

Evaluation of defatted fenugreek seed flakes (Fenuflakes™) and soluble fibers from fenugreek seeds for digestive enzyme inhibition (*in vitro*)

Prasad Thakurdesai¹, Pallavi Deshpande^{1*}, Roshani Pagar^{2,4}, Sanjeevani Deshkar^{2,4}, Asha Thomas^{3,4}

Prasad Thakurdesai¹, Pallavi Deshpande^{1*}, Roshani Pagar^{2,4}, Sanjeevani Deshkar^{2,4}, Asha Thomas^{3,4}

¹Department of Scientific Affairs, Indus Biotech Private Limited, 1, Rahul Residency, Off Salunke Vihar Road, Kondhwa, Pune-411048, INDIA.

²Department of Pharmaceutics, Dr. D. Y. Patil Unitech Society's, Dr. D. Y. Patil Institute of Pharmaceutical Sciences and Research, Pune 411018, INDIA

³Department of Pharmaceutical Chemistry, Dr. D. Y. Patil Unitech Society's, Dr. D. Y. Patil Institute of Pharmaceutical Sciences and Research, Pune 411018, INDIA.

⁴Dr. D. Y. Patil Dnyan Prasad University, School of Pharmacy and Research, Pimpri, Pune, INDIA.

Correspondence

Deshpande P

Department of Scientific Affairs, Indus Biotech Private Limited, 1, Rahul Residency, Off Salunke Vihar Road, Kondhwa, Pune-411 048, Maharashtra, INDIA.

E-mail: pallavi@indusbiotech.com

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ABSTRACT

Introduction: Diabetes mellitus is a chronic disorder marked by elevated blood glucose and related complications. Inhibiting α -amylase, α -glucosidase, and lipase helps to control postprandial glucose and lipids, with natural extracts as safer alternatives. **Objective:** The objective of the study was to explore the antidiabetic mechanisms of defatted fenugreek seed flakes (Fenuflakes™) and soluble fibers from fenugreek seeds by inhibiting key digestive enzymes involved in carbohydrate and lipid metabolism. **Methods:** *In vitro* assays were conducted to evaluate the inhibitory activity of the Fenuflakes and soluble fibers against α -amylase, α -glucosidase, and pancreatic lipase. Enzyme inhibition was assessed in a concentration-dependent manner and compared with standard reference inhibitors. **Results:** Fenuflakes and soluble fibers demonstrated inhibition of α -amylase and α -glucosidase, indicating potential suppression of carbohydrate digestion. It also showed pancreatic lipase inhibitory activity, suggesting reduced lipid hydrolysis. **Conclusion:** Fenuflakes and soluble fibers exhibit promising antidiabetic potential, primarily through multitarget inhibition of digestive enzymes, supporting their role as natural agents for glycemic and metabolic regulation.

Keywords: Alpha-amylase; alpha-glucosidase; lipase; insoluble fibers; *Trigonella foenum-graecum*

INTRODUCTION

Diabetes mellitus represents a major and escalating global public health burden, with prevalence increasing substantially from 108 million cases in 1980 to 463 million in 2019. Epidemiological projections estimate that this number may rise to approximately 700 million by 2045¹. In the Indian context, about 77 million adults are experiencing type 2 diabetes, while 25 million are prediabetic, with over 50% of cases undiagnosed.² This increases the risk of complications like cardiovascular disease and nephropathy³, highlighting the urgent need for effective medical nutrition therapy to enhance glycemic control and prevent disease progression.

In this context, dietary fiber plays a critical role in diabetes management due to its well-established physiological benefits. Soluble fibers like β -glucans and pectin form gels in the gastrointestinal tract, delay gastric emptying and modulate hormones that regulate satiety and glucose absorption³. Soluble fiber also increases intestinal viscosity, acting as a barrier to enzymes such as α -amylase and thereby slowing glucose absorption⁴. Meta-analyses show that a median intake of ~10 g/day of dietary fiber significantly reduces HbA1c and fasting plasma glucose while improving insulin sensitivity in individuals with T2D⁵. Additionally, high intake of cereal- and vegetable-derived fibers is linked with a minimal risk of emerging T2D in healthy populations⁶, underscoring fiber's preventive and therapeutic importance.

Fenugreek (*Trigonella foenum-graecum*) seeds are rich in soluble dietary fiber, particularly galactomannan, and contain bioactive

compounds including saponins, polyphenols, and flavonoids. Building on this nutritional profile, defatted fenugreek seed flakes (Fenuflakes™) offer a standardized formulation that preserves the seed's fiber- and protein-rich composition while minimizing fat content, making them an ideal ingredient for health-focused food applications. Clinical studies show that Fenuflakes™ can lower the glycemic index of foods, decrease fasting glucose levels, and improve glycemic variability in adults with type 2 diabetes (T2D)^{7, 8}. They also exhibit prebiotic effects by increasing beneficial short-chain fatty acids (SCFAs) and improving gut microbiota diversity⁹. Understanding the digestive and metabolic mechanisms behind these benefits is crucial for establishing their biological relevance. In this context, assays targeting the inhibition of α -amylase, α -glucosidase, and pancreatic lipase are vital for elucidating the effect of Fenuflakes™ and soluble fibers from fenugreek seeds on nutrient digestion and metabolic pathways. Inclusion of soluble fiber enables delineation of the contribution of the viscous galactomannan-rich fraction to enzyme inhibition and helps distinguish component-specific effects from the composite Fenuflakes™ matrix. These mechanistic approaches enhance understanding of whether intrinsic fiber properties or the overall food matrix drives the observed effects.

α -amylase and α -glucosidase are enzymes that convert dietary starch into glucose. Inhibiting these enzymes can help to manage postprandial hyperglycemia, a key strategy in diabetes care¹⁰. Fenugreek galactomannan significantly inhibits carbohydrate-digesting enzymes, enhancing intestinal viscosity and limiting enzyme-substrate

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interactions. Studies show it reduces intestinal disaccharidase activity and glucose uptake, improving glycemic control in diabetic models¹¹. Complementary *in vitro* tests reveal that fenugreek extracts inhibit alpha-amylase and alpha-glucosidase¹². Conducting alpha-amylase and alpha-glucosidase inhibition assays specifically using defatted fenugreek seed flakes and their soluble fiber fraction is particularly important, as the defatting process enriches soluble fiber and protein fractions while minimizing lipid interference and allows assessment of dose-dependent, fiber-specific inhibitory effects. Demonstration of enzyme inhibition would provide direct mechanistic evidence supporting the low glycemic index, reduced postprandial glucose response, and improved glycemic variability observed in clinical studies of Fenuflakes™^{7,8}.

Pancreatic lipase is key for fat digestion, and its partial inhibition can improve lipid metabolism and insulin sensitivity¹³. Fenugreek extracts have shown significant lipase inhibitory effects¹⁴. This inhibition also enhances gut health by delivering undigested substrates to the colon for microbiota fermentation¹⁵. Fenuflakes™ demonstrate prebiotic effects, such as increased SCFA production, enhanced microbial diversity, and reduced gas production compared to inulin⁹. This suggests that many dietary fibers resist digestion in the upper gastrointestinal tract. Inhibition of alpha-amylase, alpha-glucosidase, and lipase explains this resistance, ensuring more substrate is available for colonic fermentation. The inhibition of digestive enzymes serves as a foundational mechanism that links delayed digestion of carbohydrates and lipids, improved glycemic responses, and enhanced prebiotic effects of defatted fenugreek seed flakes and their soluble fiber fraction. Therefore, this study has been designed to evaluate the digestive enzyme inhibition activities (specifically alpha-amylase, alpha-glucosidase, and lipase) of fenuflakes dispersion and its soluble fibers in an *in vitro* setting.

MATERIALS

Sample preparation

Fenuflakes dispersion and the corresponding soluble fiber fraction were derived from fenugreek seed extract and used as the test samples for subsequent analyses. The Fenuflakes and soluble fibers used in this study were provided by Indus Biotech Private Limited (India). Analysis showed that the Fenuflakes samples contained not less than 25% protein, as well as both soluble and insoluble fibers, while containing minimal amounts of carbohydrates and fats, as determined by AOAC official methods reported previously¹⁶. The fenuflakes powder contained 26.53% soluble fiber, 34.00% insoluble fiber, 0.36% total fat, and a loss on drying of 4.29%. The soluble fiber fraction of Fenuflakes contained an equivalent amount of soluble fibers, enabling mechanistic delineation of its soluble fiber-mediated enzyme-inhibitory effects independent of the composite Fenuflakes™ matrix.

Samples were processed by centrifugal dialysis using a Thermo R8C centrifuge with Vivaspin 10 kDa MWCO units at 10,000 × g for 45 min. Both soluble fibers and fenuflakes dispersion samples were evaluated at five concentration levels, including undiluted and four serial 1:2 dilutions. Undiluted fenuflakes dispersion samples (30 mg/mL) were used directly. Subsequent dilutions (1:2 to 1:16) were prepared by mixing equal volumes (500 µL) of the preceding dilution with cell culture medium for MTT assay and with specific buffer solutions for enzyme assays to a final volume of 1.0 mL, yielding concentrations from 30 mg/mL to 1.87 mg/mL for fenuflakes dispersion and 15 mg/mL to 0.937 mg/mL for soluble fibers.

Cell cytotoxicity assay

HT-29 human epithelial cells were acquired from the National Centre for Cell Science (NCCS), Pune, India. Dulbecco's Modified Eagle

Medium (DMEM; Gibco, USA), fetal bovine serum (FBS; Gibco, USA), penicillin–streptomycin solution (HiMedia, India), phosphate-buffered saline (PBS, pH 7.4; Gibco, USA), and trypsin–EDTA (0.25%; Gibco, USA) were used for cell culture and maintenance. The MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was procured from Invitrogen (Thermo Fisher Scientific, USA). Dimethyl sulfoxide (DMSO; HiMedia, India) was utilised to solubilise formazan. Sterile syringe filters (0.22 µm; Whatman, Cytiva), 96-well sterile flat-bottom microplates (Tarsons, India; Biofil, China), T25 and T75 tissue culture flasks (Tarsons, India; Abdos, India), centrifuge tubes (15 mL and 50 mL; Tarsons, India), 1.5 mL microcentrifuge tubes (Tarsons, India), and protein concentrators (Vivaspin, 10 kDa MWCO; Thermo Scientific, USA) were used as consumables. Equipment included a centrifuge (Thermo Scientific, USA, Model R8C), CO₂ incubator (Thermo Scientific, USA, Forma Steri-Cycle 371), biosafety cabinet (IMSET, India, Class II), inverted phase-contrast microscope (Olympus, Japan CKX53), vortex mixer (Remi, India, CM-101), and microplate reader (Thermo Scientific, USA, Model 5119100).

Alpha amylase inhibition assay

Alpha-amylase enzyme, soluble starch substrate, acarbose (positive control), and 3,5-dinitrosalicylic acid (DNS reagent) were obtained (Sigma-Aldrich, USA). Sodium chloride was purchased from SRL (India), for buffer preparation, potassium dihydrogen phosphate, sodium hydroxide pellets, and potassium sodium tartrate tetrahydrate were procured from Loba Chemie (India). The assay utilized 96-well flat-bottom microplates (Nest Biotechnology, China), a microplate spectrophotometer (Biotech Instrument, Epoch), micropipettes (HiMedia, India), a vortex mixer (Remi, India), an analytical balance (Shimadzu, Japan), an incubator (Bio-TechnoLab, India, RHI-50), and a pH meter (Elico, India).

Alpha-glucosidase inhibition assay

Alpha-glucosidase (maltase), p-nitrophenyl-α-D-glucopyranoside (pNPG) substrate, and acarbose were obtained (Sigma-Aldrich, USA). Sodium carbonate was purchased from SRL (India), for buffer preparation, potassium phosphate monobasic and dibasic salts were obtained from Loba Chemie (India). The assay was conducted using 96-well flat-bottom microplates (Nest Biotechnology, China), a microplate spectrophotometer (Biotech Instrument, Epoch), micropipettes (HiMedia, India), a vortex mixer (Remi, India), an analytical balance (Shimadzu, Japan), an incubator (Bio-TechnoLab, India, RHI-50), and a pH meter (Elico, India).

Pancreatic lipase inhibition assay

4-Nitrophenyl laurate substrate, pancreatic lipase enzyme, and orlistat (positive control) were obtained from Sigma-Aldrich (USA). Sodium lauryl sulfate (SLS) was acquired from Sigma-Aldrich (from Sigma-Aldrich USA), while Triton X-100 and Tris base were purchased (Loba Chemie, India). Dimethyl sulfoxide (DMSO) was sourced (SRL, India). The assay employed 96-well flat-bottom microplates (Nest Biotechnology, China), a microplate spectrophotometer (Biotech Instrument, Epoch), micropipettes (HiMedia, India), a vortex mixer (Remi, India), an analytical balance (Shimadzu, Japan), an incubator (Bio-TechnoLab, India, RHI-50), and a pH meter (Elico, India).

METHODS

Cell cytotoxicity assay

HT 29 human intestinal epithelial cells were cultivated in DMEM supplemented with 10% FBS and maintained at 37 °C in an incubator with 5% CO₂ and humidity. The cells were seeded overnight at a density of 1 × 10⁴ cells per well in sterile, flat-bottom 96-well microplates to facilitate adhesion. Following attachment, freshly prepared culture

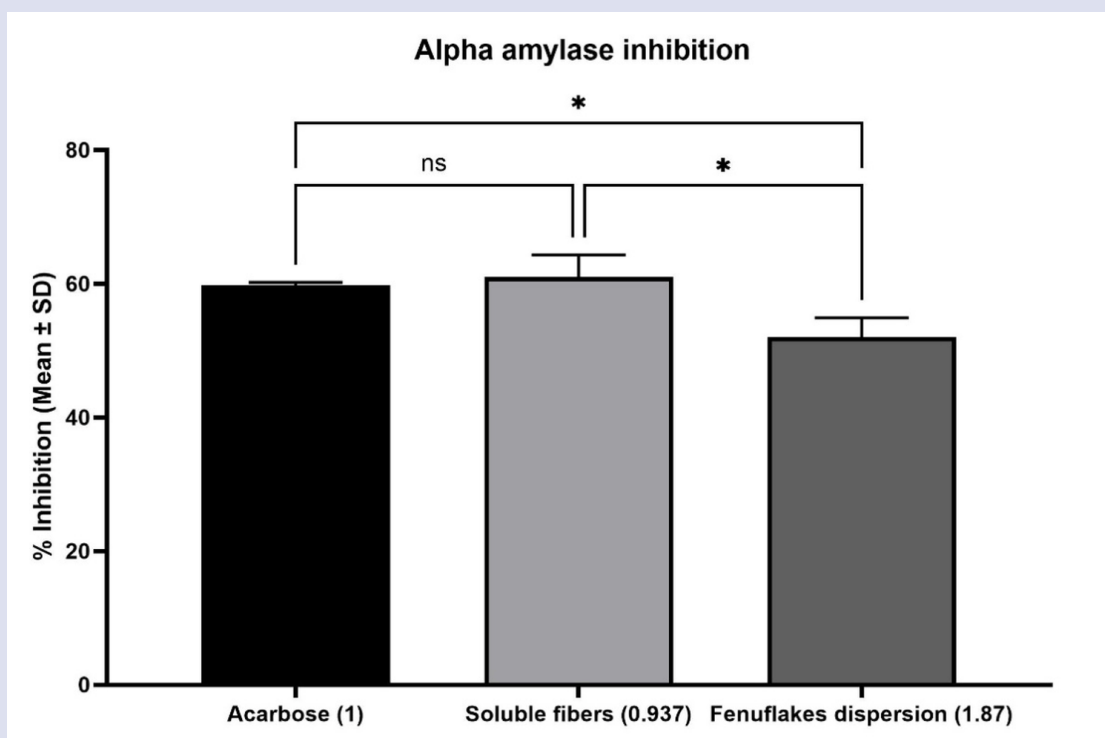


Figure 1. *In vitro* alpha amylase inhibition activity of Fenuflakes

Values are expressed as mean ± SD. Numbers in parentheses indicate dose in mg/mL. n=3; the data were examined using one-way ANOVA, followed by Tukey's test. * P < 0.05

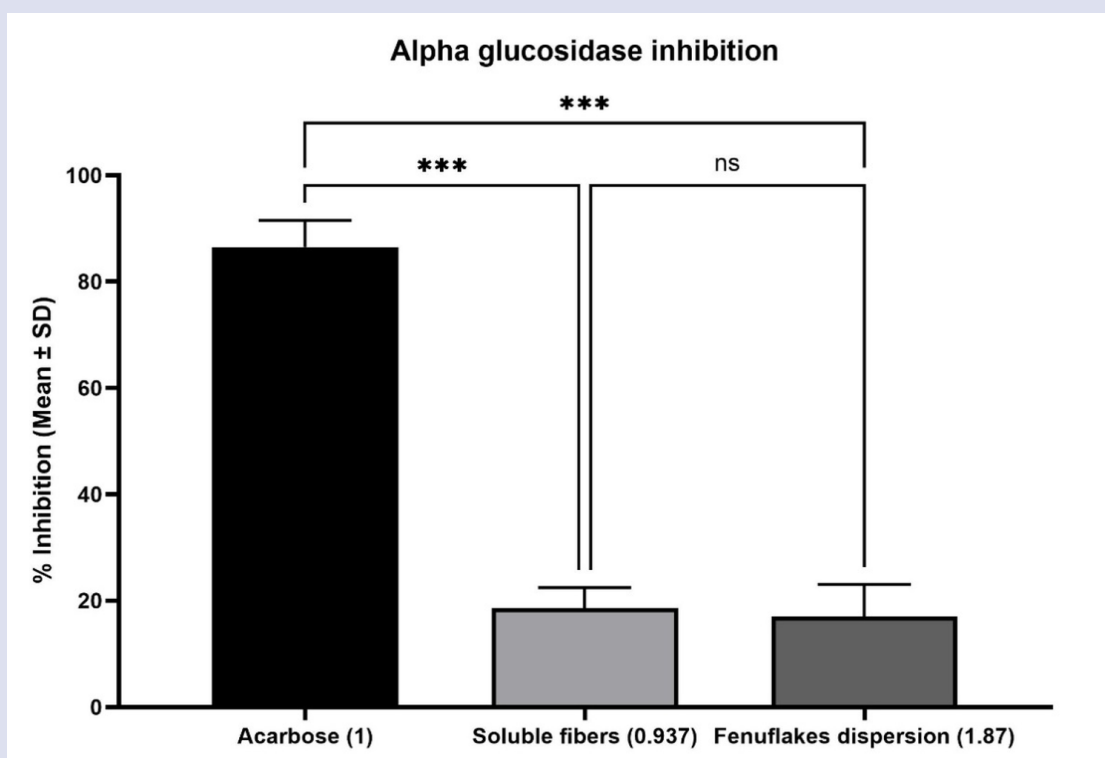


Figure 2. *In vitro* alpha glucosidase inhibition activity of Fenuflakes

Values are expressed as mean ± SD. Numbers in parentheses indicate dose in mg/mL. n=3; data were examined using One-way ANOVA followed by Tukey's test. ***P < 0.001.

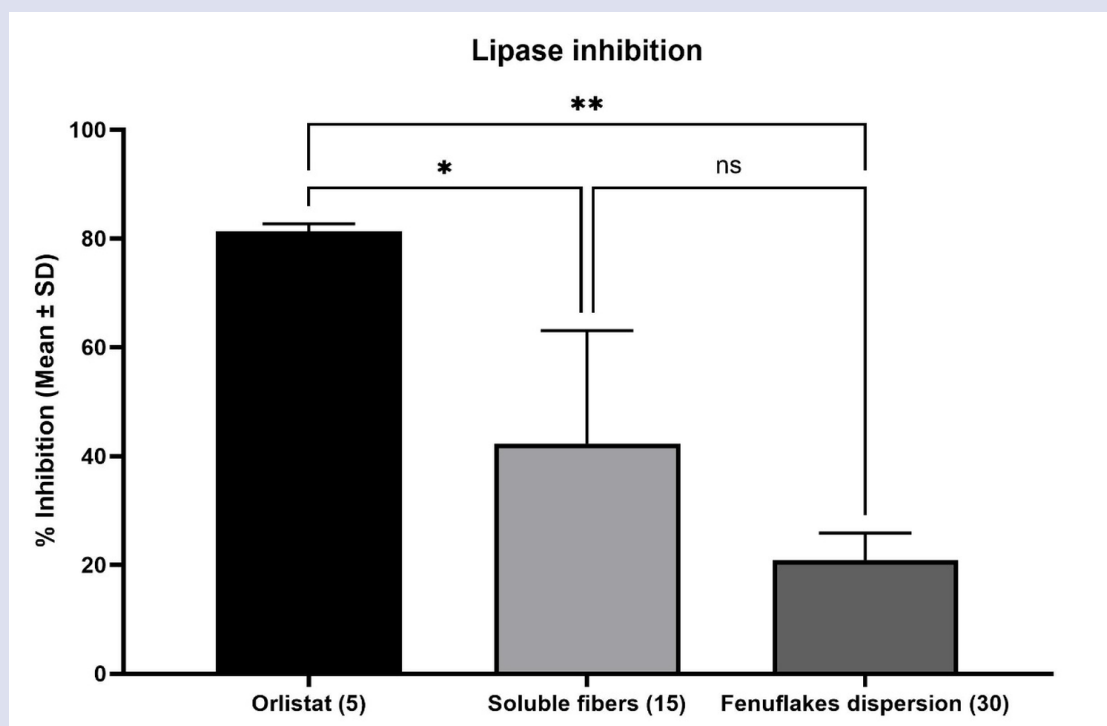


Figure 3. *In vitro* lipase inhibition activity of Fenuflakes

Values are expressed as mean ± SD. Numbers in parentheses indicate dose in mcg/mL for Orlistat and mg/mL for soluble fibers and fenuflakes dispersion. n=3; data were examined using One-way ANOVA followed by Tukey's test. * P < 0.05 and ** P < 0.01.

media containing varying concentrations of fenuflakes fractions were introduced to the culture medium. To evaluate the dose-dependent cytotoxicity, the cells were incubated at 37°C for 24 hours in a humidified 5% CO₂ environment.

After treatment, each well received 50 µL of freshly formed MTT solution (5 mg/mL in PBS) and was incubated in the dark at 37°C for 4 hours. MTT was transformed into insoluble purple formazan crystals by metabolically active cells during incubation.

After incubation, the supernatant containing residual medium and MTT was gently aspirated without disturbing the formazan precipitate, and 100 µL of DMSO was introduced to each well to solubilize the crystals. To ensure complete dissolution, the plates were agitated for 10 minutes at room temperature at 300 rpm in an orbital shaker and absorbance was measured at 560 nm using a microplate reader (Thermo Scientific™; Model 5119100). Optical density readings were proportional to the number of viable cells and normalized against untreated controls. Test samples were prepared from fenuflakes fractions using centrifugal dialysis with a 10 kDa molecular weight cut-off at 10,000 × g for 45 min. Two test samples (soluble fibers and fenuflakes dispersion) were tested at five concentrations (undiluted and four serial 1:2 dilutions), with each condition assayed in triplicate, and the cell viability (%) was evaluated using the formula as follows :

$$\text{Cell viability (\%)} = \frac{(A \text{ sample mean} - A \text{ blank mean})}{(A \text{ control mean} - A \text{ blank mean})} \times 100$$

Alpha amylase inhibition assay

The alpha-amylase inhibition of the samples was assessed using a modified DNS technique as previously reported¹⁷. 1 mg/mL of acarbose was employed as the positive control. Briefly, 100 µL of alpha-

amylase enzyme solution was mixed with 100 µL of each test sample or acarbose solution in test tubes, and the reaction mixtures were prepared in triplicate. A negative control was established by replacing the test sample with buffer. The mixtures were pre-incubated at 37°C for 20 minutes, then 100 µL of starch solution was added to initiate the enzymatic reaction. Following further incubation at 37 °C for 20 min, to terminate the reaction, 200 µL of DNS reagent was added. The resultant solutions were heated up in a boiling water bath for 5 minutes, then cooled to room temperature and diluted with 3 mL Milli-Q water. After transferring 200 µL of each reaction mixture to a 96-well microplate, the absorbance was measured at 540 nm utilising a microplate reader (BioTek Instruments, Inc., USA). Using the following formula, the % inhibition of alpha-amylase activity was determined:

$$\text{Alpha - amylase inhibition (\%)} = \frac{A - B}{A} \times 100$$

where XA represents the enzyme control absorbance (100% enzyme activity), and XB indicates the absorbance of the reaction including the test sample or acarbose.

Pancreatic lipase inhibition assay

The inhibitory activity against pancreatic lipase was assessed using a colorimetric test with p-nitrophenyl laurate (pNPL) as the substrate, as described earlier¹⁹. Briefly, a 1.6 mM pNPL stock solution was prepared by dissolving approximately 5.14 mg of p-nitrophenyl laurate in 0.5 mL DMSO and diluting to a final volume of 10.0 mL with SLS/Triton buffer. To prepare the lipase working solution (150 U/mL⁻¹), 15 mg of lipase was dissolved in 10 mL of 66 mM Tris-HCl buffer (pH 7.4). Orlistat (5 µg/mL) was employed as the positive control.

The experiment was conducted in a transparent, flat-bottomed 96-well microplate (Nest Biotechnology, China). Test samples, positive control (orlistat), and enzyme control (100% activity) were assayed in triplicate. For each reaction, 25 µL of the test sample prepared in assay buffer was added to the designated wells. For the enzyme control, the test sample was replaced with buffer, while for the positive control, 25 µL of orlistat solution was added. Furthermore, 100 µL of pNPL substrate solution was introduced into each well, followed by 50 µL of lipase working solution. The reaction mixtures were gently mixed and incubated at 37 °C for 30 minutes in a temperature-controlled incubator. (Bio-TechnoLab, India; RHI-50).

Following incubation, the release of p-nitrophenol was assessed spectrophotometrically at 405 nm by utilizing the microplate reader. The lipase inhibitory activity was determined using the following formula:

$$\text{Lipase inhibition (\%)} = \frac{A - B}{A} \times 100$$

where A signifies the absorbance at 405 nm of the control without the test sample, while B signifies the absorbance of the test sample or orlistat

Statistical Analysis

Results were provided as mean ± SD for various parameters, including *in vitro* cytotoxicity and inhibition of alpha-amylase, alpha-glucosidase, and lipase. Statistical analysis was conducted using GraphPad Prism (version 8.0.2; GraphPad Software, USA). A one-way ANOVA with Tukey's test was used to compare the groups, and significance levels were indicated as **p* < 0.05.

RESULTS

Cell cytotoxicity assay

The undiluted fenuflakes dispersion showed high cytotoxicity (56.74 ± 10.75%), but dilution improved viability to near baseline (84.34 ± 5.11%). The soluble fiber fraction was also cytotoxic when undiluted (37.62 ± 9.50%) but enhanced viability upon dilution (112.60 ± 18.19%), indicating a threshold for biological activity.

Alpha amylase inhibition assay

Fenuflakes dispersion and soluble fibers exhibited concentration-dependent inhibition of alpha-amylase activity. Fenuflakes dispersion demonstrated inhibitory activity across all tested concentrations (30, 15, 7.5, 3.75, and 1.875 mg/mL), with inhibition values of 83.24 ± 0.57%, 81.97 ± 2.38%, 78.96 ± 1.09%, 65.03 ± 6.87%, and 52.00 ± 2.92%, respectively. Similarly, soluble fibers showed inhibition of 83.88 ± 0.27%, 82.42 ± 0.16%, 80.87 ± 0.82%, 65.48 ± 3.11%, and 61.02 ± 3.28% at the (15, 7.5, 3.75, 1.875, and 0.937 mg/mL) concentrations. A gradual decrease in enzyme inhibition was observed with increasing dilution.

At a concentration of 1 mg/mL, acarbose produced 59.73 ± 0.47 % inhibition of alpha-amylase activity. At comparable concentrations, fenuflakes dispersion (1.875 mg/mL) and soluble fibers (0.937 mg/mL) exhibited inhibition values of 52.00 ± 2.92% and 61.02 ± 3.28%, respectively, with no significant difference between acarbose and soluble fibers, whereas significant differences were observed between acarbose and fenuflakes dispersion (*p*=0.02) as well as soluble fibers and fenuflakes dispersion (*p*=0.01) (Figure 1). The IC₅₀ values for fenuflakes dispersion and soluble fibers, calculated over the tested concentration range, were 0.825 mg/mL and 0.596 mg/mL, respectively.

Alpha-glucosidase inhibition assay

Fenuflakes dispersion and soluble fibers exhibited alpha-glucosidase inhibition activity. Fenuflakes dispersion demonstrated inhibitory

activity across all tested concentrations (30, 15, 7.5, 3.75, and 1.875 mg/mL), with inhibition values of 32.77 ± 20.06%, 22.78 ± 8.43%, 16.55 ± 2.43%, 26.36 ± 4.90%, and 17.10 ± 5.98%, respectively. Similarly, soluble fibers showed inhibition of 39.84 ± 5.84%, 23.10 ± 11.91%, 13.87 ± 3.96%, 26.60 ± 7.27%, and 18.69 ± 3.80% at the (15, 7.5, 3.75, 1.875, and 0.937 mg/mL) concentrations. A gradual decrease in enzyme inhibition was observed with increasing dilution except at 3.75 and 1.875 mg/mL for Fenuflakes dispersion and soluble fibers.

At a concentration of 1 mg/mL, acarbose produced 86.55 ± 4.98% inhibition of alpha-glucosidase activity. At comparable concentrations, fenuflakes dispersion and soluble fibers exhibited inhibition values of 17.10 ± 5.98% and 18.69 ± 3.80%, respectively, with a significantly less reduction in fenuflakes dispersion and soluble fibers as compared to acarbose (*p*<0.001) and no significant difference between soluble fibers and fenuflakes dispersion (Figure 2). Since the percent inhibition was very low with fenuflakes dispersion and soluble fibers, the IC₅₀ values could not be calculated.

Pancreatic lipase inhibition assay

Fenuflakes dispersion and soluble fibers exhibited lipase inhibitory activity. Fenuflakes dispersion demonstrated inhibitory activity across all tested concentrations (30, 15, 7.5, 3.75, and 1.875 mg/mL), with inhibition values of 20.92 ± 4.96%, 37.47 ± 0.63%, 22.97 ± 2.75%, 32.41 ± 4.18%, and 43.31 ± 6.45%, respectively. Similarly, soluble fibers showed inhibition of 42.31 ± 20.75%, 38.62 ± 7.73, 21.48 ± 1.98%, 36.60 ± 21.03%, and 37.93 ± 6.64% at the (15, 7.5, 3.75, 1.875, and 0.937 mg/mL) concentrations. A gradual decrease in enzyme inhibition was observed with increasing dilution in the case of soluble fibers except at 3.75 and 1.875 mg/mL, whereas dispersion demonstrated an increase in inhibitory activity with dilution.

At a concentration of 5 µg/mL, Orlistat produced 81.30 ± 1.40% inhibition of lipase activity and showed significantly higher inhibition as compared to soluble fibers (*p* = 0.02) and fenuflakes dispersion (*p* = 0.002) at their respective comparable concentrations (Figure 3). Since the percent inhibition was very low with fenuflakes dispersion and soluble fibers (below 50%), the IC₅₀ values could not be calculated.

DISCUSSION

The present study demonstrates that Fenuflakes dispersion and soluble fibers derived from *Trigonella foenum-graecum* are non-cytotoxic to intestinal epithelial cells while exhibiting inhibitory activity against key digestive enzymes alpha amylase, alpha glucosidase and pancreatic lipase involved in carbohydrate and lipid metabolism. These findings reinforce the growing body of evidence indicating that fenugreek-based dietary components can effectively modulate postprandial glucose and lipid digestion without inducing cellular toxicity^{8,20}.

The safety and efficacy of Fenuflakes dispersion and soluble fibers were evaluated using the MTT assay and enzyme inhibition assays (alpha-amylase, alpha-glucosidase, and pancreatic lipase) to provide comprehensive validation of their functional potential, in line with established approaches for functional food evaluation²¹.

The MTT assay was performed using HT-29 human epithelial cells to evaluate cytotoxicity and establish the safety of the samples toward mammalian systems. HT-29 cells are broadly utilized as an *in vitro* model of the intestinal epithelium due to their structural and functional similarity to small intestinal enterocytes, making them suitable for assessing the safety, transport, and bioavailability of food-derived compounds²². The functional efficacy of Fenuflakes dispersion and soluble fibers was assessed using enzyme inhibition assays targeting alpha-amylase, alpha-glucosidase, and pancreatic lipase. Inhibition of alpha-amylase and alpha-glucosidase was used to evaluate the potential of Fenuflakes dispersion and soluble fibers

to delay carbohydrate digestion and reduce glucose absorption. In contrast, lipase inhibition was examined to determine its effect on fat digestion and lipid regulation. Safety and efficacy were evaluated across a range of concentrations to simulate gastrointestinal dilution. Previous analysis using AOAC official methods indicated that Fenuflakes contain $\geq 50\%$ total dietary fiber, comprising both soluble and insoluble fractions¹⁶. The literature reports that maximal of alpha-amylase and alpha-glucosidase inhibition by dietary fibers occurs at 1–10 mg/mL for soluble fibers and 1–20 mg/mL for insoluble fibers, with reduced activity observed at higher concentrations^{23,24}.

Based on these findings, an initial concentration of 30 mg/mL was selected for Fenuflakes dispersions to achieve approximately 15 mg/mL of total dietary fiber. For soluble fiber fractions, a maximum concentration of 15 mg/mL was used. Serial dilutions were prepared to achieve a concentration range of 0.937–15 mg/mL, covering the reported effective range for enzyme inhibition.

In the MTT assay, reduced cell viability was observed at the highest concentrations (30 mg/mL for total dispersion and 15 mg/mL for soluble fibers), likely due to physicochemical effects rather than intrinsic cytotoxicity. High fiber concentrations increase medium viscosity and osmotic pressure, thereby limiting the diffusion of nutrients and growth factors into cells. This restriction induces metabolic stress and reduces mitochondrial activity, thereby decreasing cell viability. However, these interpretations remain preliminary, as the study did not include direct assessment of osmolarity, viscosity characterization, cellular morphology, or apoptosis/necrosis markers. Therefore, further mechanistic investigations are warranted to clarify the underlying causes of the observed concentration-dependent effects on cell viability. Similar concentration-dependent effects have been reported for polysaccharides such as chitosan and chitosan oligosaccharides, where cytotoxicity is observed above 10 mg/mL, while lower concentrations are considered safe²⁵. No cytotoxicity was observed at lower, physiologically relevant concentrations of Fenuflakes dispersion and soluble fibers, consistent with previous reports on fenugreek-derived fiber-rich fractions¹⁶. Phytochemical analyses suggest that soluble fibers (particularly galactomannan), along with saponins and polyphenols, contribute to biological activity without inducing cytotoxic effects at nutritionally relevant doses^{11,26}. Fenuflakes, with its soluble fiber fraction demonstrate a strong safety profile, being well-tolerated at 30 g/day in human clinical studies⁸ and showing a high toxicological NOAEL (>2000 mg/kg/day) corresponding to a human equivalent dose (HED) of 19.2 g/day¹⁶. This favorable safety profile supports the potential application of Fenuflakes as a functional food ingredient for long-term management of metabolic disorders.

Fenuflakes dispersion and soluble fibers exhibited clear concentration-dependent inhibition of alpha-amylase, with activity comparable to that of acarbose at equivalent concentrations. This observation aligns with earlier studies demonstrating that galactomannan-rich fractions effectively inhibit alpha-amylase through multiple complementary potential mechanisms^{23,27}. Soluble fibers, particularly galactomannan, have been reported to non-competitively bind alpha-amylase at sites other than the active site, inducing conformational alterations that minimise enzyme activity. In contrast, insoluble fibers may adsorb or entrap the enzyme–substrate complex, resulting in mixed-type inhibition²³. In addition to direct enzyme interaction, soluble fibers form viscous, gel-like structures upon hydration, creating a physical barrier between alpha-amylase and starch substrates. This barrier limits starch accessibility and slows enzymatic hydrolysis, thereby attenuating postprandial glucose release^{23,27}. Insoluble fibers, owing to their loose fibrous structure, further contribute by physically entrapping enzymes and starch granules. The extensive swelling of both soluble and insoluble fibers increases the viscosity of the gastrointestinal milieu, reducing enzyme–substrate diffusion and interaction rates. The significant

alpha-amylase inhibition observed for Fenuflakes dispersion and soluble fiber fractions is therefore attributable to the combined effects of soluble galactomannan and insoluble fibers. The observed reduction in inhibitory activity upon dilution can be explained by decreased viscosity, reduced fiber density, and dilution of phytochemicals, which collectively diminish enzyme–substrate interference.

In contrast, Fenuflakes dispersion and soluble fibers exhibited lower alpha-glucosidase inhibitory activity compared to acarbose, a trend consistently reported in the literature for fenugreek-derived materials^{23,28}. In the present study, the soluble fiber fraction and Fenuflakes dispersion showed non-significant alpha-glucosidase inhibition in undiluted form, with inhibition decreasing upon dilution. Insoluble fibers in fenuflakes dispersion primarily constrained substrate accessibility, while soluble fibers interfered directly with enzyme binding. The galactomannan-rich soluble fiber fraction inhibited alpha-glucosidase through hydrogen bonding and steric hindrance of the enzyme–substrate complex, supporting previous mechanistic observations²³. This provides a mechanistic explanation for the sustained glycemic control observed in clinical studies, rather than acute enzyme suppression, thereby minimizing gastrointestinal side effects^{7,8}.

Lipase inhibition by Fenuflakes dispersion and soluble fibers was not concentration-dependent but significantly lower than that of orlistat, consistent with previous reports on fenugreek seed extracts and fiber-rich fractions^{14,29}. The soluble fiber fraction, enriched in galactomannans, exhibited intermediate lipase inhibition by restricting enzyme access to lipid substrates and altering enzyme–substrate interactions. This inhibition declined progressively with dilution but remained measurable, reflecting the persistence of soluble fiber effects even at lower concentrations. The undigested Fenuflakes dispersion showed the lowest lipase inhibition, as insoluble fibers contribute minimally to lipase suppression. However, upon dilution, increased solubility enhanced the relative contribution of soluble fibers, resulting in improved enzyme–substrate interaction and a gradual increase in inhibition. These observations suggest that Fenuflakes modulate lipid digestion primarily through indirect, fiber-mediated mechanisms such as interference with emulsification and micelle formation, rather than direct enzyme blockade.

Unlike previously studied fenugreek preparations that primarily rely on isolated phytochemical extracts, Fenuflakes™ represents a defatted, fiber-rich whole-food matrix containing soluble and insoluble fibers along with residual bioactive constituents. This integrated composition may contribute to the modulation of nutrient digestion by attenuating the activities of α -amylase, α -glucosidase, and lipase, thereby influencing both carbohydrate and lipid metabolism. The enzyme inhibition assays were performed in technical triplicates using undigested test material to evaluate the inherent inhibitory potential of the Fenuflakes™ matrix prior to gastrointestinal transformation. The relatively higher standard deviations observed in the α -glucosidase and lipase assays may reflect matrix heterogeneity and variable hydration characteristics under non-digested conditions, as dietary fiber structure and enzyme–substrate interactions are known to change dynamically during digestion³⁰. There are several reports indicating high standard deviations in such assays using soluble and insoluble fibers^{23,31}. In addition, *in vitro* enzyme inhibition assays may not fully replicate the complex physicochemical and physiological conditions of the gastrointestinal tract, including pH transitions, digestive secretions, transit dynamics, and matrix restructuring during digestion. Future studies incorporating biological replicates and simulated gastrointestinal digestion models may further improve mechanistic understanding, physiological relevance, and assay reproducibility.

Overall, the results of this study corroborate extensive literature demonstrating that Fenuflakes and soluble fibers from fenugreek seeds

exert multitarget inhibitory effects on digestive enzymes involved in glucose and lipid metabolism. The combination of non-cytotoxicity, effective α -amylase inhibition comparable to acarbose, moderate α -glucosidase inhibition, and physiologically balanced lipase inhibition supports the potential of Fenuflakes dispersion and soluble fibers as functional food ingredients for managing postprandial hyperglycemia and dyslipidemia. These findings further reinforce the role of soluble and insoluble fibers from fenugreek seeds as a safe, natural dietary adjunct for metabolic health management, as supported by converging *in vitro*, *in vivo*, and clinical evidence.

CONCLUSION

Fenuflakes and soluble fibers from fenugreek seeds inhibited α -amylase, α -glucosidase, and pancreatic lipase, thereby reducing carbohydrate and lipid digestion and supporting glycemic control and improved metabolic regulation through enzyme-based mechanisms.

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