in an oven maintained at 50°C for 24 hours. Dried seeds were ground into fine powder to pass through a 250 μm sieve using a coffee grinder. The flour was defatted with hexane (1:5 w/v) thrice with 1 hour time intervals at room temperature and air-dried for 12 h. Defatted and dry fenugreek powder was stored not more than three weeks in an airtight container at -20°C for further analysis.
Extraction of alkaloids

Alkaloids were extracted using the caffeine extraction method reported by Barre, Akaffou, Louarn, Charrier and Hamon & Noirot [15] with few modifications. Briefly, 1 g of defatted fenugreek seed powder was mixed with 1 g of magnesium oxide and 30 ml of distilled water in a capped tube and heated for 20 minutes at 105°C in an autoclave. Extract thus obtained was centrifuged at 4000 rpm for 20 minutes and the supernatant was collected. Pectins and other non-starchy polysaccharides were precipitated from the extract by adding 70% ethanol (1:3 v/v); centrifuged at 4000 rpm for 20 minutes and the supernatant was collected. After concentrating the supernatant in a rotary evaporator, it was filtered (0.2 µm) and used for quantification of trigonelline.

Separation and identification of trigonelline

Separation and quantitative analysis of trigonelline were performed by HPLC according to the method of Campa, Ballester, Doulbeau, Dussert, Hamon & Noirot [16] with slight modifications. An Agilent 1200 series liquid chromatograph (Agilent Technologies Rising Sun, MD, USA) equipped with a quaternary pump (G1311A), a degasser (G1322A), a variable wavelength detector (G1314A) and fitted with LiChrospher 100 RP-18 column (4 × 250 mm, 5 µm; Merck, Darmstadt, Germany) was used for separation of trigonelline. All the operations and the data acquiring were controlled by an Agilent chemstation software. Elution was performed using mobile phase A [10 mM acetic acid/triethylamine (1000/1)] and mobile phase B (methanol) at a flow rate set to 1 ml/min. The solvent gradient in volumetric ratios was as follows: 0-10 min, 5% mobile phase B, isocratic; 10-20 min, 30% mobile phase B; 20-25 min, 50% mobile phase B; 25-30 min, 100% mobile phase B; linear. The column was cleaned with 100% B for an additional 5 min. Finally, the column was eluted isocratically for 5 min with mobile phase A before the next injection. Identification and quantification were carried out at room temperature using reference standard at 263 nm. Spiking and external standard methods were used for qualitative and quantitative analyses of trigonelline by comparing changes in peak area and retention time. External standards using three replicate points at concentrations of 10, 20, 30 and 40 µg/ml were used for plotting calibration curve. Peak areas were plotted against concentrations of external standards.

Extraction and quantification of total phenolic content

The phenolic compounds from defatted fenugreek seed powder (5 g) were extracted with methanol (1:10 w/v) on a rotary shaker set at 100 rpm for 12 h at 50°C. The resultant mixture was centrifuged at 4000 rpm for 20 min; the supernatant was collected for further analysis. The total phenolic content (TPC) of the extract was determined by the Folin–Ciocalteu method using gallic acid as standard [17]. Results were represented in milligram gallic acid equivalents (GAE) per 100 g of defatted flour.

HPLC quantification of phenolic acids and flavonoids

Individual phenolic acids and flavonoids were analyzed by HPLC according to the method of Pradeep & Sreerama [18]. The same Agilent 1200 series liquid chromatography system as used for trigonelline analysis was used for quantitative analysis of phenolics. The elution gradient applied for the analysis of phenolic acids, consisted of mobile phase A: 0.1% trifluoroacetic acid (TFA) in 10% acetonitrile and mobile phase B: 0.1% TFA in 80% acetonitrile. The gradient was: 0-5 min, 5% mobile phase B; 5-10 min, 15% mobile phase B; 10-20 min, 15% mobile phase B; 25-30 min, 100% mobile phase B; 30-35 min, 5% mobile phase B. Total running time was 40 min. Quantitative determination of the eluted phenolic acids was performed at 320 nm for cinnamic acid derivatives (ferulic, p-coumaric, caffeic, chlorogenic and sinapic acids) and 280 nm for benzoic acid derivatives (gallic acid and 3,4 dihydroxybenzoic acid). For flavonoids mobile phase A was 0.1% TFA in water and mobile phase B was 0.1% TFA in acetonitrile. The gradient was 100% mobile phase A in 0.01 min; 10% mobile phase B in 5 min; 36% mobile phase B in 31 min; 54% mobile phase B in 43 min; 64% mobile phase B in 50 min; 100% mobile phase B from 55-60 min; 100% mobile phase A from 65-70 min. Total running time was 70 min. Ultraviolet absorbance at 340 nm was used to detect quercetin, kaempferol, myricetin, rutin, apigenin and luteolin. Spiking and external standard methods were used for qualitative analyses by comparing peak area changes and retention times. The quantitative measurement of the individual phenolic compounds was based on matching retention time and UV/V is spectral data with the standards. The calibration curves of the analyzed phenolics were made in triplicates for each standard and were plotted separately at concentrations in the range of 10-100 µg/ml.

Determination of antioxidant properties

DPPH free radical scavenging activity: The scavenging capacity of the extracts against DPPH radical was measured using the method of Brand-Williams,
Cuvelier & Berstein [19] with slight modifications. Phenolic or alkaloid extract of known concentrations in methanol (100 µl) was mixed with 100 µl of DPPH solution (final concentration was 0.1 mM in methanol) in a 96-well plate. The mixture was shaken gently and kept in the dark at room temperature for 30 min. Thereafter, the absorbance was measured at 517 nm against methanol using a microplate reader (Varioskan Flash Multimode Reader, Thermo Scientific, USA). The radical scavenging activity of extracts was calculated from the calibration curve of trolox and expressed as mmoles of trolox equivalents (TE) per gram of defatted flour.

**Ferric reducing antioxidant power (FRAP):** This assay is based on the reduction of the Fe^{3+} - TPTZ complex to the ferrous form at low pH. This reduction is followed by measuring the absorption change at 595 nm [20]. Ferrous sulfate standard solution in the range of 1.3-6.7 μg/ml and known concentrations of phenolic and alkaloid extracts in distilled water (0.2 ml) were mixed with 1.3 ml of FRAP reagent and incubated at 37°C for 30 min. The FRAP reagent consisted of 0.3 M acetate buffer pH-3.6:10 mM TPTZ: 20 mM FeCl₂ [10:1:1; (v/v)]. The absorbance of the incubated samples was determined against blank at 595 nm. The values obtained were expressed as mmols of Fe^{2+} equivalents per gram of defatted flour.

**Ferrous ion chelating activity:** The Fe^{2+}-chelating capacities of extracts were determined as described by Dinis, Madeira & Almeida [21] with minor modifications. A known concentration of phytochemical extracts from fenugreek seeds (20 µl) was added to 2mM FeCl₂ (15 µl). The reaction was started by adding 5 mM ferrozine (60 µl), and the total volume was adjusted to 1.2 ml with distilled water. The mixture was mixed and left at 25°C for 10 min. The absorbance of the reaction mixture was measured at 562 nm. Distilled water was used instead of the extract for control. Different concentrations (4.9 to 24 nmol) dipotassium EDTA were used to prepare the standard curve. Results were expressed as mmol of EDTA equivalents per gram of defatted flour.

**Inhibition of hydroxyl radical-mediated DNA fragmentation:** Ferrous (II)-catalyzed oxidation of λ-DNA in the presence of hydrogen peroxide was determined according to the method of Ghanta, Banerjee, and Poddar & Chattopadhyay [22]. Hydrogen peroxide-metal-catalyzed oxidation system (hydroxyl radical generating system) consisted of λ DNA (0.5 µg), 1 mM FeSO₄, 25 mM H₂O₂ in Tris buffer (10 mM, pH 7.4) in a final reaction volume of 20 µl for 1 h at 37°C. Phenolic (62.5 µg) and alkaloid (171.7 µg) extracts were used to study their potential to prevent OH•-mediated DNA fragmentation. Samples were analyzed on 0.8% agarose gel prepared in tris-acetate-EDTA buffer (pH-8.5) at 100 v for 1 h at room temperature. The gels were scanned on a gel documentation system (Gel Doc XR, Bio-Rad, and Hercules, CA, USA). Bands were quantified using Image Lab™ software (Bio-Rad). Protective effects of DNA damage were expressed in percentage change in DNA band intensity compared to that of the control.

**Enzyme inhibitory activity**

**Determination of DPP-IV inhibitory activity:**
DPP-IV assay was carried out following the modified method of Al-masri, Mohammad & Tahaa [23]. Cleavage of a chromogenic substrate, Gly-Pro-p-nitroanilide by DPP-IV results in the release of p-nitroaniline, a yellow colored product, which can be measured at 405 nm. Known concentrations of fenugreek phytochemical extracts or commercial drug (sitagliptin) in 35 µl of 50 mM tris-HCl buffer, pH 7.5 was pre-incubated with 15 µl of DPP-IV (0.05 U/ml; one unit of enzyme activity is defined as the amount of enzyme that cleaves 1 µmole of substrate per minute under assay conditions) for 10 minutes at 37°C to facilitate binding of inhibitor. This was followed by the addition of 50 µl of the substrate (0.2 mM Gly-Pro-p-nitroanilide). Final incubation was done at 37°C for 30 minutes. The reaction was stopped by adding 25 µl of 25% glacial acetic acid and release of p-nitroaniline was calculated from the absorbance measured at 405 nm using a microtiter plate reader (Varioskan Flash Multimode Reader, Thermo Scientific, USA). Background absorbance of samples was corrected by replacing enzyme with buffer. Suitable solvent controls were maintained for each concentration of phenolic and alkaloid extracts. The control samples were prepared by the same procedure devoid of the extract. The percent DPP-IV inhibition was calculated as follows:

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\text{DPP-IV inhibition (\%) = } \left( \frac{\text{Absorbance of control} - \text{Absorbance of inhibitor/ Absorbance of control}}{\times 100} \right)
\]

Inhibitory activity of DPP-IV at various concentrations of phenolic and alkaloid extracts was used to derive the IC₅₀ values. IC₅₀ is defined as the concentration of extract required to inhibit 50% of the enzyme activity. The IC₅₀ values were obtained from the least squares regression line of the plots of the logarithm of the sample concentration (log) versus DPP-IV activity (%).

**Kinetics of enzyme inhibition:** The kinetic properties of DPP-IV were determined using various concentrations of Gly-Pro-p-nitroanilide as substrate
(0.05-0.5 mM) at fixed amount of enzyme. Enzyme activities were determined in the absence or presence of different concentrations of phenolic compounds (75, 100 and 126.7 µg/ml) and alkaloids (45, 90 and 112.5 µg/ml). The Michaelis-Menten constant ($K_m$), maximal velocity ($V_{max}$) and mode of inhibition were determined using Line weaver-Burk plots. $K_i$ values were calculated by adjusting the curves to the equation: $m_i = m(1+I/K_i)$ where $m_i$=slope of linear plot of inhibited reaction; $m$=slope of linear plot from reaction without inhibitor; [I]= inhibitor concentration and $K_i$=inhibitory constant [24].

**Statistical analysis:** Experimental data were presented as the mean ± standard deviation of three or more parallel measurements. The statistical analyses were performed by one-way analysis of variance (ANOVA), followed by Dennett’s t-test. A value of $p < 0.05$ was considered statistically significant. Enzyme kinetic data analysis was performed using Origin 6.0 software (Origen Lab Corporation, Northampton, MA, USA).

**Results and Discussion**

**High performance liquid chromatographic separation and determination of trigonelline in fenugreek seeds**

Trigonelline (1-N-methylnicotinic acid, nicotinic acid N-methylbetaine), an alkaloid is involved in cell cycle regulation and is very effective in inducing G2 arrest in the root apices of numerous plant species [25]. In addition, it also controls several other processes in plants, such as nyctinasty, signal transduction, detoxification and host selection [26]. The available literature indicates that trigonelline accumulates in seeds, leaves and roots of various plants such as legumes, coffee (*Coffea arabica*), orange jessamine (*Murraya paniculata*) and four o’clock flower (*Mirabilis jalapa*) [27,28]. Furthermore, trigonelline has recently attracted interest as a potentially protective dietary agent in human health [28]. Therefore quantitative analysis of fenugreek seeds was made using high-performance liquid chromatograph as described in the materials and methods. Identification and quantification of trigonelline were performed by injecting the trigonelline standard three times at four different concentrations ($R^2 \geq 0.999$). The retention time and peak area under the curve were used to make a calibration curve. Trigonelline eluted at a retention time of 11.07 min (Figure 1). The content of trigonelline in the alkaloid extract of fenugreek seeds was found to be 8.63 mg/g. However, the trigonelline content observed in the present study was higher than the reported value (1-1.5 mg/g) for fenugreek seeds [29]. The differences could be attributed to the differences in extraction methods. It was shown that trigonelline is a bitter alkaloid in coffee which serves to produce important aroma compounds [30]. Trigonelline partially degrades during roasting to produce two important compounds - pyridines and nicotinic acid - such that a very dark roast will have only a fraction of its original trigonelline content. Therefore, trigonelline present in fenugreek seeds may have a positive impact on flavor in addition to health beneficial effects.

![Figure 1: HPLC separation of trigonelline derived from fenugreek seeds extract. A. Trigonelline reference standard and B. Alkaloid extract.](image)

**Phenolic content of the extract**

Different solvents such as aqueous mixtures of ethanol, methanol, acetone and water are most commonly used to extract phenolic compounds from plant foods. However, preliminary experiments before this work revealed that absolute methanol was the best solvent under shaking at 50°C for 12 h for the extraction of phenolics from fenugreek seed flour. This solvent system and the
extraction conditions gave the maximum content of phenolics in the extract. The total phenolic content (TPC) of the fenugreek seed extract was 425 ± 31 mg GAE/100 g of defatted flour. These values were comparable to the reported values [31].

**Identification and quantification of phenolic acids and flavonoids**

**Phenolic acids:** The phenolic acid contents of fenugreek seeds extract are presented in table 1. Two hydroxybenzoic acid derivatives (gallic and 3,4-dihydroxybenzoic acids) and five hydroxycinnamic acid derivatives (chlorogenic, caffeic, p-coumaric, sinapic and ferulic acids) were detected in the extract. Among the phenolic acids, gallic and ferulic acids were the predominant phenolic acids identified in fenugreek seeds. Chlorogenic, p-coumaric, sinapic and caffeic acids were present in very low amounts. The phenolic acid composition obtained in this study was in agreement with previous work [32]. However, despite their well-established profiles, we noticed variations in the quantities of individual phenolic acids. This change could be related to the extraction procedures performed by previous analytical works. A significant amount of bioactive compounds can remain in the solid residues after such extractions and are not taken into account for further analysis. In plants, phenolic acids have been associated with diverse functions, including nutrient uptake, protein synthesis, enzyme activity, photosynthesis, structural components and allelopathy [33]. In addition, these phenolic acids have been considered to have the high antioxidant ability and inhibitory potential against the enzymes responsible for generating reactive oxygen species [34]. Epidemiological, randomized, and controlled dietary investigations on human or rodents lend a lot of evidence indicating that consumption of dietary phenolic improves the indices of diabetes risk by controlling the critical pathway of carbohydrate metabolism and liver glucose homeostases such as glycolysis, glycogenesis, and gluconeogenesis [35]. Ferulic and gallic acids, which are abundantly present in fenugreek seeds are reported to exert antidiabetic effects, consequently alleviating liver, kidney, and pancreas damage caused by streptozotocin and alloxan-induced diabetic rats [36,37].

**Flavonoids:** The flavonoid contents of fenugreek seeds are also shown in table 1. Four flavonols (quercetin, kaempferol, myricetin and rutin) and two flavones (luteolin and apigenin) were detected in fenugreek methanol extract. Predominant flavonoids were myricetin and rutin, with small amounts of quercetin, kaempferol and luteolin. Jahan et al. [32] also reported myricetin as a major flavonoid detected in fenugreek seeds. Oxidative stress, which is regulated by the balance of reactive oxygen species production and antioxidant enzyme activity, play a crucial role in the pathogenesis of diabetes mellitus and its complications. In addition, flavonoids have been proved to be ideal natural antioxidant compositions in many common plants and herbs. Epidemiological, *in vitro* and *in vivo* studies indicate the beneficial effects of dietary flavonoids on glucose homeostasis. Flavonoids act on a number of molecular targets and regulate different signaling pathways in pancreatic β-cells (insulin secretion), hepatocytes (glucose metabolism) and skeletal muscle (glucose uptake) [38].

**Antioxidant properties**

**DPPH radical scavenging activity:** Antiradical activity or hydrogen donating ability of fenugreek seed phytochemical extracts was measured using DPPH, a stable free radical by the method of Brand-Williams et al. [19]. DPPH is a purple color dye having absorption maxima of 517 nm and upon reaction with a hydrogen donor the purple color fades or disappears due to its conversion to 2,2'-diphenyl-1-picryl hydrazine resulting in a decrease in absorbance. Table 2 shows the scavenging of DPPH radical by fenugreek seed extracts. Alkaloid extract showed strong scavenging activity (0.36 mmol trolox equi/g) followed by phenolic extract (0.28 mmol trolox equi/g). Although water and various solvent extracts of fenugreek seeds are reported to scavenge DPPH radical [39], to the best of our knowledge, this is the first report describing the DPPH radical scavenging activity in the magnesium oxide (alkaloid) extract of fenugreek seed. Previous studies have shown that myricetin displayed higher free radical scavenging activity among all the flavonoids tested [40]. The 0-dihydroxy group on the ring B of flavonoid moiety of myricetin plays a crucial role in radical scavenging activity. A double bond at a 2-3 position connected with 4-oxo function and OH groups in position 3 and 5 also contribute towards the antiradical activity of myricetin [41]. Therefore, strong DPPH radical scavenging activity observed in the phenolic extract of fenugreek seeds may be associated with the higher content of myricetin in the extract (Table 1). In addition, the radical scavenging ability of alkaloid extract may also be due to the resonant structure of the pyridine ring in the niacin-related compounds like trigonelline [42]. These results suggest that antioxidants in fenugreek seeds are an effective electron or hydrogen donors and this activity contributes to the antioxidant capacity by scavenging the free radicals and may reduce diabetes complications.
Ferric reducing antioxidant power (FRAP): The ferric reducing power was determined by the FRAP method, which depends upon the reduction of ferric tripyridyltriazine (Fe3+-TPTZ) complex to the ferrous tripyridyltriazine. Fe2+-TPTZ has an intense blue color and can be monitored at 593 nm. Table 2 shows the FRAP values of alkaloid (1.78 mmol Fe2+ equiv/g) and phenolic extracts (0.43 mmol Fe2+ equiv/g). Similar to DPPH radical scavenging activity, alkaloid extract showed superior FRAP activity compared to phenolic extract. Mashkor [39] also reported the FRAP values for fenugreek seed methanolic extract. Reducing power is associated with the presence of reductones, which exert antioxidant action by breaking the free radical chain. Besides, reductones can reduce the oxidized intermediates of lipid peroxidation process, so that they can act as primary and secondary antioxidants. However, the antioxidant activity of oxidants has been attributed to various mechanisms, among which are the suppression of chain initiation, binding of transition metal catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging. Since the FRAP reaction involves a single electron or hydrogen transfer mechanisms, functional groups in predominant phenolic compounds of fenugreek seeds may play a crucial role in reducing the oxidized intermediates of peroxidation.

Ferrous ion chelating activity: The presence of transition metal ions like Fe2+ and Cu2+ in a biological system could catalyze the Haber-Weiss and Fenton-type reactions resulting in the generation of hydroxyl radicals (OH•). However, phytochemical extracts with antioxidant activities could form chelating complexes with transition metal ions, which results in the suppression of OH•-generation and inhibition of peroxidation processes of biological molecules. Table 2 shows the ferrous ion chelating activity of fenugreek phytochemical extracts. Similar to DPPH radical scavenging activity and FRAP assays, alkaloid extract showed strong chelating activity (4.03 mmol EDTA equiv/g) than phenolic extract (0.14 mmol EDTA equiv/g). Previous studies have shown that myricetin has some potential metal binding sites. These include positions between the 4-oxo and the 3-or 5-OH groups that can form complexes with a binding stoichiometry of 1:1 or 1:2, metal/myricetin [43,44]. Similarly, a glycoside, rutin ameliorated oxidative stress induced by iron overloaded in hepatic tissue of rats [45]. It is to be noted that myricetin and rutin were the major flavonoids detected in fenugreek seeds (Table 1). The high metal chelating properties of alkaloid and phenolic extracts of fenugreek suggest that they play a vital role in metal-overload diseases and all oxidative stress conditions involving a transition metal ion.

Inhibition of hydroxyl radical-mediated DNA fragmentation: Hydroxyl radicals (OH•) generated by Fenton reaction (Fe2+ + H2O2 → Fe3+ + OH• + OH-) may cause nicks in DNA. Therefore, to get further insight into the antioxidant and radical scavenging activities of fenugreek phytochemical extracts, we have used Fe2+/H2O2 model oxidizing system to investigate protective abilities of these extracts on OH•-mediated DNA fragmentation. Incubation of λ-DNA with FeSO4 and H2O2 resulted in oxidative damage and DNA fragmentation as indicated by total disappearance of the λ-DNA band (Figure 2, lane 2). These results suggest that the oxidation of λ-DNA induced by H2O2 is due to OH• formed by a Fe2+-dependent Fenton-like reaction. However, oxidative damage to λ-DNA under H2O2 treatment was prevented by both the phenolic and alkaloid extracts (Figure 2, lane 3 & 4) indicating their strong divalent metal chelating capabilities. The band intensities of λ-DNA in the presence of phenolic and alkaloid extract were only 15.4 and 18.2%, respectively of the control DNA without phytochemical extracts (Figure 2). These results indicate that the fenugreek seeds phytochemical extracts are effective inhibitors of OH•-mediated DNA fragmentation and played an important role in the protection of DNA against oxidative damage.

Enzyme inhibitory activity

DPP-IV inhibitory activity of fenugreek seed phytochemical extracts: Fenugreek seed phytochemical extracts and commercial drug sitagliptin (Januvia) were evaluated for the inhibition of DPP-IV. Fenugreek phytochemicals showed a dose-dependent inhibition of DPP-IV activity. Although sitagliptin (commercial drug, Merck, Germany) was very effective in inhibiting DPP-IV activity (IC50 value of 0.072 µg/ml), strong inhibition was also noted in phenolic (IC50, 153.01 µg/ml) and alkaloid extracts (IC50, 664.8 µg/ml). It was reported that myricetin binds at the active site of α-amylase and interacts directly with the catalytic residues notwithstanding its bulky planar nature [46]. Strikingly, it reduces the normal conformational flexibility of the adjacent substrate binding cleft. Likewise, direct interaction of fenugreek myricetin with the active site via hydrogen bonds may have contributed to the inhibition of DPP-IV activity. In addition, it was reported that kaempferol and quercetin exhibited lowest binding energy (∆G) and differing dissociation constant (Kd) compared to sitagliptin in insilico docking studies against...
Table 1: Compositional analysis and quantification of phenolicsa.

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<tr>
<th>Flavonoids</th>
<th>Content (µg/g)</th>
<th>Phenolic acids</th>
<th>Content (µg/g)</th>
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<tr>
<td>Apigenin</td>
<td>0.73 ± 0.05</td>
<td>3,4-Dihydroxybenzoic acid</td>
<td>21.67 ± 0.35</td>
</tr>
</tbody>
</table>

aResults are mean ± standard deviation of 3 independent determinations

Table 2: DPPH radical scavenging activity, FRAP values and metal chelating activity of phytochemical extractsa.

<table>
<thead>
<tr>
<th>Sample</th>
<th>DPPH radical scavenging activity (mmol Trolox equi/g)</th>
<th>Metal chelating activity (mmol EDTA equi/g)</th>
<th>FRAP  values (mmol Fe2+equi/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenolic extract</td>
<td>0.28 ± 0.00b</td>
<td>0.14 ± 0.01b</td>
<td>0.43 ± 0.03b</td>
</tr>
<tr>
<td>Alkaloid extract</td>
<td>0.36 ± 0.01a</td>
<td>4.03 ± 0.08a</td>
<td>1.78 ± 0.02a</td>
</tr>
</tbody>
</table>

aResults are mean ± standard deviation of 3 independent determinations. Means followed by different letters (a and b) within the same column are significantly different (p ≤ 0.05)

Table 3: Kinetic properties of fenugreek seeds phytochemical extract on DPP-IV inhibition.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Phenolic extracta</th>
<th>Alkaloid extractb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Km mM</td>
<td>0.71</td>
<td>0.71</td>
</tr>
<tr>
<td>Vmax (µmole pNA/min)</td>
<td>102.04 × 10⁻⁴</td>
<td>102.04 × 10⁻⁴</td>
</tr>
<tr>
<td>Vmax¹ (µmole pNA/min)</td>
<td>83.3 × 10⁻⁴a</td>
<td>90.9 × 10⁻⁴ab</td>
</tr>
<tr>
<td>Vmax² (µmole pNA/min)</td>
<td>52.6 × 10⁻⁴a</td>
<td>62.5 × 10⁻⁴ab</td>
</tr>
<tr>
<td>Vmax³ (µmole pNA/min)</td>
<td>43.5 × 10⁻⁴a</td>
<td>52.63 × 10⁻⁴ab</td>
</tr>
<tr>
<td>IC₅₀ (µg/ml)</td>
<td>152.01 ± 12.72</td>
<td>664.8 ± 46.6</td>
</tr>
<tr>
<td>Kᵢ (µg)</td>
<td>34.93 ± 0.14</td>
<td>32.3 ± 4.8</td>
</tr>
<tr>
<td>Mode of inhibition</td>
<td>Mixed Non-competitive</td>
<td>Non-competitive</td>
</tr>
</tbody>
</table>

aPhenolic extract: Vmax¹, Vmax², Vmax³ represents 75 µg/ml, 100 µg/ml and 126.7 µg/ml inhibitor concentrations, respectively, while corresponding values for balkaloid extract are 45 µg/ml, 90 µg/ml and 112.5 µg/ml, respectively.

Crystal structure of DPP-IV [47]. Furthermore, binding site analysis of the rigid and flexible mode of docking suggests that these two flavonoids occupy the active site with the maximum flexibility in their positioning. These parameters and analysis through computational studies, explain the inherent DPP-IV inhibitory characteristics of kaempferol and quercetin although they are present in low concentration in the extract.

Plant alkaloids are nitrogen containing organic constituents, which are well-known for antidiabetic properties such as insulin mimetic, minimizing insulin resistance, glycolysis promotion and enhancing the GLP-I release. Vasicine and Vasicinol, which were isolated from Adhatoda vasica Nees, showed a high sucrase inhibitory activity with IC₅₀ values 125 and 250 µM, respectively [48]. Besides, Bharti et al. [49] showed that the three major alkaloids from Castanospermum australe seeds extract showed strong DPP-IV inhibition with an IC₅₀ value of 13.96 µg/ml and a low binding affinity. In addition, berberine, an alkaloid isolated from different plant species exhibited DPP-IV inhibition [23]. Therefore, trigonelline, a major component of alkaloid extract of fenugreek seed may form favorable electrostatic interactions bridging the...
positively charged nitrogen atom of its pyridinium ring and negatively charged active site amino acid residue of DPP-IV enzyme. This may be one such mechanism to explain the anti-hyperglycemic activity of trigonelline from fenugreek seeds through DPP-IV inhibition. These findings suggest that both phenolic and alkaloid extracts of fenugreek seeds exhibit promising therapeutic effects on hyperglycemia by inhibiting a key regulatory enzyme linked to diabetes.

![Figure 2](image)

**Figure 2:** Prevention of oxidative DNA damage by fenugreek seeds phytochemical extracts. Histogram (A) and agarose gel electrophoretogram (B) showing the protective effect of fenugreek seeds phytochemical extracts on hydroxyl radical-mediated DNA damage. Numbers on the bars indicate percentage change in DNA band intensity quantified using Image Lab™ software as described in materials and methods. Lane 1: Control DNA (0.5 µg); Lane 2: DNA 0.5 µg + 1 mM FeSO₄ + 25 mM H₂O₂; Lane 3: 0.5 µg DNA + 1 mM FeSO₄ + 25 mM H₂O₂ + 62.5 µg fenugreek seeds phenolic extract; Lane 4: 0.5 µg DNA + 1 mM FeSO₄ + 25 mM H₂O₂ + 171.7 µg fenugreek seeds alkaloid extract.

**Kinetics of enzyme inhibition:** The mode of inhibition of DPP-IV activity by fenugreek seed phytochemicals was determined using Michaelis-Menten and Line weaver-Burk equations. In the case of phenolic extract, the slope of the straight lines in the double-reciprocal plot increased with increasing concentrations of phenolics. The straight lines were joined at a single point in the second quadrant suggesting a mixed non-competitive type of inhibition (Figure 3A). A similar mode of inhibition was proposed earlier for the porcine kidney DPP-IV- (−)-vitisin B complex [50]. This type of inhibition indicates that phenolics can bind to the enzyme irrespective of whether the enzyme is previously bound to the substrate or not. Binding of inhibitor changes the affinity for the substrate, resulting in increasing Kₘ and decreasing Vₘₐₓ proportionate to the concentration of phenolics. However, in the presence of alkaloids, double-reciprocal plots for the uninhibited and partly inhibited enzyme exhibited different slopes and intercepted in various locations in the vertical axis without affecting the Kₘ or indicating non-competitive inhibition (Figure 3B). The non-competitive inhibition shown by fenugreek phytochemicals suggest that the active component of the extract binds to a region excluding the active site of the enzyme or combines with either free enzyme [E] or enzyme-substrate complex [ES], possibly interfering with the action of both. Since these inhibitors are not competing with the substrate to bind to the active site of the enzyme, they offer significant advantage over sitagliptin, which is a competitive inhibitor [51]. Inhibitory action of these inhibitors would not be affected at a higher concentration of substrate.

Kinetic constants for the inhibition of DPP-IV by fenugreek seed phytochemicals are listed in Table 3. The DPP-IV has a Michaelis-Menton constant (Kₘ) of 0.71 mM for Gly-Pro-p-nitroanilide and a Vₘₐₓ value of 102.04 × 10⁻⁴ µmoles of p-nitroaniline released/min. The apparent Vₘₐₓ values decreased with increasing concentrations of both phenolic and alkaloid inhibitors (Table 3). The inhibitory constants (K) of enzyme-inhibitor complexes presented in Table 3 indicate strong affinity of phenolic (Kᵢ, 34.93 ± 0.14 µg) and alkaloid extracts (Kᵢ, 32.3 ± 4.8 µg) for DPP-IV. It is important to highlight that despite large differences in their IC₅₀ values of phenolic and alkaloid extracts (Table 3), they displayed similar Kᵢ values (Table 3). Although IC₅₀ and Kᵢ are both measures of an inhibitor’s ability to block the action of an enzyme, they are not equivalent. Kᵢ values, which are a true equilibrium constant, are considered a more accurate measure since the Kᵢ of an enzyme-inhibitor complex is a constant. IC₅₀ values, in contrast, can vary since they depend on the substrate concentration (single concentration) used in the IC₅₀ determination.

Inhibition of DPP-IV by different classes of phenolic compounds is described in the literature. Fan et al. [52] showed that resveratrol and flavone competitively inhibited DPP-IV activity whereas luteolin and apigenin exhibited non-competitive inhibition. The differences in
inhibitory mechanisms of these phytochemicals should be attributed to their molecular structures. Furthermore, the results of this investigation suggest that the phenolic compounds and alkaloids present in the fenugreek seed may suppress blood glucose elevation by inhibiting the degradation of active GLP-1 by DPP-IV. This, in turn, improves glucose homeostasis with a lower risk of hypoglycemia. Further experiments in mouse models and clinical trials would be necessary to support these in vitro findings.

**Figure 3:** Line weaver-Burk plots for the inhibition of porcine dipeptidyl peptidase-IV by fenugreek phenolic (A) and alkaloid (B) extracts. Different concentrations of phenolics and alkaloids were incubated in the presence of various concentrations of Gly-Pro-p-nitroanilide (0.05-5 mM) as substrate. Data are expressed as the mean of three independent experiments.

**Conclusion**

This study revealed the composition, free radical scavenging and metal chelating activities of fenugreek seed phytochemicals. Noticeably, these phytochemicals protected OH• radical-induced DNA damage and displayed strong inhibition of DPP-IV, a new enzyme target for type-2 diabetes treatment option. Collectively, the studies highlight the fact that fenugreek seeds and its derived products have potential application in the management of glucose homeostasis and might represent a novel therapy for the treatment of hyperglycemia as well as the excessive oxidative stress in individuals with type 2 diabetes. Given the numerous adverse effects of synthetic drugs, the use of phenolics and alkaloids from fenugreek seeds may represent an alternative therapeutic route for the regulation of glycemic index of food products.

**Declarations**

**Ethics approval and consent to participate**

Not applicable. The article does not include any animal or human data or tissue.

**Consent for publication**

Not applicable. The article does not include a clinical study.

**Availability of data and materials**

The datasets used and analysed during this study are available from the corresponding author on reasonable request.

**Competing interests**

The authors declare that they have no competing interests. This manuscript is original and has not been submitted for possible publication to another journal neither has it been published elsewhere.

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**Authors’ contributions**

MAR was responsible for performing experiments, made significant contributions to development of enzymatic assays, acquisition and analysis of data and writing article. YNS conceived the project, designed experiments, supervised execution of the project, analysed and interpreted the data and critically revised the article. Both the authors analysed the results, discussed and approved the final version of the manuscript.

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