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Investigation of Antioxidant Potential of Black Beans Due to Phytochemical and Globulin Content for Nutraceutical Application

Sanjana Patel[#], Porshia Sharma[#], Mansi Supat[#], Swati Mali[$], Angurbala Bafna[$], Tasneem Rangwala[$], Rashmi Limaye[!,] and Payal Puri[!*]

[#,!]*Institute for Innovative Learning and Research Academy, Indore - 452 010, Madhya Pradesh, India*

[$]*Department of Biochemistry, Government Holkar Science College, Indore - 452 001, Madhya Pradesh, India*

[*]*Email: iilracademy@gmail.com*

ABSTRACT

The study aims to elucidate the antioxidant and anti-inflammatory properties of black beans and identify the key contributors within the beans. Black beans boast high protein and phytochemical content, suggesting potential antioxidant activity. This work delves into this possibility, focusing on the role of globulins (a protein fraction) and various phytochemicals in black bean's bioactivity. Globulins were isolated from black beans by salt precipitation. Ethanolic and aqueous extracts were prepared, followed by phytochemical screening for phenolics, flavonoids, alkaloids, etc. Quantification of phenolics and flavonoids was performed by colorimetric assays. SDS-PAGE and mass spectrometry identified the isolated globulin. Ferric reducing potential and total antioxidant capacity tests assessed antioxidant capacity, while erythrocyte membrane stabilisation inhibition evaluated anti-inflammatory potential. Black beans exhibited a diverse range of phytochemicals. The ethanolic extract displayed the highest flavonoid content (11.18±0.11mg QE/gram extract), while the aqueous extract was richest in phenolics (0.71±0.14 mg GAE/gram extract). The aqueous extract displayed the strongest antioxidant activity (2.87 ± 0.98 mg AAE/gram extract), followed by the globulin fraction (1.23± 0.43 mg AAE/gram extract). The ethanolic extract demonstrated superior anti-inflammatory activity. Mass spectrometry identified globulin as a 68.7 kDa legumin protein from *Phaseolus vulgaris*. Black beans display significant antioxidant and anti-inflammatory activity due to globulins and diverse phytochemicals. By informing dietary practices and facilitating the development of nutraceutical interventions, this work has the potential to contribute to a healthier population.

Keywords: Anti-oxidant; Black beans; Phytochemical; Protein; SDS-PAGE

NOMENCLATURE

°C	: Degree Celsius
M	: Molar
gm	: Gram
µg	: Microgram
µl	: Microliter
mM	: Milimolar
ml	: Mililiter
mg	: Miligram
nm	: Nanometer
rpm	: Rotations Per Minute
%	: Percentage
m/z	: Mass/Charge
BSA	: Bovine Serum Albumin

1. INTRODUCTION

The recent popular shift towards plant-based and vegetarian diets results from the health risks associated with saturated fat in animal-derived products[1]. Plant-based diets are lower in calories and saturated fat, but rich in fiber, vitamins, minerals, and antioxidants[2]. Beans are particularly valuable due to their high nutritional and nutraceutical content[3,4], offering a significant source of plant-based protein as a substitute for meat. Research has shown that Black beans, known as Phaseolus vulgaris, are rich in high-quality proteins and phytochemicals such as polyphenols like phenols and flavonoids[5]. These small, lustrous beans are popular in Latin American cuisine and are native to the Americas but are now cultivated worldwide[6,7].

They contain significant amounts of phenols like ferulic acid and flavonoids like catechin, which exhibit antioxidant activity and possess therapeutic effects[8]. It has been established that the flavonoids such as anthocyanins, quercetin glycosides, and condensed tannins present in bean seed coats have significant antioxidant activity with protective and therapeutic effects towards disorders linked to oxidative stress and cell damage[9,10]. Additionally, plant proteins in beans, such as albumin and globulin, have been used in various therapeutic measures[11]. Black beans are a nutritious choice, as they are cholesterol-free, low in fat, and easily digestible. With a very low glycemic index, they help regulate blood glucose levels, support bone health, and aid digestion. Black beans also contain selenium, uncommon in fruits and vegetables, providing detoxifying and anti-tumor

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properties. Additionally, they are a good source of fiber, quercetin, and saponins, which may contribute to preventing cardiovascular diseases[12].

There is a lack of understanding about the antioxidant properties of common beans despite extensive studies on their nutritional and pragmatic properties. Previous research focused on the antioxidant activity of black beans due to their phytochemical content[13-15], while recent studies revealed their potential in managing sickle cell disease[16]. The present study aimed to assess the combined antioxidant potential and in vitro anti-inflammatory activity of polyphenols, flavonoids, and globulin protein content in black beans. The findings could promote the consumption of black beans for their nutraceutical benefits.

2. MATERIAL AND METHODS

2.1 Collection of Black Beans

Black bean seeds used in this study were purchased from Neelam Foodland Grocery Store, Mumbai (FSSAI License No.-11517006000318).

2.2 Preparation of Crude Extracts

The extract of black beans was prepared by homogenisation[17]. Fine flour was made from ground seeds, and amalgamated with water and ethanol, respectively. To enable the phytochemicals to soak into the solvent, the homogenised samples were left overnight. The extracts were filtered, and yields were recorded along with the extracts' color.

2.3 Phytochemical Analysis of Black Bean Extracts

Black bean extracts (ethanolic and aqueous) were analysed using established qualitative methods from Harbone and Raman[18,19] to identify phytochemicals. Table 1 details the employed tests for various classes of compounds, including steroids/terpenoids, flavonoids, polyphenols, tannins, saponins, glycosides, and anthocyanins.

Table 1. Phytochemical screening procedure

Phytochemicals	Procedure	Observation
Phenols	Extract+ 5% $FeCl_3$	Blue-black coloration
Tannins	Extract +10% $FeCl_3$	Blue-black coloration
Flavonoids	Extract+10% NaOH	Yellow coloration
Alkaloids	Extract + Wagner's reagent	Brown precipitation
Saponins	Extract + Water	Foam production
Glycosides	Extract + acetic acid+ 5% $FeCl_3$ Warm and add con H_2SO_4	Reddish-brown coloration
Terpenoids and Steroids	Extract+ $CHCl_3$ + con H_2SO_4	Red coloration
Anthocyanin	Extract + 10% NaOH Extract + con HCl	Green coloration Red coloration

2.4 Quantification of Phytochemicals

2.4.1 Total Phenolic Content

Black bean TPC was determined using the Folin-Ciocalteu method[20] with gallic acid (1mg/ml) as standard. This method measures phenolics' ability to reduce phosphomolybdate/tungstate to a blue complex. A gallic acid calibration curve represented TPC in Gallic Acid Equivalents (mg GAE) per mg extract. All measurements were performed in triplicate.

2.4.2 Total Flavonoid Content

The Aluminum Chloride assay quantified Total Flavonoid Content (TFC) using quercetin (1mg/ml) as a standard[21]. Flavonoid interaction with Al^{3+} yields a yellow complex. A quercetin calibration curve enabled the determination of TFC as mg Quercetin Equivalents (QE) per gram extract.

2.5 Extraction of Globulin from Black Bean

2.5.1 Defattening of Black Bean Seeds

Black bean seeds were ground in ether. They were allowed to sit overnight and filtered. The ether was then evaporated and the defatted powder was allowed to dry.

2.5.2 Fractionation of Globulin

The method outlined by Osborne[22] was implemented to obtain the globulin fraction. Defatted seeds' powder was suspended in distilled water. It was shaken vigorously for 20 minutes and, was allowed to centrifuge for 5 minutes at 2000 rpm. The pellets were subsequently washed with distilled water. They were then dissolved in 0.5 N NaCl, shaken gently for 20 minutes, and centrifuged again. Globulin present in the resulting supernatant (Figure 1) was tested by Biuret reagent (0.5 % $CuSO_4$ and 10 % NaOH) for the appearance of a purple tint which indicated the presence of protein. The globulin sample was dialysed against distilled water and used for the determination of protein content, identification, and antioxidant potential.

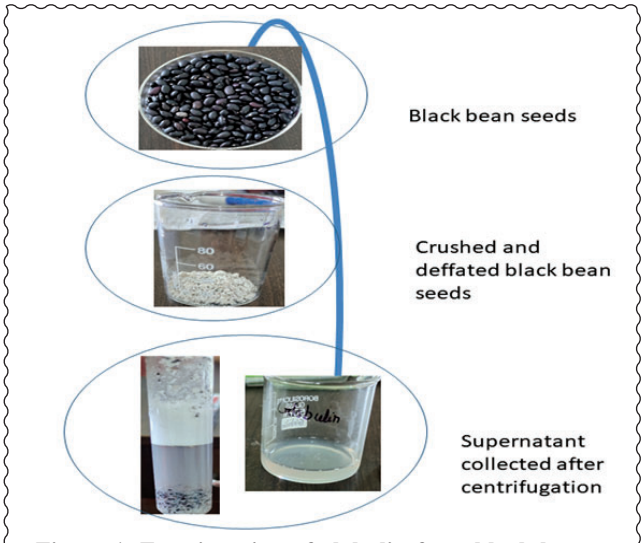

Figure 1. Fractionation of globulin from black beans.

2.5.3 Determination of Protein Content

The protein content of globulin fraction was determined by the Folin-Lowry method[23]. The concentration was estimated in terms of protein per gram of defatted powder with the help of standard protein-BSA (Bovine Serum Albumin).

2.6 Antioxidant Activity Assays of Crude Extracts and Globulin Fraction from Black Beans

2.6.1 Total Antioxidant Capacity

The phosphomolybdate assay[24] assessed the Total Antioxidant Capacity (TAC) of extracts and globulins. Antioxidants reduce a Mo(VI) complex to Mo(V), forming a greenish-blue complex at acidic pH. Absorbance at 765 nm was measured to determine TAC in mg Ascorbic Acid Equivalents (AAE)/g extract (triplicate assays).

2.6.2 Ferric Reducing Power Assay (FRPA)

Black bean extract and globulin reducing power, another antioxidant indicator, was measured using a modified potassium ferricyanide-ferric chloride method[25]. This method detects the reduction of ferric ions (Fe^{3+}) by antioxidants to ferrous ions (Fe^{2+}), which form a Prussian blue complex with ferricyanide. Absorbance at 700 nm was measured and compared to ascorbic acid (1mg/ml) standards to assess reducing power.

2.7 In-Vitro Anti-inflammatory Activity of Crude Extracts

2.7.1 Human Red Blood Cell Membrane Stabilisation (HRBC)

Erythrocyte membrane stabilisation, an alternative for lysosomal membrane effects, was used to assess the anti-inflammatory potential of phytoconstituents. With a few minor adjustments, the technique was implemented to assess the in-vitro anti-inflammatory efficacy of the extracts[26,27].

In test tubes, the crude extracts, the standard drug, and the control (saline) were mixed individually with 10 % RBC suspension, and 10 mM PBS, pH 7.4. The tubes were incubated at 37 ºC for 10 min. Heat-induced hemolysis was carried out at 54 ºC for 20 min in a water bath. After cooling the reaction mixture, it was centrifuged for 5 min at 2500 rpm, and the supernatant's absorbance was measured at 560 nm. Aspirin was used as a standard drug. For every test sample, the experiment was run in triplicate. We estimated the drug's and extracts' % inhibition of hemolysis by the approach.

Percentage inhibition = (Abs control – Abs sample) X 100/ Abs control

2.8 SDS-PAGE Analysis

The globulin fraction was analysed under non-reducing and reducing conditions using discontinuous SDS-PAGE[28]. The electrophoresis was conducted in 12 % separating gel and 4 % stacking gel. Approximately 10ug of globulin fraction and defatted bean protein was loaded separately in wells with 1:1 dilution with sample buffer (50 mM Tris pH 6.8, 2 % SDS, 20 % glycerol, 2 % 2-mercaptoethanol, and 0.04 % bromophenol blue) after boiling. The gel was stained with 0.2 % (w/v) Coomassie brilliant blue R-250 following electrophoresis. A 30 % methanol and 10 % acetic acid solution was used to destain the gel. Utilising a prestained Protein Ladder containing a mixture of 10 red, green, and blue-colored proteins (ranging in size from 11 to 135 kDa), the molecular weight of the globulin fraction was determined.

2.9 Protein Identification by Orbitrap-High Resolution Liquid Chromatography Mass Spectrometry

A high-resolution Orbitrap mass spectrometer (Q-Exactive Plus Biopharma, Thermo Fisher Scientific) was used to identify globulin in a sample. Before analysis, the sample was filtered with a 0.2 micrometer nylon membrane. Chromatographic separation employed a PepMap RSLC C18 analytical column (2 µm, 100 Å x 50 cm) with a pre-column (Acclaim PepMap 100, 100 µm x 2 cm nanoviper). The mobile phase consisted of solvent A: 0.1% FA in milliq water, solvent B: 85:15 (ACN: milliq water) + 0.1 % FA. The flow rate was set at 5 µL/min under a pressure of 700 bar.

Mass spectra were acquired in positive ionisation mode (API) within a mass range of 200-2000 m/z. Protein identification relied on matching the obtained spectra to known globulin fragmentation patterns within a proteomics software suite (Thermo Proteome Discoverer 2.2).

2.10 Statistical Analysis

Each experiment was conducted in triplicate, and the mean ± standard deviation (SD) was provided as the final result.

Standard Deviation: $s = \sqrt{\frac{1}{N-1}\sum_{i=1}^{N}(x_i - \bar{x})^2}$, Mean($\bar{x}$) = $\frac{\sum x}{n}$

(x_i, $\bar{x}$ and **N** = Sample value, mean, and size).

For the development of conventional graphs and statistical computations, Microsoft Excel was utilised.

3. RESULTS AND DISCUSSION

3.1 Yield of Black Bean Extracts

Table 2 displays the color and extractive yield of the ethanolic and aqueous extracts. The ethanolic extract yielded the most, suggesting that ethanol is more effective in extracting phytoconstituents from black beans.

Table 2. Extraction yield of black bean extracts

Extract	Final volume (ml)	Color	Yield
Ethanolic	12	Transparent	60%
Aqueous	10	White turbid	50%

3.2 Phytochemical Screening

Black bean extract analysis (Table 3) revealed the presence of various phytochemicals. Compared to the aqueous extract, the ethanolic extract had lower levels of terpenoids, steroids, and glycosides. Saponins were only detected in the aqueous extract. The absence of detectable polyphenols and tannins might indicate the presence of masked (conjugated) forms. These identified phytochemicals, known for their antioxidant properties, are linked to potential anti-inflammatory and antitumor activities.

Table 3. Result of phytochemical screeing of black bean extracts

S.No.	Phytochemicals	Ethanolic	Aqueous
1.	Flavonoids	++	+
2.	Terpenoids	+	++
3.	Glycosides	+	++
4.	Saponins	-	++
5.	Tannins	-	-
6.	Phenols	-	-
7.	Steroids	+	++
8.	Alkaloids	+	+
9.	Anthocyanins	+	+

++, indicates high presence; +, indicates faint presence; –, indicates the absence

3.3 Polyphenol Content

Table 5 shows black bean Total Phenolic Content (TPC) measured in aqueous medium to focus on polar, antioxidant phenolics. As shown in Figure 2 (gallic acid calibration curve), the aqueous extract had significantly higher TPC (0.71 ± 0.14 mg GAE/g extract) compared to the ethanolic extract. This suggests a correlation between TPC and antioxidant activity in the aqueous extract.

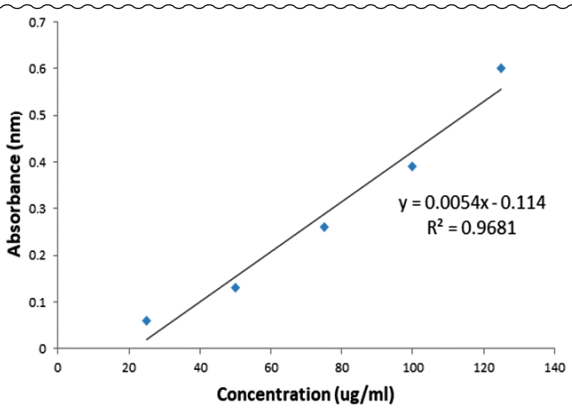

Figure 2. Standard graph of gallic acid

3.4 Flavonoid Content

Black bean flavonoid content (Table 5) was determined using a quercetin calibration curve (Figure 3). The ethanolic extract exhibited a significantly higher TFC (11.18 ± 0.11 mg QE/g extract) compared to the aqueous extract. This suggests a potential link between high flavonoid content and the ethanolic extract's antioxidant activity.

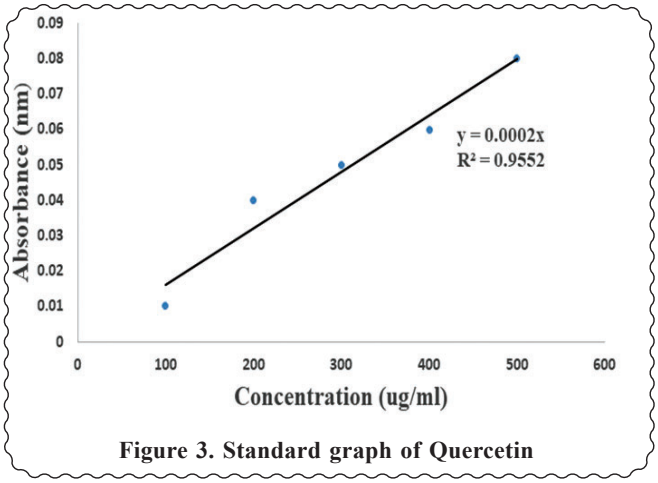

Figure 3. Standard graph of Quercetin

3.5 Globulin Identification from SDS-PAGE and O-HRLCMS

The study investigated the globulin content of defatted black beans. The isolated globulin fraction (15.52 ± 0.231 mg/g) represented a significantly smaller portion compared to the total crude protein (90 ± 0.768 mg/g) as measured by the Folin-Lowry method. SDS-PAGE analysis revealed a single band around 63-75 kDa under non-reducing conditions, suggesting a possible globulin complex. Under reducing conditions, two distinct bands appeared at approximately 32 and 48 kDa, potentially representing globulin subunits. (Figures 4A & 4B).

High-resolution liquid chromatography coupled with mass spectrometry (HRLCMS) was employed to identify proteins within the isolated globulin fraction. The analysis (Figure 5) revealed a total of 10 distinct proteins (Table 4). Legumin, a protein from Phaseolus vulgaris, was the most abundant with a high score sequence (86.36) and 14 unique peptides identified (e.g., m/z 21.96, m/z 28.47, *m/z* 28.87, *m/z* 20.81, *m/z* 21.94, *m/z* 28.43 and m/z 28.99 etc.). Since globulin is known to contain legumin, which itself is comprised of legumin subunits, the isolated protein fraction is strongly supported as globulin[2]

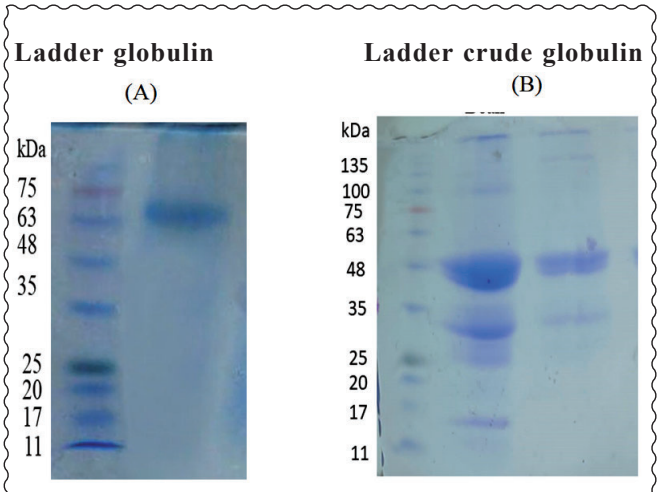

Figure 4. SDS-PAGE profile of bean proteings under (A) non-reducing and (B) reducing conditions

Table 4. List of proteins identified from black beans using O-HR- LCMS

Master Protein	Accession	Description	Coverage (%)	Peptides	Unique peptides	Amino acids	MW (kDa)	Cal. pI	Score	Peptides by search engine
1	A9S5G7	Predicted protein (Fragment) OS=Physcomitrella patens subsp. patens OX=3218, GN=PHYPADRAFT_162951 PE=4 SV=1	50	1	1	50	5.2	9.04	2.4	1
2	A0A327XM89	Purine-binding chemotaxis protein CheW OS=Rhizobium etli OX=29449 GN=BCL34_11110 PE=4 SV=1	41	1	1	92	9.5	5	2.3	1
3	A0A0U3JUJ4	Uncharacterised protein OS=Rhizobium leguminosarum bv. viciae OX=387 PE=4 SV=	36	1	1	45	4.8	8.98	7.49	1
4	F8QXP7	Legumin OS=Phaseolus vulgaris OX=3885 PE=2 SV=1	35	14	17	606	68.7	5.92	86.36	14
5	A0A2K1JTD5	Uncharacterised protein (Fragment) OS=Physcomitrella patens subsp. patens OX=3218 GN=PHYPA_014550 PE=4 SV=1	33	1	2	33	4.1	8.87	3.96	1
6	A0A2R6QGQ3	Peroxisomal membrane protein (Fragment) OS=Actinidia chinensis var. chinensis OX=1590841 GN=CEY00_ Acc18142 PE=4 SV=1	31	1	1	102	11.2	6.1	3.39	1
7	E5B8Z3	Uncharacterised protein OS=Erwinia amylovora ATCC BAA-2158 OX=889211 GN=EAIL5_3129 PE=4 SV=1	29	1	1	42	4.7	11.36	2	1
8	W1PAR5	Uncharacterised protein OS=Amborella trichopoda OX=13333 GN=AMTR_ s00141p00055890 PE=4 SV=1	28	1	1	128	14.3	10.36	3.48	1
9	A0A1Q4TMY0	Uncharacterised protein OS=Mycobacterium sp. SWH-M1 OX=1490485 GN=EB72_15315 PE=4 SV=1	28	1	1	139	14.8	7.09	3.87	1
10	W1PRE2	Uncharacterised protein OS=Amborella trichopoda OX=13333 GN=AMTR_ s00028p00164740 PE=4 SV=1	28	1	1	100	10.5	5.19	5.19	1

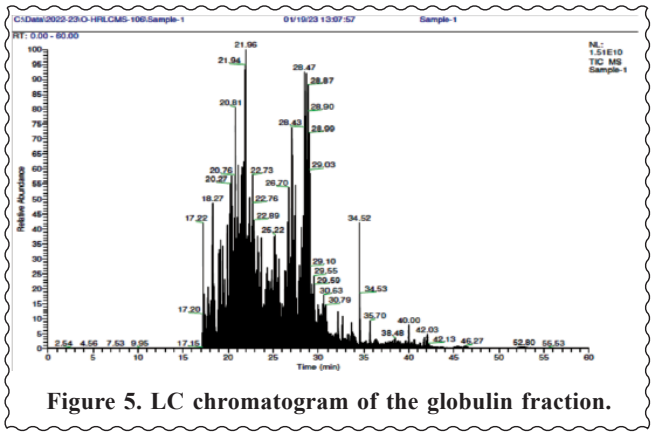

Figure 5. LC chromatogram of the globulin fraction.

3.6 TAC (Total Antioxidant Capacity)

The phosphomolybdate assay assessed overall antioxidant capacity (Table 5). Both ethanolic (0.925 ± 0.03 mg AAE/g) and aqueous (0.84 ± 0.09 mg AAE/g) extracts showed similar TAC, suggesting they can manage oxidative stress by reducing free radicals. Globulin also exhibited this activity (0.3 ± 1.2 mg AAE/g).

3.7 FRPA (Ferric Reducing Power Assay)

The FRAP assay measured the reducing power of black bean extracts and globulin (Table 5). The aqueous extract displayed significantly higher antioxidant activity (2.87 ± 0.98 mg AAE/g extract) compared to the ethanolic extract, likely due to its greater polyphenol content. Globulin also showed notable free radical scavenging activity (1.23 ± 0.43 mg AAE/g extract).

3.8 In-vitro Anti-inflammatory Activity

Linking oxidative stress to inflammation and cell lysis, this study investigated black bean extract's protective effect. The ethanolic extract displayed significantly less hemolysis compared to the control, indicating superior erythrocyte membrane stabilisation. This suggests its potential benefit in inflammation management, exceeding even aspirin's effect. Table 6 displays the proportion of hemolysis inhibition from the two separate extracts.

4. DISCUSSION

Motivated by the growing interest in natural antioxidants for health benefits, this study investigated the antioxidant properties of black beans, known for their protein and phytochemical content. The study intended to assess the antioxidant capacity of globulin protein and phytochemicals from black bean seeds. The screening of black beans' aqueous and ethanolic extracts for phytochemicals revealed that the beans are a rich source of several phytochemicals, including glycosides, terpenoids, alkaloids, polyphenols, flavonoids, and saponins. The results are consistent with previous studies done by Fonseca-Hernández[31], et al. and Ikezu[32], *et al.,* It is believed that dietary polyphenols and flavonoids are key players in antioxidant action for protection against free radicals-induced oxidative stress-related diseases[33].

The flavonoid content was significantly the highest in ethanolic bean extract with a greater degree of hemolytic inhibition indicating its substantial total antioxidant capacity and anti-inflammatory activities. Our results demonstrated a robust relationship between the aqueous extract's polyphenol

Table 5. Total phenolic, flavonoid content, and antioxident assay of black bean fraction

Extract	Total phenolics	Total flavonoids	TAC	FRPA
Ethanolic	0.29±0.21 mg/GAE/g	11.18±0.11 mg/QE/g	0.925±0.03 mgAAE/g	0.88±0.54 mg/AAE/g
Aqueous	0.71±0.14 mg/GAE/g	3.12±0.67 mg/QE/g	0.84±0.09 mgAAE/g	2.87±0.98 mgAAE/g
Globulin			0.3±1.2 mgAAE/g	1.23±0.43 mgAAE/g

Values are expressed as mean ± SD for three determinations

Table 6. Results for the effect of black bean extract on inhibition of hemolysis

Sample	Volume (µl)	Absorbance	% Hemolysis inhibition
Control	200	0.25	Maximum hemolysis
Ethanolic	200	0.03	88±0.678
Aqueous	200	0.18	28±0.34
Aspirin(15mg/ml)	200	0.20	20±0.89

Values are expressed as mean ± SD for three determinations

concentration as assessed by the FRPA test and its antioxidant activity. The preponderance of globulin as the major antioxidant protein in black bean seeds was confirmed by the present study. The black bean showed globulin content of 15.52±0.231mg /g defatted matter with the antioxidant value of 1.23 ±0.43 mg AAE/gram extract. Moreover, a sharp protein band in the 63-75kDa region was observed in the isolated protein fraction. The mass spectrometric analysis and database search identified this band as legumin (Acc.No. F8QXP7) related to globulin. These results are as per Di-Francesco**,** *et al.,* who showed proteins in the range of 45-65kDa for legumin[34]. The molecular weight of bands under reducing conditions correlates with those reported by Derbyshire[35], *et al.,* The differences in molecular weight in various studies depend on the method of extraction and characterisation. From this study, it was observed that globulin along with phytochemicals from black beans showed promising antioxidant activity. The findings of Chen, *et al.,* and Garcia-Cordero, et al. directly support the proposition that black beans possess promising antioxidant activity[36,30]. Their research demonstrates that enzymatic hydrolysis of black bean protein concentrates this activity within specific peptide fractions, highlighting the potential of black beans as a source of natural antioxidants.

5. CONCLUSION

Based on the outcomes of the research, it was discovered that the presence of globulin protein and many pharmacologically active phytochemicals in black beans offers potential anti-inflammatory and antioxidant properties. These findings emphasise the potential use of black beans as nutraceutical food with biomedical applications in the prevention of chronic diseases that are on the increase worldwide. However, in vivo antioxidant studies are required further for future research implications.

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CONTRIBUTORS

Ms Sanjana Patel is currently pursuing an MSc (Biochemistry) from Devi Ahilya Vishwavidyalaya, Indore. Her area of interest is plant biochemistry and molecular biology.
For this work, she carried out the experiments in the laboratory, tabulation, calculations, and compilation of work under the guidance of the corresponding author.

Ms Porshia Sharma obtained Post Graduate Diploma in Microbiology from Rajasthan University in 2022. Her area of

research is microbiology and agricultural science.
For this work, she has contributed to the experimentation.

Ms Mansi Supat completed her MSc (Biotechnology) in 2023 from Government Holkar Science College, Indore. Her area of interest is molecular biology and bioinformatics.
for this work, she was involved in the preparation of laboratory reagents, instrumentation, and experimental work.

Ms Swati Mali is a faculty member at Government Holkar Science College, Indore. Currently, she is pursuing a PhD in Biochemistry from Holkar Science College. Her area of interest is plant biochemistry.
For this work, she was involved in protein analysis by SDS-PAGE.

Dr Angurbala Bafna is working as Head of Department Biochemistry in Government Holkar Science College, Indore. She is a registered guide in the Department of Biochemistry, Devi Ahilya Vishwavidyalaya, Indore. Her research area is plant biochemistry and medical biochemistry.
She was involved in the preparation of the manuscript for the study and made final approval for this work to be published.

Dr Tasneem Rangwala is working as an Assistant Professor at Government Holkar Science College, Indore. She has a PhD in Biochemistry with a specialisation in plant biochemistry.
She contributed to the analysis of data and preparation of the manuscript.

Mrs Rashmia Limaye has done MSc (Microbiology) in 2008 from Devi Ahilya Vishwavidyalaya, Indore. Currently, she is working as a Research Faculty in the Institute for Innovative Learning and Research (IILR) Academy, Indore. Her field of research is phytochemistry and microbiology.
For this work, she guided the students in experimentation, collected data, and performed its analysis.

Dr Payal Puri received her PhD (Biochemistry) from DRDE, Gwalior in 2011. She is the Founder and Director of Innovative Learning and Research (IILR) Academy, Indore. She has research experience of more than 10 years and published more than 14 research papers in scientific journals. Her research specialisation includes proteomics, toxicology, and photochemistry.
She contributed to the planning of the study, supervised the experiments, and prepared the manuscript.