Received: 18 April 2024 | Revised: 16 August 2024 | Accepted: 3 September 2024

DOI: 10.1111/1750-3841.17411

Journal of Food Science WILEY

ORIGINAL ARTICLE

Food Chemistry

Effect of fenugreek (*Trigonella foenum-graecum* L.) seed extract on glycemic index, in vitro digestibility, and physical characterization of wheat (*Triticum aestivum* L.) starch

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Funding information
Taif University, Saudi Arabia, Grant/Award Number: TU-DSPP-2024-07; Director of the Experiment Station, G.B. Pant University of Agriculture & Technology, Pantnagar −263,145, Uttarakhand, India

Abstract: Diabetes is a major health concern and is approaching epidemic proportions worldwide. In 2021, diabetes mellitus was responsible for 6.7 million deaths across the globe. Mortality due to diabetes is predicted to rise nearly 10-fold by 2030 and 783 million by 2045. Wheat starch, which constitutes about 70% of the endosperm, is a key component of wheat grain. The rapid hydrolysis of wheat starch can result in elevated postprandial glucose levels, leading to diabetes. The increase in blood glucose levels is primarily due to carbohydrate hydrolysis, catalyzed by the enzymes α-amylase and α-glucosidase. Although various medications are available for treating diabetes, most of them are costly and may lead to adverse effects. Natural herbs like fenugreek are recommended in traditional medicine for regulating blood glucose levels. This investigation aimed to study the effect of fenugreek seed extract (FSE) on in vitro starch hydrolysis by pancreatic α-amylase and the ultrastructure of starch. Wheat cultivars were characterized for their total starch, amylose content, and resistant starch content, and were screened for their predicted glycemic index. Microscopic studies were conducted to analyze the size and shape of starch granules and to compare native starch with starch treated with FSE. Significant inhibition of enzymatic starch hydrolysis was observed with FSE, with the maximum inhibitory effect caused by 0.2% FSE. These findings suggest that fenugreek could

play a role in controlling blood glucose levels by reducing wheat starch hydrolysis and could be effective in managing diabetes.

KEYWORDS
amylopectin, amylose, fenugreek seed extract, resistant starch, α-amylase

1 | INTRODUCTION

Wheat (*Triticum aestivum* L.) is one of the most widely consumed cereal crops. It serves as a dietary staple for a large population, providing higher protein and calorie content in the diet compared to many other staple grains. Wheat starch makes up about 70% of the endosperm (K. H. Kim & Kim, 2021) and is a crucial component of the wheat grain serving as the primary ingredient in products like bread, noodles, cookies, and many other food products. The rapid hydrolysis of this starch can result in elevated postprandial glucose levels, potentially affecting insulin sensitivity and leading to the clinical condition of diabetes. Diabetes mellitus is a metabolic disorder characterized by chronic hyperglycemia due to deficiencies in insulin secretion and/or its action. This can lead to disruptions in carbohydrate, fat, and protein metabolism (Dilworth et al., 2021; N. Kaur et al., 2021). Carbohydrates are hydrolyzed by salivary α-amylase into disaccharides and oligosaccharides, which are further broken down by α-glucosidase into monosaccharides such as glucose (Taslimi et al., 2018). The rate of carbohydrate hydrolysis is monitored by the predicted glycemic index (pGI) (Goñi et al., 1997). High glycemic index (GI) foods can contribute to serious health issues, including diabetes and obesity. The pGI of wheat is 70 (Chaturvedi et al., 1997), and it can vary among cultivars. Whole wheat flour chapatti, which is commonly consumed in India, has a GI of 61.24 (K. Kaur et al., 2020), which is still considered high (>55). This can lead to an increase in postprandial glucose levels associated with the clinical condition of diabetes. Inhibiting α-amylase could reduce hyperglycemia, as α-amylase inhibitors can decrease glucose absorption rates, reducing the postprandial rise in plasma glucose and the risk of long-term diabetes complications (Dandekar et al., 2021; Gazali et al., 2023; Sidar et al., 2023). Currently, α-amylase inhibitors, such as acarbose, are marketed as treatments for diabetes. However, these drugs can cause many side effects.

Secondary metabolites in plants have emerged as major sources of bioactive agents, playing a crucial role in diabetes treatment and prevention. Bioactive compounds from plants could suppress the action of carbohydrate digestive enzymes, thereby reducing the absorption rate of glucose (Gao et al., 2008; Jeng et al., 2015). Fenugreek, widely accepted in Ayurveda medicine, is recognized for its potential in diabetes treatment. In vivo studies have shown that fasting blood glucose levels were significantly reduced in diabetic rats treated with fenugreek extract compared to the control group (Baset et al., 2020). The ethanolic extracts of fenugreek seeds contain polyphenols, flavonoids, and antioxidant compounds with the potential to inhibit α-amylase activity, suggesting fenugreek extract's potential in combating type 2 diabetes and its complications (Singleton et al., 1999; Kamtekar et al., 2014). Fenugreek seeds are also rich in alkaloids, free unnatural amino acids (4-hydroxy-isoleucine), dietary fiber, and furostanols like diosgenin, gitogenin, yamogenin, and so on. Some studies have indicated that flavonoid compounds could inhibit α-amylase enzyme activity and are currently being explored for their potential as natural alternatives (Takahama & Hirota, 2018; Kashtoh & Baek, 2023). Khenifi et al. (2023) have studied α-amylase inhibition and molecular docking of flavonoids from fenugreek seeds and identified 27 flavonoid compounds, consisting of 22 flavones and three flavanols. It was observed that higher levels of phenolic compounds in fenugreek correlated with greater inhibitory potency against the α-amylase enzyme. Moreover, molecular docking analysis showed that the binding scores and patterns of the studied flavonoids were very similar to those of acarbose with human α-amylase, indicating a promising binding affinity and potential for potent inhibition activity.

Adding fenugreek seed extract to wheat flour in adequate amounts inhibits α-amylase activity, reducing postprandial blood glucose and decreasing the risk of diabetes. US patent (US20100266665A1) by Losso et al. (2010) have developed a method to produce fenugreek flour from fenugreek seeds, which can be incorporated into baked goods and other food products and does not affect the taste or texture of the final product, which can be included in everyday foods, promoting increased use of fenugreek to help prevent diabetes and obesity. The consumption of this fenugreek bread was shown to reduce serum insulin levels, indicating that the bioactivity of fenugreek was retained in the bread. With these considerations, it is crucial to evaluate the impact of fenugreek seed extract on the rate of wheat starch hydrolysis. The study aimed to screen wheat cultivars for total starch, resistant starch (RS), amylose–amylopectin ratio, and pGI. Electron micro-

TABLE 1 Wheat cultivars selected for experimental studies.

Sr. No.	Wheat cultivars	Sr. No.	Wheat cultivars	Sr. No.	Wheat cultivars
1	22nd HRWYT 241	9	Raj 4250	17	PBW 754
2	9th STEMRRSN 41	10	HI 1588	18	PBW 343
3	35th ESWYT 113	11	HPW 360	19	UP 2843
4	22nd SAWYT 323	12	PHS 1106	20	UP 2871
5	DBW 71	13	JAUW 598	21	UP 2691
6	DBW 110	14	K 1006	22	UP 2672
7	DBW 88	15	HD 3234	23	UP 262
8	GW 2010-288	16	WL 711		

scopic studies were conducted to observe the impact of gelatinization, retrogradation, and fenugreek seed extract on the ultrastructure of wheat starch. This study will help in understanding the effect of fortification of wheat with fenugreek seed extract (FSE), enhancing its nutritional benefits, and potentially aiding in the prevention of diabetes and associated complications.

2 | MATERIALS AND METHODS

2.1 | Wheat cultivars and experimental materials

In this study, 23 wheat cultivars were selected from the northern parts of India (Table 1) and procured from the Department of Genetics and Plant Breeding of the G.B. Pant University of Agriculture and Technology (GBPUAT), Pantnagar.

Fenugreek seeds were obtained from the Vegetable Research Centre of GBPUAT. Porcine pancreas α-amylase (10–25 U/mg) and glucoamylase ex. Rhizopus sp. (30 U/mg) used in this study were purchased from SRL Co. The glucose oxidase/peroxidase (GOPOD) kit was purchased from Sigma-Aldrich.

Starch was extracted from all wheat cultivars, and the starch yield was estimated. The extracted starch was then treated with FSE to assess the inhibition of α-amylase. Total starch content, apparent amylose content (AAC), and amylose–amylopectin ratio were determined in all wheat cultivars. Depending upon the starch and AAC, six wheat cultivars were selected out of 23 wheat cultivars.

2.2 | Extraction of starch from wheat flour

Starch was extracted using the alkaline extraction method (H. Y. Kim et al., 2012). Wheat flour (5 g) was suspended in 30 mL of 0.5% NaOH, stirred for 30 min, and allowed to stand at 4°C for 24 h. The suspension was centrifuged at 5000 × *g* for 10 min at 20°C, and the supernatant was discarded. The pellet was then washed with a 0.5% NaOH solution, and the alkaline slurry was centrifuged again under the same conditions. This process was repeated until the upper yellowish layer was completely removed. The alkaline pellet was neutralized with 1.0 M HCl and centrifuged again. The resulting starch slurry was dried in a convection oven at 35–40°C for 48 h. The dried material was ground gently in a mortar and pestle and stored in airtight containers for future use.

2.3 | Determination of total starch

Total starch from wheat flour was determined by the acid hydrolysis method. Wheat flour (100 mg) was extracted in 80% ethanol, centrifuged, and the supernatant was discarded. The process was repeated thrice with residue. The pellet was mixed with 2 mL distilled water, and the tube was placed in a water bath at 95°C for 1 h followed by the addition of 2 mL of 70% perchloric acid. The contents were shaken for 15 min, diluted to about 10 mL with water, and centrifuged at 5000 × *g* for 20 min. The supernatant was diluted to 25 mL and used to determine the starch content (Albalasmeh et al., 2013; DuBois et al., 1956).

2.4 | Determination of apparent amylose content and amylopectin in starch

AAC in starch was determined by using the method described by McCready et al. (1950). In this method, 100 mg starch was transferred to a 100-mL volumetric flask, mixed with 1 mL of 95% ethanol and 9 mL of 1 N NaOH. The flask was kept in a boiling water bath to gelatinize the starch for 10 min and later allowed to cool for 1 h at room temperature. The volume was made to 100 mL with distilled water. From this, 5 mL of starch solution was taken and one drop of 0.1% phenolphthalein indicator was added, which

turned the solution pink. Using 1 N acetic acid, the pH was adjusted to near neutrality. Iodine solution (0.2 g I_2 + 2 g KI in 100 mL water) measuring 2 mL was added, and the volume was made to 100 mL with distilled water. The absorbance was measured at 620 nm after 20 min using a UV-VIS spectrophotometer (model Systronics 117). A standard curve for amylose was made to determine the AAC of each sample, and amylopectin content was calculated by subtracting the amylose content percentage from 100% (Aristizábal et al., 2007).

2.5 | Determination of soluble protein fraction

Albumin, globulin, and prolamins were extracted by the method of Villareal and Juliana (1978) with some modifications. 100 mg of flour was weighed and added into 10 mL of distilled water for 1 h at 25°C, after that samples were centrifuged at 1000 × *g* for 15 min, and the supernatant was collected for albumin estimation. For globulin estimation, 0.5 M NaCl was used for extraction, while the rest of the steps were similar to albumin extraction. The residue obtained from globulin extraction was added to 70% ethanol containing 0.6% β-mercaptoethanol for prolamins extraction. The supernatants from all extractions were analyzed for soluble protein concentration by the Bradford method using the standard curve of bovine serum albumin.

2.6 | Determination of resistant starch

The RS content in wheat starch was estimated (Goñi et al., 1997). In this experiment, samples were heated, and then protease (2.5 U) was added to break the protein molecules bound to starch granules. The samples were centrifuged, and α-amylase (2.6 U) and glucoamylase (3.0 U) were added. The mixture was then incubated for 16 h at 37°C to hydrolyze the digestible starch. The residue containing RS was obtained after centrifugation and dissolved in 2 N KOH. Sodium acetate buffer (1.2 M, pH 4.75) was then added, followed by the addition of glucoamylase (3.0 U). The mixture was incubated for 35 min at 60°C. The residue remaining represents crude RS. This residue was solubilized in 2 N KOH. After removing the insoluble material, which primarily consists of non-starch polysaccharides, the pH of the supernatant was adjusted to 4.7 and incubated with enzymes. The released glucose was then measured, and the RS content was calculated using the formula RS = glucose × 0.9 (Tharanathan & Tharanathan, 2001).

2.7 | Preparation of gelatinized and retrograded starch

The gelatinized and retrograded starch were developed by using the method of Chung et al. (2006). For gelatinization, starch samples were kept in distilled water at 65°C for 20 min with stirring. After cooling to room temperature, the gelatinized starch samples were used to study in vitro starch hydrolysis by α-amylase. For retrogradation, the gelatinized starch paste was allowed to stand at 4°C for 24 h and was used as a substrate for enzymatic hydrolysis.

2.8 | Preparation of fenugreek seed extract

Fenugreek seed powder (10 g) was macerated with ethanol and transferred to a rotary shaker at 120–140 rpm for 72 h. The contents were centrifuged at 4000 × *g* for 10 min. The supernatant was collected and allowed to evaporate in a water bath (95°C) and stored at 4°C (Pandey & Awasthi, 2015). The obtained extract was used to obtain 0.05%, 0.1%, and 0.2% solutions for use in α-amylase inhibition. The wheat flour was supplemented with the FSE with the desired test concentrations (0.05%, 0.1%, and 0.2%, respectively).

2.9 | In vitro starch hydrolysis

The in vitro starch digestion (native starch, thermally modified starch such as gelatinized and retrograded starch, and starch + FSE) was performed by Englyst et al. (1992) and Gularte et al. (2012) with some modifications. 250 µg flour was suspended in 50 µL water and incubated with 50 µL of porcine pancreatic α-amylase (15 U/mL) and 50 µL of α-glucoamylase (31.25 U/mL) in sodium acetate buffer (0.2 M, pH 5.9), at 37°C for 180 min. Aliquots were drawn at intervals of 0, 30, 60, 90, 120, and 180 min. The reaction mixture was heated at 100°C for 10 min to stop the reaction. After centrifugation at 5000 × *g* for 5 min, the supernatant was measured for glucose content using the GOPOD kit. In vitro hydrolysis of starch was studied at the intervals of 30, 60, 90, 120, and 180 min and the amount of the total starch (TS) hydrolyzed was estimated.

2.10 | Inhibition of α-amylase by FSE

The α-amylase inhibitory assay was performed by the method adapted from Adisakwattana et al. (2012) with some modifications. Wheat flour (250 µg) was suspended

in 50 μL of water and kept in a boiling water bath for 10 min. It was mixed with 100 μL of FSE in 0.2 M sodium acetate buffer (pH 5.9) followed by the addition of 50 μL of porcine pancreatic α-amylase (15 U/mL) dissolved in 0.2 M sodium acetate buffer. The mixture was diluted to 250 μL with 0.2 M sodium acetate buffer. After incubation at 37°C for 10 min, the sugar released was determined by 3,5-dinitrosalicylic acid reagent (Miller, 1959). The control (C) was prepared without FSE. The blank (B) was without α-amylase. Absorbance was measured at 540 nm. The percentage of α-amylase inhibition was calculated by the following formula. Inhibition (%) = $[(OD_C - OD_B) - (OD_{sample} - OD_B)]/(OD_C - OD_B) \times 100$.

2.11 | Predicted glycemic index

pGI were calculated from starch hydrolyzed at 0, 30, 60, 90, 120, and 180 min, and the area under curves (AUC) were determined for each wheat cultivar in both control and the presence of 0.1% and 0.2% FSE. The hydrolysis index (HI) was obtained by dividing the AUC of each sample by the corresponding area of a reference sample (white bread). The pGI was then calculated using the equation: pGI = 39.71 + 0.549 HI (Goñi et al., 1997).

2.12 | Biophysical characterization of starch under different conditions

2.12.1 | Scanning electron microscopy (SEM)

The small amount of starch sample was placed on an aluminum stub with the help of double-sided adhesive tape, and the starch was coated with gold (using JFC 1600 gold coater). The starch was characterized with respect to its shape, size, and surface by SEM. Micrographs of scanning electron microscopy were recorded with the model JEOL JSM-6601 ALV. An accelerating potential of 5 kV was used under a high vacuum. Magnification ranges of 250x, 500x, 1000x, and 2200x were used to observe wheat starch granules (Singh & Singh, 2001).

2.12.2 | Confocal scanning laser microscopy

For confocal microscopy samples were prepared by the method of van de Velde et al. (2002). The starch suspension was made by dissolving wheat starch (200 mg/mL) in double distilled water and heating this suspension for 30 min at 68°C. Stock solutions of fluorescent dyes were prepared at concentrations of 2 and 5 mg/mL for rhodamine and safranin, respectively.

2.13 | Statistical analysis

The experiments were conducted in triplicate, and results were analyzed as mean ± standard error of mean (SEM) using a completely randomized design. Analysis of variance by Tukey's test ($p < 0.05$ for statistical significance) were conducted using GraphPad Prism 8.0.

3 | RESULTS AND DISCUSSION

3.1 | Starch yield, amylose content, total starch, and amylose–amylopectin ratio

Starch is the major constituent of cereal grains and a key determinant of grain yield. The wheat cultivars studied showed considerable variation in starch content, ranging from 35% to 56%. Amylose content varied from 13.3% to 21.87% (Table 2). High-amylose starches are particularly interesting as they can be processed into RS, which offers nutritional benefits (Richardson et al., 2000). For the subsequent studies described in this paper, six wheat cultivars were selected based on their amylose content: WL 711 (low); UP 262, UP 2672, 9th STEMRRSN, PHS 1106 (intermediate); and UP 2691 (high).

3.2 | Resistant starch content and its correlation with amylose content

The RS content estimated in selected wheat cultivars is shown in Figure 1a. The RS is found to be highest (1.32%) in UP 2691 followed by 9th STEMRRSN 41 (1.1%) and the lowest (0.79%) in WL 711. The RS is also compared against the amylose content (Figure 1b). As evident, a positive correlation between amylose and RS content is observed with the Pearson correlation coefficient of 0.8872. This observation is consistent with observations related to various cereal products documented in the literature (Vaidya & Sheth, 2011). While this suggests amylose content as the major factor influencing the RS content, there are other factors such as the quantity of lipids and proteins, and the formation of complexes with long-chain fatty acids that have also been demonstrated as crucial contributors to RS (Benmoussa et al., 2007; Copeland et al., 2009; Putseys et al., 2010). Similarly, structural elements such as starch crystallinity, and the organization of the crystalline regions in both amorphous and crystalline lamellae of the granule, are crucial factors influencing starch digestibility. These characteristics significantly contribute to the RS content (Jane et al., 1997; G. Zhang et al., 2006).

TABLE 2 Starch yield (%), total starch (%), amylose content, and amylose/amylopectin ratio of 23 wheat cultivars.

Wheat cultivars	Starch yield (%)	Total starch (%)	Amylose content (%)	Amylopectin(%)	Amylose/amylopectin	Type
WL 711	40.40 ± 0.90	45.64 ± 0.60	13.13 ± 0.18	86.87 ± 0.18	0.15 ± 0.03	Low
RAJ 4250	39.90 ± 0.30	42.53 ± 0.65	15.61 ± 0.30	84.39 ± 0.30	0.18 ± 0.03	Low
PBW 754	40.25 ± 0.95	44.86 ± 1.83	15.86 ± 0.23	84.14 ± 0.23	0.18 ± 0.00	Low
PBW 343	35.15 ± 0.45	38.94 ± 0.67	16.98 ± 0.25	83.03 ± 0.25	0.20 ± 0.00	Low
22nd SAWYT 323	31.95 ± 0.45	48.58 ± 0.74	17.53 ± 0.29	82.48 ± 0.29	0.21 ± 0.05	Low
UP 262	37.60 ± 1.20	36.08 ± 0.80	17.77 ± 0.02	82.24 ± 0.02	0.22 ± 0.01	Int
HD 3234	41.10 ± 0.40	49.97 ± 0.52	17.99 ± 0.10	82.02 ± 0.10	0.22 ± 0.01	Int
UP 2672	38.70 ± 0.90	55.00 ± 1.12	18.05 ± 0.35	81.95 ± 0.35	0.22 ± 0.05	Int
UP 2843	37.10 ± 0.30	53.14 ± 1.96	18.14 ± 0.51	81.87 ± 0.51	0.22 ± 0.05	Int
UP 2871	36.85 ± 0.45	46.47 ± 2.39	18.1 ± 0.632	81.91 ± 0.63	0.22 ± 0.01	Int
HI 1588	38.10 ± 0.40	45.56 ± 2.43	18.25 ± 0.40	81.75 ± 0.40	0.22 ± 0.05	Int
DBW 110	40.00 ± 0.50	44.47 ± 1.52	18.47 ± 0.51	81.53 ± 0.51	0.22 ± 0.08	Int
JAUW 598	35.80 ± 0.70	54.58 ± 1.90	18.52 ± 0.40	81.48 ± 0.40	0.23 ± 0.05	Int
K 1006	37.80 ± 0.70	53.22 ± 1.92	18.72 ± 0.43	81.28 ± 0.43	0.23 ± 0.05	Int
DBW 88	41.80 ± 0.30	44.69 ± 1.15	18.81 ± 0.70	81.20 ± 0.70	0.23 ± 0.01	Int
35th ESWYT 113	37.90 ± 0.80	53.03 ± 3.04	19.03 ± 0.45	80.98 ± 0.45	0.23 ± 0.08	Int
DBW 71	38.40 ± 1.00	40.78 ± 1.16	19.25 ± 0.52	80.86 ± 0.52	0.23 ± 0.08	Int
9th STEMRRSN 41	39.20 ± 0.60	44.86 ± 1.53	19.42 ± 0.79	80.58 ± 0.79	0.24 ± 0.01	Int
GW2010-288	37.60 ± 0.80	44.89 ± 1.26	19.71 ± 0.23	80.29 ± 0.23	0.24 ± 0.03	Int
HPW 360	31.80 ± 0.60	35.78 ± 2.49	19.76 ± 0.30	80.24 ± 0.30	0.24 ± 0.03	Int
22nd HRWYT 241	39.20 ± 0.80	56.56 ± 3.29	19.77 ± 0.43	80.24 ± 0.43	0.24 ± 0.08	Int
PHS 1106	36.00 ± 0.20	44.44 ± 1.30	20.14 ± 0.21	79.87 ± 0.21	0.25 ± 0.03	Int
UP 2691	40.80 ± 0.90	42.56 ± 0.42	21.87 ± 0.10	78.13 ± 0.10	0.28 ± 0.01	High

Note: Results are expressed as mean ± SE (standard error); Int, intermediate

3.3 | Soluble protein fraction in selected wheat cultivars

Total protein fractions (albumin, globulin, and prolamin) of wheat cultivars were determined (Figure 2), and the total protein content of PHS 1106 (13.33%) was the highest among the studied wheat cultivars, while UP 2691 had the lowest protein content (9.58%). Albumin, globulin, and prolamin content varied significantly among selected wheat cultivars. Starch and protein molecules have electrostatic interaction due to negatively charged groups of starch and positively charged groups of the protein. These positive and negative interactions have a great impact on the stability and rate of digestion (Scott & Awika, 2023). Protein can inhibit or promote starch retrogradation based on its exposed residues. Charged residues promote charge–dipole interactions between starch-bound phosphate and protein, hydrophobic groups restrict amylose release and reassociation, while hydrophilic groups impact water/molecular mobility. Covalent bonds (disulfide linkages) formed between proteins may enhance starch retrogradation, while glycosidic bonds formed between starch and protein during high-temperature processing may limit starch retrogradation. Functional properties of starch, such as gelatinization and starch digestion rate, are affected by proteins (Zhang et al., 2022) as a contiguous layer surrounding the starch granules is formed by the proteins. Wong et al. (2009) observed the same phenomenon in sorghum.

3.4 | Phenolics and flavonoid content in fenugreek

The total phenolic content of the aqueous FSE was estimated by the folin-ciocalteau method. The content of the phenolic compounds and flavonoids in the FSE was 91.02 ± 0.12 mg GAE/gm (gallic acid equivalent) extract and 9.50 ± 0.19 mg quercetin equivalents/g extract, respectively, which indicates that fenugreek is a rich source of these antioxidants (Pant et al., 2017).

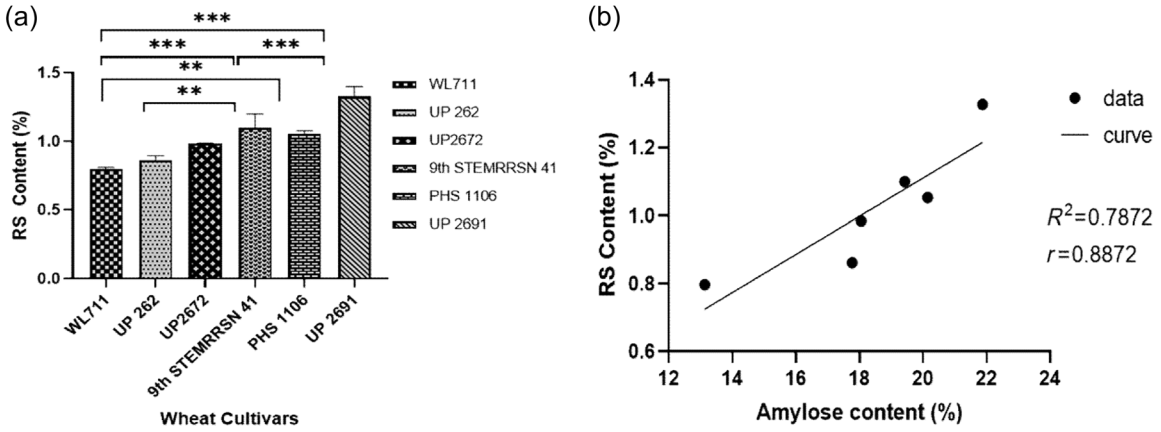

FIGURE 1 (a) Resistant starch (RS) content was estimated in selected wheat cultivars. The highest RS content is observed in UP 2691 and the lowest in WL 711; values are expressed as mean ± SEM (standard errors of mean) and (b) Relationship between amylose and resistant starch content in wheat cultivars. A positive correlation is observed with the Pearson correlation coefficient of 0.8872. ***$p < 0.001$; **$p < 0.01$ significance as per Tukey's test.

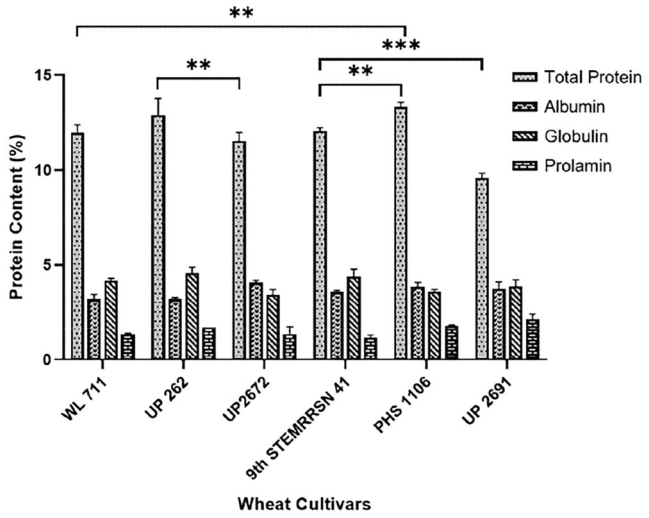

FIGURE 2 Soluble protein fraction (albumin, globulin, and prolamins) in selected wheat cultivars. Values are expressed as mean ± SEM (standard errors of mean). ***$p < 0.001$; **$p < 0.01$ significance as per Tukey's test.

3.5 | Percentage inhibition of pancreatic α-amylase by FSE-treated starch

To understand if FSE affects starch hydrolysis by the pancreatic α-amylase, wheat starch treated with FSE was used as a substrate for the enzyme. The rate of hydrolysis was measured in terms of maltose released. Maximum starch hydrolysis is observed in the control. The enzymatic activity decreased with the increasing concentration of FSE. Maximum inhibition of α-amylase activity was observed with 0.2% FSE in all wheat cultivars, which varied from 69% to 82% (Table 3).

The results indicate that the addition of 0.2% FSE caused the highest inhibition of starch digestion, which could be attributed to the high flavonoid content of FSE (Figure 3). In all cultivars, a similar hydrolysis pattern was observed. The hydrolysis in these cultivars varied from 28.2% to 34.2% at 30 min. The least hydrolysis (28.26%) was observed in UP 2691. FSE could reduce starch hydrolysis effectively in all wheat cultivars. This could be attributed to the bioactive constituents of FSE. It has been found to directly interact with starch, altering its physicochemical properties and molecular configurations. It has also been associated with an increase in RS content as well as the inhibition of digestive enzymes such as α-amylase and α-glucosidase (Sun et al., 2020). Maibam et al. (2023) studied the inhibitory effects of Euryale ferox seed shell extract (EFSSE) and observed that the interactions between the bioactive constituents of EFSSE with α-amylase and α-glucosidase could reduce in vitro starch digestibility.

3.6 | Predicted glycemic index

Table 4 shows the H90, HI, AUC, and pGI of wheat starch for selected cultivars. The pGI values of UP 2672 (68.51) and PHS 1106 (68.48) are the lowest, while WL 711 had the highest pGI (71.33). The variation of pGI with RS content is shown in Figure 4. As observed, with an increase in the RS, the pGI decreases. A strong negative correlation is found between RS and pGI with a Pearson correlation coefficient of −0.7974 (Figure 4). This is consistent with earlier studies which found that a higher RS content is associated with a lower GI due to the gradual release of glucose (Englyst et al., 1992). Foods with elevated RS undergo slower digestion, leading to a reduced

TABLE 3 The percentage of pancreatic α-amylase inhibitory activity of fenugreek seed extract (FSE).

FSE	Inhibition in different wheat cultivars (%)					
	WL 711	UP 262	UP 2672	9th STEMRRSN 41	PHS 1106	UP 2691
0.05% (w/v)	33.80 ± 0.23	37.72 ± 0.24	35.80 ± 0.22	38.02 ± 0.41	34.72 ± 0.21	35.95 ± 0.13
0.1% (w/v)	64.48 ± 0.42	63.33 ± 0.13	67.52 ± 0.90	63.70 ± 0.63	65.33 ± 0.11	52.30 ± 0.34
0.2% (w/v)	82.02 ± 0.43	69.44 ± 0.51	83.13 ± 0.40	69.00 ± 0.70	70.24 ± 0.49	76.87 ± 0.45

Note: Results are expressed as mean ± SE (standard error).

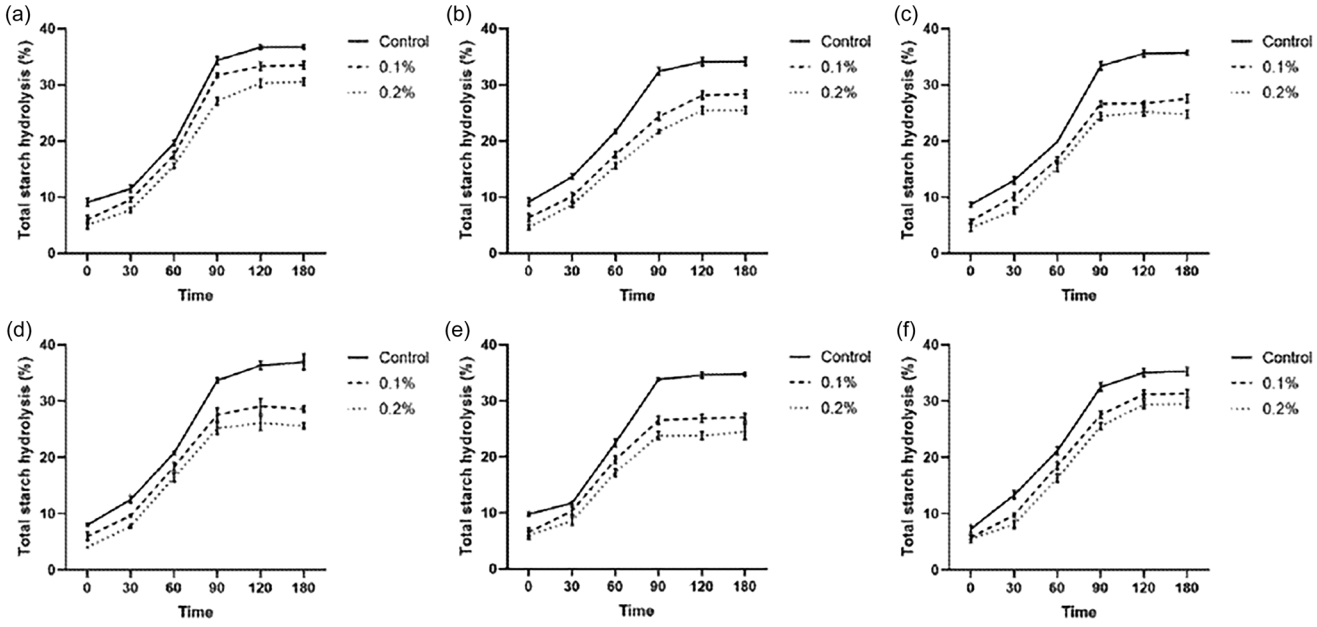

FIGURE 3 Effect of 0.1% and 0.2% fenugreek seed extract (FSE) on starch hydrolysis in comparison with the control: (a) cultivar WL 711, (b) cultivar UP 262, (c) cultivar UP 2672, (d) cultivar 9th STEMRRSN 41, (e) cultivar PHS 1106, and (f) cultivar UP 2691. Values are expressed with respect to time in an interval of 0, 30, 60, 90, 120, and 180 min. The addition of FSE causes the inhibition of starch digestion, and treatment with 0.2% FSE resulted in the maximum reduction in the rate of starch hydrolysis as compared to the control (without treatment with FSE).

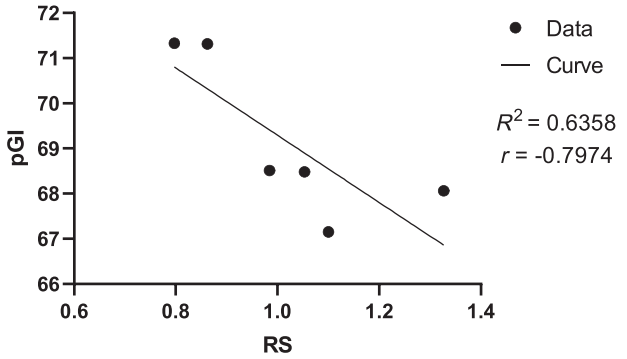

FIGURE 4 Variation of predicted glycemic index (pGI) with resistant starch (RS) content and linear regression curve fit where a strong negative correlation is found between RS and pGI with a Pearson correlation coefficient of −0.7974.

rate of glucose release, resulting in a lower GI and subsequently a diminished insulin response when compared to low RS foods. This is particularly beneficial for individuals with type 2 diabetes (Cummings et al., 2004). These observations align with the findings in studies by A. Kumar et al. (2018), Fuentes-Zaragoza et al. (2010), Hu et al., 2013 and Asp et al. (1996). Furthermore, we observed that the fenugreek reduces the pGI (Table 4). Earlier studies have also found similar observations, where fenugreek has been found to have a GI-lowering effect when added to rice and wheat diets digestion (V. S. Kumar et al., 2011). One of the reasons for this effect is attributed to decreased glucose absorption and inhibition of starch digestion (V. S. Kumar et al., 2011).

3.7 | In vitro starch hydrolysis of native, gelatinized, and retrograded starch in selected wheat cultivars

The in vitro hydrolysis of starch was observed in six wheat cultivars at intervals of 30, 60, 90, and 120 min

TABLE 4 Predicted glycemic index (pGI) of selected wheat cultivars.

Wheat cultivars	Treatment	H90	HI	AUC	pGI
WL 711	Control	40.01	40.01	4733	71.33
	0.1% FSE	31.42	31.42	3717	65.71
	0.2% FSE	27.66	27.66	3272	63.38
UP 262	Control	40.01	40.01	4733	71.31
	0.1% FSE	31.42	31.42	3717	64.44
	0.2% FSE	27.66	27.66	3272	61.42
UP 2672	Control	36.48	40.49	4790	68.51
	0.1% FSE	33.01	36.15	4277	65.71
	0.2% FSE	30.11	32.04	3790	63.38
9th STEMRRSN 41	Control	34.8	39.46	4668	67.15
	0.1% FSE	30.81	34.05	4028	63.95
	0.2% FSE	29.04	31.3	3703	62.52
PHS 1106	Control	36.48	40.49	4790	68.48
	0.1% FSE	33.01	36.15	4277	64.44
	0.2% FSE	30.11	32.04	3790	61.42
UP 2691	Control	35.94	40.52	4793	68.06
	0.1% FSE	28.21	32.13	3825	61.86
	0.2% FSE	25.22	28.63	3387	59.46

Note: pGI of selected wheat cultivars in control, in the presence of 0.1% and 0.2% FSE (control-without treatment with FSE). FSE reduces the predicted glycemic index in wheat cultivars with the maximum reduction observed with 0.2% FSE in UP 2691. H90, starch hydrolysis at 90 min.

Abbreviations: AUC, Area under curve; FSE, fenugreek seed extract; HI, Hydrolysis index; pGI, predicted glycemic index.

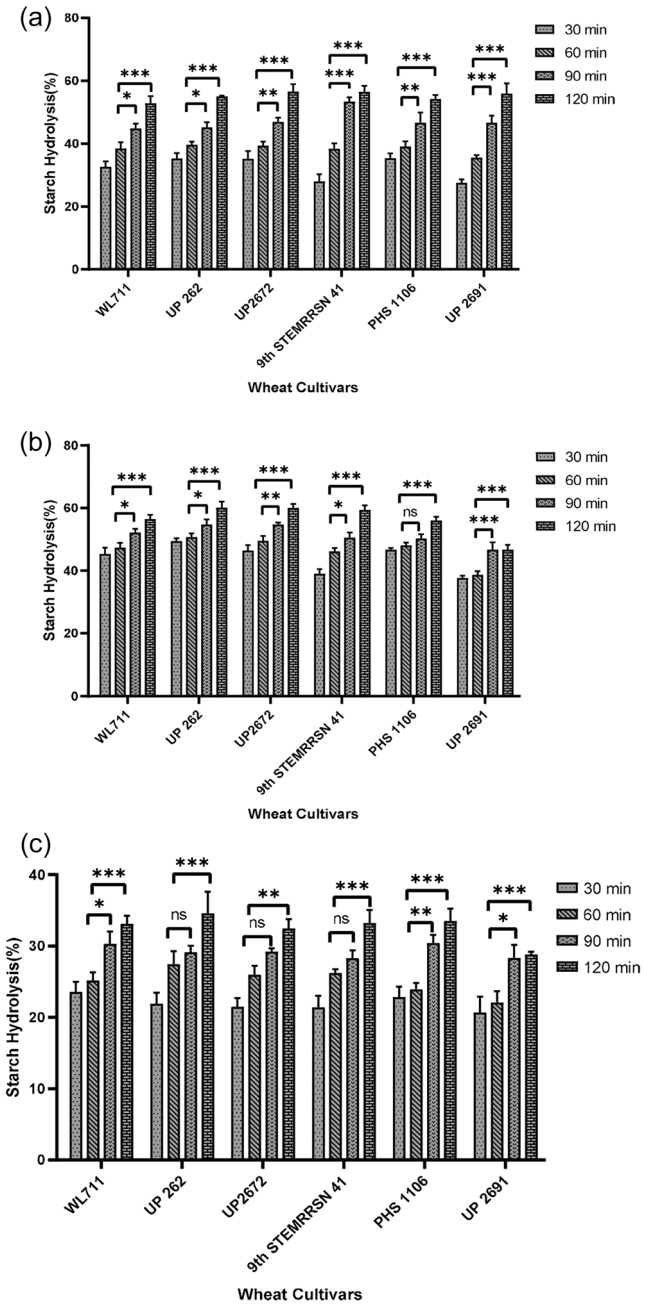

FIGURE 5 In vitro starch hydrolysis of (a) native, (b) gelatinized, and (c) retrograded starch observed at intervals of 30, 60, 90, and 120 min in selected wheat cultivars. Values are expressed as mean ± SEM. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; ns: $p > 0.05$ significance as per Tukey's test. The rate of gelatinized starch was higher than that of native starch, showing an increase of 7%–12% in hydrolysis rate. However, following retrogradation of starch, the total starch hydrolyzed decreased by 15%–20% as compared to the native starch (control).

(Figure 5). Additionally, in vitro hydrolysis of both gelatinized and retrograded starch was performed, and total starch hydrolyzed was estimated at the same intervals. It was noted that the rate of hydrolysis of gelatinized starch was higher than that of native starch, showing an increase of 7%–12% in hydrolysis rate (Figure 5b). However, following retrogradation of starch, the total starch hydrolyzed decreased by 15%–20% (Figure 5c). Understanding the relationship between the rate of starch digestion, glucose production, and risk factors associated with diet-related diseases is crucial in nutritional research. Most of the starch consumed by humans undergoes cooking or processing, which leads to the gelatinization of starch granules. During gelatinization, the hierarchical structure of native starch granules undergoes disruption, leading to the loss of granular morphology, melting of crystallites, and unwinding of double helices (Lehmann & Robin, 2007).

Native starch granules are initially resistant to attack by α-amylases, but cooking or processing significantly enhances starch digestibility (Chung et al., 2006; Juansang et al., 2012; Tester & Sommerville, 2001). Previous studies indicate that the rate of digestion of gelatinized starch by α-amylase is significantly higher compared to native starch. This difference arises from the disruption of the organized structure of gelatinized starch, which facilitates easier access of digestive enzymes to starch molecules. However, there is growing evidence suggesting that the

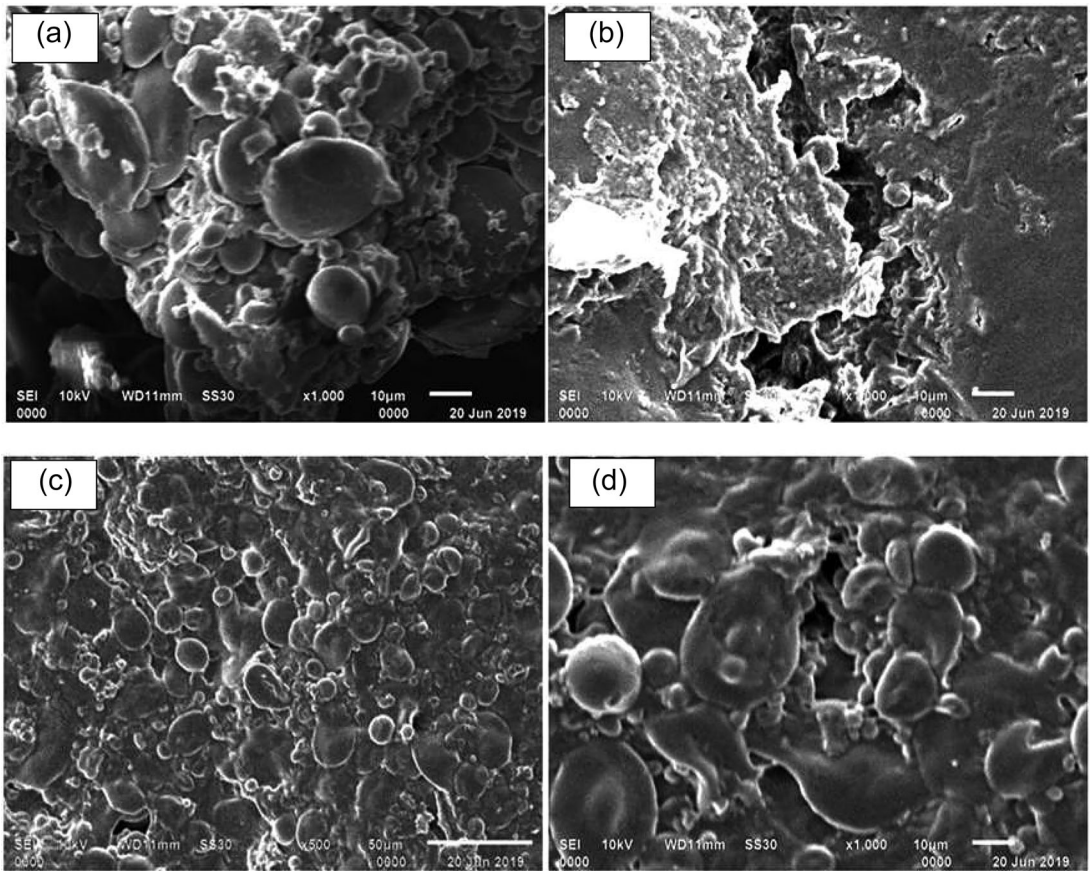

FIGURE 6 Physical characterization of wheat starch granules. Scanning electron micrographs of different starches (a) native starch at 1000x indicating large A-type and small B-type granules, (b) gelatinized starch at 1000x showing disruption of starch granules and loss of morphology, (c) retrograded starch at 500x showing a coalescence of swollen, compact, and hard granules, and (d) fenugreek extract-treated starch at 1000x also appears like retrograded starch and the increase in the firmness or hardness of the starch.

digestibility of cooked starch is not solely determined by the extent of gelatinization or structural disruption. For instance, the digestibility of starch in cooked rice may be influenced more by the mechanical processing that disrupts the grain structure than by the degree of starch gelatinization caused by cooking (Tamura et al., 2016).

Although many studies have reported the relationship between the degree of gelatinization and in vitro digestibility, there is a limited understanding of how the degree of cooking influences the disassembly of the starch structure at multiple scales concerning starch digestibility. When starch is heated to around 50–65°C in the presence of water, the amylose in the granule swells, the crystalline structure of the amylopectin breaks down, and the granule ruptures. This process, known as gelatinization, causes the polysaccharide chains to adopt a random configuration, leading to the swelling of the starch and thickening of the surrounding matrix, making the starch easily digestible (Sajilata et al., 2006). The greater the gelatinization, the more viscous the starch becomes, which increases its GI and can also affect its glycemic load. Gelatinized starch samples are significantly more susceptible to degradation by α-amylase compared to native starch granules (Dona et al., 2010; Vesterinen et al., 2002).

3.8 | Biophysical characterization of starch under SEM and confocal scanning laser microscopy

Wheat has a bimodal size distribution of starch granule populations and has two types of size groups, that is small granules (below 10–14 µm) and large granules (above 14–36 µm). Granule sizes in the range of 5.9–14.6 µm are called B-type and 14.6–31.0 µm are called A-type. The B-type granules are spherical and polygonal in shape, whereas A-type granules are disc-like or lenticular in shape. Some of the native starch granules are also broken due to the severity of grinding (Figure 6a).

Similar results were observed by Singh et al. (2009) who classified starch granules into three distinct categories based on scanning electron micrographs, that is,

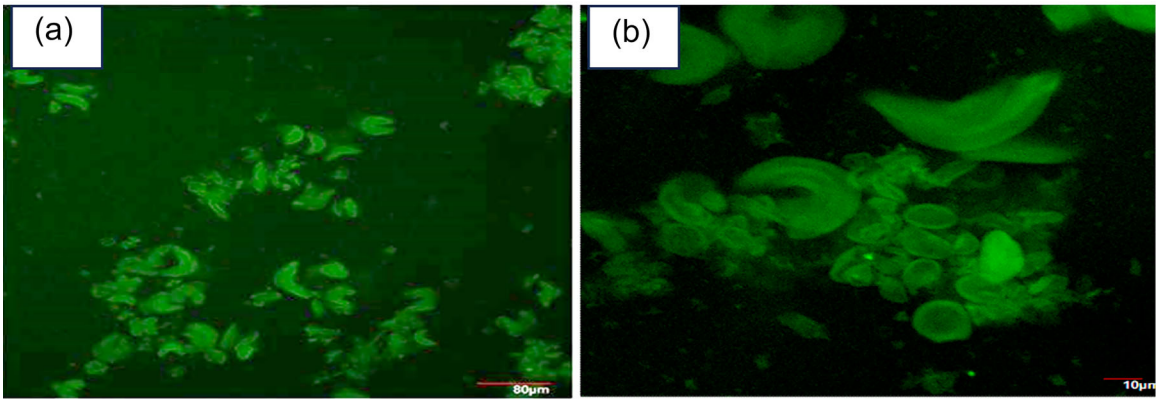

FIGURE 7 Morphology of native starch granules stained with rhodamine; size bars (a) 80 µm and (b) 10 µm, respectively, observed under confocal laser scanning microscope.

small, medium, and large. Starch granules from native starch showed regular shape and small size granules (Figure 7a,b), whereas those from gelatinized starch had larger sized granules (Figure 6b) (Svegmark & Hermansson, 1991). An increase in temperature to 60°C resulted in the disruption of starch granules with the complete loss of granular morphology, which suggests the full gelatinization of the wheat starch followed by a substantial disruption of the starch-ordered structure at different levels. Gelatinization occurs when water diffuses into the granule, which then swells substantially due to hydration of the amorphous phase causing loss of crystallinity and molecular order (Donovan, 1979; Jenkins et al., 1993).

Starch granules, freeze-dried (retrograded) as shown in Figure 6c and on interaction with FSE (Figure 6d) appeared to be a coalescence of swollen, compact, and hard granules. The process of retrogradation increases the firmness or hardness of the starch (Shrestha & Halley, 2014). In baking, this process is known as staling and is used to describe the increasing firmness of breadcrumbs over storage time (Gray & Bemiller, 2004).

Amylose crystallizes over a period of minutes to hours, while amylopectin retrogrades over hours or days (Bulkin et al., 1987). Retrogradation is the key process to manufacture RS (type 3), in which amylose chains, solubilized during gelatinization, aggregate forming crystalline double helices (Haralampu, 2000). Thus, the high amylose starches are chosen as common sources for the RS preparation (Lee et al., 1997; Sievert & Pomeranz, 1990). By comparing images observed by scanning electron microscopy of native, gelatinized, retrograded, and interaction with FSE, it can be observed that after gelatinization, starch granules can be easily hydrolyzed because of their swollen and disrupted structure, as there is an increase in surface accessibility of hydrolyzing enzymes to starch granules. On the contrary, in retrograded and interaction with FSE, starch granules become firm and hard and cannot be easily hydrolyzed, as it hinders the access of hydrolyzing enzymes to starch granules.

4 | CONCLUSION

The interaction of bioactive compounds in FSE could inhibit starch hydrolysis by targeting digestive enzymes, suggesting its potential to control blood glucose levels by slowing down starch hydrolysis. This effect also lowers the pGI of wheat, which may explain the use of fenugreek seeds for diabetes treatment. Incorporating the appropriate amount of FSE could help develop functional food products made up of wheat. This study also suggests gelatinization and retrogradation alter the starch morphology. Further studies are needed to examine the interaction between protein–starch and its effect on RS. Additionally, research should focus on elucidating the mechanisms of action and identifying the specific bioactive components present in fenugreek seed extract responsible for enzyme inhibition involved in starch digestibility.

AUTHOR CONTRIBUTIONS

Payal S. Mate: Conceptualization; investigation; writing—original draft; methodology; validation; visualization; software; formal analysis; data curation. **Vivek Chandra Verma**: Conceptualization; investigation; writing—review and editing; supervision; project administration; validation; methodology; funding acquisition. **Sanjeev Agrawal**: Conceptualization; writing—original draft; investigation; validation; visualization. **Jai Prakash Jaiswal**: Conceptualization; investigation; writing—original draft; methodology; validation; visualization; resources. **Venugopalan Visha Kumari**: Conceptualization; writing—review and editing; formal analysis; software; data curation; resources; methodology. **Rajeev Kumar**: Conceptualization; investigation; writing—

original draft; methodology; validation; visualization; resources. **Mala Kumari**: Conceptualization; investigation; writing—original draft; methodology; validation; visualization. **Ahmed Gaber**: Funding acquisition; writing—review and editing; software; formal analysis; data curation; resources. **Akbar Hossain**: Writing—review and editing; software; data curation; formal analysis; funding acquisition.

ACKNOWLEDGMENTS

The authors are thankful to the Director, Experiment Station, G.B. Pant University of Agriculture & Technology, Pantnagar, Uttarakhand, India for providing logistic support for carrying out the investigation. The authors also extend their appreciation to Taif University, Saudi Arabia, for supporting this work, Project No. (TU-DSPP-2024-07).

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data will be made available on request.

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How to cite this article: Mate, P. S., Verma, V. C., Agrawal, S., Jaiswal, J. P., Kumari, V. V., Kumar, R., Kumari, M., Gaber, A., & Hossain, A. (2024). Effect of fenugreek (*Trigonella foenum-graecum* L.) seed extract on glycemic index, in vitro digestibility, and physical characterization of wheat (*Triticum aestivum* L.) starch. *Journal of Food Science*, 11111. https://doi.org/10.1111/1750-3841.17411