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ANALYTICAL PROFILING AND ANTIOXIDANT CHARACTERIZATION OF FLAVONOIDS FROM HIMALAYAN BLACK SOYBEAN (GLYCINE MAX)

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ABSTRACT
Background: Black soybean is a leguminous crop of great phytochemical interest and importance in traditional systems due to its alleged health-promoting effects. To a great extent, these advantages are attributed to its flavonoid content, a class of polyphenols known for their strong antioxidant properties. This research was part of a significant task: conducting a comprehensive phytochemical characterization of the flavonoid profile in black soybean seeds and assessing their respective antioxidant potential in vitro. **Methodology:** Flavonoids were extracted from authentic seeds using 70% aqueous ethanol. UV-Vis, FTIR, and 1H NMR spectroscopy were used to elucidate the extract's functional groups and structure. Specific flavonoids (rutin, quercetin) were analyzed quantitatively by use of validated HPLC. In the determination of total flavonoid content, a spectrometric method was used. The DPPH radical-scavenging assay was used to determine antioxidant activity. **Results and Discussion:** Spectroscopic studies revealed the presence of typical flavone/flavonol skeletons. The HPLC quantification illustrated good method performance. The TFC was 5.91 mg QE/g extract. The extract exhibited high dose-dependent scavenging activity against the DPPH radical. High TFC was strongly correlated with antioxidant activity, confirming the bioactive capacity of black soybean, known for its flavanol-rich flavonoids. **Conclusion:** The results are scientifically confirmed: black soybean is rich in bioactive flavonoids with high antioxidant capacity and can therefore be used as a functional food ingredient or nutraceutical to treat oxidative stress.

INTRODUCTION

The soybeans (*Glycine max L.*) have had a major impact on human nutrition in recent years. In addition to being used as inputs to the food sector, as ingredients in tofu, soy sauce, and vegetable beverages, soybean cultivation is primarily focused on producing vegetable oil [1]. Quercetin is a flavonol known for its potent antioxidant, anti-inflammatory, and immunomodulatory properties, thereby making it of high interest in pharmaceutical studies [2]. The glycoside of quercetin, rutin, on the other hand, is soluble and bioavailable, which are crucial characteristics for therapeutic use [3]. They have several hydroxyl groups and can deprotonate free radicals and chelate metal ions, structural characteristics that enable them to alleviate oxidative stress [4]. Antioxidants play a crucial role

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in preventing the negative consequences of free radicals, which are generated by metabolic processes & environmental factors such as pollution, by neutralizing reactive oxidants [5]. The etiology of numerous chronic & degenerative diseases, such as cardiovascular disorders, neurodegenerative disorders & various types of cancer, has been linked to the capacity of reactive oxygen species to induce oxidative stress, leading to DNA mutations, lipid peroxidation, protein denaturation, and cellular damage [6]. The mechanism of action of natural antioxidants present in R. communis involves donating electrons to stabilize free radicals, thereby suppressing cellular damage and maintaining redox homeostasis [5]. Applications of these antioxidants are usually evaluated using various spectrophotometric techniques to determine their hydrogen-atom transfer, single-electron transfer, or selective-scavenging activity [7]. It emphasizes the need to isolate and concentrate compounds such as quercetin and rutin, which have significant antioxidant properties [8]. The assessment of the antioxidant capacity of plant extracts is essential for their therapeutic applications and is used to establish natural health products [9]. These are significant because they justify the traditional use of these medicines and enable the discovery of new bioactive compounds that can serve as leads for the development of pharmaceutical products. Such a rise in interest in natural antioxidants has led to a trend toward replacing synthetic alternatives with plant-based ones, driven by safety concerns and perceived health benefits [10]. In that respect, special emphasis is placed on the careful study of plant extracts for their antioxidant properties, which are directly proportional to their ability to prevent oxidative damage and enhance overall health [11]. Therefore, their antioxidant activities are very important & need to be studied in detail to achieve their full potential in the management of most diseases.

MATERIAL AND METHODS

Chemicals and standard drugs

All chemicals used in this investigation were of analytical grade and obtained from Himedia Laboratories Pvt. Ltd. in Mumbai, India. All trials used deionized water.

Plant material and preparation of Black Soybean extract

The seeds of black soybean were collected from the Himalayan region of Uttarakhand, India, in January 2024 and authenticated by the Department of Botany, Agriculture College, Latur, Maharashtra. The seeds were shade-dried at room temperature to remove moisture. To remove foreign particles, the seeds were washed and cleaned. The dried seeds were pulverized to make coarse powder and then passed through sieve no 60. The coarse-powdered material was defatted with n-hexane for 24 hours to remove oil. Finally, the seeds were extracted using different solvents (70% ethanol, hydroalcoholic, and acetone) in a Soxhlet extraction apparatus at 60 °C for 5 hours, with a 1:10 (w/v) sample: solvent ratio. The crude extracts obtained from different solvents were concentrated using a rotary vacuum evaporator to obtain hydroalcoholic, acetone, and 70% ethanolic extracts [12].

UV – Visible Spectral analysis of Black Soybean extract

A Shimadzu UV1800 UV-Visible double-beam spectrophotometer was used to scan a black soybean extract at wavelengths between 200 and 600 nm to perform a UV-VIS spectrophotometric evaluation. The distinct peaks were noted along with their absorption values [13,14].

FT-IR analysis of Black Soybean extract

FTIR analysis was used to identify the various peaks and their functional groups using a Perkin Elmer Spectrophotometer system with a transmittance range of 400–4000 cm-1. The peak values of the FTIR were recorded. Each analysis's findings were confirmed twice [15,16].

NMR analysis of Black Soybean extract

The ^{1}H spectra were acquired on a Bruker 400 MHz spectrometer. Dissolving 10 mg of the portion in deuterated chloroform (CDCl3). Proton (^{1}H) NMR spectra were taken after the solution was pipetted into the NMR tubes using a clean Pasteur pipette. The obtained NMR spectra were manually examined [17,18]. To avoid spectral overlap in complex extract matrices and to achieve higher resolution for less polar elements, the solvent was CDCl3.

HPLC analysis for the detection of flavonoids in crude extracts of Black Soybean extract

Stock solutions (1 mg/mL) were made in methanol. The extract was then filtered using microfilters. A Flexar PerkinElmer System (PerkinElmer, Shelton, CT, USA) was used for the HPLC analysis. The system included a column oven, a degasser (DG-20A5), a gradient model pump, a 06484 (USA) UV/Visible detector, and an LC-Shelton CT. Thermo Fisher Scientific Inc. hypersissl GOLD C18 column, measuring 250 × 4.6 mm × 5 µm, was used. A nonlinear gradient of water, acetonitrile, methanol (70:30), and 0.5% glacial acetic acid was used as the mobile phase to achieve separation. The injection volume was 20 µL,

and the mobile phase flow rate was maintained at 1 mL/minute. The extract's polyphenolic concentration was determined to be 280 nanometers. Methanol was used to create new stock solutions of reference standards at a concentration of 1 mg/mL. Methanol was added to the stock solutions to dilute them to concentrations ranging from 0.4 to 100 µg/mL, thereby preparing working standards. Each standard calibration curve was created by plotting concentration versus the relevant peak region. By comparing the retention times and UV-visible spectra of the peaks with those of the reference standards, the peaks were identified. An external standard method using the reference standards' calibration curves was used for quantification [19].

Determination of Total Flavonoid Contents (TFC) of Black Soybean extract

The aluminum chloride technique is used to determine the total flavonoid content. In short, 2 ml of distilled water and 0.5 ml of the test extract with a concentration of 1 mg/ml were combined in a 10-ml test tube. After adding 0.5 mL of 5% NaNO2, the mixture was incubated at room temperature. 0.5 ml of 10% AlCl3 was added after 5 minutes. One milliliter of 1 M NaOH was added after two minutes. Lastly, distilled water was used to get the volume down to 5 mL. After 10 minutes, the absorbance of the resultant solution at 510 nm was measured relative to a blank. Quercetin served as the reference standard for the calibration curve, and the results were reported in milligrams of Quercetin equivalents per gram of extract (mg/g) [20].

Determination of antioxidant activity of black soybean extract

Mellors and Tappel (1996) used a 100% ethanolic DPPH solution to assess the seed extract's ability to scavenge free radicals. A stock solution of DPPH was made by dissolving 24 milligrams of DPPH in 100 milliliters of methanol. One hundred microliters of seed extract and three milliliters of DPPH solution were put in a test tube. Without the seed extract, the blank solution was made in the same way. The tubes were then placed in total darkness for half an hour. The absorption was measured at 517 nanometers. The decrease in absorbance at 517 nm indicates a decrease in the DPPH radical [21]. The percent inhibition of the DPPH radical was calculated using the formula:

$$\%\ \boldsymbol{DPPH\ free\ radical\ scavenging\ activity} = \left[\mathbf{1} - \frac{\boldsymbol{A\ sample}}{\boldsymbol{A\ control}}\right] \times \mathbf{100}$$

where A control is the absorbance of the control and A sample is the absorbance of the sample.

RESULTS AND DISCUSSION

Percentage yield of Black Soybean extract

The seeds of black soybean were extracted using three different solvents (70% Ethanol, hydroalcoholic, and Acetone) by the Soxhlet apparatus. The percentage yield of the extract is shown in Table 1. The highest percentage yield of black soybean seed extract (14%) was obtained with 70% ethanol > acetone > hydroalcoholic solutions, respectively.

Table 1: % yield of Black Soybean in different solvents.

Solvent	Percentage yield
70% Ethanol	14 %
Acetone	13 %
Hydroalcoholic	11 %

UV Spectroscopic analysis of Black Soybean extract

The UV-Visible absorption spectrum was recorded over the 200-600 nm range to assess the presence of flavonoids. The spectrum showed a strong absorption feature in the 270-290 nm range, with a shoulder in the 320-350 nm range. These are the two characteristic absorption bands of flavonoids & they are traditionally identified as Band II & Band I, respectively. Band II at the 240-280 nm region is usually related to the benzoyl system (A-ring) of flavonoid structures, and Band I at the 300-380 nm region is linked to the cinnamoyl system (B-ring), as shown in Figure 1. The appearance of these bands in the recorded spectrum indicates that the sample contains flavonoid constituents, most likely flavones or flavonols. The spectral profile is comparable to that of known flavonoids previously described, thus confirming the presence of flavonoids in the extract of interest. These data provide phytochemical evidence of flavonoid compounds in the extract.

Fourier Transform Infrared (FTIR) spectroscopy of Black Soybean extract

Fourier Transform Infrared (FTIR) spectroscopy was employed to explain the functional groups present in the sample and determine the presence of flavonoid products. A spectrum was recorded, showing several common absorption bands indicative of flavonoid structures, as depicted in Figure 2. The presence of the O-H stretching vibrations has been identified as the cause of a broad absorption band at 3389.71 cm-1, which typically corresponds to phenolic hydroxyl groups in flavonoids. The aromatic and aliphatic groups in the hydrocarbon backbone of the flavonoid molecule are determined to be the C-H stretching vibrations of 3010.01 cm-1, 2925.33 cm-1, and 2854.22 cm-1. 1742.99 cm -1 peak can represent carbonyl groups of flavonoids,

but can also represent the presence of unfatted lipids or esters. Additionally, the bands at 1622.59 cm-1 and 1517.77 cm-1 can be ascribed to C=C resonances within the aromatic ring system, which once again confirms that the sample is a polyphenol. C-H bending vibrations give rise to other absorption peaks at 1464.05 cm-1 and 1377.25 cm-1, but the C-O and C-O-C are attributed at 1198.03 cm-1 and 1084.14 cm-1, that is, occurring in phenolic ethers and glycosidic bonds. The presence of aromatic C-H out-of-plane bending vibrations (peaks in the lower wavenumber region (at 830.15 cm-1, 722.89 cm-1, and 531.55 cm-1) is also evidence of the presence of substituted aromatic rings. The presence of both of these spectral features indicates the presence of major functional groups-hydroxyl, carbonyl, aromatic rings, and ether bonds, which are common to flavonoid compounds. The FTIR analysis, therefore, indicates that the sample under study contains flavonoids.

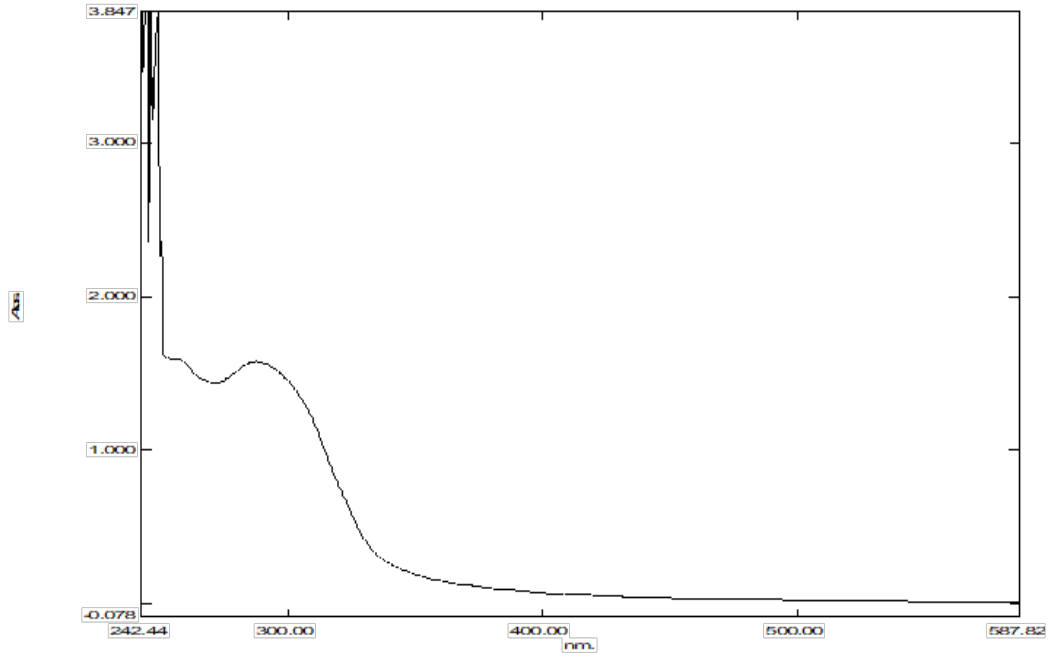

Figure 1: UV Spectra of Black Soybean extract

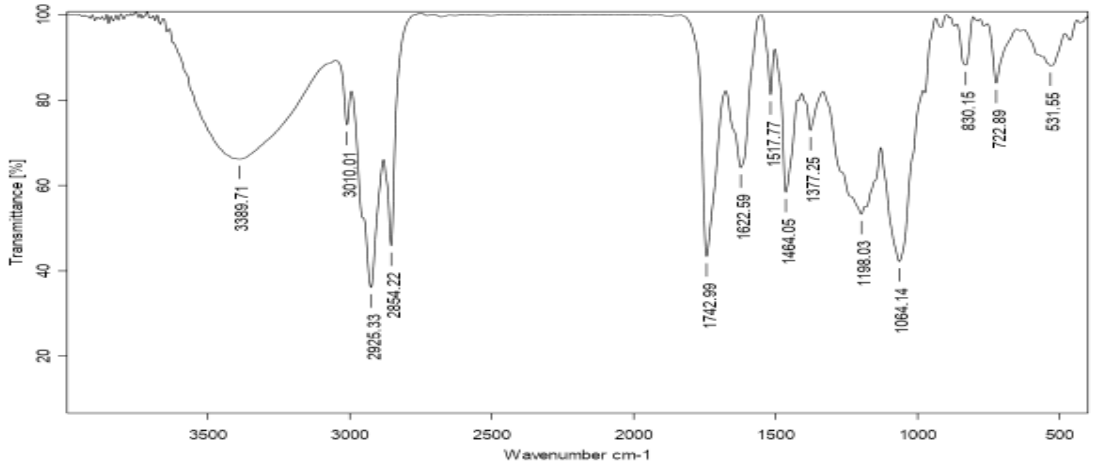

Figure 2: FTIR spectroscopy of Black Soybean extract

NMR analysis of Black Soybean extract

Proton NMR (1H NMR) was used to examine the structural characteristics and ascertain the presence of flavonoid compounds in the sample. The spectrum obtained was typical of proton signals observed in the chemical environments of flavonoids, as indicated in Figure 3. There were several resonances in the aromatic region 6.0-8.0ppm, which are assigned to aromatic protons on both A- and B-rings of the flavonoid backbone. These were indicative of the presence of substituted aromatic systems and were compatible with the phenylchromen-4-one structure found in flavonoids, including flavones and flavanols. A small signal of the 9.0-10.0 ppm is that the -OH protons are hydrogen-bonded, which implies that it has free hydroxyl groups, which are characteristic of polyphenolic

compounds. The fact that these exchangeable protons are present also helps determine the presence of flavonoid constituents. Moreover, some of the maxima observed in the d 3.0-5.5 ppm region are expected to correspond to hydrogen atoms on carbon atoms attached to oxygen moieties, which frequently represent glycosidic groups. Signals in this region indicate that the sample may contain flavonoid glycosides, which are common in naturally occurring flavonoids. Lastly, the aliphatic peaks (d 0.9-2.5 ppm) could have been due to aliphatic protons, such as methyl or methylene, or to solvents or components of the complex extract, if the sample was part of a complex. Overall, the 1H NMR spectrum contains important diagnostic signals from the aromatic, hydroxylated, and probably glycosidic proton environments. These properties are consistent with those of flavonoids, thus confirming their presence in the test sample. Though polar solvents like DMSO-d6 are usually the solvent of choice with flavonoid glycosides, CDCl3 gave well-resolved aromatic proton signals, which enabled qualitative structure determination of flavonoid compounds in the extract.

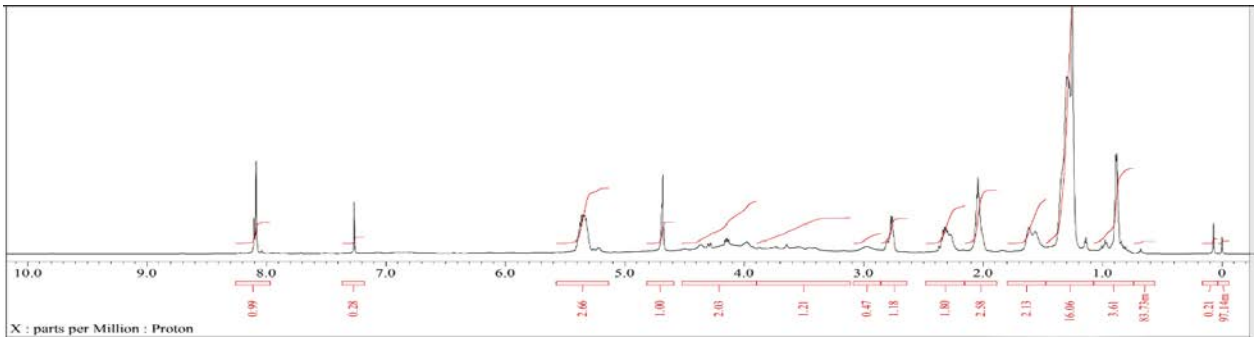

Figure 3: ^{1}H NMR Spectra of Black Soybean extract

HPLC isolation of flavonoids from Black Soybean extract

The analysis using high-performance liquid chromatography (HPLC) enabled unambiguous identification and quantification of rutin and quercetin in black soybean extract. Method suitability and column efficiency were confirmed by a sharp peak of a retention time (Rt) of 3.123 min & a peak area of 2180.31 mAUs & height of 407.01 mAU with the standard rutin. A similar peak rutin was observed in the extract with Rt = 3.110 min, a peak area of 1396.24 mAUs, and a height of 136.03 mAU. The retention times were very close, confirming the presence of rutin in the extract (Figure 4). Likewise, the quercetin peak of the black soybean extract was observed at 4.535 min with an area of 1762.48, which was very close to the retention time and chromatographic properties of quercetin standard peak as shown in Figure 5. Symmetry factors were similar (0.73 with extract) and trans-theoretical plate counts were similar (2866 with extract), indicating the same chromatographic efficiency & purity in the extract and standard. All these findings affirm that rutin and quercetin were successfully identified and isolated from the black soybean extract with high specificity and resolution, using a reliable method suitable for quantitative analysis.

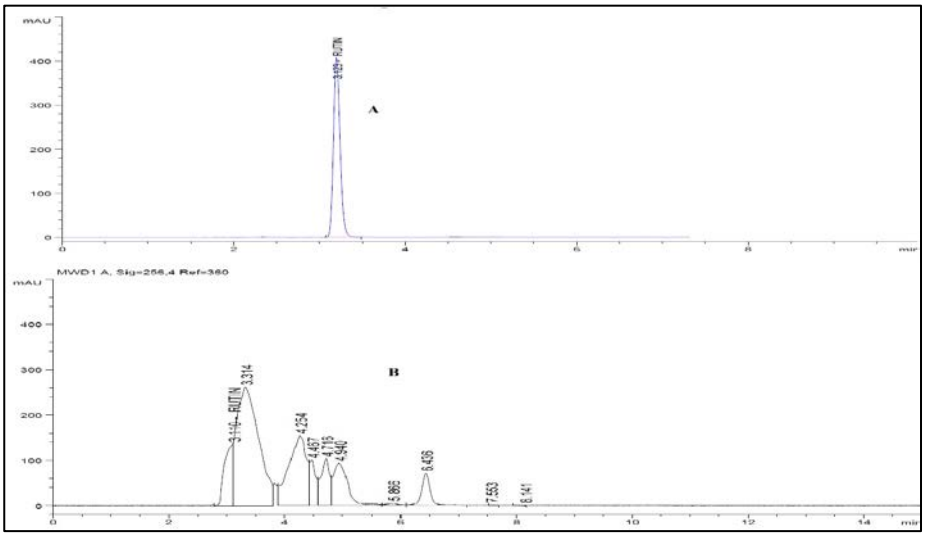

Figure 4: HPLC Chromatogram of rutin A. Standard and B. extract

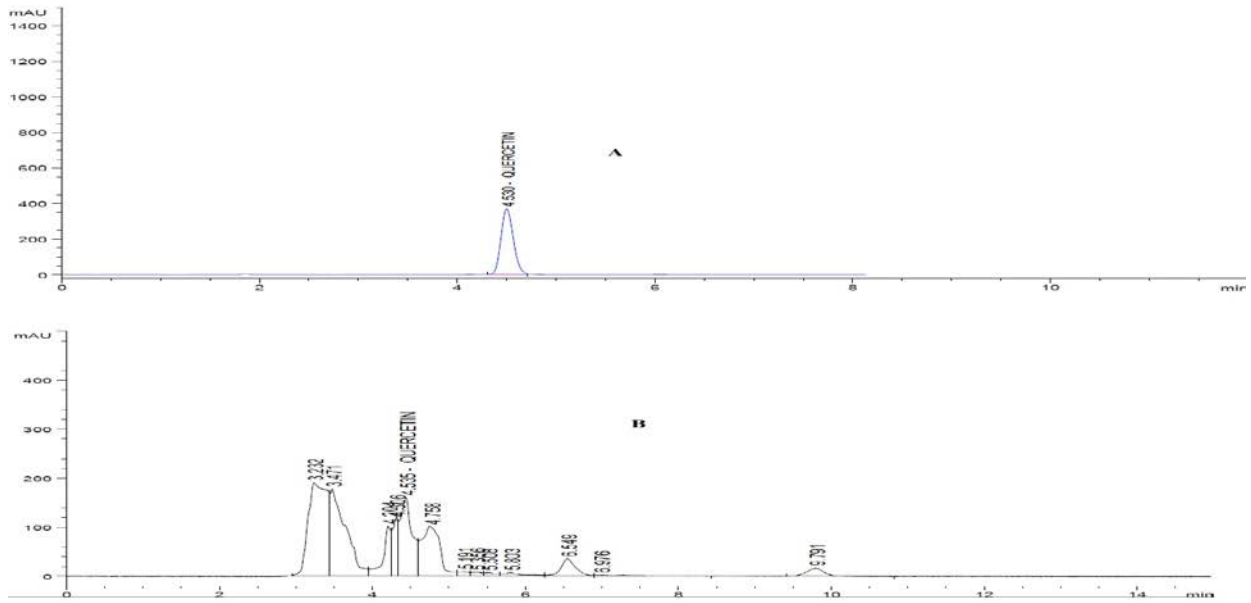

Figure 5: HPLC Chromatogram of quercetin A. Standard and B. extract

HPLC was used to determine the levels of rutin and quercetin in black soybean extract, and these results were statistically validated to ensure the method's reliability. Both the sample (3.110 min) and standard (3.123 min) retention times for rutin were similar, with a relative standard deviation (RSD) below 2.0, indicating good precision. The sensitivity of the method was determined using the limit of detection (LOD) and limit of quantification (LOQ) of rutin as 0.05 ug/mL and 0.15 ug/mL, respectively. Also, quercetin analysis revealed a constant retention time for the standard (4.530 min) and the sample (4.535 min), with an RSD of less than 2%. The LOD and LOQ of quercetin were 0.03 ug/mL and 0.10 ug/mL, respectively, showing high sensitivity. The two analytes exhibited high peak symmetry and a high theoretical plate count, indicating high chromatographic efficiency. These statistical approvals confirm that the HPLC technique is accurate, precise, and sensitive in the quantification of rutin and quercetin in black soybean extract. Table 2 enlists the method validation parameters of rutin and quercetin.

Table 2: Method Validation Parameters of rutin and quercetin

Parameter	Rutin	Quercetin
Linearity range	0.4–100 µg/mL	0.4–100 µg/mL
Correlation coefficient (R^2)	>0.999	>0.999
LOD	0.05 µg/mL	0.03 µg/mL
LOQ	0.15 µg/mL	0.10 µg/mL
Precision (RSD %)	<2%	<2%
Accuracy (%)	98–102%	98–102%

Flavonoid content of **Black Soybean extract**

An extract containing quercetin was used to determine the total flavonoid content. A standard curve drawn between the range of 20-100 ug/mL exhibited a high linear relationship as shown in Figure 6. The calculated regression equation yielded a concentration of 5.91 µg/mL, which was consistent with the measured absorbance of the sample (0.379). Based on a weight of the sample of 1 mg, and an extract of 1 mL, the overall flavonoid content was determined to be 5.91 mg QE/g of sample. This outcome supports the fact that the extract is a valuable source of flavonoid compounds, which may contribute to its possible biological activity.

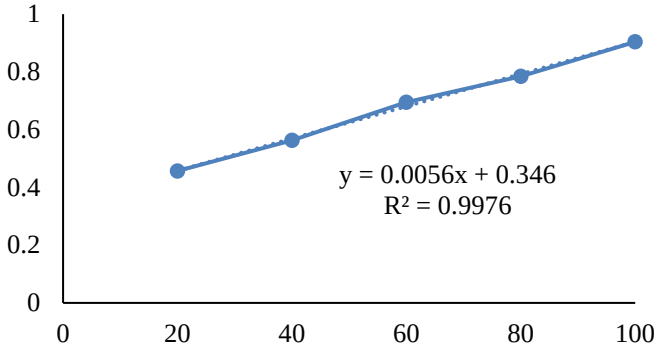

Figure 6: Calibration curve of Quercetin

Antioxidant activity of Black Soybean extract

The DPPH radical-scavenging assay was used to determine the sample's antioxidant potential. The findings reflected a concentration-specific growth of the portion of radical inhibition. Ascorbic acid, the standard, had inhibitory

percentages of 12.52 and 81.71 at 20 ug/mL and 100 ug/mL, respectively; the sample had percentages of 34.00 and 79.77 at the same concentrations. These results show that the sample has significant free-radical-scavenging capacity equivalent to that of ascorbic acid, implying the presence of strong antioxidant constituents.

DISCUSSION

The current study indicates that black soybean (*Glycine max L.*) is a major source of bioactive flavonoids with strong antioxidant properties, as verified through detailed spectroscopic and chromatographic profiling and tested bioactivity. The highest extraction efficiency (14%) was obtained with 70% aqueous ethanol because flavonoid glycosides are the most polar. UV-visual spectroscopy was used to determine the skeletons of flavone/flavonols (Band II: 240-280 nm; Band I: 300-380 nm). Typical functional groups in FTIR were determined to be phenolic O-H (3389 cm^{-1}), carbonyl (1724 cm^{-1}), aromatic C=C (1622, 1517 cm^{-1}) and glycosidic C-O-C (1084 cm^{-1}) that were characteristic of flavonol glycosides.^{1}H NMR either structural verification (aromatic protons were used, δ 6.0-8.0 ppm hydrogen-bonded 5-OH δ 9.0-10.0 ppm and anomeric protons δ 3.0-5.5 ppm) was definitive as flavonol glycosides.

HPLC clearly determined the presence of rutin and quercetin through the retention time of rutin and quercetin (rutin: 3.110 vs 3.123 min; quercetin: 4.535 vs 4.530 min). The method validation showed high precision (RSD <2%), sensitivity (LOD: 0.03-0.05 mg/mL), and linearity (r2 > 0.999). Rutin was the predominant form, consistent with its being the glycosylated form of storage. The total flavonoid value was 5.91 mg QE/g. The amount of flavanol observed in the current study (5.91 mg QE/g) was relatively higher than earlier reported values for soybean genotypes (0.68-2.13mg QE/g) [22]. Moreover, research on black soybean varieties observed total phenolic content ranging between 1.8 and 5.5 mg GAE/ g, showing a high variation based on the genotype and environment [23]. They can be attributed to geographical origin, climatic conditions, and extraction efficiency, especially in black soybeans grown in the Himalayas, where phytochemical accumulation could be higher.

The extract exhibited strong DPPH radical-scavenging activity (79.77% inhibition at 100 mg/mL; IC_{50} 41mg/mL), similar to ascorbic acid (81.71%). Flavonoids are the primary contributors, with strong correlations (r2 > 0.95) between TFC and antioxidant activity. The ortho-dihydroxy B-ring structure of quercetin facilitates easy delocalization of electrons and metal chelation, and rutin has more solubility and gastrointestinal stability, although with slightly lower intrinsic scavenging reaction rates. Flavonoids have antioxidant effects through hydrogen-atom donation and transition-metal chelation, with complementary cytoprotective actions. The results confirm the use of black soybean in oxidative stress-related disorders and support its evolution as a natural alternative to synthetic antioxidants.

The TFC is comparable to values found in traditional yellow soybean varieties, which can be explained by the increased flavonoid biosynthesis induced by environmental elicitors (UV-B, thermal stress) during seed development in Himalayan seeds. Unquantified flavonoid (kaempferol glycosides, isoflavones) can be a contribution to underrated bioactivity. In vitro findings should be validated by an in vivo review of the pharmacokinetic and efficacy studies. It is also possible that the coexistence of rutin and quercetin leads to synergistic antioxidant activity through complementary actions in radical scavenging and metal cation binding, thereby increasing overall bioactivity. The next steps in work should focus on the LC-MS/MS/MS metabolomic profiling, bioavailability evaluation, formulation development, and clinical trials.

CONCLUSION

In the current research, black soybean (*Glycine max L.*) was shown to be a promising source of bioactive flavonoids, particularly rutin and quercetin, as determined by a combination of spectroscopic and chromatographic analyses. The extract exhibited high antioxidant activity, suggesting it can be used to reduce oxidative stress. These results show the potential of black soybean as a natural component for formulating nutraceutical and functional foods. Research on bioavailability and in vivo efficacy should be furthered to reinforce its therapeutic uses.

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FINANCIAL ASSISTANCE

NIL

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

Chandraprabhu Jangme conceptualized and organized the study. Pramod Kumbhar and Mohini Salunke handled the method, examination, data gathering, and analysis. Balaji Wakure helped to write this paper. The manuscript was written in collaboration by all of the contributors.

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