

OPTIMIZATION OF FENUGREEK SEED POWDER FERMENTATION AND CHARACTERIZATION OF THE FERMENTED PRODUCT

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ABSTRACT

Trigonella foenum graecum (Fenugreek) seed is well known for their phytochemical content and diverse biological activities. This study focused on optimization of aqueous fenugreek seed powder fermentation using yeast. Response surface technique was used for optimizing the fermentation process. The independent influencing variables, temperature (°C) addition of quantity of yeast (%), and fermentation period (day) were modelled to correlate the number of viable cells as the response. The response surface model was further optimized for maximized viable cell count. To study the impact of fermentation in enhancing the nutritional and bio-functional properties, the fermented and unfermented product at optimal condition were comparatively investigated for phenolic content, antioxidant potential, chemical composition, amino acid profiling, and cytotoxicity assay. Both fermented and unfermented seed samples were analysed using Gas Chromatography-Mass Spectrometry (GC-MS) analysis to compare the bioactive compounds that involved in bioconversion of substrate by yeast fermentation. The results indicated that the significant beneficial changes in the volatile component profile. Furthermore, high-performance liquid chromatography (HPLC) was utilized to analyse amino-acid profiling of both unfermented and fermented fenugreek seed powder using selected essential amino acids as standard positive control which was apparent that the fermented product showed increased amino acids level; highlighting its nutritional potential. In addition, the cytotoxicity capability of yeast-fermented product was assessed using an in vitro cytotoxicity assay on MCF -7 cancer cell lines.

Keywords: *Trigonella foenum* seed, yeast fermentation, optimization, total phenolics, anti-oxidant, GC-MS, amino-acid, and cytotoxicity

INTRODUCTION

Trigonella foenum-graecum, commonly known as fenugreek, is a versatile herb extensively used for its culinary, medicinal, and agricultural benefits. In cuisine, both its seeds and leaves are used to enhance flavor in a variety of dishes, particularly within Indian, Middle Eastern, and Mediterranean traditions (Basu *et al.*, 2019). The fenugreek seeds are notable for their relatively high content of protein, saponins, total polyphenols (Vishwakarma *et al.*, 2024). Many studies have investigated the pharmacological properties of fenugreek seed extracts, including their antiviral and antioxidant activities (Niknam *et al.*, 2021). These therapeutic effects are largely attributable to their distinctive chemical constituents, such as lysine and L-tryptophan (Hadi & Mariod, 2022). Clinical researches have shown that ingesting fenugreek intake significantly reduces blood levels of low-density lipoprotein (LDL) cholesterol, triglycerides, and total cholesterol in humans (Badiie Gavarti *et al.*, 2025). Traditionally, fenugreek has also been applied topically to reduce inflammation and to promote lactation in postpartum animals. Due to its broad pharmacological activity, it is used as a food ingredient, animal fodder, and medicinal herb (Thakur *et al.*, 2023). Historically, it was used as medicinal purpose to treat ulcers, bronchitis, wounds, abscesses, digestive disorders, demulcent, laxative, and galactagogue (Salman & Qadeer, 2021). It exhibits hypocholesterolaemia, hypolipidemic, and hypoglycaemic effects in both healthy and diabetic animals and human subjects (Ruwali *et al.*, 2022). These wide-ranging applications make fenugreek a valuable plant in both health-related and industrial contexts.

Fermentation enhances the breakdown of complex carbohydrates, improving digestibility, reducing bitterness and enhancing flavor. Fermented products have been explored for their role in promoting gut health, managing cholesterol, and regulating metabolic functions. Furthermore, they are gaining popularity in the food and nutraceutical industries as functional ingredients in beverages, supplements, and probiotic formulations, offering natural, health-promoting alternatives to synthetic additives. Fermentation with yeast, in particular, increases the bioavailability of bioactive compounds, thereby enhancing the antioxidant and probiotic potential of the final product.

Recently, fermented of fenugreek seed powder (FSP) using yeast has attracted attention for its improved nutritional and functional properties. However, to date, no studies have been conducted on the fermentation of FSP using yeast. Therefore, the present study aimed to optimize the fermentation conditions of FSP using yeast. Response surface methodology (RSM) was employed to optimize key independent variables, temperature, yeast concentration, and fermentation time that influence the fermentation process (Tofighi *et al.*, 2023). The fermented product obtained under optimal conditions was subsequently evaluated for its total phenolic content, antioxidant potential, chemical composition, amino acid profile, and cytotoxicity activity (Salam *et al.*, 2023).

MATERIALS AND METHODS

Seed sample collection

The Fenugreek seeds (Figure 1) were obtained from the native market in Coimbatore, Tamil Nadu, India. Samples were stored at room temperature in polythene bags before processing. The seeds were carefully cleansed with clean water, and dried. The dried seeds were thoroughly grinded using a mortar and pestle to create a fine powder. Mortar and pestle, conical flask, beaker petri plates, T-flask, well plate, and test tubes were utilized in the present study that were procured from Tarson products Ltd., India). Sodium carbonate (20%), methanol, glacial acetic acid, ethyl acetate, anti-oxidant reaction mixture, DMEM, N-butanol, MTT dye, MCF 7 cell line, DMSO, trypsin, acetonitrile (HPLC grade), sodium acetate, and amino acids standards were utilized in this study that were belongs to Hi Media Lab., India. Centrifuge (Remi), UV visible spectrophotometer (make & model, country), CO₂ incubator (Thermo Fischer, model), Gas chromatography coupled with mass spectroscopy (Agilent Technologies, model with country), and High-performance liquid chromatography connected with an array of diode detectors (Agilent Technologies 1200, country) were utilized for analytical purposes.



Figure 1 Fenugreek seeds used for this study

Fermentation

Trigonella foenum seed powder (5g) was taken in 100ml of deionized water in a 250ml conical flask, and 5% glucose (taken from glucose stock solution which was prior filter sterilized) was added to the mixture as a supplement for enhancing the fermentation process. Based on the predetermined design of experiment, a required quantity of yeast (*Saccharomyces cerevisiae*) culture was inoculated into the mixture. Further, the fermentation was carried out for predetermined time. After fermentation, the fermented broth was separated using centrifuge. Similarly, an unfermented seed powder of *Trigonella foenum* seed powder (without glucose addition and inoculation) was taken as a control for comparative studies in terms of their properties and activities (Shu et al., 2022).

Response surface methodology (RSM)

RSM, a powerful tool, which can be employed to develop a statistical model via correlating the multiple influencing variables and analyse the corresponding response. Herein, during fermentation, the addition of the amount of inoculum (%), the fermentation period (day), and temperature (°C) were considered the independent influencing variables for optimizing the fermentation process. The viable microbe count (CFU/mL) reflects the present of living microorganisms after applying a fermentation condition. This directly shows the microbial efficiency of the tested condition. CFU/ml provides a quantifiable and continuous response variable, which can be ideal for RSM response. It enables fitting a regression model and generating response surfaces for optimization. Thus, the viable microbe count in terms of CFU/ml was considered as the response value for the experiments. The Box-Behnken Design (BBD) is a response surface methodology used to optimize processes with multiple independent variables. It evaluates the effects of these variables and their interactions on a response, using a minimal number of experiments. BBD involves three levels for each factor and does not include extreme combinations, enhancing safety and efficiency. Key parameters include independent variables [temperature (°C), time (d), and addition of yeast quantity (% v/v)] and the response [number of viable microbe, (CFU/mL)] measured. The design fits a quadratic model to predict and optimize the response surface. Based on BBD, the three variable and three levels design were adopted and executed in this study. Table 1 presents the levels of considered variables that were coded as -1, 0 and +1. Design Expert 13.0 was employed to develop the experimental plan and regression analysis. Using the software, a quadratic equation was fitted using a quadratic regression and interaction effects were developed. To assess each factor's and its interaction's effect on the response value, the regression equation's variance analysis was performed. The ideal condition, that is, the level of each factor at which the response value was highest was identified by the regression model, further, it was confirmed by experimental trials (Dejene et al., 2023).

Table 1 Values of considered factors for optimization and their limits for Box–Behnken design

Coded level of independent variables	Temperature (°C)	Time (d)	Addition of yeast quantity [% (v/v)]
-1	29	12	8
0	32	14	9
1	35	16	10

Comparative characterization of fermented and unfermented FSP

Total phenol content

Total phenol content plays a significant role in antioxidant or free radical scavenger property. In the present study, Folin-Ciocalteu's analysis technique was used to analyse two samples which include unfermented FSP (control) and yeast-

fermented FSP. To analyse TPC, 0.2 mL of the sample was blended with 10% Folin-Ciocalteu's reagent in two separate test tubes, which were shaken, and kept for 5 min at 37°C. Then, 3 mL of the reaction mixture was prepared by adding 1 mL sodium carbonate (20%) and sterilized water. Then, the mixture was incubated at 45 °C for 45 min. To measure the sample's absorbance at 765 nm, a UV-visible spectrophotometer was used. The gallic acid equivalent (GAE)/g measure, was used to find the sample's total phenolic content, methanol was added as a diluent to the extract at concentrations of 20, 40, 60, 80, and 100 GAE mg/ml (Shori et al., 2021).

DPPH assessment

The scavenging action of antioxidants was examined using the DPPH (2,2-Diphenyl-1-picrylhydrazyl) assay. In brief, 0.5mL of unfermented and yeast-fermented FSP was taken in two different test tubes, and 0.2 mL of 0.1 mM DPPH was poured in both tubes. Later, the tubes were incubated at normal temperature (37°C) for 5 minutes. 0.4 mL of 50 mM Tris HCl was mixed, and incubation was carried out at normal temperature (27°C) for 30 min. A spectrophotometer (Thermo Scientific™, A51119500C, India) was used to quantify the sample at optical density (OD) 517 nm for the test and control sample. The antioxidant activity was computed utilizing the equation (1) as given below (Akyüz et al., 2023).
 Antioxidant activity (%) = [(Absorbance of control – Absorbance of Sample)] / (Absorbance of blank) × 100 (1)

GC-MS analysis

The GC-MS profiling was examined using GC system (Agilent Technologies, 7820A, country), fitted with DB-5 column (30 m in length, 0.25 mm internal diameter, and 0.25 µm film thickness). The GC system was connected to the MSD via an appropriate interface in order to facilitate mass spectrum detection. Initially, 100°C temperature was kept and it was designed to elevate by 10°C every sixty seconds till it attained 270°C. Helium serves the role as carrier gas in the mobile phase, which flowed at a rate of 1.2 mL min⁻¹. Using mass hunter software (Agilent Technologies), the mass spectra were collected and analysed by comparing unfermented control with yeast-fermented FSP (Yolin Angel et al., 2024).

Amino acid profiling

To prepare samples for free amino acids profiling, 2.5 mL of each sample (including control) was thinned by the addition of sterilized water. After centrifuging the solution for 10 min at 15,000 rpm, it was filtered through filter paper into a 10 mL glass tube. The tube was then filled with developed solution (0.5 mL), methanol (0.75 mL), diethyl ethoxy methylene malonate (DEEMM) (3 µL), and 1 M borate buffer (1.748 ml) at 9.0 pH for the conversion process. The lid of the tube was then tightened followed by shaking and it was left in a room-temperature ultrasonic bath for half an hour. Derivatized samples were characterized using HPLC (Agilent Technologies) that was coupled to a Diode Array Detector at 280 nm after being filtered via a 0.22 µm cellulose membrane filter. An analysis of chromatography was carried out using a C18 column (4.6 mm × 250 mm × 5 µm). The flow rate through the column was 1.0 ml/min, and it was thermostated at 18°C. The intended injection quantity was 20 µL. The gradient conditions were followed when using acetonitrile and acetate buffer (0.1 M, pH 6.0) as the mobile phase. Retention durations derived from pure chemicals were used to identify the samples. The amino acids (primary and secondary) were excited and emitted at different wavelengths. For the primary amino acids, these were 340 nm and 450 nm, and for the secondary amino acids, 266 nm and 305 nm. A calibration curve (6.25 to 100 µM) was used to perform quantification, and the internal standard was used to normalize the data (Peixoto et al., 2021).

FT-IR analysis

Both the fermented and unfermented samples were diluted into absolute ethanol at a 1:10 ratio before the analysis. Using KBr, the transparent discs of fermented and unfermented samples were created. The Anton Paar (Lyza 7000, India) FT-IR spectrometer, which has a scan range of 500–4000 cm⁻¹, was loaded with discs.

Cytotoxicity assessment of fermented product

In this study, the MCF-7 cell line was used to examine the cytotoxicity activity of the fermented product. The MCF-7 lineage was acquired at the National Centre for Cell Science in Pune, India. The MCF-7 cell lines were bought and then sub-cultured in a Dulbecco's Modified Eagle Medium (DMEM) medium with 10% Bovine serum albumin (BSA), glucose, and sodium carbonate. The T flask was seeded with 10µL cell line culture along with the chemical reagents and was inoculated in the medium, followed by incubation in a CO₂ incubator for 24-72 hours. The ideal conditions were pH 7.0, temperature 37°C, and moisture content around 70–80%. Further, the work was carried out after its proliferation which was confirmed under an inverted microscope. For the cytotoxicity experiment, samples were placed in 96-well plates, and then left to adhere to one another for an entire day at 37 °C and with 80% humidity. The cell line was subjected to different

sample concentrations and incubated again for 24 h, with DMSO as a blank and a cell line devoid of sample as the control. After the incubation, the cells were cleansed with 50µL of DMSO and trypsin. After washing, each well was added with 20 µL of MTT dye. After blending the mixture well, it was incubated in CO₂ incubator for 24 h at 37°C. Afterwards, the cautious extraction of the reaction mixture, 100µL of DMSO was mixed and thoroughly dissolved the formazan crystals. Using a 96-well ELISA plate, the OD was evaluated at 570 nm after 24 hours. The potency was evaluated using the formula (2) to get the cell death and viability using an ELISA reader (Robonik, India) (Kis et al., 2022).

$$\text{Cellviability (\%)} = [(OD_{\text{Control}} - OD_{\text{Total}})]/OD_{\text{(Control)}} \times 100 \dots\dots\dots (2)$$

RESULTS AND DISCUSSION

RSM based optimization of fermentation process

In the present study, the RSM analysis was opted out for determination of optimal fermentation condition. In this regards, Box-Behnken optimization method was used to obtain the results of target response as given in table 2. The second-order model equation presenting the target response as number of viable microbe (10¹⁰ CFU/ml)] was developed as the function of the independent variables, such as temperature (°C), yeast addition(%), and fermentation period (d). The generated model (Eq. 3) was statistically analyzed by multiple regression analysis.

$$\text{Number of viable microbe, (10}^{10}\text{CFU/mL)} = -130.12966 + 4.23663 \times A + 7.84994 \times B + 4.17756 \times C - 0.051667 A \times B - 0.023155 \times A \times$$

$$C + 0.04625 \times B \times C - 0.054782 \times A^2 - 0.371964 \times B^2 - 0.137634 \times C^2 \dots\dots (3)$$

The Table 3 shows the data obtained from ANOVA on the developed model. The higher F value (25.04) and acceptable p-value (0.0002) were explicated the significance of the model. The statistical significance of the developed design was validated by the R² value (0.9699). The p- value 0.9298 (> 0.05) for lack of fit was not significant; suggesting that the acquired model had very good accuracy and reliability (Kumar et al., 2019). The value of R²_{Adj} for the model was 0.9312, indicating that the developed correlation of model can explain 93.12% of the changes of the response value. The value reflects the universality and accuracy and of the equation. Based on the interaction effect analysis, three response surface plots were generated by the software (Fig. 2). The plots intuitively explain the relationship between test variables and target response (number of viable microbe). They also describe the interactions between two selected variables and response within the experimental levels. According to reports, RSM could potentially be used in related experimental domains for the statistical modeling and optimization of fermentation systems, and this work confirms this finding. In the present study, the number of live microbes was considered to be the determining factor in the quality of fermentation products and the perception of fullness of utilization in fermented products (Fig. 3). Table 4 presents the predicted optimized condition for maximized number of viable microbes for fermentation. Statistically, the optimized condition was determined as temperature: 31.4 °C, yeast addition quantity: 9%, and fermentation period 14 d which resulted the highest number of viable microbe (1.97%, v/v), further, was confirmed by experimental run.

Table 2 The experimental design combination of selected parameters under BBD and corresponding number of viable microbes during fermentation

Run No.	Temperature (°C)	Yeast addition (%)	Fermentation time (d)	Number of viable microbe (10 ¹⁰ CFU/ml)
1	29	8	14	0.95
2	29	10	14	1.59
3	35	10	14	0.91
4	32	9	14	2.04
5	35	8	14	0.89
6	32	8	16	0.81
7	32	8	12	0.98
8	35	9	16	0.59
9	32	9	14	1.9
10	32	10	16	1.26
11	29	9	16	1.21
12	32	9	14	1.71
13	29	9	16	1.21
14	35	9	12	0.89
15	32	9	14	1.94
16	32	9	14	2.16
17	32	10	12	1.06

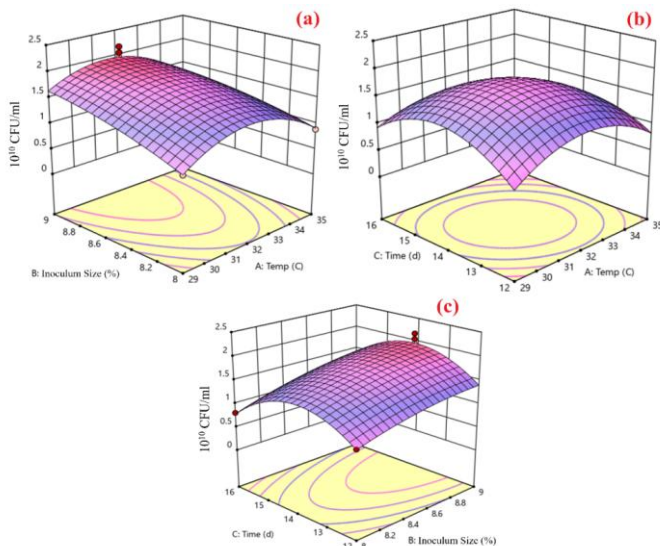


Figure 2 Response surface interaction plots among different combination of independent factors (a) inoculum size vs temperature, (b) fermentation period vs temperature, and (c) fermentation period vs inoculum size.

Table 3 The ANOVA data on the developed model of equation

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	3.73	9	0.414	25.04	0.0002	significant
A-Temp	0.04	1	0.04	2.42	0.1637	
B-Inoculum Size	0.6517	1	0.6517	39.42	0.0004	
C-Time	0.0096	1	0.0096	0.579	0.4716	
AB	0.0961	1	0.0961	5.81	0.0467	
AC	0.0416	1	0.0416	2.51	0.1568	
BC	0.0342	1	0.0342	2.07	0.1934	
A ²	0.8351	1	0.8351	50.52	0.0002	
B ²	0.4753	1	0.4753	28.75	0.0011	
C ²	1.04	1	1.04	62.99	< 0.0001	
Residual	0.1157	7	0.0165			
Lack of Fit	0.0033	2	0.0017	0.0738	0.9298	not significant
Pure Error	0.1124	5	0.0225			
Cor Total	3.84	16				

Table 4 Predicted optimized condition by RSM for maximized number of viable microbes

Temperature (°C)	Yeast addition (%)	Fermentation time (d)	Predicted number of viable microbes (10 ¹⁰ CFU/ml)
31.457	9	14.041	1.966

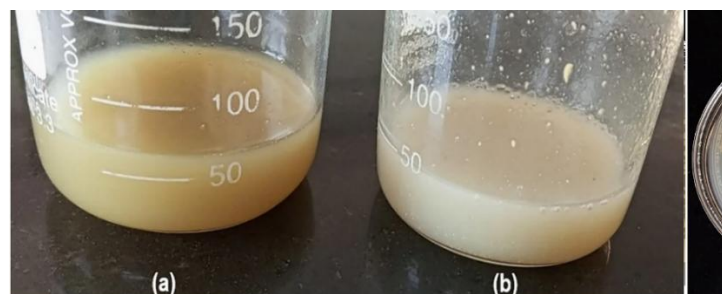


Figure 3 (a) Unfermented FSP, (b) yeast fermented FSP, and (c) number of viable microbes (10¹⁰ CFU/ml) at optimal condition.

Comparative analysis of fermented FSP at optimized condition and unfermented FSP

Total phenolic content

The quantification of total phenol content in both fermented and unfermented samples was ascertained by adopting the method reported by Kis *et al.* (Kis *et al.*, 2022) through gallic acid the standard. From the results, the total phenol content from yeast-fermented sample and unfermented sample was determined to be 69.1 mg GAE/mL and 57.5 mg GAE/mL, respectively. It was apparent that the yeast-fermented product had a higher total phenolic content than unfermented sample (Kis *et al.*, 2022; Kumar *et al.*, 2019). In studied elsewhere, it was reported different values of total phenol content ranging from 45 mg GAE/g to 80 mg GAE/g for the unfermented fenugreek seed with respect to variables that can influence a plant species' secondary metabolite composition like origin, age of seed, etc. (Kumar *et al.*, 2019).

DPPH assessment

Antioxidants are the chemical substances that counter the negative effects of reactive oxygen species. Antioxidants are given to reactive oxygen species, and have the capacity to stop oxidation reactions at their source, thereby reducing the quantity of reactive oxygen species in the body. While comparing yeast-fermented FSP and unfermented sample, the yeast-fermented FSP yielded a total antioxidant capacity of 83.31% which was quite higher when compared to the unfermented sample which was determined to be 54.71%. A similar study was reported using solvent extraction of fenugreek seeds in which DPPH assay yield ranged from 67.30% to 43.61% of total antioxidant capacity (Khushboo Jyot *et al.*, 2022). The observed increase in antioxidant activity in the fermented *Trigonella foenum-graecum* seed product indicates a higher concentration of phenolic and other antioxidant compounds on comparison with the unfermented sample. Nutritionally, this suggests that the fermented product may offer greater protection against oxidative stress by neutralizing free radicals, thereby contributing to cellular health and reducing the risk of chronic diseases. Pharmacologically, enhanced antioxidant potential may support therapeutic applications in preventing or managing oxidative stress-related conditions such as inflammation, cardiovascular disorders, neurodegeneration, and cancer. This highlights the fermented product's promise as both a functional food and a nutraceutical agent.

GC-MS Analysis

The GC-MS chromatogram of the unfermented and fermented *Trigonella foenum-graecum* seed powder revealed, 19 and 30 peaks, respectively as shown in Figure 4 and 5. The obtained peaks were identified using NIST libraries. The compound name, retention time (RT), molecular formula and area % of the unfermented and fermented samples are presented in Table 5 and 6, respectively. According to the results, the unfermented sample contained γ -Sitosterol, 9,19-Cyclolanost-24-en-3-ol, (3 β)-, and Glycidyl palmitoleate as major components, whereas the fermented product contains 1,2,3-propanetriyl ester, Dodecanoic acid, Linoleic acid ethyl ester, and (E)-9-Octadecenoic acids ethyl ester as the major components. The bioactive compounds present in the fermented product exhibits various pharmaceutical properties like antibacterial, antifungal, antioxidant, antitumor etc. Dodecanoic acid, 1,2,3-propanetriyl ester, a major compound present in the fermented product was reported for its antifungal, antiepatotoxic, lubrication, antioxidant and antibacterial properties (HANIF *et al.*, 2022; Reddy *et al.*, 2018). In addition, Linoleic acid ethyl ester, and (E)-9-Octadecenoic acids ethyl ester are known for their significant anti-inflammatory and anti-tumor properties (Manoharan *et al.*, 2023; Yang *et al.*, 2024). The other compounds present in the fermented sample, like γ -Sitosterol, 1,3-Benzenedicarboxylic acid, bis (2-ethylene, Diosgenin, 7,10,13-Hexadecatrienoic acid, Linoelaidic acid, Hexadecanoic acid, ethyl ester are reported for its antibacterial, antioxidant and anti-tumor potentials (Sundarraj *et al.*, 2012; Valarmathi R *et al.*, 2023; Yolín Angel *et al.*, 2024). The reduction of sugar and the increased ester content in the fermented product was observed as a result of bioconversion process which occurred on the yeast addition for fermentation. The majority of the GC-MS compounds identified from the fermented sample were evaluated for Lipinski's Rule of 5. Several compounds such as 2,7-Octadiene-1,6-diol, Hexane, 3,3-dimethyl-, and 7,10,13-Hexadecatrienoic acid fully obey with the rule, indicating good drug-likeness. Although a few compounds slightly exceeded the ideal LogP or molecular weight but their pharmacological activities such as antibacterial, antioxidant and antitumor activity justify their therapeutic potential. These findings support the bioactive value of the fermented product. While comparing with the unfermented sample, there was an increase in the bioactive compounds present in the fermented sample. These bioactive compounds in the fermented sample has various therapeutic properties which on their individual and synergetic interaction which can enhance the therapeutic values of the fermented product.

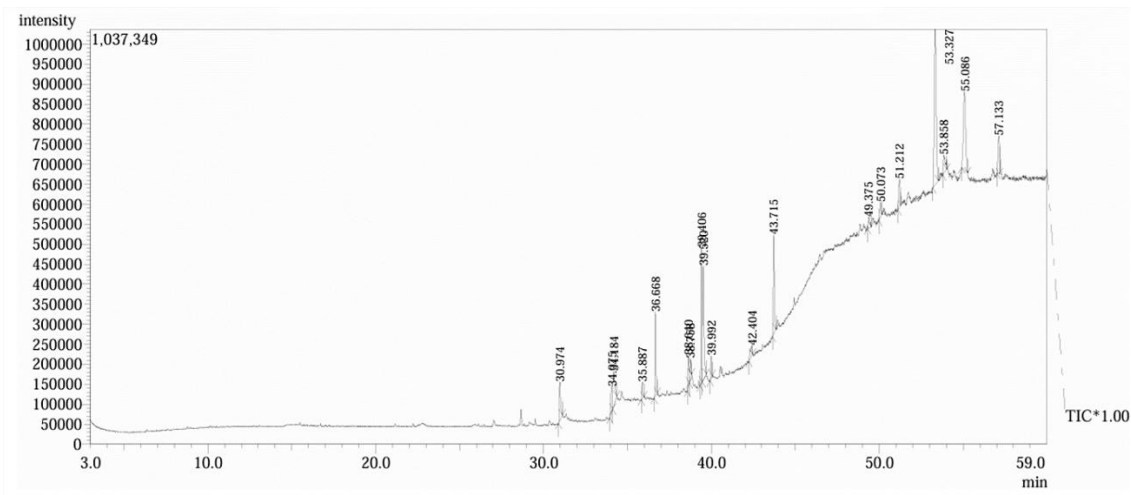


Figure 4 Chromatogram of GC-MS studies for unfermented sample.

Table 5 Bioactive compounds detected from the unfermented sample.

Compound Name	Molecular formula	Retention Time	Area %	Biological activity
n-Hexadecanoic acid	C ₁₆ H ₃₂ O ₂	30.974	3.84	Antibacterial (Yolin Angel et al., 2024)
5-Decen-1-ol, (Z)	C ₁₀ H ₂₀ O	34.075	3.25	Antifungal, antioxidant, hypercholesterolemic (Campbell et al., 2003)
1-Hexadecanol	C ₁₆ H ₃₄ O ₂	34.184	4.58	Hemolytic, 5-alpha reductase inhibitor (Rehman et al., 2024)
Glycerol 1-palmitate	C ₁₉ H ₃₈ O ₄	35.887	1.14	Penetration enhancer of drugs in the skin (Faria-Silva et al., 2022)
Octadecanoic acid, 2-hydroxy-1,3-propane	C ₃₉ H ₇₆ O ₅	36.668	5.06	Antimicrobial, Anticancer, Hepatoprotective (Elkahoui et al., 2024)
9,12-Octadecadienoyl chloride, (Z,Z)-	C ₁₈ H ₃₁ ClO	38.640	2.15	Anti-microbial, Hepatoprotective, antihistaminic, hypocholesterolemic, anti-eczemic (Hussein et al., 2024)
7-Tetradecenal, (Z)-	C ₁₄ H ₂₆ O	38.758	1.54	radical scavenger (Hemachandra et al., 2021; Pandiyan & Ilango, 2023)
9,12-Octadecadienoyl chloride, (Z,Z)	C ₁₈ H ₃₁ ClO	39.406	9.65	Anti-microbial, Hepatoprotective, antihistaminic, hypocholesterolemic, anti-eczemic (Hussein et al., 2024)
Glycidyl palmitoleate	C ₁₉ H ₃₄ O ₃	39.520	10.74	Penetration enhancer of drugs in skin (Agnisia et al., 2024)
Myristic acid glycidyl ester	C ₁₇ H ₃₂ O ₃	39.992	1.4	Radical scavenger (Wang et al., 2022)
9,12-Octadecadienoyl chloride, (Z,Z)	C ₁₈ H ₃₁ ClO	42.404	1.5	Anti-microbial, Hepatoprotective, antihistaminic, hypocholesterolemic, anti-eczemic (Hussein et al., 2024)
1,4-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	C ₂₄ H ₃₈ O ₄	43.715	7.09	Antibacterial and antioxidant (Valarmathi et al., 2023)
Vitamin E	C ₂₉ H ₅₀ O ₂	49.375	1.33	Antibacterial, Antioxidant and Anticancer (Manimaran et al., 2021; Rishikesan & Basha, 2020)
Dodecanoic acid, ethenyl ester	C ₁₄ H ₂₆ O ₂	50.073	1.58	Antifungal, Antioxidant, cancer preventive, hypercholesterolemic (Helal et al., 2019)
Campesterol	C ₂₈ H ₄₈ O	51.212	3.14	antibacterial activity, antioxidants, anti-inflammatory, cholesterol lowering and anticarcinogenic (Buř et al., 2024; Kmiecik et al., 2011)
γ-Sitosterol	C ₂₉ H ₅₀ O	53.327	22.44	Antimicrobial (Rios-Chavez et al., 2019)
Octanoic acid, 4-tridecyl ester	C ₂₁ H ₄₂ O ₂	53.858	2.69	Antidiabetic, Anti-cancer, Anti-atherosclerotic (Oganezi & Onyejekwe, 20231)
9,19-Cyclolanost-24-en-3-ol, (3.β.)-	C ₃₀ H ₅₀ O	55.086	12.87	Antioxidant (Mazhar et al., 2022)
β-Sitosterol acetate	C ₃₁ H ₅₂ O ₂	57.133	4.03	Antimicrobial (Hidayathulla et al., 2018)

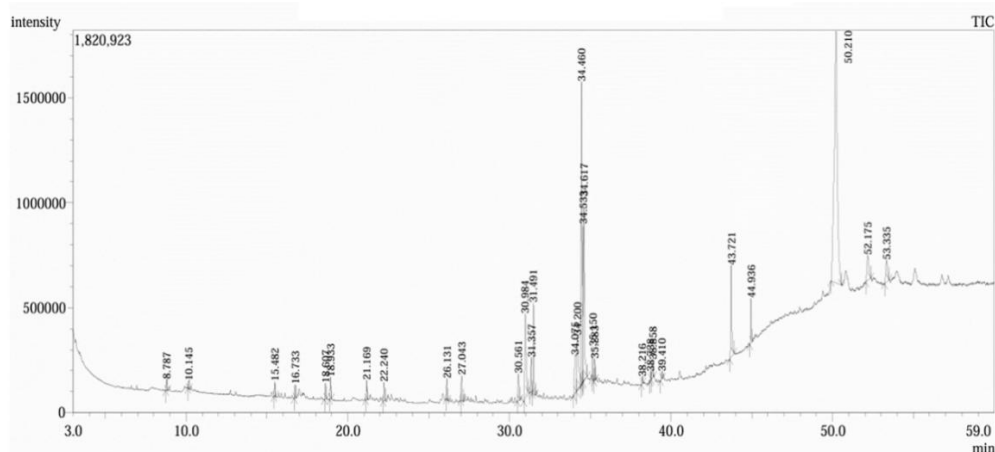


Figure 5 Chromatogram of GC-MS studies of yeast-fermented sample

Table 6 Bioactive compounds detected from the yeast-fermented sample

Compound Name	Molecular formula	Retention Time	Area %	Biological activity
Hexane, 3,3-dimethyl-	C ₈ H ₁₈	8.787	0.33	Antioxidant, antimicrobial (Wright et al., 2016)
Octane, 6-ethyl-2-methyl-	C ₁₁ H ₂₄	10.145	0.2	Antioxidant, pesticide, antimicrobial (Fan et al., 2023)
Decane, 3,7-dimethyl-	C ₁₂ H ₂₆	15.482	0.43	Antifungal, Antioxidant, cancer preventive (López-Ramos et al., 2021).
Undecane, 3,7-dimethyl-	C ₁₃ H ₂₈	16.733	0.4	Anti-diabetic, antioxidant, antimicrobial (Alotaibi et al., 2024)
Caryophyllene	C ₁₅ H ₂₄	18.607	0.56	Anti-inflammatory (Santana et al., 2024)
Aromandendrene	C ₁₅ H ₂₄	18.933	0.66	Antibacterial, Antifungal, analgesic, and anti-inflammatory (Yan et al., 2024)
Decane, 3,7-dimethyl-	C ₁₂ H ₂₆	21.169	0.64	Antifungal, Antioxidant, cancer preventive (López-Ramos et al., 2021).
Undecane, 3,7-dimethyl-	C ₁₃ H ₂₈	22.240	0.75	Anti-diabetic, antioxidant, antimicrobial (Alotaibi et al., 2024)
Nonane, 1-iodo-	C ₉ H ₁₉ I	26.131	0.6	Food additives
Decane, 3,7-dimethyl-	C ₁₂ H ₂₆	27.043	0.88	Antifungal, Antioxidant, cancer preventive (López-Ramos et al., 2021).
2-Methyltetracosane	C ₂₅ H ₅₂	30.561	1.11	cytotoxicity on malignant cells, Laxative, antibacterial, antidiarrheal, cardiotoxic, anthelmintic, and fatigue-removal anti-inflammatory (Das et al., 2024; Uddin et al., 2024)
n-Hexadecanoic acid	C ₁₆ H ₃₂ O ₂	30.984	5.52	Antibacterial (Yolin Angel et al., 2024)
Hexadecane	C ₁₆ H ₃₄	31.357	1.25	Cytotoxicity and Antimicrobial. (Ferdosi et al., 2023)
Hexadecanoic acid, ethyl ester	C ₁₈ H ₃₆ O ₂	31.491	3.34	Antimicrobial (Ochieng Nyalo et al., 2022)
Linoelaidic acid	C ₁₈ H ₃₂ O ₂	34.075	3.38	Antimicrobial (Thakur et al., 2024)
Erucic acid	C ₂₂ H ₄₂ O ₂	34.200	4.56	antimicrobial activity (Raynor et al., 2004)
Linoleic acid ethyl ester	C ₂₀ H ₃₆ O ₂	34.460	10.69	Hypocholesterolemic, Nematicide (Susanti et al., 2022)
7,10,13-Hexadecatrienoic acid	C ₁₆ H ₂₆ O ₂	34.533	5.85	Antimicrobial (Qiu et al., 2025; Sari et al., 2023)
(E)-9-Octadecenoic acid ethyl ester	C ₂₀ H ₃₈ O ₂	34.617	7.95	Antiviral and anti-obesity properties (Singh et al., 2023)
Octadecanoic acid, ethyl ester	C ₂₀ H ₄₀ O ₂	35.150	1.07	anti-tumourogenic, Antidiabetic, Anticancer, Haemolytic, Pesticide, Skin irritant (Youssef et al., 2023)
Hexadecane	C ₁₆ H ₃₄	35.283	0.64	Cytotoxicity and Antimicrobial. (Ferdosi et al., 2023)
Nonane, 5-(2-methylpropyl)-	C ₁₃ H ₂₈	38.216	0.23	Antimicrobial, Antioxidant, Anticancer (Awan et al., 2023)
2,7-Octadiene-1,6-diol, 2,6-dimethyl-	C ₁₀ H ₁₈ O ₂	38.738	0.28	Antifungal, antibacterial, and/or antiviral properties (Nasr et al., 2024)
Octadecane, 3-ethyl-5-(2-ethylbutyl)-	C ₂₆ H ₅₄	38.858	0.96	Antioxidant, pesticide, antimicrobial, lubricant (Sharnika et al., 2024)

7-Hexadecenal, (Z)-	C ₁₆ H ₃₀ O	29.410	0.33	Antibacterial, Anti-inflammatory, Fungicide (Lykholat et al., 2021)
1,3-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	C ₂₄ H ₃₈ O ₄	43.721	4.61	Antibacterial and antioxidant (Valarmathi et al., 2023)
Squalene	C ₃₀ H ₅₀	44.936	1.67	Antibacterial, and Antioxidant (Cheng et al., 2024; Lykholat et al., 2021)
Dodecanoic acid, 1,2,3-propanetriyl ester	C ₃₉ H ₇₄ O ₆	50.210	36.96	Antifungal activity (Arasu et al., 2019)
Diosgenin	C ₂₇ H ₄₂ O ₃	52.175	2.44	Good anti-tumor activity (Chen et al., 2018; Huang et al., 2022)
γ-Sitosterol	C ₂₉ H ₅₀ O	53.335	6.95	Antimicrobial (Cagnini et al., 2022)

Amino acid profiling

The presence of amino acid in both, fermented and unfermented samples were quantitatively analysed by HPLC system (Agilent Technologies 1200 series) coupled to a Diode Array Detector. The amino acids present in the unfermented sample were found to be Aspartic acid, L-Glutamic acid, L-Histidine, L-Serine, L-Threonine, Glycine, L-Arginine, L-Tyrosine, L-Alanine, L-Cystine, L-Methionine, L-Valine, L-Isoleucine, L-Phenylalanine, L-Lysine, L-Leucine, and L-Proline (Fig. 6). There was a significant increase in amino acids quantification for L-Serine, L-Histidine, Glycine, L-Threonine, L-Alanine, L-Tyrosine, L-

Cystine, L-Valine, L-Methionine, L-Phenylalanine, L-Isoleucine and L-Proline (Fig. 8) in the yeast-fermented sample. Because of the increased breakdown of protein through the fermentation process, the amino acid content of the fermented product increases making it simpler to absorb by the human gut (**Adebo et al., 2022**). Thus, the intake of fermented food promotes our immune system, promotes digestion, maintains proper cholesterol, equilibrate blood pressure and the increased antioxidants helps in antiaging (**Gaur, 2024**). While comparing with the control, these findings highlighted the nutritional and therapeutic value of yeast-fermented FSP.

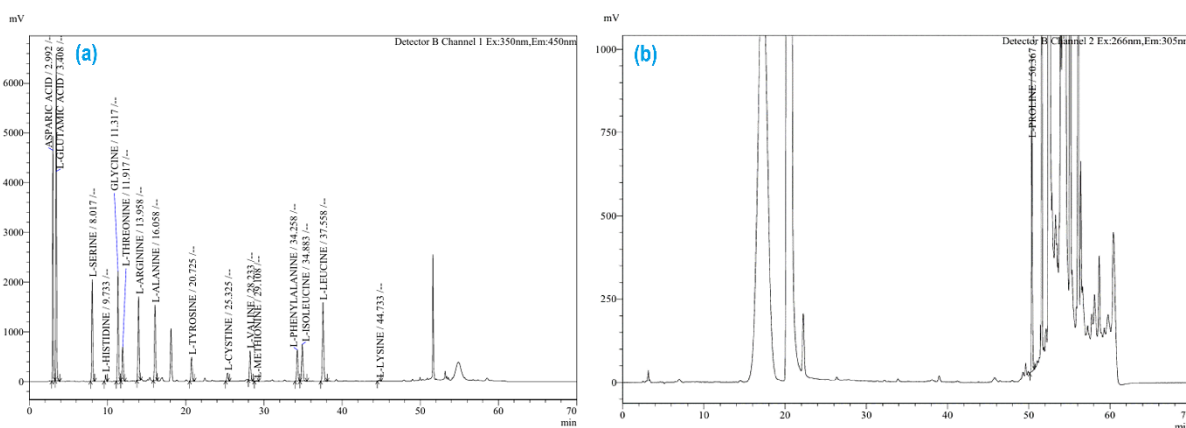


Figure 6 Chromatogram of amino acid profiling unfermented sample (a) Channel 1 and (b) Channel 2

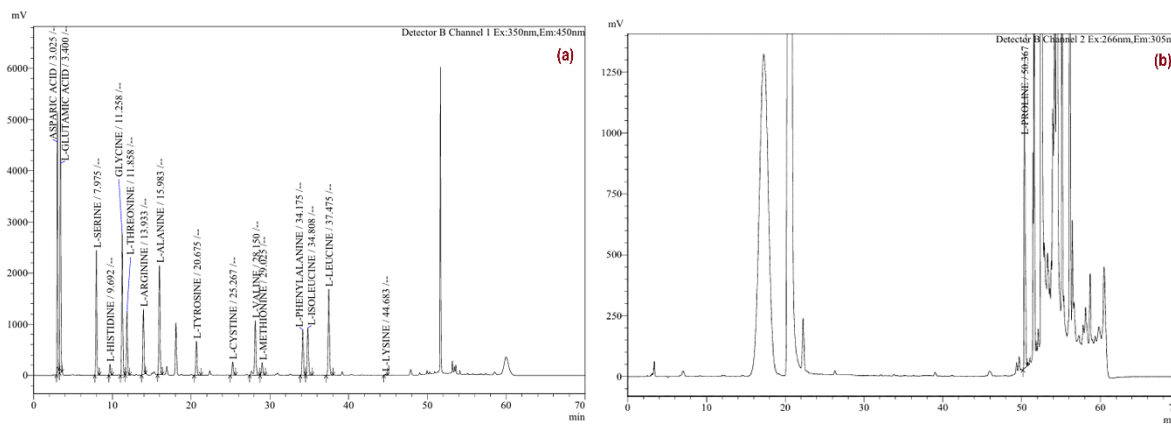


Figure 7 Chromatogram of amino acid profiling for fermented sample (a) Channel 1 and (b) Channel 2

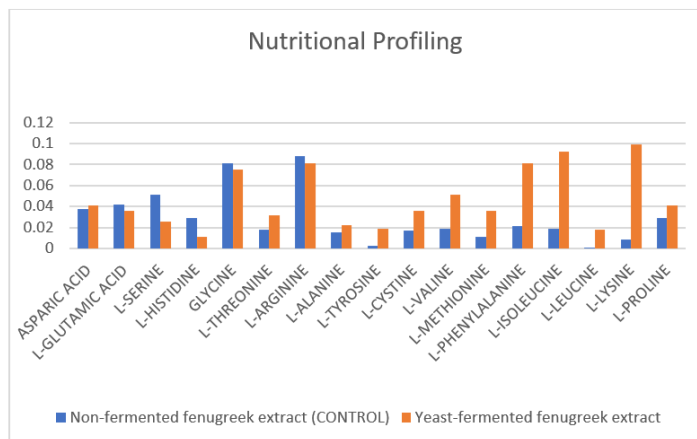


Figure 8 Representation of amino-acid profiling for yeast-fermented and unfermented sample.

FT-IR characteristics

The FT-IR studies on the unfermented and fermented samples showed the various available functional groups. According to results, the unfermented sample shows 9 prominent peaks, 3317, 2121, 1904, 1635, 686, 601, 555, 486, 455 and 416 cm⁻¹ and the fermented sample showed 10 peaks, 3317, 1635, 1095, 686, 601, 555, 493, 470, and 432 cm⁻¹ as shown in the fig 9 and 10, respectively. The peaks, 3317 and 1635 cm⁻¹ represent the N-H stretch and C=O stretch which indicated the presence of amino acid and ketone group (de Albuquerque Mendes et al., 2018; Tejamukti et al., 2020). The peak at 2121 cm⁻¹ represents to C≡C stretching, notifying the presence of alkyne group (Tan et al., 2018). In the fermented sample a new peak at 1095 cm⁻¹, corresponding to C-N stretch was observed which might be due to emergence of new amino acids or protein hydrolysis during fermentation process. The peaks, 686 and 601 cm⁻¹, attribute to C-H and OH groups which related to ketones and ester (Tariq et al., 2024). The peaks at 555, 486, 455, and 416 cm⁻¹ in the unfermented sample correspond to the C-H group, whereas in the fermented sample there was a peak shift to 493, 470, and 432 cm⁻¹ which indicated the presence of amino acid like arginine, O-H group and bending vibration of C=N (Salleh et al., 2021), respectively. The peak shift observed in the fermented product may be due to presence of amino acid as a result of fermentation.

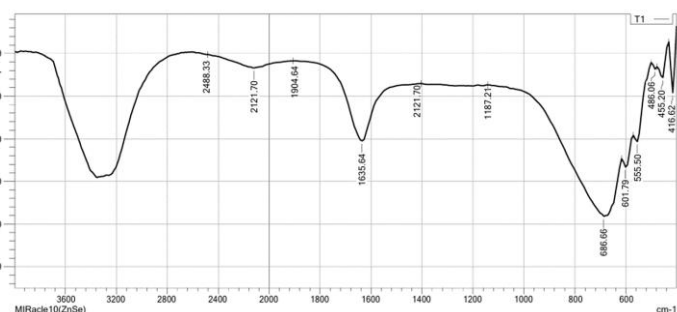


Figure 9 FTIR analysis of unfermented powder

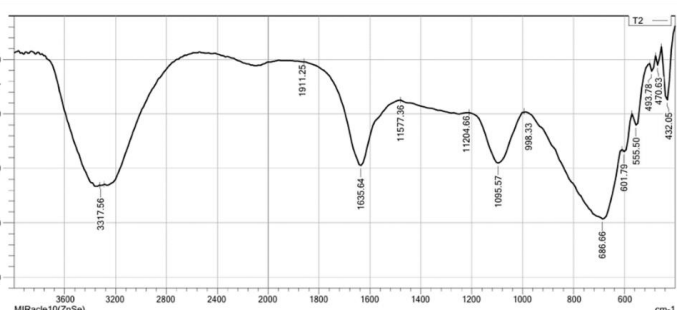


Figure 10 FTIR analysis of fermented product

Cytotoxicity evaluation of the fermented product

Cytotoxicity potency was assessed for the yeast-fermented product against mammary gland breast cancer cell lines (MCF-7). The cytotoxicity characteristic was recorded using an ELISA plate analyser set at 570 nm. The outcome indicates a positive possibility to affect cancer cell viability. Table 7 illustrates the concentration-dependent cytotoxicity effect of varying the fermented sample concentration from 2 to 10µL. At 10µL of sample, the cytotoxicity was more

pronounced, resulting in 35.249% of cell viability. The strong ability of the fermented sample against the cell viability was observed from the results. As the intensity of the yeast-fermented seed powder was differed from 2 to 10µL, as revealed in Figure 10; representing a cytotoxic effect depending on the concentration.

Table 7 Impact of fermented Trigonella foenum graecum seed powder on MCF-7 cell lines viability.

Concentration (µL)	Cell viability (%)
0 (control)	100
2	15.453
4	22.349
6	25.925
8	31.034
10	35.249

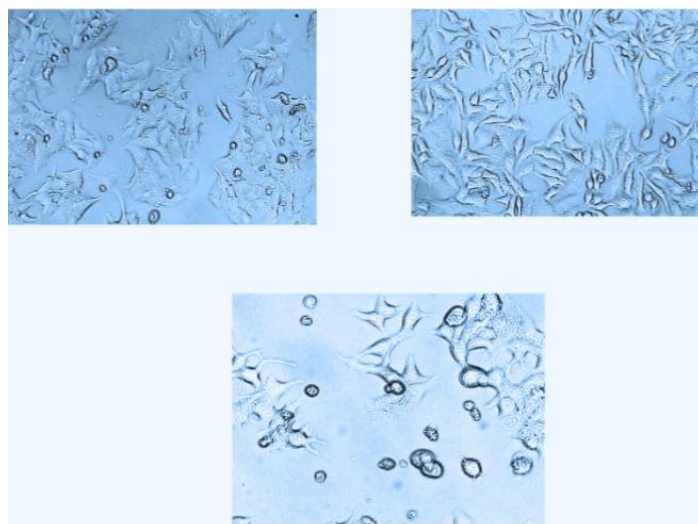


Figure 11 Microscopic image of MCF cells treated with yeast-fermented sample in the range of (A) control (B) 2µL (C) 10µL

CONCLUSION

In the present study, an investigation had been carried out on the phytochemical properties of yeast-fermented *Trigonella foenum graecum* seed powder which provided valuable insights. Response surface technique was performed for optimizing the independent influencing parameters, such as temperature, addition of quantity of yeast, and fermentation period for ameliorating the fermentation process. The fermented product at optimal condition was further investigated for its phenolic content, antioxidant potential, chemical composition, amino acid profiling, and cytotoxicity assay. Results revealed that the presence of a significant amount of phenol components, which were well-known for their antioxidant property. The findings from the antioxidant assays showed that the fermented seed powder had potent free radical scavenging activity, indicating that it could be useful in mitigating oxidative stress. The GC-MS analysis identified different volatile compounds, which helped us to understand more significant bioactive compound components present in fermented sample when compared to unfermented sample. Furthermore, amino acid profiling revealed there was a raise in the net concentration of essential amino acids levels after fermentation, highlighting the nutritional value. In addition, the cytotoxicity assays revealed promising results for the fermented extract's effects of cytotoxicity on MCF 7 cell lines. These findings support yeast-fermented *Trigonella foenum graecum's* potential with chemo preventive and other significant therapeutic properties. This broad-spectrum analysis highlights the health benefits of fermented *Trigonella foenum graecum*.

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Data availability: All data are fully available without restriction.

Competing interest: The authors declare no competing interests.

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