Antioxidant and Pharmacognostic Evaluation of *Achyranthes Aspera*: Therapeutic Potential and Analytical Validation of Secondary Metabolites

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Abstract

The therapeutic potential of plants in human health has been recognized since ancient times, with Achyranthes aspera (A. aspera) being historically used to treat various ailments. This study aimed to standardize A. aspera by evaluating multiple pharmacognostic parameters, including microscopic analysis, organoleptic assessment, and forthcoming analytical techniques. Dry powdered plant material was extracted using methanol, followed by liquid-liquid extraction with dichloromethane. The extracts were screened for different phytoconstituents, and their total phenolic and flavonoid contents were assessed using gallic acid and quercetin standards, respectively. The antioxidant activity of the methanolic and dichloromethane extracts was tested in vitro against the free radical DPPH. The methanolic extract exhibited maximum antioxidant efficacy, with an IC50 value of 7.61 µg/ml, while the dichloromethane extract showed an IC50 of 14.25 µg/ml, compared to standard ascorbic acid. Scanning electron microscopy (SEM) analyzed the surface morphology of the dried powder, and atomic absorption spectroscopy (AAS) confirmed the absence of toxic metals in both extracts. High-performance liquid chromatography (HPLC) revealed 55.965% quercetin content, indicating a high concentration of flavonoids. Fourier transform infrared spectroscopy (FTIR) identified functional groups like ethyl amines, alkenes, alkanes, primary nitro groups, and esters, contributing to the plant's enhanced stability. These findings highlight A. aspera's potential as a source of bioactive compounds with antioxidant properties, confirming its historical use and paving the way for future pharmacological applications.

Keywords: Achyranthes aspera, Pharmacognostic evaluation, Secondary metabolites, Analytical techniques, Herbal medicine.

Significance | The study demonstrated Achyranthes aspera's therapeutic properties, supporting its traditional use through pharmacognostic analysis and modern analytical techniques.

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Editor Md Shamsuddin Sultan Khan, And accepted by the Editorial Board Mar 15, 2024 (received for review Jan 18, 2023)

Introduction

Medicinal plants have played a vital role in healthcare across human communities for centuries. A significant portion of ethno-medicinal products consists of plant extracts, and approximately half of the medicinal products used in clinical settings are derived from natural substances or their derivatives (Balunas & Kinghorn, 2005). Herbal medicines fulfill 80% of global health needs, with 20-25% of medical prescriptions involving herbal formulations or semi-synthetic drugs derived from plants (Balunas & Kinghorn, 2005).

Ethnopharmacology, a multidisciplinary field, investigates and validates the use of traditional natural and plant-based products, aiming to document and preserve cultural traditions (Gilani, 2005). Civilizations such as Greco-Arab Egyptian, Chinese, Western, and

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Please cite this article:
Muhammad Rashad, Muhammad Athar Adil et al. (2024). Antioxidant and Pharmacognostic Evaluation of Achyranthes Aspera: Therapeutic Potential and Analytical Validation of Secondary Metabolites, Australian Herbal Insight, 7(1), 1-14, 9692

Kampo have practiced folk remedies since ancient times. For instance, quinine derived from cinchona bark was traditionally used to alleviate malaria symptoms even before the disease was formally identified (Eid et al., 2013). Despite significant advancements in synthetic chemistry, many traditional treatments remain irreplaceable, highlighting the importance of cultural knowledge passed down through generations (Gilani, 2005).

If a plant extract demonstrates beneficial activity in disease prevention or treatment, there is potential to isolate and test its active compounds, which could eventually lead to the development of synthetic or semi-synthetic treatments (Gilani, 2005). Classical medicines often contain plant extracts with varying potencies of chemical constituents. While traditional medicines may have broader therapeutic effects, conventional medicines formulated with well-characterized active ingredients tend to have narrower therapeutic windows (Vaghasiya et al., 2011).

During growth and development, plants produce primary metabolites that are further converted into secondary metabolites through biosynthesis. These secondary metabolites play a significant role in combating microbes and pests and serve as essential components in various pharmaceutical formulations (Saadullah et al., 2022). Numerous herbal medicines have demonstrated their effectiveness in treating microbial infections, cancer, inflammatory diseases, pain relief, immune system stimulation, and digestive improvements. These phytochemicals, including polyphenols, saponins, flavonoids, and vitamins, are pharmacologically important (Garrido et al., 2016). There is scientific evidence supporting their beneficial roles in conditions such as coronary artery disease, various cancers, diabetes mellitus, psychiatric disorders, infections, and spasmodic conditions (Michalak, 2006).

Plants are considered a convenient source of antioxidants and essential molecules. Methods such as the Total Oxidant Scavenging Capacity (TOSC) and DPPH (free radical scavenging) are widely used due to their excellent repeatability, reproducibility, and ease of implementation (Michalak, 2006). The objective of this study is to identify the secondary metabolites present in the plant and validate various analytical techniques for their detection.

2. Materials and methods

2.1 Plant collection

The plant was collected in September 2018 from Rehman Garden, Lahore, Punjab, Pakistan. Its verification was conducted by Dr. Zaheer-ud-Din Khan, a Professor in the Botany Department at Government College University Lahore. A voucher specimen with the number 3590 was allocated, and the plant specimen was submitted to the herbarium.

2.2 Preparation of extract

The experimental research was carried out at the Faculty of Pharmacy, The University of Lahore, Pakistan. The collected plant material was thoroughly examined to eliminate any extraneous weeds, washed with distilled water, and then dried in the shade. After drying completely, the plant material was ground with an electric grinder. The resulting dried plant powder was macerated in analytical-grade methanol at room temperature for three days and then filtered. The extract obtained was concentrated using a rotary evaporator under reduced pressure at 37°C. This procedure was repeated with dichloromethane as the solvent for another three days.

2.3 Pharmacognostic features of A. aspera

The plant standardization process involved assessing critical pharmacognostic parameters according to World Health Organization guidelines. The organoleptic evaluation of A. aspera was carried out by observing its appearance, shape, color, texture, odor, taste, and fracture. Microscopic examination was performed on appropriate portions of shade-dried and finely powdered leaves using a microscope. Small samples of plant material were placed on glass slides, mounted with a few drops of chloral hydrate, and covered with cover slips. These slides were then observed under a digital microscope to identify specific diagnostic features, with photographic documentation captured using a camera Lucida.

2.4 Physicochemical analysis of Acyanthes aspera

The salient physicochemical metrics, including ash values, moisture content, swelling index, and foaming index, were analyzed following the guidelines provided by the World Health Organization (WHO) in 1998.

2.4.1 Moisture content

To determine the moisture content, 2 grams of powdered plant material was placed into a clean china dish that had been pre-dried in an oven, cooled in a desiccator, and accurately weighed using an electric balance. The china dish containing the powdered sample was then dried in an oven at 105°C for approximately 30 minutes. This process was repeated until a constant weight was achieved (Michalak, et al, 2006). The moisture content was then calculated using the following equation:

$$\text{Dry matter}(\%) = \left(\frac{\text{Weight of dried sample}}{\text{Weight ofsample before drying}}\right) \text{X } 100$$

$$\text{Moisture Content } (\%) = 100 - \text{Dry matter } (\%)$$

2.4.2 Ash values

To determine the percentage of the impurities in a synthetic substance such as silicate, phosphate, and carbonate. Ash values were calculated using the procedures recommended in Indian Pharmacopoeia (total ash, insoluble acid ash, and water-soluble ash) (Patel et al, 2012). Dried test sample powder was prepared and sifted and further processed via sieve No. 20.

2.4.3 Total ash

A clean silica crucible was dried and kept in a desiccator at room temperature until a constant weight was achieved. The empty crucible was then weighed, and approximately 2 grams of powdered plant material was added to it. The crucible was heated in a furnace for about 15 minutes until carbon-free ash was obtained (Saadullah et al, 2023). After removing the crucible from the furnace, it was cooled in a desiccator until a constant weight was reached. The final weight of the crucible was recorded, and the percentage of total ash was calculated as follows:

$$Ash\ (\%) = Weight\ of\ \frac{Ash}{Powder\ sample}\ X\ 100$$

2.4.4 Acid insoluble ash

A minimum of 25 ml of 2 N diluted HCl was added to the crucible, and the mixture was boiled for approximately 5 minutes. The insoluble residue was then collected on filter paper, washed with warm water, and transferred back to the crucible. The crucible was heated repeatedly until a constant weight was achieved.

2.4.5 Water soluble ash

Add 25 ml of distilled water to the total ash, and simmer for 5 minutes. The insoluble residue was first deposited on an ashless filter pad, then washed with hot water and transferred to a crucible. The crucible containing the insoluble ash was heated at 450°C for 15 minutes and weighed. This process was repeated until a constant weight was obtained. The weight of this residue was subtracted from the total weight of ash, and the difference in weight was used to determine the water-soluble ash content .

2.4.6 Determination of foaming index

Plant material (1 g) was transferred to a 100 ml conical flask (distilled water) and the aqueous decoction was boiled for about 30 minutes. Hence, the elimination of impurities was first filtered and then sequentially transferred to 10 test tubes, i.e. 1 ml, 2 ml, 3 ml-10 ml. Added water to alter the volume of each test tube to 10 ml. After measuring foam height, each test tube was carefully stopped and shaken vigorously for 15 minutes, then allowed to stay in each test tube for 15 minutes.

The foaming index was measured using the proceeding formula

$$Foaming\ Index = \frac{1000}{a}$$

a = Volume (ml) of decoction used for preparing the dilution in the tube where foaming of height of 1 centimeter observed.

2.4.7 Determination of swelling index

The following swelling index of plant content was used to assess the foaming index, often associated with significant biological utilities. The swelling index measures the amount of mucilage in a specific herbal product, defined as the volume in milliliters occupied by the swelling of 1 gram of plant material under given conditions (Sosa et al, 2016).

To determine the swelling index, 1 gram of accurately weighed powdered plant sample was transferred to a 25 ml stoppered, alcohol-rinsed measuring cylinder, and 25 ml of distilled water was added. The measuring cylinder was securely stoppered and the mixture was shaken at ten-minute intervals for about sixty minutes. The sample was then allowed to stand for 180 minutes. Three consecutive measurements were taken, and the volume occupied by the plant material and adhering mucilage was recorded in milliliters.

2.4.8 Fluorescence analysis

The fluorescence characteristics were calculated by taking 1 g of precisely weighted powdered plant in each of the 8 test tubes. Every test tube was then filled with 10 ml of chloroform, methanol, distilled water, 5% ferric chloride, 1 N H2SO4, HCl, NaOH and HNO3, so that each test tube was shaken gently and allowed to remain for 45 minutes. Each test tube was then placed in the UV chamber and examined at wavelengths of 254 nm (short) and 365 nm (long) for change of color at daylight as well as UV light (Sosa et al, 2016).

2.5 Preliminary phytochemical screening of Acyranthes aspera

Phytochemical evaluation for thwereetection of essential phytoconstituents in plant extracts (methanol and dichloromethane) was conducted following standard procedures explained in Supplementary data.

2.6 Quantitative analysis of A. aspera

2.6.1 Estimation of total phenolic content

The evaluation of total phenols in various A. aspera extracts (methanol and dichloromethane) was conducted with some modifications to the procedure.

A stock solution (1 mg/ml) was prepared by dissolving 4 mg of each plant extract (methanol and dichloromethane) in 4 ml of methanol. To prepare the standard gallic acid solution, 5 mg of gallic acid was dissolved in 5 ml of methanol and further diluted to create serial dilutions (10, 20, 40, 80, 100, and 120 µg/ml).

For the quantitative estimation of total phenols in each plant extract, 1 ml of plant extract was dissolved in 9 ml of distilled water. To this mixture, 1 ml of Folin-Ciocalteu (FC) reagent was added, followed by vigorous shaking for 5 minutes. Then, 10 ml of 7% sodium carbonate solution was added, and the final volume was adjusted with distilled water up to 25 ml. The same protocol was applied to the regular gallic acid standards (10, 20, 40, 80, 100, and 120 µg/ml) as well as a blank solution.

These solutions were then incubated at room temperature in a dark place for 1.5 hours, and the absorbance was measured at 750 nm using a UV/Vis spectrophotometer (UV-1800, Shimadzu, Tokyo, Japan) (Telagari & Hullatti, 2015).

The concentration of phenols was determined using calibration curves constructed from the standard gallic acid solutions, and the total phenolic content was expressed as mg of gallic acid equivalent per gram of plant extract (mg gallic acid/g plant extract).

2.6.2 Estimation of total flavonoid content

The colorimetric aluminum chloride method was used to estimate total flavonoids in A. aspera extracts. Initially, 4 mg of each plant extract was dissolved in 4 ml of methanol to create a stock solution. From this stock solution, 200 µl was taken and mixed with 100 µl of aluminum chloride and 1 M potassium acetate, followed by dilution with 4.6 ml of distilled water. Similarly, standard quercetin was prepared at a concentration of 1 mg/ml and diluted to generate serial dilutions (10, 20, 40, 80, 100, and 120 µg/ml). Both the sample and standard solutions were then left at room temperature for 30 minutes of incubation. After incubation, the absorbance was measured at 415 nm using a UV/Vis spectrophotometer. The content of flavonoids in the plant extracts was calculated based on the calibration curve constructed using the standard quercetin solutions. The results were expressed as mg of quercetin equivalent per gram of plant extract (mg quercetin/g extract).

2.6.3 Antioxidant activity by DPPH free radical scavenging method

In each test tube, both the sample solution and standard solutions with specific concentrations (62.5, 125, 250, 500, and 1000 µg/ml) were prepared. To each tube, 2 ml of freshly prepared DPPH (2,2-diphenyl-1-picrylhydrazyl) solution was added, followed by the addition of 1 ml of methanol to reach a total volume of 4 ml per tube. The mixture was thoroughly shaken and then incubated at room temperature for 30 minutes in the dark. A standard solution of ascorbic acid was used as a positive control, while a negative control was prepared by adding 2 ml of DPPH solution to 1 ml of methanol. The absorbance of each sample was measured at 517 nm using methanol as a blank. This entire process was conducted in triplicate to ensure accuracy and reliability of the results (Vaghasiyam & Chanda, 2011).

In terms of percentage inhibition of DPPH, the plant extract's antioxidant capacity was calculated using the following equation.

$$Inhibition\ (\%) = \left[\left(A(control) - \frac{A(test)}{A(control)}\right)\right] \times 100$$

While

Control = absorbance of negative control (DPPH + methanol)

Test = Absorbance of sample or standard

Nonetheless, by comparing inhibition rate to extract concentrations, IC50 was calculated from graph.

2.7 Analytical techniques

2.7.1 High performance liquid chromatography

The High-Performance Liquid Chromatography (HPLC) analysis of methanolic extract was conducted using a Chromatographic system (YL 9100, Korea), equipped with an autosampler (YL 9150) featuring a 100 µl fixed loop, and a YL9120 UV-Visible detector. The separation was achieved on an SGE Protecol PC18GP120 (250mm × 4.6mm, 5µm) column maintained at ambient temperature.

The mobile phase comprised a methanol to water ratio of 70:30 (v/v), and the separations were performed using an isocratic mode. The elution process was carried out at a flow rate of 1 ml/min. Samples were analyzed over a 15-minute run time, and detection was performed at 254 nm using the UV detector. All chromatographic data were meticulously recorded and processed using the Autochro-3000 software.

This method was employed to ensure precise separation and detection of the components present in the methanolic extract. The choice of a methanol-water mixture in a 70:30 ratio as the mobile phase was critical in achieving optimal separation efficiency. The isocratic mode, which maintains a constant composition of the mobile phase throughout the analysis, provided consistent and reproducible results.

The autosampler (YL 9150) played a vital role in ensuring accurate sample injection, reducing the potential for human error, and enhancing the repeatability of the analysis. The 100 µl fixed loop was used to introduce the sample into the system, ensuring consistent sample volume and improving the reliability of the results.

The SGE Protecol PC18GP120 column, with its dimensions of 250mm × 4.6mm and particle size of 5µm, was selected for its ability to provide high-resolution separation. The ambient temperature condition was maintained to prevent any thermal degradation of the analytes, ensuring the integrity of the sample components during the analysis.

Detection at 254 nm using the UV detector (YL9120) was chosen due to the strong absorbance of many organic compounds at this wavelength, allowing for the sensitive detection of the methanolic extract components. The UV detector provided quantitative data on the concentration of the analytes, which were crucial for subsequent data analysis and interpretation.

The chromatographic data collected were processed using the Autochro-3000 software, which facilitated efficient data handling, integration, and analysis. This software ensured accurate peak identification, quantification, and provided a comprehensive overview of the chromatographic profile of the methanolic extract.

The High-Performance Liquid Chromatography (HPLC) analysis of methanolic extract was meticulously carried out using the Chromatographic system (YL 9100, Korea). This system featured an autosampler (YL 9150) equipped with a 100 µl fixed loop, and a YL9120 UV-Visible detector. The separation process utilized an SGE Protecol PC18GP120 column (250mm × 4.6mm, 5µm), operating at ambient temperature. The mobile phase comprised a methanol to water ratio of 70:30 (v/v), and the separations were executed using an isocratic mode. The elution was conducted at a flow rate of 1 ml/min. The samples were analyzed for a duration of 15 minutes, with detection carried out at 254 nm by the UV

detector. All chromatographic data were recorded and processed using the Autochro-3000 software.

The mobile phase of methanol and water in a 70:30 ratio was carefully selected to optimize the separation efficiency. Methanol, a common organic solvent in HPLC, provides good solubility for many organic compounds, while water helps in modulating the polarity of the mobile phase, ensuring adequate separation of analytes. The isocratic mode, maintaining a constant mobile phase composition throughout the run, was chosen for its simplicity and reproducibility, making it ideal for routine analysis.

The flow rate of 1 ml/min was established to balance between sufficient time for separation and overall run time efficiency. At this flow rate, the analytes had ample time to interact with the stationary phase, facilitating effective separation without causing excessive backpressure that could damage the column or the system.

The 15-minute runtime was deemed optimal for the separation process, allowing for the complete elution of analytes within a reasonable timeframe. The detection wavelength of 254 nm was selected based on the absorption characteristics of the compounds of interest, many of which absorb UV light strongly at this wavelength. This choice ensured high sensitivity and specificity in detecting the analytes.

The autosampler (YL 9150) played a crucial role in enhancing the precision and accuracy of the analysis. By automating the sample injection process, it minimized human errors and variability, ensuring consistent sample volumes and improving the reproducibility of the results. The 100 µl fixed loop facilitated the introduction of a precise and consistent sample volume into the chromatographic system, which is vital for reliable quantification and comparison of results.

The SGE Protecol PC18GP120 column, with its dimensions and particle size, was selected for its high-resolution capabilities. The 250mm length provided sufficient surface area for interaction between the analytes and the stationary phase, while the 4.6mm internal diameter ensured a manageable flow rate and column backpressure. The 5µm particle size offered a good balance between resolution and column efficiency, allowing for sharp and well-defined peaks.

Maintaining the column at ambient temperature was crucial to prevent thermal degradation of sensitive analytes, ensuring their integrity throughout the analysis. Temperature control is important in HPLC as fluctuations can affect the retention times and peak shapes, potentially compromising the accuracy of the analysis.

Detection at 254 nm using the YL9120 UV-Visible detector was a strategic choice. UV detection is widely used in HPLC due to its sensitivity and ability to detect a wide range of compounds. The selected wavelength of 254 nm corresponds to the absorption maxima of many organic compounds, making it ideal for the analysis of methanolic extracts. The UV detector provided quantitative data on the concentration of the analytes, which was essential for further data analysis and interpretation.

The chromatographic data collected were processed using the Autochro-3000 software, which facilitated efficient data handling, integration, and analysis. This software ensured accurate peak identification and quantification, providing a comprehensive overview of the chromatographic profile of the methanolic extract. The ability to process and analyze the data accurately is critical in HPLC analysis, as it directly impacts the reliability and validity of the results.

Methanolic extract analsyis

The methanolic extract was analyzed using HPLC (High Performance Liquid Chromatography) on a Chromatographic system (YL 9100, Korea), which included an autosampler (YL 9150) with a fixed loop of 100 µl and an YL9120 UV-Visible detector. The separation was conducted on a SGE protocol, PC18GP120 column (250mm × 4.6 mm, 5µm), maintained at room temperature. The mobile phase consisted of a mixture of methanol and water in a ratio of 70:30 (v/v), and the separation was carried out in an isocratic mode with a flow rate of 1 ml/min. Each sample was analyzed over a 15-minute duration, and detection was performed at 254 nm using the UV detector. All chromatographic data were recorded and subsequently processed using the Autochro-3000 software.

Amount of Quercetin was calculated by following formula

$$Amount\ of\ Quercetin = \frac{Concentration\ of\ Sample}{Concentration\ of\ Standard} \times \frac{weight\ of\ Standard}{weight\ of\ Sample} \times Potency$$

2.7.2 Scanning Electron Microscopy

In this research study, Scanning Electron Microscopy (SEM) was performed on various plant parts using SEM Quanta 650 FEG. The SEM instruments were equipped with a Bruker QUANTAX EDS XFlash 6 detector for elemental analysis.

Fresh plant samples were carefully sectioned to approximately 4-8 mm in size. These samples were then placed onto a drop of water on the Peltier cooling stage, which was equipped with a specialized fat cylindrical brass sample holder. The use of water on the cooling stage allowed for the observation of samples in their fully hydrated state.

During the SEM imaging of fully hydrated samples, the environmental conditions were set as follows: the sample temperature was maintained at 3 °C, and the pressure was controlled at 760 Pa of water vapor. The accelerating voltage used for the imaging process was 20 kV, and the probe current was set to 80 pA. Additionally, the environmental distance between the sample surface and the second pressure limiting aperture was fixed at 8.5 mm.

These specific conditions were chosen to ensure optimal imaging quality and prevent artifacts during the SEM analysis. The combination of SEM instruments, the EDS XFlash 6 detector, and the controlled environmental parameters allowed for comprehensive imaging and elemental analysis of the plant samples, providing valuable insights into their microstructural features and elemental composition. (Vaghasiya & Chanda, 2011).

2.7.3 Fourier Transform Infrared Spectroscopy

FTIR fingerprinting was used to do qualitative analysis on crude powdered plant extract. A powdered sample of A. aspera was introduced on a sample plate in Bruker's FTIR. This yielded an FTIR spectrum in the 4000-400cm-1 IR band. The peaks representing different regions were compared to typical organic compound peaks (Veeresham et al, 2012).

2.7.4 Atomic Absorption Spectroscopy

A fine powder of A. aspera was utilized for the determination of major, minor, and trace elements. Ten grams of the finely ground powder were taken in a pre-cleaned silica crucible. The crucible was heated in an oven at a temperature range of 300-400°C for a duration of 2-3 hours. After heating, the crucible was allowed to cool to room temperature inside a desiccator. The resulting ash obtained from the heating process was used to prepare a solution.

One gram of the obtained ash was accurately weighed and mixed with 25 ml of concentrated HCl and 25 ml of double distilled deionized water. The mixture was stirred thoroughly to obtain a clear solution and then filtered using Wattman filter paper 41. Subsequently, 950 ml of deionized double-distilled water was added to the filtered solution to make a total volume of one liter (1000 ml) of solution.

The prepared solutions were then utilized for the quantitative measurement of minerals and trace elements using the Atomic Absorption Spectroscopy (AAS) technique. AAS is a powerful analytical method that enables the determination of the concentration of various elements in the solution (Yao et al. 2004).

3. Results

3.1 Percentage yield of Acyranthes aspera

The dried leaves of A. aspera (400 g) were subjected to cold maceration using analytical grade methanol as the solvent. This extraction process yielded approximately 400 grams of powdered leaf material. From this powder, a crude methanol extract was obtained, weighing 20.75 grams. This represents a percentage yield of 5.1% based on the initial weight of the dried leaf powder. Subsequently, 15 grams of the crude methanol extract underwent further fractionation using dichloromethane as the solvent. The percentage yield from this fractionation step was calculated and recorded. The percentage yield of both the extracts are enlisted in Table 1, and physical features in Table 2.

For the determination of percentage yields of Acyranthes aspera extracts obtained using various solvents, the following formula was employed:

Percentage yield = [(Actual yield/Theoretical yield)] × 100

3.2 Pharmacognostic studies of A. aspera

3.2.1 Organoleptic evaluation

The organoleptic properties, encompassing characteristics such as color, odor, taste, texture, and appearance, of both fresh and desiccated leaves of the A. aspera plant were meticulously observed and documented. The empirical findings pertaining to these sensory attributes were systematically cataloged and presented in Table 3 for comprehensive analysis and comparison.

3.2.2 Microscopic evaluation

The powdered material derived from A. aspera was subjected to microscopic examination utilizing a digital microscope equipped with a 10X eyepiece and a 40X objective lens. The various microscopic features of the plant material, including the xylem, phloem, and pitted fibers, were meticulously observed with the aid of the software integrated into the digital microscope. The observed microscopic characteristics were subsequently authenticated by cross-referencing with the Atlas of American Herbal Pharmacopoeia (AAHP, 1994). The results of this microscopic analysis were compiled and visually represented in Figure 1.

3.3 Proximate analysis of Acyranthes aspera

A comprehensive analysis was undertaken to evaluate the diverse physicochemical properties of A. aspera. The investigation encompassed an array of parameters, including moisture content, total ash value, acid-insoluble ash, water-soluble ash, alcohol-soluble extractive value, and water-soluble extractive value. The experimental procedures adhered to established methodologies outlined in recognized pharmacopoeial references. The resultant data obtained from this rigorous examination of the physicochemical characteristics were meticulously recorded and presented in Table 4.

3.4 Fluorescence analysis

The desiccated and pulverized plant material was subjected to treatment with various distinct chemical reagents. The resultant interactions were meticulously observed for any discernible color changes under three distinct lighting conditions: daylight, short-wavelength ultraviolet (UV) light, and long-wavelength UV light. The outcomes of these observations, which provide insights into the potential phytochemical constituents present in the plant material, were methodically documented and visually represented in Figure 2. This approach facilitates the identification and characterization of the phytochemical profile of the plant species under investigation (Table 5).

3.5 Phytochemical analysis of Acyranthes aspera

A systematic qualitative phytochemical screening was conducted to investigate the presence of various phytochemical constituents in the extracts obtained from A. aspera. This comprehensive analysis involved the application of established chemical tests and reagents to detect the existence of diverse phytochemical classes, including alkaloids, glycosides, phenolic compounds, flavonoids, saponins, steroids, tannins, and terpenoids, among others. The extracts were subjected to a sequential series of specific chemical reactions, and the resulting observations, such as color changes, precipitate formation, or other characteristic responses, were meticulously documented. The outcomes of this qualitative phytochemical screening provided valuable insights into the phytochemical profile of the plant species under investigation, laying the foundation for further quantitative analyses and potential identification of bioactive compounds. Results are shown in Table 6.

3.6 Quantitative analysis of Acyranthes aspera

3.6.1 Total phenolic content

Total phenolic content of Acyranthes aspera (methanol and dichloromethane) extracts were determined by folin-ciocalteau method results were calculated by applying equation obtained by standard gallic acid curve (Figure 3).

$y = 0.0021x + 0.0562$, $R^2 = 0.9758$

The results obtained showed that crude methanol extract exhibited the highest phenolic content followed by dichloromethane extract (Table 7). (GAE 59.52 and 31.55 mg / g, respectively)

3.6.2 Total flavonoid content

Total flavonoid content in crude methanolic and dichloromethane extract of A.aspera were checked by applying standard quercetin curve (Figure 4) equation in terms of quercetin equivalent.

$y = 0.0051x + 0.2984$, $R^2 = 0.9638$

According to estimated results (Table 8) it was observed that total flavonoid content in different extracts of A. aspera was in order as methanol> dichloromethane (GAE 59.52 and 31.55 mg / g, respectively) respectively (Figure 5).

3.7 In vitro antioxidant activity of Acyranthes aspera by DPPH radical scavenging method

The antioxidant potential of different polarity extracts of A. aspera (methanol and dichloromethane) was evaluated at concentrations of 62.5, 125, 250, 500, and 1000 µg/ml. The percentage inhibition and IC50 values for all extracts were determined and summarized in Figure 6 and Figure 7, respectively. From the results, it was observed that all extracts exhibited lower activity compared to the standard ascorbic acid (IC50 8.242 µg/ml). However, the crude methanolic and dichloromethane extracts demonstrated significant antioxidant activity with IC50 values of 10.60 µg/ml and 12.24 µg/ml, respectively. It is important to note that a lower IC50 value indicates higher inhibitory activity against oxidation.

3.8 Analytical techniques

3.8.1 High performance liquid chromatography

The quantification of quercetin in the methanolic extract of A. aspera was performed using high-performance liquid chromatography (HPLC) with a reversed-phase C18 column and UV detection at 370 nm (Figure 8). The amount of quercetin was calculated by area under the peak.

Amount of Quercetin = 1334287 / 1175426 × 24.85 / 49.89 × 98.98 = 55.965 %

3.8.2 Fourier transform infrared spectroscopy

FTIR spectroscopy is a powerful analytical technique used to identify chemical bonds in a sample based on their absorption of infrared light. In our results we found different functional groups based on different peaks (Figure 9).

3.8.3 Scanning electron microscopy

The surface morphology and ultrastructural features of the powdered plant material derived from A. aspera were examined using scanning electron microscopy (SEM). Prior to analysis, the samples underwent meticulous preparation, including dehydration and sputter coating with a thin conductive layer to enhance image quality and resolution. The SEM analysis was performed under high vacuum conditions, employing an accelerating voltage optimized for the specific sample characteristics.

The SEM micrographs, acquired at various magnifications, provided detailed insights into the intricate surface topography, revealing the presence of intricate microstructures, such as trichomes, stomata, and other distinguishing features. These high-resolution images facilitated the identification and characterization of the plant material, potentially aiding in the authentication process and supporting further phytochemical investigations.

The SEM analysis complemented the microscopic observations conducted using light microscopy, offering a comprehensive understanding of the plant's structural characteristics at multiple scales, from the macroscopic to the nanoscale level (Figure 10).

3.8.4 Atomic absorption Spectroscopy

Atomic absorption is used for the detection of traces of metal elements. It follows the Beer's Law which states that absorption is directly proportional to concentration of solution.

Metals detected in both methanol & DCM extracts of A. aspera are expressed in Figure 11.

4. Discussions

Pharmacognostic studies are widely recognized as effective and objective methods for ensuring the safety and reproducibility of plant-based medicines. This study aims to evaluate the therapeutic potential of Achyranthes aspera (A. aspera) through various pharmacognostic procedures and to establish criteria that support its medicinal validity.

The extraction of secondary metabolites from A. aspera was performed using cold maceration, yielding significant amounts of phytonutrients, particularly when methanol and dichloromethane

Table 1. Percentage yield of crude methanolic extract and its fractions of A. *aspera*

Extract/Fraction	Dry weight (g)	Percentage yield (%w/w)
Crude methanolic extract	23.64	5.3
Dichloromethane fraction	13.89	2.96

Table 2. Physical features of *Acyranthes aspera* extracts (methanol and dichloromethane)

Extract	Consistency	Color	Odor
Methanol	Semi solid	brownish Green	Aromatic
Dichloromethane	Semi solid	brownish Green	Aromatic

Table 3. Organoleptic features of *A. aspera*

Features	Fresh	Dry
Taste	Salty	Bitter
Texture	Smooth	Smooth
Shape	Scale like	Scale like
Size	1.5-2 mm long	1.5-2 mm long
Fracture	Brittle	Brittle
Odor	Odorless	Odorless
Color	Bluish green	Bluish green

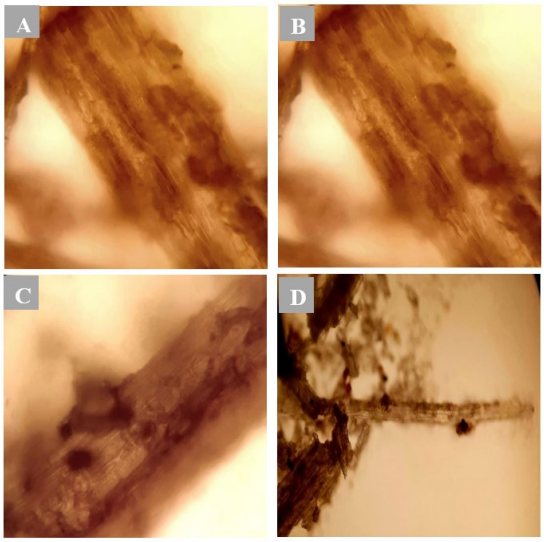

Figure 1. Microscopic studies of *A. aspera* (A) Xylem (B) Phloem (C) calcium oxalate crystals (D) pitted fibers

Table 4. Physicochemical features of *A. aspera*

Parameters	Observed Values	Official Limit
Total ash	6.9%	< 13%
Acid insoluble ash	6%	<10%
Water soluble ash	3%	0.5-5%
Moisture content	8.5%	8-14%
Swelling index	4.1 ml	Up to 5 ml
Foaming index	104.05	100-1000

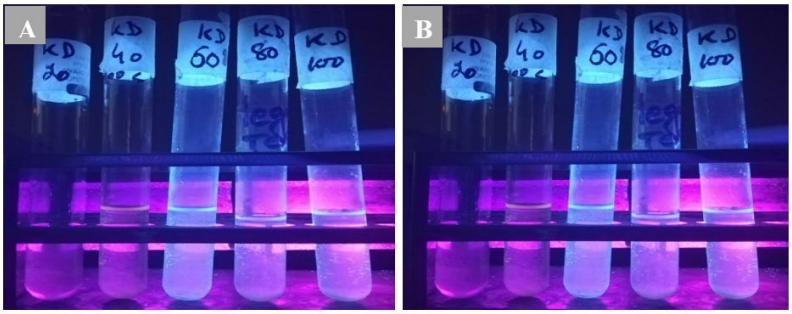

Figure 2. Fluorescence analysis of *A. aspera* under UV light (A, B).

Table 5. Fluorescence analysis of *A. aspera* under UV light at 245 nm and 365 nm.

(Sr #.)	Regents	Visible range	Short wavelength (245 nm)	Long wavelength (365 nm)
1	Drug +HCl	dark brown	dark brown	bluish black
2	Drug+distilled water	brown	dark brown	dark blue
3	Drug + methanol	light brown	light pink	dark pink
4	Drug + NaOH	Blackish brown	dark blue	bluish green
5	Drug+ Conc HNO_3	dark orange	light blue	dark blue
6	Drug+Conc H_2SO_4	Black	Blue	Blue
7	Drug + Chloroform	light brown	Pink	bright pink
8	Drug+Ferric Chloride	bottle green	bluish black	Black

Table 6. Qualitative phytochemical screening of *A. aspera* extracts

Chemical constituents	Test performed	Methanolic extract	Dichloromethane Extract
Glycosides	Legal's test	+	+
	Keller-Killani's test	+	+
Carbohydrates	Molisch test	-	+
	Barfoed's test	-	-
	Benedict's test	-	-
Alkaloids	Fehling's test	+	+
	Mayer's test	+	-
	Hager's test	+	-
	Wagner's test	+	-
Phenolics and tannins	Ferric chloride test	+	+
	Lead acetate test	+	+

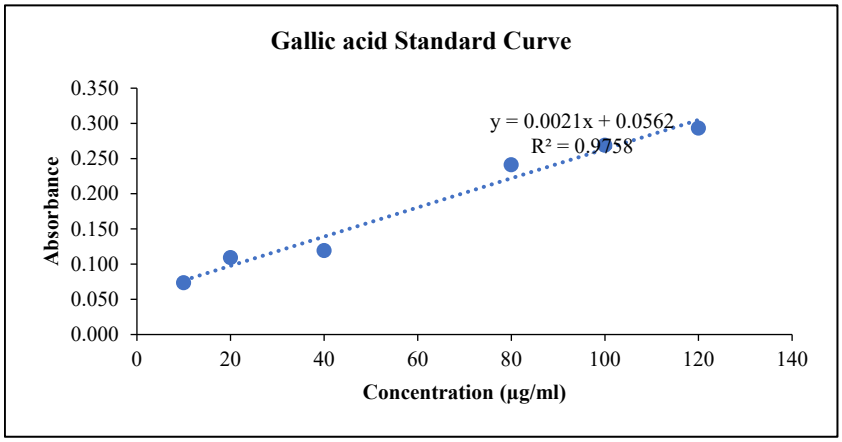

Figure 3. Gallic acid standard curve

Table 7. Total phenolic contents in *A. aspera*

Plant Extracts	Total phenolics (mg/g GA)± SD
Methanol	59.52 ± 1.402
Dichloromethane	31.55 ± 0.728

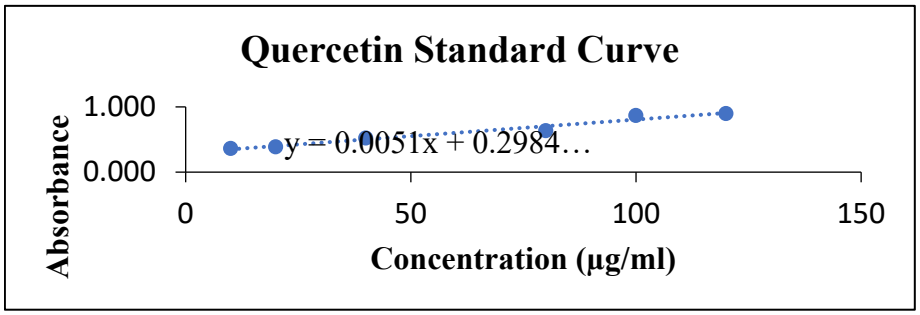

Figure 4. Quercetin standard calibration curve

Table 8: Total flavonoid content (mg/ gQE) ± SD

Extracts	Total flavonoid content (mg/g QE)± SD
Methanol	55.083 ± 0.300
Dichloromethane	43.633 ± 0.540

The values enlisted in above table shows mean± S.D of three values of flavonoids expressed as QE equivalent

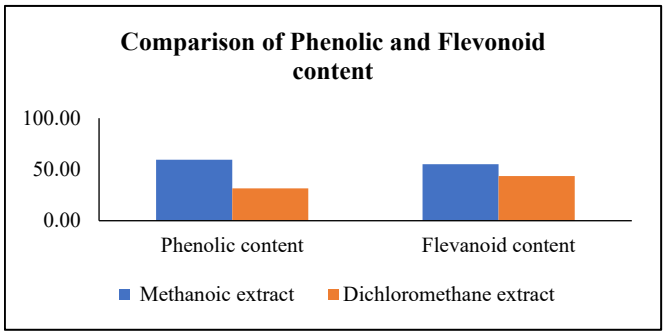

Figure 5. Total phenolic and flavonoid contents of *A. aspera* extracts

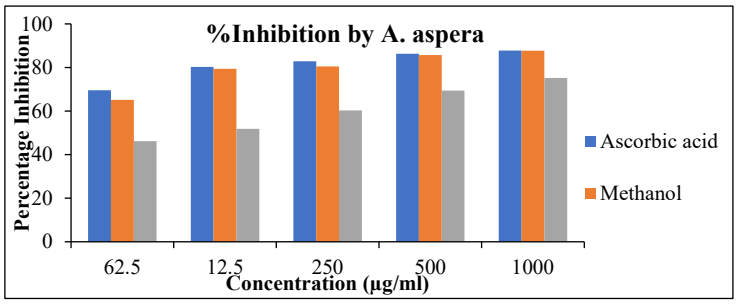

Figure 6. Free radical scavenging activity of *A. aspera* extracts and ascorbic acid

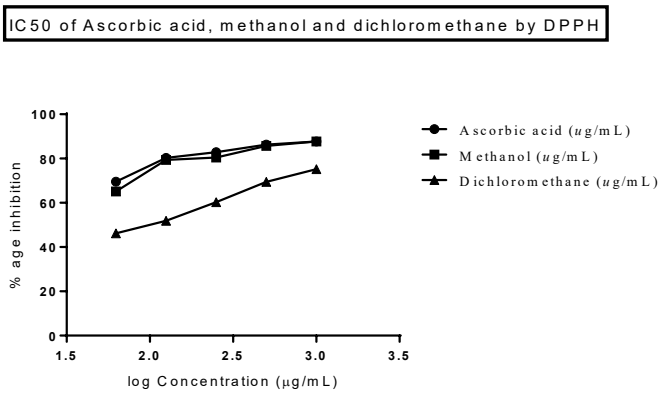

Figure 7. IC_{50} of ascorbic acid, methanol and dichloromethane

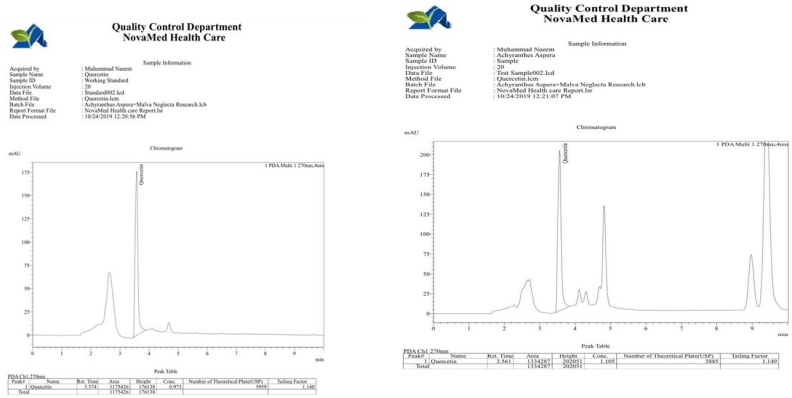

Figure 8. High performance liquid chromatographic spectrum of *A. aspera* and standard Quercetin

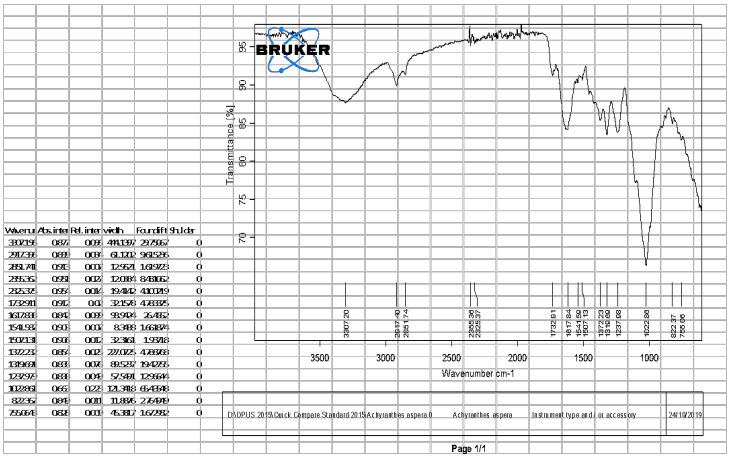

Figure 9. Fourier transform infrared spectrum of *A. aspera*

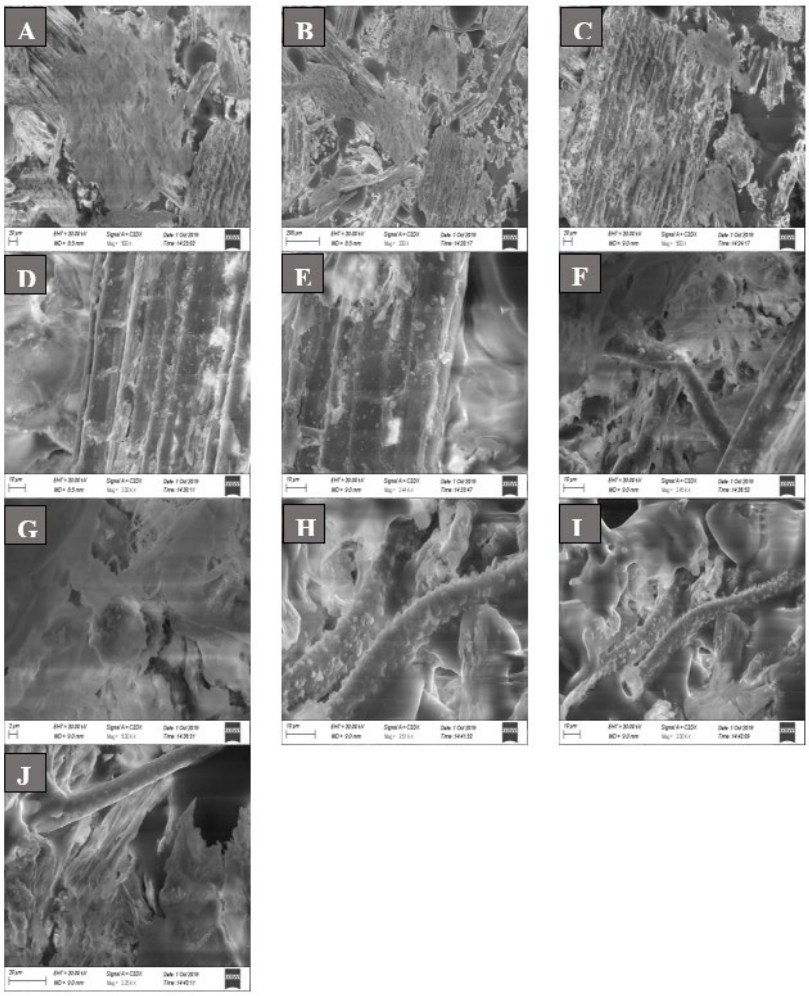

Figure 10. SEM analysis of A. *aspera*

were utilized as solvents. These solvents were found to be especially effective in extracting bioactive compounds due to their differential polarity, a finding consistent with other studies highlighting the optimal nature of methanol and dichloromethane for phytonutrient extraction (Veeresham, 2012; Santhiya et al., 2016).

Microscopic examination of the plant's outer portions revealed the presence of xylem, phloem, calcium oxalate crystals, and pitted fibers, corroborating findings from previous morphological studies (Telagari & Hullatti, 2015). Additionally, physicochemical parameters, which are critical for determining the purity and consistency of herbal medicines, were assessed. The moisture content of the dried powdered plant was measured at 8.5%, within the acceptable range of 8-14%, indicating good storage stability and low risk of fungal contamination, aligning with findings from other pharmacognostic studies of medicinal plants (Patel et al., 2012).

Ash content analysis showed values for total ash, acid-insoluble ash, and water-soluble ash at 6.9%, 6%, and 3%, respectively. These values exceed the limits set by the World Health Organization (WHO), indicating the presence of significant inorganic impurities, a concern noted in previous studies evaluating crude drug purity (Saadullah et al., 2023). The foaming index was determined to be 104.05, suggesting the presence of saponins, which was further confirmed by phytochemical screening. This finding is consistent with other studies on plants with known saponin content (Saadullah et al., 2022).

The swelling index, a measure of mucilage content, was found to be 4.1 mL, within the acceptable limit of 5 mL. Fluorescence analysis, an essential pharmacognostic parameter, was conducted on the powdered plant material under various lighting conditions, revealing characteristic fluorescence that assists in plant identification and authentication. These results are in line with studies on other medicinal plants, where distinct fluorescence patterns are used for authentication (Mari et al., 2016).

Phytochemical analysis of methanol and dichloromethane extracts of A. aspera identified the presence of phenols, tannins, terpenoids, flavonoids, glycosides, alkaloids, saponins, fixed oils, fats, and carbohydrates—compounds known for their antioxidant, antidiabetic, antimicrobial, and antitumor activities (Kumar & Lalitha, 2017). Methanol extracts demonstrated the highest phenolic (59.52 ± 1.402 mg GAE/g dry weight) and flavonoid (55.083 ± 0.300 mg QAE/g dry weight) contents, which is consistent with research indicating that methanol extracts often yield higher levels of these compounds due to the solvent's polarity (Muthukrishnan & Sivakkumar, 2018).

The antioxidant capacity of A. aspera extracts was evaluated using the DPPH assay, with methanol extracts showing the highest antioxidant activity (IC50 = 10.60 µg/mL), followed by dichloromethane extracts (IC50 = 12.24 µg/mL). This significant free radical scavenging activity is attributed to the high levels of phenolic compounds and flavonoids in the extracts, a finding that aligns with other studies on the antioxidant potential of phenolic-rich plant extracts (Edwin et al., 2008).

Scanning Electron Microscopy (SEM) provided detailed images of the surface morphology of A. aspera, revealing a high formulation density with spherical and ovoid shapes, which are similar to morphological traits observed in SEM studies of other medicinal plants. Atomic Absorption Spectroscopy (AAS) detected trace metals, including calcium (Ca), copper (Cu), iron (Fe), potassium (K), manganese (Mn), zinc (Zn), and magnesium (Mg) in the methanol extract, findings that are consistent with other studies identifying essential mineral content in medicinal plants.

Fourier-transform infrared (FTIR) spectroscopy identified various functional groups, confirming the presence of phenolic and alcoholic compounds, aliphatic amines, alkenes, and nitro groups, which contribute to the plant's stability and bioactivity. These findings are in agreement with FTIR results reported in studies of other medicinal plants (Fakhar-E-Alam et al., 2024).

Conclusion

In conclusion, our comprehensive pharmacognostic evaluation of A. aspera confirms its therapeutic potential and establishes a scientific basis for its use in traditional medicine. The consistency of our findings with previously published data reinforces the validity of our methodologies and provides a benchmark for future research. However, further attention is needed at molecular level for isolation and purification of active constituent and validation of its mechanism of action.

Author contributions

M.R. conceptualized the study and drafted the manuscript. M.A.A. and S.P. contributed to data analysis and interpretation. M.B.S. and M.S. assisted in writing sections of the manuscript and provided critical revisions. All authors reviewed and approved the final version of the manuscript.

Acknowledgment

Author was grateful to their department.

Competing financial interests

The authors have no conflict of interest.

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