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Original Article

Harnessing the Health Benefits and Biological Impact of Fermented Galactomannan from *Trigonella foenum-graecum* L. (Fenugreek) Seeds

Sarumathi Chandrasekaran, Sai Sakthi Sridevi Kumaran, Bhuvaneswari Shanmugam*, Ahamed Rofiya Begam Mohamed Samsudeen

Department of Chemistry and Biosciences, Srinivasa Ramanujan Centre, SASTRA (Deemed University), Kumbakonam, Tamil Nadu, INDIA.

ABSTRACT

Introduction: Recently, polysaccharides from plant sources have been playing a crucial role in various therapeutic applications. Indeed, galactomannan from fenugreek seeds has garnered significant attention from researchers due to its vast pharmaceutical and medical applications. The chemical variation of this compound has a significant impact on numerous biological activities. **Objectives:** The primary objective of this investigation was to isolate and identify the most potent Galactomannan (GM) fermenting strain among three tested microbes: *Lactococcus lactis* MTCC 440, *Saccharomyces cerevisiae* MTCC 170 and *Calocybe indica* MTCC 109481. **Materials and Methods:** Among them, *Lactococcus lactis* fermented GM metabolites were subjected to characterize using FT-IR, GC-MS and ^{1}H NM due to high production of ethanol. **Results:** The *Lactococcus lactis* MTCC 440-fermented GM metabolites demonstrated high *in vitro* biological activities, including antioxidant, anti-inflammatory, antimicrobial, anti-diabetic, and anti-ulcer activities when compared to other fermented GM metabolites. Additionally, these fermented metabolites exhibited strong antimicrobial activity against foodborne pathogens, including *Escherichia coli, Staphylococcus aureus*, and *Candida albicans*. **Conclusion:** In summary, the fermented galactomannan shows robust biological activity, suggesting that they could be a potential tool for improving human health.

Keywords: Biological activities, Fenugreek, Fermentation, Galactomannan, *Saccharomyces cerevisiae*.

Correspondence:
Dr. Bhuvaneswari Shanmugam
Assistant Professor, Department of Chemistry and Biosciences, Srinivasa Ramanujan Centre, SASTRA (Deemed University), Kumbakonam-612001, Tamil Nadu, INDIA.
Email: bhuvaneswari@src.sastra.edu

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INTRODUCTION

Medicinal plants have been recognized as promising therapeutic agents due to the presence of biological active molecules. They include a diverse range of secondary metabolites, each having specific pharmacological characteristics, including terpenoids, polyphenols, alkaloids, and flavonoids. The development of new chemicals with potential medicinal applications is made possible by this diversity. The biological activities of these substances are well-documented, showcasing their antioxidant, antibacterial, anti-inflammatory, and anti-cancer potentials.[1] Fenugreek is an annual herb with angular, yellowish seeds that have a number of health advantages. Fibre, glycolipids, phospholipids, linoleic acids, oleic acids, choline, nicotinic acid, Vitamin A, B_1, B_2, C, niacin, minerals including copper, potassium, and calcium, as well as numerous other useful components, are all present in significant amounts in fenugreek seeds. In addition to this, GM, diosgenin, quercetin, trigonelline, 4-hydroxy isoleucine and phenolic mixtures are the principal chemical components that found in fenugreek.[2] Numerous therapeutic uses, including anti diabetic, anticarcinogenic, hypocholesterolemia, hypoglycemia, stomach stimulant, and anti-anorexia, have been discovered in recent investigations and research for fenugreek.

Galactomannan (GM) is a polysaccharide primarily found in plants like *Ceratonia siliqua* (locust bean), *Cyamopsis tetragonoloba* (guar), *Caesalpinia spinosa Kuntze* (tara), and *Trigonella foenum-graecum* L. (fenugreek), commonly utilized in food products. GMs are heteropolysaccharides, often derived from the seeds of these plants, and they hold significant potential for various industrial applications. Galactomannans (GMs) are the most commonly used polysaccharides, with cellulose and starch following in prevalence. The primary component of fenugreek seeds is GM, which has a structure consisting of a β-D-mannosyl backbone (1→4) with a single galactose unit attached α-glycosidically at the C_6 position.[3] The mannose, backbone of the GM has been grafted with galactose units. GM from fenugreek has been shown to have a number of positive effects on human

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health, including decreasing blood pressure, blood lipids, and fibrinolysis in males maintains good health and decreasing the levels of LDL cholesterol in hypercholesterolemic patients. It is essential for both type 1 and type 2 diabetics to improve the balance of glucose by delaying the breakdown and uptake of carbohydrate.[4]

Microbial fermentation has been increasingly employed as a bioprocessing strategy to enhance the functional and biological properties of plant-derived polysaccharides. During fermentation, microbial enzymes could partially depolymerize polysaccharide chains, reduce molecular weight, and modify side-chain substitutions. Therefore, fermentation is expected to improve the bioactivity of galactomannan through both structural modification of the polymer and the formation of fermentation-derived metabolites. The present study focusses on the assessment of various *in vitro* biological activities. The results of this investigation have the potential to pave the way on the enhanced or modified biological activities of fermented GMs, offering valuable insights into their therapeutic potential. This scope aligns with the growing interest in natural and plant-based remedies, as consumers and the pharmaceutical industry seek alternatives to synthetic drugs, emphasizing the need for well-founded research on the biological activities of natural compounds like GM.

MATERIALS AND METHODS

Extraction, purification and chemical analysis of GM

Fenugreek seeds (*Trigonella foenum - graecum* L.) were acquired from a local market in Kumbakonam, Tamil Nadu, India. The extraction, and purification of GM from the seeds of fenugreek were carried out using the procedure described by Rashid *et al.*[5] with little modification. The seeds were washed, macerated, and immersed in 5% sodium chloride solution. The pH was adjusted to 3, and the mixture was incubated for 24 h at 50°C with continuous stirring at 300 rpm, followed by filtration through muslin cloth to remove the GM-containing crude gum. After that, the crude solution was vigorously mixed with 90% ethanol and 10% Isopropanol (IPA-spirit) in a 3:1 by volume ratio, then centrifuged at 5000 rpm for 10 min. The GM-containing gum was deposited at the bottom of the tube. To improve the purity, the precipitated compound was re-seeded with IPA (isopropyl alcohol)-spirit, prompting it to precipitate again. This process was carried out thrice to minimize the salt and acid content in the products. The purified GM polysaccharides were dried in an oven at 50°C for 24 h and then kept in airtight containers for storage. The protein and ash contents of purified samples were further analyzed for their chemical constituents, denoted by the AOAC-authorized protocols.[6] Similarly, nitrogen and mineral concentrations were also measured by the standard protocol given by AOAC.[7]

Fermentation and Extraction

The three strains, *Calocybe indica* MTCC 109481, *Saccharomyces cerevisiae* MTCC 170 and *Lactococcus lactis* MTCC 440, were procured from the Microbial Type Culture Collection (MTCC), CSIR-IMTECH, Chandigarh, India. The fermentation and extraction process for the GM polysaccharides was conducted according to the approach outlined by Majeed *et al.*[3] with minor modifications. Briefly, a single isolated colony of each strain was inoculated in individual Nutrient and Potato Dextrose Agar (PDA) broth. It was then incubated at 37°C with 150 rpm for overnight. GM from fenugreek seeds alone in 0.5%, 1.0%, 1.5% and 2.0% (w/v), and along with Nutrient and PDA broth in 0.5%, 1.0%, 1.5% and 2.0% (w/v) were prepared. The pH of the medium was adjusted to 7.0, with fructooligosaccharide used as a reference control for comparison with GM derived from fenugreek seeds. The medium was freshly prepared, autoclaved, and inoculated with 10% of each strain, then incubated at 37°C with constant stirring at 110 rpm for 24 h. pH was observed at 0^{th} h of incubation and 24^{th} h of fermentation. The total sugar was estimated by Dinitrosalicylic Acid (DNS) method using glucose as standard. Using Bovine Serum Albumin (BSA) as the reference, the total protein was calculated using Lowry's method. The polysaccharides were confirmed by TLC using strong acid alcohol reagent and presence of sugar by Fehling's test.

Detection of Ethanol by Potassium Dichromate Method

After cleaning and drying the test tubes, 2% absolute alcohol was added to each one (0, 0.2, 0.4, 0.6, 0.8, 1 mL). then, distilled water was added to bring it down to 3 mL. Then, 4 mL of potassium dichromate ($K_2Cr_2O_7$) reagent was added. 1 mL of conc. sulfuric acid was added along the sides of the test tubes very carefully after the completion of reaction. For the test samples, 4 mL of the sample and 4 mL of the $K_2Cr_2O_7$ reagent were added to the dried tubes. The mixture was incubated in a water bath at 60°C for 20 min, then allowed to cool to room temperature. Finally, distilled water was added to bring the total volume up to 5 mL. The blank solution was made using distilled water and used to plot the linearity curve with concentrations ranging from 1 to 10% ethanol (v/v). UV analyses a straight curve at 620 nm to figure out the amount of ethanol in the test sample.

Characterization of Fermented GM Polysaccharides

Lactococcus lactis Fermented GM (LLFGM) was subjected to characterize using FT-IR, GC-MS and ^{1}H NMR due to the results of end product analysis of fermented medium. Using the KBr technique, the isolated LLFGM' FT-IR spectrum was obtained. Samples of bio polymers were crushed at a ratio of 1:100 into KBr pellets. The Fourier transform-infrared spectra were obtained within the range of 4000-400 cm^{-1} at a resolution of 4 cm^{-1} utilizing a JASCO FTIR-6000 spectrometer. The GC-MS analysis was performed using a Perkin Elmer Clarus 500 system equipped

with a mass spectrometer-interfaced gas chromatograph and an AOC-20i auto sampler. The analysis was performed with a 0.5 µL injection volume (split ratio: 10:1), a constant helium flow rate of 1 mL/min, and injector and ion source temperatures set to 250°C and 280°C, respectively, using helium as the carrier gas. The oven was programmed to begin at 110°C (isothermal) for 2 min, rise to 200°C at a rate of 10°C/min, fall to 280°C at a rate of 5°C/min, and stay at that temperature for 9 min. A Bruker 600 M spectrometer (Rheinstetten, Germany) operated at 25°C was used to perform a ^{1}H NMR structural investigation of LLFGM. 1 mL of 99.9% water was used to dissolve 30 mg of the powdered samples. A 300 MHz frequency (field of 7.1 T) was used to record ^{1}H NMR. Data analysis was performed using the MestRe Nova 5.3.0 program (Mestrelab Research S.L.). In the chemical shift, PPM refers to parts per million.

Assessment of Biological Activities *in vitro*

DPPH Radical Scavenging Assay for Antioxidant Activity

DPPH radical scavenging assay was performed in accordance with the prior study by Ahmad,[8] with some modifications. The different concentrations (µg/mL) of GM polysaccharides (SCFGM, CIFGM, and LLFGM) were made up with distilled for 3 mL. About 2.4 mL of 0.1 mM methanolic solution of 2,2-Diphenylpicrylhydrazyl (DPPH) was mixed 1.6 mL of galactomannan polysaccharides in methanol at various concentrations. For the control, 3 mL of DPPH was used, while ascorbic acid served as the reference standard. The reaction mixture was incubated in the dark at room temperature for 30 min, after which the absorbance was measured at 517 nm using a spectrophotometer. The experimental procedure was performed in triplicate. The results were calculated by comparing the % radical scavenging capacities of test samples with solvent and positive control. The radical scavenging efficiency of the GM

$$\text{DPPH scavenging activity}(\%) = \left[\frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})}{\text{Abs}_{\text{control}}}\right] \times 100$$

Where, Abs of control is the absorbance of DPPH free radical + methanol and Abs of sample is the absorbance of DPPH free radical + test sample at different concentrations. The results were reported as IC_{50} values.

Anti-inflammatory assessment by inhibition of protein denaturation

Anti-inflammatory protein denaturation was performed in accordance with the prior study by Wei *et al.*[9] with some modifications in order to evaluate protein denaturation inhibition. The test fermented GM polysaccharides were present in the reaction mixture in varying quantities, along with 1% BSA (aqueous solution). To adjust the pH of the reaction mixture, 1 N HCl was employed. The samples were subjected to heating for 20 min at 37°C, followed by an additional 20 min at 57°C, and then allowed to cool. Diclofenac sodium served as the reference drug. The turbidity of the samples was measured at 660 nm and the experiment was conducted in triplicate. The following formula was used to assess the inhibition of protein denaturation by the fermented GM polysaccharides.

$$\%\text{ of protein denaturation inhibition} = \left[\frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})}{\text{Abs}_{\text{control}}}\right] \times 100$$

Where,

Abs of control is the absorbance of Diclofenac sodium and Abs of sample is the absorbance of the test sample.

Antimicrobial activity by disc diffusion method

The antimicrobial activity for the three fermented GMs using the disc diffusion method was carried out according to the previous study by Abdulkareem *et al.*[10] with some modifications. The overnight culture of each microbial isolate was emulsified with nutrient broth to achieve a turbidity of 0.5 McFarland (10^5 CFU/mL). A 100 µL aliquot of the test culture was evenly spread over the surface of the solidified agar to evaluate the antibacterial efficacy of the extracts. A sterile disc loaded with extract was placed over agar surface. An antibiotic such as ofloxacin was used for positive controls for determining the inhibition zone. The previously loaded plates with the appropriate extracts and test microorganisms were cultured for one day at 37°C for bacteria and 48°C for fungi. The inhibitory zone was measured in millimeters after incubation.

Anti-diabetic activity by α-amylase inhibitory assay

Pancreatic α-amylase enzyme preparation: The lysate was clarified by centrifuging the pancreatic cells after they had been lysed in a modified RIPA (Radioimmunoprecipitation assay) buffer. After measuring the protein content, cell lysate was separated and kept at -20°C. The anti-diabetic efficacy of fermented GM polysaccharides was assessed by an α-amylase inhibitory assay utilizing the starch-iodine technique, consistent with previous research by Oluwagunwa *et al.*[11] with some modifications. After fermentation of GM polysaccharides at varying concentrations of 25-100 µg/mL (w/v) along with positive control Acarbose (50 µg/mL), 500 µl of pancreatic lysate (pancreatic α-amylase enzyme) of 0.02 M sodium phosphate buffer (pH 6.9) were added to the assay mixture. It was then incubated at 37°C for 10 min. After the pre-incubation phase, 250 µL of a 1% (v/v) starch solution was added to each tube and incubated for 15 min at 37°C in the previously specified buffer. Iodine solution was added, diluted, and the resulting solution was heated to a boiling point for 5 min to terminate the reaction process. At 540 nm, the absorbance was then measured. There was nothing in the control, as evidenced by the 100% enzyme activity in the control reaction.

$$\%\text{ of Relative enzyme activity} = \left[\frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})}{(\text{Abs}_{\text{control}})}\right]$$

Where,

Abs of control is the absorbance of the blank and Abs of sample is the absorbance of the test sample.

Anti-ulcer activity by H^+, K^+ -ATPase inhibition assay

Preparation of H^+, K^+ -ATPase Enzyme: The H^+, K^+ -ATPase enzyme was prepared by extracting parietal cells. Fresh sheep stomachs were obtained from the local slaughterhouse (Karimnagar abattoir). The gastric mucosa of the fundus was excised and opened, and the inner lining of the stomach was scraped to isolate the parietal cells. After being homogenized in 200 mM Tris HCl buffer with a pH of 7.4, the stomach parietal cell was centrifuged for 10 min at 6000 rpm. Bradford's method was used to assess the H^+, K^+ -ATPase inhibition protein concentration in the supernatant after centrifugation, using BSA as a standard.

Determination of gastric H^+, K^+ -ATPase inhibition activity: H^+, K^+ -ATPase activity was performed in accordance with the prior study by Reyes-Chilpa *et al.*[12] with slight modifications. The parietal cell extract was pre incubated with various concentrations of the test materials and standards (chloroform and methanol extract) for 30 min. The reaction was initiated by the addition of 2 mM ATP, 20 mM Tris-HCl (pH 7.4), 2 mM $MgCl_2$, and 2 mM KCl, followed by incubation for 30 min at 27°C. The reaction was then terminated by adding 10% trichloroacetic acid. Centrifugation was performed at 2500 rpm for 10 min. The amount of inorganic phosphate produced from ATP was measured using spectral analysis at 640 nm. The results were compared to Omeprazole (50 µg/mL), a well-known Proton Pump Inhibitor (PPI).

Statistical Analysis

All experiments were performed in triplicate (n=3), and results are expressed as Mean±Standard Deviation (SD). Statistical analysis was carried out using GraphPad Prism (Version 7.04).

RESULTS

Isolation of GM

GM from fenugreek seed was extracted by carrying out the selective extraction using isopropanol and NaCl mediated ultrasonication. The seeds were processed, and polysaccharide was separated. The net yield of polysaccharide is 54% (Table 1).

Fermentation Analysis

The growth of three cultured strains on Nutrient and PDA broth was observed under light microscope. The gram-positive cocci in chains of probiotic confirmed as *L. lactis*. Formation of budding cells observed in *S. cerevisiae*. In PDA media, *C. indica* demonstrated a stripe with a well-differentiated pileus and the quickest mycelium growth. All the three strains were capable to grown on fermentation medium and the pH of fermentation medium during end of the day was acidic by *Lactococcus lactis* and 6-7 in case of yeast and mold.

Table 2 showed the data of fermented medium component analysis. It described how to determine the amount of ethanol in alcoholic beverages through an obvious response. The process involves colouring ethanol with sodium dichromate. In accordance with the use of ethanol and sodium dichromate as limiting reactants with sulfuric acid and an acetate buffer with a pH of 4.3, calorimetric quantification was performed. The maximum absorbance for the ethanol was discovered to be at 578 nm. At the end of fermentation 26.2±0.06 and 24.6±0.07% of ethanol was produced by both *S. cerevisiae* and *C. indica*, respectively. But slightly higher significant amount of alcohol 42.4±0.01% was detected in *L. lactis* fermentation. During fermentation the breakdown of GM leads to produce reducing sugars molecule quantified by DNS Method. Based on the conventional regression with the equation, the colour change in the DNS reagent that caused the initial colour of yellow to turn reddish orange was determined and it was estimated at 2.1±0.11 mg in *S. cerevisiae* fermentation, 1.2±0.24 mg in *C. indica* and 0.1±0.01 mg by *L. lactis* fermentation.

Characterization of fermented GM polysaccharides

FT-IR analysis

FT-IR analysis is an important tool used to gain initial insights and identify biopolymers from different sources. The FT-IR spectra of the fractions of fermented GMs showed various band stretches ranging from 4000 cm^{-1} to 400 cm^{-1} (Figure 2a). The band stretching observed at 3436.76 cm^{-1} is attributed to the presence of O-H bonds, as indicated by the broad peaks. These indicate the existence of hydroxyl groups in carbohydrates, together with a little quantity of moisture. The Peak at 2924.78 and 2854.26 cm^{-1} are the indication of C-H stretch; whereas peak at 2057.43 cm^{-1} is indication for C-H bending. It is noted that some of the proteins have been associated to the polysaccharides, which may enact and improve the biological activities. The absorption peak at 874.22 cm^{-1} indicates strong C=C bending, confirming the presence of an alkene group. Similarly, the peak at 847.73 cm^{-1} corresponds to medium C=C bending. The absorption peak at 780.38 cm^{-1}, indicative of medium C=C bending, may suggest the presence of an alkene or trisubstituted group. Additionally, the peak at 606.03 cm^{-1} corresponds to strong C-I stretching. The presence of these functional groups may contribute to unique biological activities, potentially enhancing the distinctiveness of the fermented GMs.

Table 1: Physicochemical characterization of Galactomannan (GM) extracted from fenugreek seeds.

Sl. No.	Parameters	Content (%, w/w)
1.	Galactomannan	54.24±0.9
2.	Total carbohydrate	68.71±1.5
3.	Protein	24.68±0.24
4.	Ash	6.73±0.21
5.	Water soluble	81.59±2.2

Table 2: Analysis of fermentation end products after 72 h of incubation.

Sl. No.	Fermentation by organisms	Total sugar (mg/L)	% of Ethanol
1.	*Saccharomyces cerevisiae*	2.1±0.11	26.2±0.06
2.	*Calocybe indica*	1.2±0.24	24.6±0.07
3.	*Lactococcus lactis*	0.1±0.01	42.4±0.01

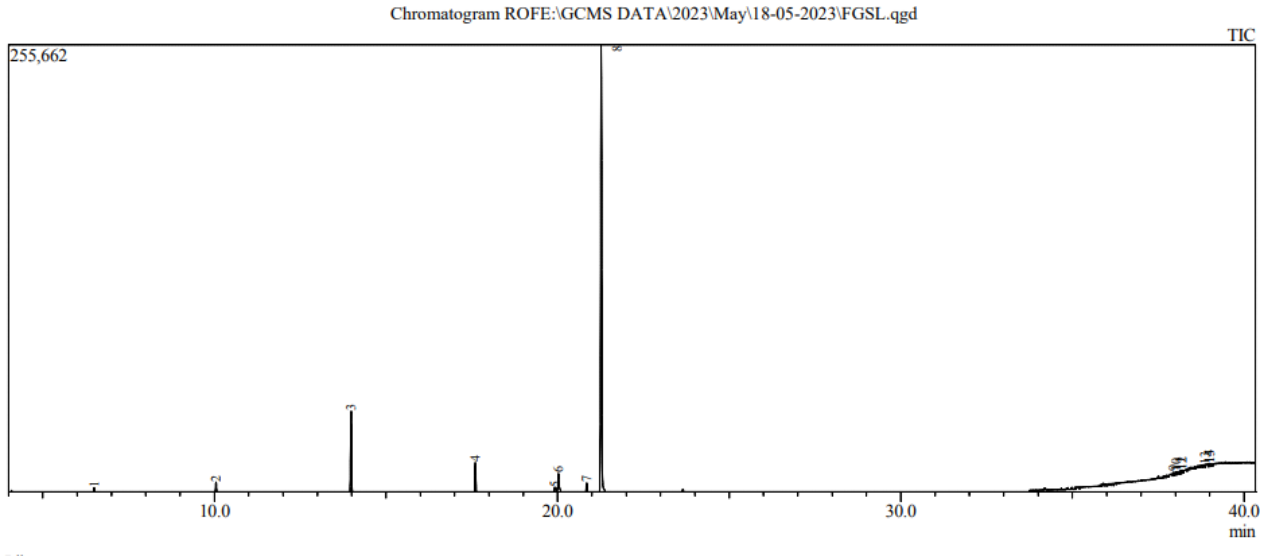


Figure 1: GC-MS chromatogram of metabolites extracted from LLFGM.

GC-MS analysis

GC-MS analysis revealed the presence of several low-molecular-weight metabolites eluting between 6.49 and 39.07 min. A total of fifteen peaks were identified by comparison with the NIST library. The detected compounds included organic acids, ester derivatives, and siloxane-related compounds. These metabolites are considered fermentation-associated or analytical artifacts rather than direct structural components of galactomannan. Therefore, GC-MS analysis was used to provide a qualitative metabolite profile of the fermented extract. The separated components name, molecular weight and structure were determined and provided in Table 3. The first compound eluted was lactic acid (0.32%), and the last was 2-((trimethylsilyl) ethynyl) heptamethyltrisilane. Figure 1 illustrates the GC-MS spectra, which shows 15 distinct peaks. The predominant compounds identified include 1,2,3-propanetricarboxylic acid, 2-hydroxy-, trihexyl ester (72%), followed by 12.13%-Cyclohexasiloxane, dodecamethyl-; 4.09%-3-Butoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy) tetrasiloxane; 3.55%-Ethyl 1,2,3,4,5,6,7,8-octahydro -8-oxo-1-naphthalenecarboxylate; 2.37%-Ethanone, 2-(4-chlorophenyl)-1-cyclohexyl-2-(1-piperidinyl); 1.31%-Tri-o-trimethylsilyl, n-pentafluoropropionyl derivative of terbutaline; 1.19%-Benzoic acid, 2,6-bis(trimethylsiloxy)-, trimethylsilyl ester; 1.11%-Furo[2,3-c]pyridine, 2,3-dihydro-2,7-dimethyl; 0.93%-(trimethylsilyl)ethynyl)heptamethyltrisilane; and 0.52%-4-tert-Octylphenol, TMS derivative detected in significant quantities. Although GC-MS is a powerful analytical tool for identifying volatile and semi-volatile compounds, it has inherent limitations in the characterization of intact high-molecular-weight polysaccharides such as galactomannan. However, GC-MS data were interpreted as metabolite-profiling information, while FT-IR and ^{1}H NMR spectroscopy were relied upon for confirming functional groups and glycosidic linkages in galactomannan.

^{1}H NMR

The ^{1}H NMR spectrum of the compound EHI was recorded in DMSO-d6 (500 MHz), and the ^{1}H NMR spectrum of the isolated GM is presented in Figure 2b. The fermented GM polymer (LLFGM) consists of two monomeric six-membered ring systems: one is the galactose (α-D-galactopyranose) ring, and the other is the mannose (β-D-mannopyranose) ring. The presence of terminal α-D-galactopyranose and β-D-mannopyranose in the main chain is supported by the two distinct anomeric proton chemical shifts observed at 4.99 ppm and 4.69 ppm, respectively

In vitro biological activities

Antioxidant activity

The DPPH radical-scavenging technique is a frequently used method used for investigating on antioxidants. At various concentrations, the property against oxidant towards DPPH was investigated. The hydrogen-donating properties of extracted metabolites, which convert the stable violet DPPH radical to the yellow DPPH, may be the cause of their antioxidant actions on DPPH radical scavenging. Figure 3a shows the free radical scavenging activity of the LLFGM, CIFGM, SCFGM and standard

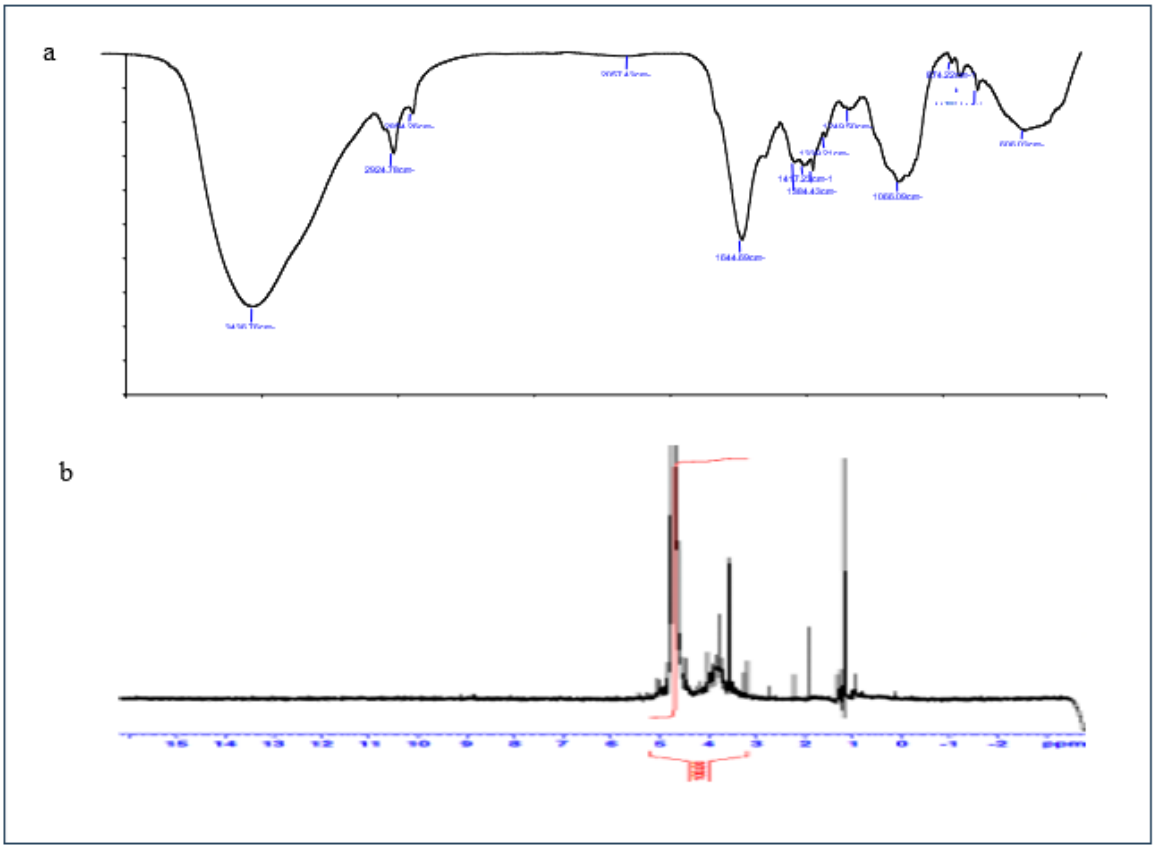


Figure 2: (a) FT-IR spectrum of LLFGM. (b) ^{1}H NMR spectrum of LLFGM.

ascorbic acid. Among the various fermented polysaccharides, LLFGM possessed the highest activity at 100 µg/mL. The free radical scavenging activity of fermented GMs and ascorbic acid was in the following order: ascorbic acid > LLFGM > CIFGM > SCFGM. Consuming probiotics with variant-specific living microorganism demonstrates numerous positive health impacts that can prevent oxidative damage and offer antioxidant property. The results of the current study demonstrate that GM fermented by *L. lactis* exhibits the most effective free radical scavenging activity at 100 µg/mL, outperforming other fermented GMs, and is identified as a potent antioxidant. This may be attributed to the presence of functional groups identified in the FT-IR analysis, such as -COOH, -OH, C=O, and -O-. Additionally, fenugreek seed constituents have demonstrated certain molecules with antioxidant and anti-inflammatory properties. Since the compounds having numerous functional and therapeutic compounds, it is considered as the better antioxidant agent.

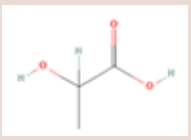
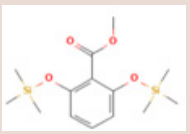
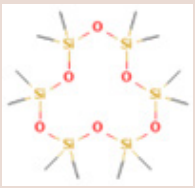
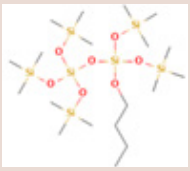
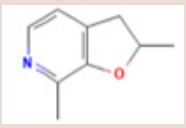
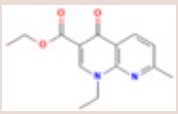
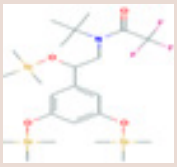
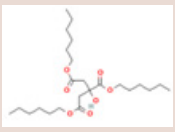
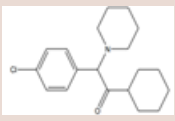
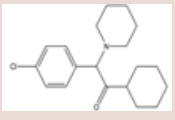
Anti-inflammatory Activity

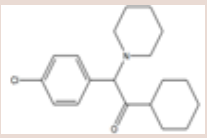
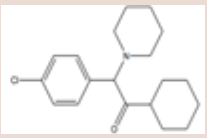
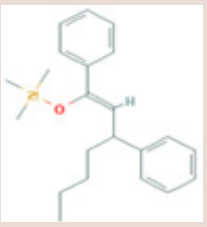
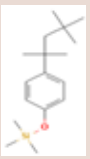
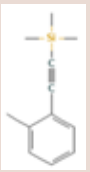
Anti-inflammatory activity for all three fermented GMs was evaluated against denaturation of egg albumin method. Protein denaturation occurs via an unforeseen mechanism, involving alterations to hydrophobic interactions, disulfide linkages, and electrostatic hydrogen bonds. The results showed that GM fermented by *L. lactis* had notable anti-inflammatory activity at 125 µg/mL, whereas *S. cerevisiae* and *C. indica* demonstrated minimal activity for denaturation inhibition on albumin protein (Figure 3b). According to the findings, *L. lactis* can be used to make nutraceuticals and as a possible probiotic strain. It is interesting to note that the extracted metabolite from *L. lactis* showed 66.17±0.54% activity for denaturing albumin protein than the standard paracetamol at 62.10±0.41% activity. In comparison to *C. indica*, yeast metabolites demonstrated a less anti-inflammatory response. Additionally, at high concentrations of *F. racemosa* extracts, the impact against albumin denaturation was much greater than that of reference medications. Anti-inflammatory medications with minimal gastrointestinal side effects that selectively inhibit COX-2 and have little to no influence on COX-1 activity are more highly valued as safe medications. Since, fermented GM (by *L. lactis* 125 µg) exhibited strong anti-inflammatory activity and suggests that it may be used as anti-inflammatory drugs.

Antimicrobial Activity

The antibacterial assays performed using the disc diffusion method revealed the ability of *L. lactis* metabolites to inhibit the growth of bacterial pathogens such as *Escherichia coli, Staphylococcus aureus*, and *Candida albicans* (Table 4). The antibacterial results demonstrated a strong and distinct inhibition of all of the examined bacterial pathogens' development. *E. coli*

Table 3: GC-MS identified metabolites of fermented galactomannan (LLFGM) with retention time, molecular weight and structure.

Peak	Name	Retention time	Molecular weight	Structure
1	Lactic acid	6.495	90.08	
2	Benzoic acid, 2,6-bis(trimethylsiloxy)-, trimethylsilyl ester	10.047	312.51	
3	Cyclohexasiloxane, dodecamethyl-	13.983	444.92	
4	3-Butoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy)tetrasiloxane	17.604	591.2	
5	Furo[2,3-c]pyridine, 2,3-dihydro-2,7-dimethyl-	19.917	149.19	
6	Ethyl 1,2,3,4,5,6,7,8-octahydro-8-oxo-1-naphthalenecarboxylate	20.025	252.31	
7	Tri-o-trimethylsilyl, n-pentafluoropropionyl derivative of terbutaline	20.849	637	
8	1,2,3-propanetricarboxylic acid, 2-hydroxy-, trihexyl ester	21.27	444.60	
9	Ethanone, 2-(4-chlorophenyl)-1-cyclohexyl-2-(1-piperidinyl)-	37.94	331.85	
10	Ethanone, 2-(4-chlorophenyl)-1-cyclohexyl-2-(1-piperidinyl)-	38.025	331.85	

Peak	Name	Retention time	Molecular weight	Structure
11	Ethanone, 2-(4-chlorophenyl)-1-cyclohexyl-2-(1-piperidinyl)-	38.139	331.85	
12	Ethanone, 2-(4-chlorophenyl)-1-cyclohexyl-2-(1-piperidinyl)-	38.19	331.85	
13	1,3-diphenyl-1-((trimethylsilyl)oxy)-1(e)-heptene	38.86	338.6	
14	4-tert-Octylphenol, TMS derivative	38.971	292.49	
15	2-((trimethylsilyl)ethynyl)heptamethyltrisilane	39	274.63	

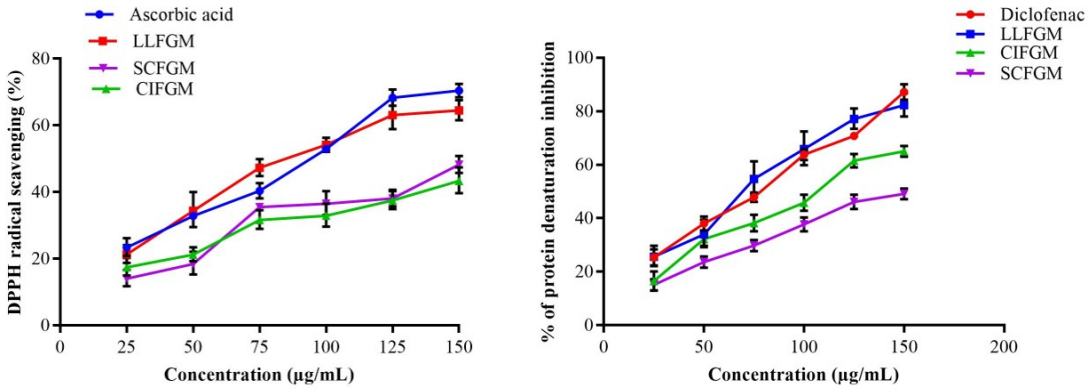


Figure 3: (a) *In vitro* antioxidant activity of fermented galactomannan determined by DPPH radical scavenging assay. (b) *In vitro* anti-inflammatory activity determined by inhibition of protein denaturation assay.

showed maximum inhibitory activity 17 mm followed by 16 mm by *S. aureus* and moderately by *C. albicans* (14 mm). As compared to standard Ofloxacin, the compound fermented by *L. lactis* showed similar bacterial inhibition. Since the results of LLFGM at a concentration of 50 µg closely related to the standard drug Ofloxacin. The presence of certain compounds may elevate the antimicrobial properties. Owing to this, the presence of certain compounds aid to exhibit such activity. It is considered that LLFGM has strong antimicrobial activity against three species and may be used for antimicrobial agent.

Anti-diabetic activity

The prevalence of DM has been increasing globally. Although insulin injections and oral anti diabetic medications are reducing diabetes, they also have harmful side effects and toxicity, such as obstructive jaundice, hyponatremia, sensitivity to alcohol, weight

gain, haematological and dermatological responses, nausea, and vomiting. This study used pancreatic lysate as source of α-amylase to assess the anti-diabetic effect of the isolated, fermented polysaccharides (Figure 4a). Acarbose was used as the standard drug to assess pancreatic α-amylase inhibition, with α-amylase inhibition quantified using an iodometric (amyloclastic) method. Figure 4a showed the anti-diabetic activity of fermented GMs. LLFGM exhibited strong anti-diabetic activity when increasing the concentration. Additionally, it is noteworthy to observe the enzyme inhibition activity at 100 µg/mL, which exhibited strong anti-diabetic effect than the standard drug acarbose. Furthermore, the results of present study showed that α-amylase was not inhibited by SCFGM and CIFGM metabolites at concentrations of 10-100 µg. It is observed to note that not all the fermented GMs were exerted the activity. *L. lactis* has capable of producing metabolites during fermentation and thus leads to the highest enzyme activity. According to these findings, fermented fenugreek GM show promise as treatments for diabetic mellitus. Investigation of its potential as an anti-diabetic drug *in vivo* with diabetes necessitates more study.

Anti-ulcer activity

Gastric ulcers are defined as breaches in the stomach mucosa that extend beyond the muscularis mucosa, typically with a diameter greater than 5 mm. Colorimetric analysis was used to measure the release of phosphate and measure the antiulcer effect. Calculations based on standards were made to determine how much phosphate from treatments and control groups emitted. Figure 4b shows the calculated phosphate quantity and the percentage of ATPase blocking, respectively. Phosphate release in the saline control H^+, K^+ -ATPase was measured at 47.61±0.37 (µ mol Pi/mg prot/hour) considered as 100% enzyme activity. Concerning the saline category, the concentration for phosphate from the *L. lactis* category was much lower recorded as 8.13±0.14 (µ mol Pi/mg prot/hour), indicating an 82.16±0.34% inhibition. In course of the group by metabolite of yeast H^+ -K^+ ATPase expression was 77.49±0.57% suppressed, and the free phosphate level was 10.47±0.32 µmoles. Treatment with the *C. indica* metabolite resulted in a 62.31±0.41% ATPase inhibition and the phosphate released was 17.35±0.42 (µ mol Pi/mg prot/hour), whereas the standard Omeprazole has 80.14±0.81 and the phosphate released was measured as 9.44±0.71 (µ mol Pi/mg prot/hour). Since, the results of the amount of phosphate released and ATPase inhibition is very closely related to the standard Omeprazole (50 µg), it can be effectively used as anti-ulcer agent. The findings demonstrate that fermented GM inhibits the proton pump, which has the effect of exerting an antiulcer potential. On H^+, K^+ ATPase, it has a focused graded response in opposition to the common medication omeprazole. Thus, fermented GM demonstrates antiulcer activity by inhibiting H^+, K^+ ATPase, a key enzyme involved in gastric acid secretion.

DISCUSSION

The cultural and physical traits of the milky mushroom fruiting bodies seen in this study are comparable to those mentioned by Karketta *et al.*[13]

GMs have been previously characterized in the literature through FT-IR analysis.[14-17] Numerous studies have acknowledged in the literature that the biopolymer's carbohydrate composition accounts for the peaks mentioned. [17,18] The indication of amides was confirmed in the region 1644.69 cm^{-1} (C=O stretching), confirms the presence of protein in the fermented GMs moieties which was similar to the results of earlier reported study.[5]

The proton signals of the 1,6-linked D-galactopyranose residue may overlap with those of the terminal units. Previous studies have successfully assigned the majority of the ring.[19]

Free radicals are the substances, which generated naturally by the individual in consequence to regular breakdown, other internal activities, or contact to certain contaminants in the environment.[20] -COOH, -OH, C=O, and -O- groups could either interact with free radicals to terminate the radical chain reaction or donate electrons to stabilize the radicals into a more stable form.[21] Interestingly, various studies showed the presence of protein moieties that are associated with the polysaccharides have ability to reduce the free radicals and exhibit strong antioxidant activity.[22] Similarly, the FT-IR results showed the peak 1644.69 cm^{-1}, which confirms the presence of protein that is associated to the fermented GMs. Thus, it enacts as one of the antioxidant-contributing factors to exhibit antioxidant activity. Moreover, the presence of compounds cyclohexasiloxane, and dodecamethyl that are identified by GC-MS also adding the antioxidant properties to the fermented GMs.[23]

In certain arthritic conditions, auto-antigen generation may be triggered by denaturation of tissue proteins. Therefore, tissue

Table 4: Inhibition zone diameters (mm) of fermented GM polysaccharides (50 µg) against *Escherichia coli, Staphylococcus aureus* and *Candida albicans* using disc diffusion assay.

Sample (50 µg)	*Escherichia coli*	*Staphylococcus aureus*	*Candida albicans*
Ofloxacin (Standard - 10 µg)	18 mm	19 mm	22 mm
LLFGM	17 mm	16 mm	14 mm
CIFGM	5.6 mm	4.4 mm	5 mm
SCFGM	0 mm	0 mm	0 mm

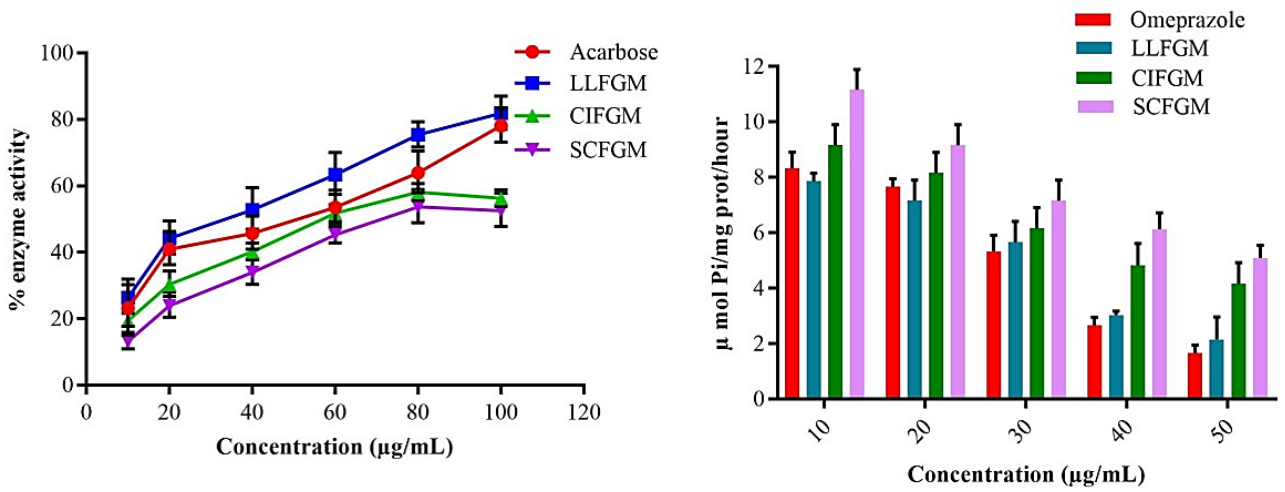


Figure 4: (a) *In vitro* anti-diabetic activity of fermented galactomannan determined by pancreatic α-amylase inhibition assay. (b*) In vitro* anti-ulcer activity determined by H^+, K^+-ATPase inhibition assay.

protein denaturation could be considered a biomarker for arthritic and inflammatory conditions.[24] According to Mandal *et al.*[25] 400 mg/kg of *Ficus racemosa* Linn. leaf extract showed about 32% of its anti-inflammatory efficacy. Drug discovery combined with natural products, particularly medicinal plants, has proven to be a very effective method of discovering new therapeutic compounds.[26]

It has been previously reported that fenugreek has strong antimicrobial agents.[27] Li *et al.*[28] reported a maximal antibacterial activity with an inhibition zone of 15.4 mm. Similarly, the results were closely related to Gashaw and Getu,[29] against *E. coli* (18.6 mm) at 100 µL concentration, which is higher than to our results.

Studies reported that fenugreek has anti-diabetic activity.[30] It has been evident that galactomannan has strong *in vivo* anti-diabetic activity.[31] But there are no reports were available for the fermented GM isolated from fenugreek seeds.

Alterations in the stomach's defensive mechanisms can compromise the gastric mucosa, potentially leading to erosion and, ultimately, ulcer formation.[32]

CONCLUSION

The present study's results demonstrate the outstanding potential of fermented fenugreek polysaccharides, notably *Lactococcus lactis*-Fermented GM (LLFGM), as a valuable source of bio active components. FT-IR, GC-MS, and ^{1}H NMR analysis has provided insight into the variety of chemical compounds found in LLFGM. Additionally, it has been shown to exhibit outstanding performance in *in vitro* biological activities across various parameters, including antioxidant, anti-inflammatory, antibacterial, anti-diabetic, and anti-ulcer properties. The results of these experiments, which are constantly better than those of other fermented GMs (SCFGM and CIFGM), highlight LLFGM's potential for therapeutic use. It is evident that these fermented polysaccharides possess numerous health-promoting qualities, making them excellent choices for a range of therapeutic uses. They have promise for improving human health and well-being and should be further investigated and developed as possible drugs and therapeutic agents due to their special qualities. However, the present study is limited to *in vitro* investigations, and further *in vivo* studies are required to validate the therapeutic efficacy and safety of fermented galactomannan formulations.

ABBREVIATIONS

mM: Millimolar; **mL:** Millilitre; **mg:** Milligram; **µg:** Microgram; **mm:** Millimeter; **MHz:** Megahertz; **°C:** Degree Centigrade; **Kg:** Kilogram; **nm:** Nanometer.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

SUMMARY

This study explores the extraction, fermentation, and biological properties of Galactomannan (GM) from fenugreek seeds. The polysaccharide was extracted using isopropanol and NaCl, yielding 54% GM. Three microbial strains, *S. cerevisiae, C. indica*, and *L. lactis*, were used for fermentation, while *L. lactis* producing the highest ethanol concentration. The fermented GM exhibited significant antioxidant, anti-inflammatory, antimicrobial, anti-diabetic, and anti-ulcer activities. Characterization through FT-IR, GC-MS, and NMR confirmed the presence of bioactive compounds responsible for these effects. The results highlight the potential of fermented GM as a natural therapeutic agent, suggesting the need for further *in vivo* investigation for clinical applications.

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