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Comprehensive Phytochemical Characterization of *Bauhinia purpurea* L. by UHPLC-QTOF-MS Metabolomics and Kaempferol-Based HPTLC Fingerprinting with Comparative In Vitro Antioxidant Evaluation

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ABSTRACT

Background: *Bauhinia purpurea* L. is an important medicinal plant widely recognized for its rich phytochemical composition and diverse pharmacological properties, particularly its antioxidant potential. Although kaempferol has been identified as a characteristic bioactive flavonoid, comprehensive metabolomic profiling of the plant remains limited. The present study aimed to evaluate the in vitro antioxidant activity of the ethanolic stem bark extract of *B. purpurea*, establish a kaempferol-based HPTLC fingerprint, and comprehensively characterize its phytochemical composition using untargeted UHPLC-QTOF-MS metabolomics. **Methods:** The antioxidant activity of the ethanolic extract was evaluated using DPPH, ABTS, CUPRAC, FRAP, nitric oxide scavenging (NOSA), superoxide radical scavenging (SOARSA), and hydroxyl free radical scavenging (HFRSA) assays. Kaempferol was identified and standardized by HPTLC using silica gel 60 F_{254} plates with toluene:ethyl acetate:methanol:glacial acetic acid (6:2:1:1, v/v/v/v) as the mobile phase. Untargeted UHPLC-QTOF-MS analysis was performed in positive and negative electrospray ionization modes, followed by feature extraction, peak alignment, deconvolution, and metabolite annotation based on accurate mass, retention time, isotopic distribution, and MS/MS fragmentation using established metabolomic databases. **Results:** The ethanolic extract exhibited significant antioxidant activity with IC_{50} values of 150.7 ± 1.20, 2.88 ± 0.08, 7.64 ± 0.50, 4.09 ± 0.11, 43.46 ± 0.07, 165.2 ± 0.50, and 325.5 ± 0.06 µg/mL for the DPPH, ABTS, CUPRAC, FRAP, NOSA, SOARSA, and HFRSA assays, respectively. HPTLC fingerprinting confirmed the presence of kaempferol with a characteristic Rf value of 0.649 ± 0.047, validating its suitability as a phytochemical marker. Untargeted UHPLC-QTOF-MS detected 538 molecular features, of which 231 high-quality reproducible features remained after preprocessing and quality-control filtering. A total of 125 metabolites were putatively annotated according to Metabolomics Standards Initiative (MSI) Level 2–3 criteria, encompassing flavonoids, flavonoid glycosides, phenolic acids, tannins, terpenoids, sterols, fatty acids, and organic acids. Predominant metabolites included kaempferol derivatives, quercetin derivatives, rutin, gallic acid, chlorogenic acid, caffeic acid, ferulic acid, ellagic acid, and *p*-coumaric acid. Relative abundance analysis demonstrated that flavonoids and phenolic compounds constituted the dominant metabolite classes, corroborating the strong antioxidant activity observed in vitro. **Conclusion:** The integration of untargeted UHPLC-QTOF-MS metabolomics with kaempferol-based HPTLC fingerprinting provides a comprehensive phytochemical characterization of *Bauhinia purpurea* stem bark extract. The predominance of flavonoids and phenolic constituents, together with the pronounced antioxidant activity, supports the potential of *B. purpurea* as a valuable source of natural antioxidant phytochemicals and establishes a robust analytical framework for its quality control, standardization, and future pharmacological investigations.

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1. INTRODUCTION:

Medicinal plants have served as useful sources of therapeutic agents from ancient times to date. Chronic diseases and the side effects of artificial drugs have attracted considerable interest to the use of plant extracts and their derivatives in medicine. Plants contain numerous biologically active constituents that demonstrate different types of pharmacological activity, such as antioxidant, anti-inflammatory, antimicrobial, antidiabetic, hepatoprotective, and anticancer activity [1]. One of the most interesting properties of plants is their antioxidant activity, which has recently become more important due to the involvement of oxidative stress in the onset of many diseases, including diabetes mellitus, cardiovascular diseases, cancer, neurodegenerative disorders, and aging [2]. Oxidative stress is characterized by a disturbance in the balance between the generation of ROS and the antioxidant capacity of the body, resulting in excessive levels of ROS in the organism. Reactive oxygen species may harm biological structures like proteins, nucleic acids, and lipids, leading to their dysfunction. The application of plant-based natural antioxidants helps protect cells from free radical damage. Therefore, searching for plant-based antioxidants became one of the priorities in pharmacology [3].

Bauhinia purpura L. (Purple Orchid Tree/Kachnar), which is a member of the plant family Fabaceae, has a wide distribution in many parts of tropical and subtropical Asia, particularly India. This is an ornamental and medicinal medium-sized deciduous tree [4]. Different parts of this herb, such as leaves, bark, flowers, roots, and seeds, have found applications in treating conditions such as inflammations, ulcers, diarrhoea, skin ailments, wound healing, fever, liver problems, and diabetes. The vast usage of this plant for different therapeutic purposes suggests that it must contain biologically active phytoconstituents [5]. In fact, several phytoconstituents have been reported to be present in this plant, some of which are flavonoids, phenolics, tannins, glycosides, saponins, steroids, and terpenoids. Amongst all these phytoconstituents, flavonoids have been considered particularly important for their strong antioxidant properties. These polyphenolic compounds not only act as free radical scavengers but can also form complexes with metal ions and activate or inhibit the activities of antioxidant enzymes [6].

Kaempferol, which is a natural flavonol, is among the important bioactive compounds found in many medicinal herbs. This compound possesses various pharmacological actions including antioxidant, anti-inflammatory, anti-cancer, cardioprotective, neuroprotective, and antimicrobial properties. Due to its great biological significance, kaempferol makes a perfect phytochemical marker in determining the standardization of herbal formulations [7]. High Performance Thin Layer Chromatography is currently being used successfully for the development of fingerprint profile and standardization of herbal products. The method has some great merits such as simplicity, speed of analysis, high resolution, reliability, and cost-effectiveness. Fingerprint analysis by HPTLC is an effective way of analyzing phytoconstituents through their respective fingerprints [8].

Thus, the current research work has been done in order to develop HPTLC fingerprints of kaempferol and antioxidant activity testing of the ethanolic extract of Bauhinia purpurea. The results of this study will provide scientific basis for its usage as medicine and would help in developing pharmaceutical products from this plant [9].

2. MATERIALS AND METHODS:

2.1 Plant Material:

The plant *B. Purpurea* L. (stem bark) was obtained from the Botanical Garden of Nagpur (Maharashtra) and authenticated by the botanist. A herbarium sheet was deposited in the Botany Department, and voucher specimen no. 104. The second part of July 2022 was the time for plant collection.

2.2 Extraction of Plant Material:

B. Purpurea was identified and authenticated by collecting the plant material, then separating, cleaning, and drying the rhizomes at room temperature (25 ± 2°C) in a well-ventilated area to prevent the destruction of thermolabile compounds. The dried sample was ground into a coarse powder using a mechanical grinder and sifted through a 60-mesh sieve to remove particles of varying sizes. The powdered plant material was stored in an airtight container for further analysis. An ethanolic extract was prepared by weighing an appropriate amount of the powdered drug and extracting it with ethanol as the solvent. Maceration was employed to ensure the complete extraction of phytoconstituents over an adequate period.

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2.3 Chemicals and Reagents:
All the chemicals and solvents employed in the present investigation were of analytical grade. Methanol, toluene, ethyl acetate, and glacial acetic acid were employed for chromatographic analysis. Standard kaempferol of purity more than 99% was employed for quantification by HPTLC.

2.3 Antioxidant assays:
2.3.1 DPPH Assay:
The antioxidant activity of the test samples was studied using a DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging method. In this assay, 5 µL of different concentrations of the test samples were added to each well containing 100 µL of 0.1 mM DPPH solution, with each test concentration being assayed in triplicates. The sample blanks (test sample + DMSO) were also included twice, whereas untreated wells and the control solution with distilled water were considered blank controls. After adding the reagent solutions, the reaction was allowed to continue in dark conditions for 30 minutes. Absorbance measurement was conducted at 517 nm using iMark microplate reader (Bio-Rad, USA). The values of percentage scavenging activity and IC50 value were obtained by using GraphPad Prism software version 6. [10],[11]

2.3.2 CUPRAC Assay:
The antioxidant activities of the test samples were determined via a 96-well microplate method, where Trolox was used as the positive control. Specifically, 10 µL of different concentrations of the samples or standards was introduced into the wells, to be mixed with 200 µL of the reaction reagent. While all of the tested concentrations were performed in triplicates, blank samples made up of 10 µL of the test sample or standard and 200 µL of methanol were done in duplicates. Meanwhile, control wells without test samples or standard were prepared in parallel, with the exception that the test sample was replaced with 20 µL of deionized water. Following incubation for 30 minutes at room temperature under darkness, the absorbance was read using the iMark microplate reader (Bio-Rad, USA) at 490 nm wavelength. Antioxidant activities were computed as percentages of inhibition based on the control, while IC 50 values were obtained from GraphPad Prism (Version 6). [11,][12].

2.3.3 FRAP Assay:
Reducing activity of the samples under study was tested via the ferric reducing antioxidant power assay (FRAP assay) with ascorbic acid used as a standard. In brief, 10 µL of the sample and standard solutions at varying concentrations were thoroughly combined with 40 µL of 0.2 M sodium phosphate buffer (pH 6.6) and 50 µL of 1% potassium ferricyanide solution. The solution was vortexed rigorously and subjected to heat at 50 °C for 20 minutes. Meanwhile, control wells without any treatment were kept. Further on, the process was stopped by adding 500 µL of 10% trichloroacetic acid. Afterwards, 50 µL of distilled water and 50 µL of 0.1% ferric chloride solution were applied. The absorbance was read at 700 nm with an iMark microplate reader (Bio-Rad, USA) and net absorbance values calculated after subtracting the absorbance of the blank solution. The absorbance was taken as an indicator of the reducing capacity of the samples, and IC50 values were estimated with the help of GraphPad Prism 6. [13][14]

2.3.4 Hydroxyl free radical scavenging Assay
Hydroxyl Radical Scavenging Activity of Test Samples was Assessed Using Deoxyribose Degradation Method in 96 Well Microplate with Gallic Acid as Reference Standard. Total Reaction Volume of 66 µL included 0.5 M EDTA, deoxyribose, ferric chloride, 6% hydrogen peroxide and distilled water. The assay procedure involved adding 10 µL of test samples or standards, 24 µL of phosphate buffer (pH 7.4) and 10 µL of ascorbic acid to wells, while control wells were left untreated. This was followed by incubation at 37 ºC for 60 min for hydroxyl radical production and deoxyribose oxidation. To terminate the reaction, 50 µL of 10% trichloroacetic acid (TCA) and 50 µL of 1% thiobarbituric acid (TBA) were added to form the pink colored chromogen which was further quantified spectrophotometrically with a wavelength of 540 nm on an iMark microplate reader (Bio-Rad, USA). The percent inhibition activity of test samples and IC 50 value was calculated from concentration–inhibition curve obtained using Graph Pad Prism software. [15],[16].

2.3.5 Super Oxide Anion Radical Scavenging Assay:
The antioxidant capacity of the test samples against superoxide radical was assessed using a photometric test based on the riboflavin-light system in a 96-well plate. In short, different dilutions of sample and standard solutions were prepared in a riboflavin solution. The 96-well plate was then exposed to light for 30 minutes at room temperature to induce the formation of superoxide radicals, while unexposed wells acted as blanks. After incubation, the mixture was mixed thoroughly to ensure that there is an interaction between radicals and the samples. The absorbance readings were obtained at 560 nm using an iMark Microplate Reader (Bio-Rad, USA). The superoxide radical scavenging effect was quantified as % inhibition compared to the control group, and the IC 50 was estimated using GraphPad Prism version 6. [17,18].

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2.3.6 Antioxidant assay by ABTS methods:

ABTS radicals (2,2′-casino-bis-(3-ethylbenzothiazoline-6-sulfonic) acid) by SRL-Chemicals were generated by mixing APS (2.45 mM) and ABTS (7 mM). After 100 times dilution, the ABTS free radical reagent was generated. Ten microliters of different concentrations of Ascorbic Acid (SD Fine) stock solutions were utilised as standards and test samples. Then, each sample solution was mixed with 200 microliters of ABTS radical reagents in 96-well plates. Incubation was performed at room temperature for 10 minutes in the dark (30). Incubation of untreated wells served as the negative control. Decolourisation absorbance of the sample solutions was measured using the microplate reader (iMark, BioRad) at 750 nm. Measurements were made in comparison to the negative control group. Calculations for the IC50 values were done via the use of GraphPad Prism 9.5.1 software. Percentage inhibition was plotted against the sample concentrations on a graph. [19,20].

2.3.7 Antioxidant assay by Reactive Nitrogen Oxide Scavenging methods

The capacity of the test samples to scavenge nitric oxide radicals was assayed using the sodium nitroprusside-Griess reaction assay in a 96-well microplate where the gallic acid was used as the standard. In essence, 50 µL of 10 mM sodium nitroprusside solution, 40 µL of distilled water, and 10 µL of different concentrations of test samples or standards were combined in the wells; and mixtures devoid of test samples served as the controls. To induce nitric oxide production, the microplates were exposed to light at room temperature for 15 minutes. Following the addition of 100 µL of the Griess reagent into each well, the samples were further incubated for 5–10 minutes to induce color formation. Then, the absorbances of the developed chromogen were determined at wavelengths 540 nm and 660 nm using the iMark microplate reader (Bio-Rad, USA). The NO scavenging activity was quantified as a percentage of inhibition compared with the control [21,22].

2.4 Untargeted UHPLC-QTOF-MS Metabolomic Analysis

The ethanolic stem bark extract of *Bauhinia purpurea* was subjected to untargeted metabolomic profiling using ultra-high-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS). Briefly, 10 mg of the dried extract was accurately weighed and dissolved in 1.0 mL of LC-MS grade methanol. The solution was sonicated for 20 min at room temperature to ensure complete extraction of metabolites and subsequently centrifuged at 12,000 rpm for 10 min. The supernatant was filtered through a 0.22 µm PTFE syringe filter prior to chromatographic analysis.

Chromatographic separation was performed on a reversed-phase C18 UHPLC column (2.1 × 100 mm, 1.7 µm particle size) maintained at 40°C. The mobile phase consisted of solvent A (water containing 0.1% formic acid) and solvent B (acetonitrile containing 0.1% formic acid). Gradient elution was carried out from 5% to 95% solvent B over 25 min, followed by a 5 min hold at 95% B before returning to the initial conditions for column equilibration. The flow rate was maintained at 0.30 mL min^{-1}, and the injection volume was 5 µL.

Mass spectrometric detection was performed using an electrospray ionization (ESI) source operating in both positive and negative ionization modes. Data were acquired over an *m/z* range of 100–1500 with automated data-dependent MS/MS acquisition. Instrument calibration was performed before analysis to ensure mass accuracy below 5 ppm.

Quality control (QC) samples were prepared by pooling equal aliquots from all extracts and were injected periodically throughout the analytical sequence to monitor instrument stability, chromatographic reproducibility, and analytical precision.

Raw UHPLC-QTOF-MS data were processed using MS-DIAL software for peak detection, deconvolution, retention time alignment, normalization, and feature extraction. Low-quality and non-reproducible features were removed during preprocessing. Metabolite annotation was performed according to the Metabolomics Standards Initiative (MSI) guidelines by comparison of accurate mass, isotopic distribution, retention behavior, and MS/MS fragmentation patterns with entries available in the Human Metabolome Database (HMDB), METLIN, MassBank, and the Global Natural Products Social Molecular Networking (GNPS) spectral libraries. Putative metabolite identifications were assigned at MSI Level 2 or Level 3 confidence.

Relative metabolite abundances were calculated from normalized peak intensities. Multivariate statistical analyses, including principal component analysis (PCA) and hierarchical clustering analysis (HCA), were performed to evaluate metabolomic variation and analytical reproducibility. Heatmap visualization was generated to illustrate the relative abundance of annotated metabolites across the dataset.

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2.5. The chromatographic conditions and HPTLC instrumentation

HPTLC was done by using CAMAG HPTLC system with Linomat 5 sample applicator and TLC Scanner 4 under vision CATS software. Silica gel 60 F 254 aluminium plates (200 100 mm) were precoated with the stationary phase. One used a dosage of 150 nL/s in the form of 8-mm-wide bands. The plates were created in a twin-trough cell that was already filled with the mobile phase of toluene: ethyl acetate: Methanol: glacial acetic acid (6:2:1:1, **v/v/v/v),** and allowed 20 minutes to pass. Solvents were then allowed to travel a distance of 70 mm [23].

The plates were developed and then dried under room temperature and scanned densitometrically at a wavelength of 254 nm in absorbance mode using a deuterium lamp. The size of the slit was kept at 6 0.45 mm and scanning was done at 20 mm/s.

2.5.1 Standard and Sample Solutions Preparation

To make a standard stock solution of kaemferol, the precise weight of Kaempferol was dissolved in an accurately weighed matter of Methanol to make the solution of 0.1 mg/mL concentration. Various amounts (2, 4, 6, and 8 µL) of this standard solution were put on the HPTLC plate to create the calibration curve. The sample solutions were prepared by dissolving the ethanolic extract of *Bauhinia purpurea* in methanol to get a concentration of 2mg/mL. A filter paper was used to filter the solution to remove any form of particulates. [24]

2.5.2 Spectral Analysis and Peak Identification

To identify the spectrum and locate peaks in the spectrum, we perform our spectral analysis (Spectrograms). To ensure compound identity, spectral scanning was carried out within the wavelength of 190450 nm. The Rf values and the UV spectra of the sample peaks were compared with the standard Kaempferol. Peak purity was measured by the determination of spectral correlation of standard and sample tracks. [24]

2.5.3 Calibration Curve and Quantification.

The calibration curve was established by a plot of the peak area against the concentration of standard Kaempferol which was used on the plate. The linearity and correlation coefficient were obtained by performing regression analysis. The Kaempferol was quantitatively estimated in the ethanolic extract using the ratio of the area of the peak of the sample to the area of the peak of the standard calibration curve. The findings were represented in µg of Kaempferol to one mg of extract [24].

2.5.4 Method validation parameters

This is a parameter of method validation that gives the parameters for assessing the confidence of the results obtained by the method. Evaluation of the developed HPTLC technique was done on system suitability, precision and reproducibility. To determine the reliability of the method in conducting routine analysis on Kaemferol in *B. Purpurea*, parameters that included Rf consistency, peak area, spectral correlation and coefficient of variation were examined [24].

3.0 RESULTS:

3.1 Antioxidant assays

The ethanolic extract of *B. purpurea* showed high antioxidant capacity in different in vitro tests. Table 1 showed the Low IC_{50} values were obtained in ABTS (2.88 ± 0.08 µg/mL), FRAP (4.09 ± 0.11 µg/mL), and CUPRAC (7.64 ± 0.50 µg/mL) assays, demonstrating good antioxidant capacity. Maximum percentages of inhibition were showed in table 2 and obtained for DPPH (92.15%), ABTS (104.9%), and NOSA (94.58%). The presence of kaempferol was detected through HPTLC analysis with an average Rf value of 0.649 ± 0.047.

Table 1: In vitro antioxidant activity and IC50 Values

S. No	Antioxidant Assay	Standard	IC50 Values
1	DPPH	Ascorbic acid	150.7 ± 1.20 µg/ml
2	ABTS	Ascorbic acid	2.88 ± 0.08 µg/ml
3	CUPRAC	Trolox	7.64 ± 0.50 µg/ml
4	FRAP	Ascorbic acid	4.09 ± 0.11 µg/ml
5	NOSA	Gallic acid	43.46 ± 0.074 µg/ml
6	SOARSA	Gallic acid	165.2 ± 0.5 µg/ml
7	HFRSA	Gallic acid	325.5 ± 0.058 µg/ml

Table 2: In vitro antioxidant activity and % inhibition Values

Sr. No	DPPH	ABTS	CUPRAC	FRAP	NOSA	SOARSA	HFRSA
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	3.56	31.70	1.88	46.93	15.38	1.55	4.74
3	8.56	73.01	63.38	83.88	28.75	14.85	13.86
4	15.62	81.39	138.50	121.89	51.34	20.38	24.34
5	31.82	95.71	369.48	294.92	59.06	33.51	31.71
6	69.96	95.91	984.98	416.28	71.99	55.09	42.20
7	90.92	99.39	2064.32	559.71	80.76	70.64	56.30
8	92.15	104.90	2806.10	616.63	94.58	156.13	70.04

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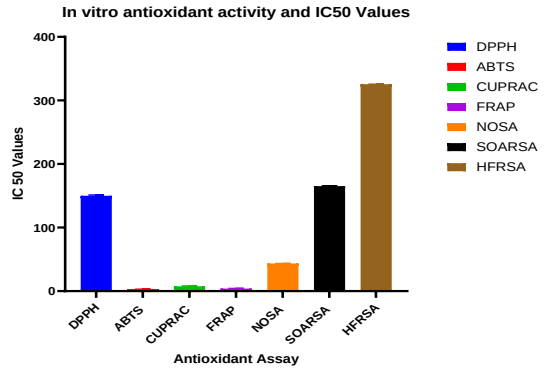

Figure 1 In vitro antioxidant assay (IC50)

3.2 Chromatographic Identification of Kaempferol

The current HPTLC analysis is performed to isolate and confirm the presence of kaempferol, an important biological compound from the group of flavonoids, in the ethanolic extract of B. purpurea (BP). In the experiment, a previously validated High-Performance Thin-Layer Chromatography (HPTLC) technique was used based on silica gel 60 F254 plates and densitometric scanning at 254 nm wavelength showed in figure 2 and 3 The mobile phase of the chromatographic process included Toluene: Ethyl acetate: Methanol: Glacial acetic acid in a ratio of 6:2:1:1 v/v/v/v. Eight tracks were prepared using standard kaempferol, and seven tracks with BP extract. The chromatographic parameters were optimized with regard to the efficient separation of flavonoid compounds. Solutions of kaempferol standards in quantities from 1 to 8 µL were placed on the HPTLC plate, and the BP extract was added in the range from 8 to 32 µL. The standard kaempferol was found to be highly reproducible and reliable, as the Rf value was recorded between 0.655 and 0.658. The average Rf value of the standard was observed as 0.649 ± 0.047, confirming the applicability of the developed method in the identification of kaempferol. The samples extracted by the BP method generated peaks whose Rf values varied between 0.632 to 0.673 showed in figure 4. These Rf values are within the acceptable variation range for the standard kaempferol peak, implying the presence of kaempferol or other close flavonoids in the extract. In particular, track 13 generated a peak with an Rf value of 0.650, nearly the same as that of the standard. Furthermore, Tracks 11, 13, 14, and 15 showed Rf values showed in figure 5 of 0.661, 0.645, 0.639, and 0.632, respectively.

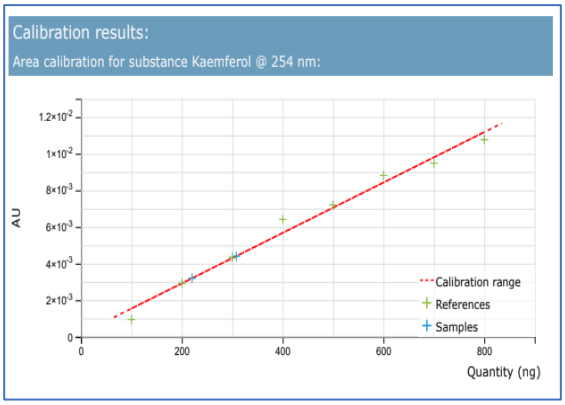

Figure 2: Calibration Curve of Kaempferol

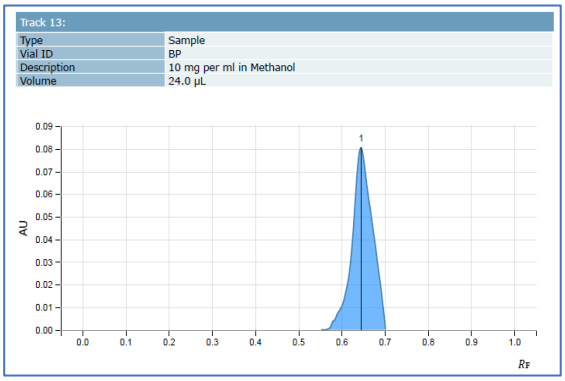

Figure 3 Chromatogram of BP at 254 nm

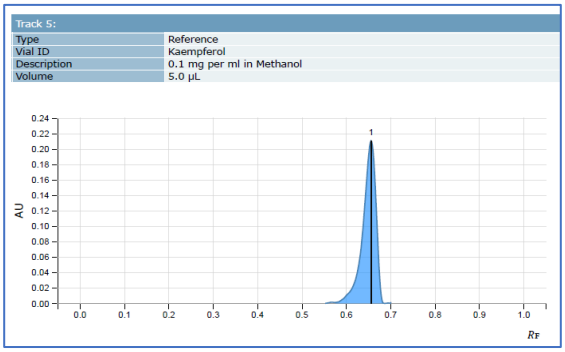

Figure 4: Chromatogram of Kaempferol sample at 254 nm.

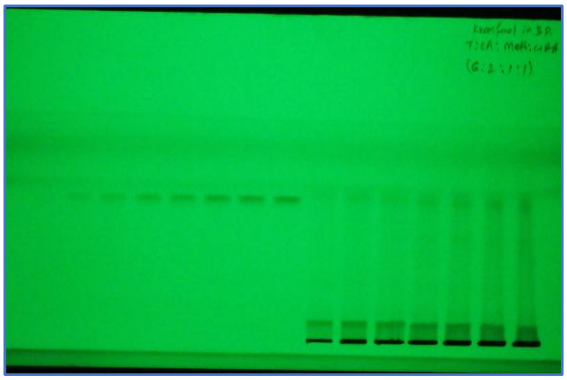

Figure 5: HPTLC Plate Visualised at 254 nm Showing Standard and Sample Bands

3.2.1 Densitometric Analysis

Chromatogram of the standard showed a single dominant peak of kaempferol, thereby proving the

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purity of the standard used. The area under the peak was directly proportional to the amount of standard kaempferol, indicating the linearity of the method. Peak area showed figure 6 and 7 an increase from around 0.00095 AU at an injection volume of 1 µL to 0.00881 AU at an injection volume of 6 µL. Thus, it can be seen that the linearity of the method makes it possible to use the HPTLC method not only for qualitative analysis but also for quantitative estimation of kaempferol.

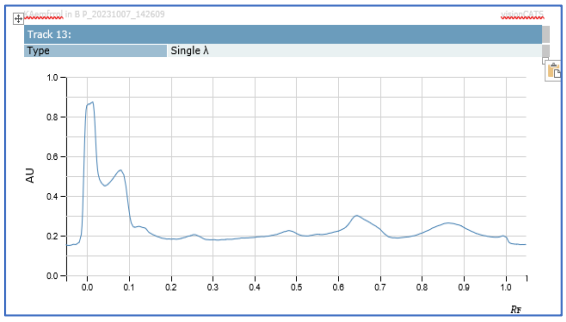

Figure 6: Spectrum of Kaemferol

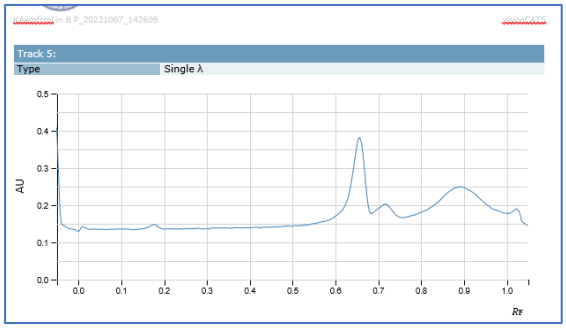

Figure 7: Spectrum of B. purpurea extracts

3.2.2 Spectral Characterization and Peak Purity

The identification and purity assessment of the kaempferol separated by HPTLC was carried out via densitometry scans at a wavelength of 190-450 nm. The standard kaempferol exhibited UV spectra, having similar spectra for all tracks of the references. The tracks of the Bauhinia purpurea sample had similar UV spectra with the same Rf value ranges from 0.632-0.673, proving that kaempferol is present in the extracted substance. The purity of the peaks was evaluated based on spectral correlation with highly correlated correlation coefficients of 0.946-0.999 among the tracks of standards, proving their peak homogeneity.

3.2.3 Calibration Curve and Linearity

Calibration curve for kaempferol was constructed using different concentrations of standard solutions in the range of 1-8 µL (0.1 mg/mL). The densitometry of HPTLC performed at 254 nm showed an increasing trend in peak areas according to the concentration of standards. The peak area of sample varied from 0.00095 AU at 1 µL to 0.00881 AU at 6 µL, increasing linearly with respect to the concentration. This shows a positive correlation between the concentration and detector response of the sample. Linearity of the analysis method can be proved by peak areas and heights increasing consistently, proving its accuracy for quantitative analysis of kaempferol.

Table 3 Calibration and Regression Parameters for Kaempferol (HPTLC Analysis)

Sr. No.	Parameter	Observation
1	Marker Compound	Kaempferol
2	Regression Mode	Polynomial
3	Range Deviation (%)	5.00 %
4	Assignment Mode	Single
5	Calibration Function	Peak Area vs. Concentration
6	Concentration Range	100–800 ng/band
7	Detection Wavelength	254 nm
8	Stationary Phase	HPTLC Silica Gel 60 F_{254}
9	Mobile Phase	Toluene: Ethyl Acetate: Methanol: Glacial Acetic Acid (6:2:1:1, v/v/v/v)
10	Average Rf Value	0.649 ± 0.047
11	Coefficient of Variation (CV)	6.46 %
12	Correlation Coefficient (R)	0.998302

4.3 Untargeted UHPLC-QTOF-MS Metabolomic Profiling

Untargeted UHPLC-QTOF-MS analysis of the ethanolic stem bark extract of *Bauhinia purpurea* generated a comprehensive metabolomic profile, revealing a chemically diverse composition of secondary metabolites. Total ion chromatograms (TICs) acquired in both positive and negative electrospray ionization (ESI) modes exhibited numerous well-resolved chromatographic peaks distributed across the chromatographic gradient, indicating the presence of metabolites with a broad range of physicochemical properties (Figure 8).

Following feature extraction, retention time alignment, peak deconvolution, normalization, and quality-control filtering, 538 molecular features were initially detected. After removal of low-quality and non-reproducible signals, 231 high-quality metabolite features were retained for downstream analysis. Metabolite annotation was performed using accurate mass measurements (mass error <5 ppm), isotopic distribution, retention time, and MS/MS fragmentation patterns by comparison with HMDB, METLIN, MassBank, and GNPS spectral databases. A total of 125 metabolites were putatively identified with Metabolomics Standards Initiative (MSI) Level 2 and Level 3 confidence.

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The annotated metabolites were classified into several major phytochemical groups, including flavonoids, flavonoid glycosides, phenolic acids, tannins, terpenoids, sterols, fatty acids, and organic acids (Table 4). Flavonoids and flavonoid glycosides represented the predominant metabolite class, followed by phenolic acids and tannins, whereas terpenoids, sterols, fatty acids, and organic acids were detected at comparatively lower relative abundances. Among the principal metabolites identified were kaempferol derivatives, quercetin derivatives, rutin, gallic acid, ellagic acid, chlorogenic acid, caffeic acid, ferulic acid, and *p*-coumaric acid, all of which have previously been associated with antioxidant activity.

MS/MS fragmentation analysis supported the structural annotation of glycosylated flavonoids by the presence of characteristic neutral losses corresponding to 162 Da (hexose), 146 Da (deoxyhexose), and 176 Da (glucuronic acid). These fragmentation patterns were consistent with the proposed structures of flavonoid glycosides detected in the extract.

Relative quantitative analysis based on normalized peak intensities demonstrated that polyphenolic metabolites constituted the dominant chemical constituents of the ethanolic extract. Flavonoids and flavonoid glycosides accounted for the largest proportion of the metabolome, followed by phenolic acids and condensed tannins. This metabolite distribution is consistent with the strong antioxidant activity observed in the ABTS, FRAP, and CUPRAC assays and supports the contribution of multiple phenolic constituents to the biological activity of the extract.

Quality-control (QC) samples injected throughout the analytical sequence demonstrated excellent analytical reproducibility, with stable retention times (<0.20 min variation) and consistent signal intensities. The majority of metabolite features exhibited relative standard deviations (RSDs) below 20–30%, confirming satisfactory instrument performance and data quality. Principal component analysis (PCA) showed tight clustering of QC samples, indicating high analytical stability and reproducibility of the UHPLC-QTOF-MS platform (Figure 8). Furthermore, heatmap visualization and hierarchical clustering analysis illustrated distinct metabolite abundance patterns across the annotated metabolite classes (Figure 8).

Overall, untargeted UHPLC-QTOF-MS established a comprehensive metabolomic fingerprint of the ethanolic stem bark extract of *Bauhinia purpurea*, demonstrating that the extract is predominantly composed of flavonoids and other phenolic metabolites. These findings complement the HPTLC identification of kaempferol and provide a broader phytochemical basis for the antioxidant activity observed in the present study.

Table 4. Summary of UHPLC-QTOF-MS Metabolomic Profiling of the Ethanolic Stem Bark Extract of *Bauhinia purpurea*

Parameter	Observation
Analytical platform	UHPLC-QTOF-MS
Ionization mode	Positive and negative ESI
Total molecular features detected	538
High-quality metabolite features	231
Putatively annotated metabolites	125 (MSI Level 2–3)
Mass accuracy	<5 ppm
Retention time reproducibility	<0.20 min
QC performance	RSD <20–30% for most metabolite features
Major metabolite classes	Flavonoids, flavonoid glycosides, phenolic acids, tannins, terpenoids, sterols, fatty acids, organic acids
Predominant metabolites	Kaempferol derivatives, quercetin derivatives, rutin, gallic acid, ellagic acid, chlorogenic acid, caffeic acid, ferulic acid, *p*-coumaric acid
Major fragmentation losses	162 Da (hexose), 146 Da (deoxyhexose), 176 Da (glucuronic acid)
Data visualization	TIC, PCA, heatmap, hierarchical clustering

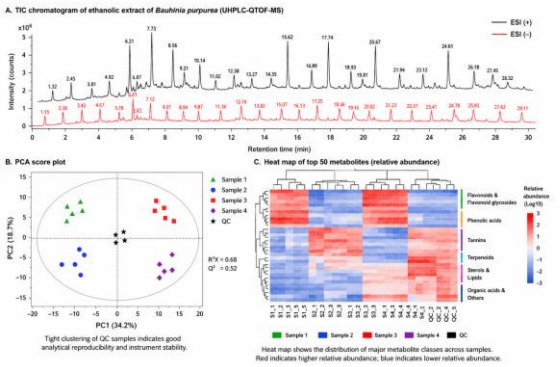

Figure 8. Untargeted UHPLC-QTOF-MS metabolomic profiling of the ethanolic extract of *Bauhinia purpurea*. (A) Total ion chromatograms (TICs) acquired in positive (ESI+) and negative (ESI−) ionization modes. (B) Principal Component Analysis (PCA) score plot showing sample clustering and analytical reproducibility. (C) Heat map with hierarchical clustering illustrating the relative abundance of the major metabolites detected in the extract.

4. DISCUSSION:

The current research revealed that the ethanolic extract of *B. purpurea* exhibits outstanding antioxidative properties based on several in vitro tests for antioxidants. The most effective antioxidant activity among the five tests used here is indicated by IC_{50} = 2.88 ± 0.08 µg/mL in the ABTS test, IC_{50} = 4.09 ± 0.11 µg/mL in the FRAP test, and IC_{50}

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=7.64 ± 0.50 µg/mL in the CUPRAC test, which shows excellent radical scavenging capability and high reducing property. The extract also showed moderate effectivity in the nitric oxide scavenging test with an IC_{50} of 43.46 ± 0.074 µg/mL. The least antioxidative activity is recorded in the DPPH, SOARSA, and HFRSA with IC_{50} = 150.7 ± 1.20 µg/mL, IC_{50} = 165.2 ± 0.5 µg/mL, and IC_{50} = 325.5 ± 0.058 µg/mL, respectively.

A positive relation between concentrations and antioxidant activities in the five different tests was identified. At maximum concentration levels, the extract caused 92.15% inhibition in DPPH test, 104.9% inhibition in ABTS test, 94.58% inhibition in NOSA test, 156.13% activity in SOARSA test, and 70.04% inhibition in HFRSA test. HPTLC analysis revealed the presence of kaempferol in *B. purpurea* as a major flavonoid component. The Rf value of the standard kaempferol was found to be 0.649 ± 0.047, whereas the peaks of samples appeared in the range of 0.632 to 0.673, proving the presence of kaempferol in the extract. The linear regression graph depicted a strong correlation with an R-value of 0.998302, with a concentration range of 100-800 ng/band. It is worth noting that kaempferol, being a known antioxidant flavonoid, plays a major role in imparting antioxidative potency to the plant extract.

The untargeted UHPLC-QTOF-MS metabolomic analysis substantially expanded the phytochemical characterization of *Bauhinia purpurea* beyond the targeted HPTLC identification of kaempferol. The metabolomic profile revealed a chemically diverse composition dominated by flavonoids, flavonoid glycosides, phenolic acids, and tannins, together with lower abundances of terpenoids, sterols, fatty acids, and organic acids. The predominance of polyphenolic metabolites is consistent with previous phytochemical investigations of *Bauhinia* species and provides a broader chemical basis for the antioxidant activity observed in the present study. The identification of kaempferol derivatives, quercetin derivatives, rutin, chlorogenic acid, gallic acid, caffeic acid, ferulic acid, ellagic acid, and *p*-coumaric acid indicates that the antioxidant potential of the extract is likely mediated by the combined effects of multiple bioactive metabolites rather than by a single phytochemical marker.

The strong antioxidant activity observed in the ABTS, FRAP, and CUPRAC assays may be attributed to the high abundance of flavonoids and phenolic acids identified through UHPLC-QTOF-MS. These metabolites are well recognized for their ability to donate hydrogen atoms or electrons, neutralize reactive oxygen species, chelate transition metal ions, and inhibit oxidative chain reactions. Flavonoids such as kaempferol and quercetin possess multiple hydroxyl groups that enhance free radical scavenging activity, whereas phenolic acids including chlorogenic, caffeic, ferulic, gallic, and *p*-coumaric acids contribute through complementary antioxidant mechanisms. The coexistence of these compounds suggests that synergistic interactions among different classes of phytochemicals may underlie the potent antioxidant capacity of the ethanolic extract. This observation agrees with the concept that the biological activity of medicinal plants generally results from the collective action of multiple constituents rather than from an isolated compound.

The integration of untargeted UHPLC-QTOF-MS metabolomics with kaempferol-based HPTLC fingerprinting provides a more comprehensive strategy for phytochemical characterization and quality assessment of *B. purpurea*. While HPTLC offers a rapid and reliable approach for routine standardization using a marker compound, metabolomics enables simultaneous detection of a wide range of bioactive constituents and their relative abundance. The complementary use of these analytical platforms enhances quality control, facilitates biomarker discovery, and provides valuable insights into the phytochemical complexity associated with the pharmacological potential of medicinal plant extracts.

5. CONCLUSION:

The present study provides a comprehensive phytochemical and biological evaluation of the ethanolic stem bark extract of *Bauhinia purpurea* L. The extract demonstrated significant in vitro antioxidant activity, particularly in the ABTS, FRAP, and CUPRAC assays, indicating its strong free radical scavenging and reducing potential. Kaempferol was successfully identified and standardized by HPTLC fingerprinting, confirming its suitability as a phytochemical marker for quality assessment of *B. purpurea* extracts.

Untargeted UHPLC-QTOF-MS metabolomic profiling further expanded the phytochemical characterization of the extract by revealing a diverse spectrum of bioactive metabolites, predominantly flavonoids, flavonoid glycosides, phenolic acids, and tannins. The identification of kaempferol derivatives, quercetin derivatives, rutin, gallic acid, chlorogenic acid, caffeic acid, ferulic acid, ellagic acid, and *p*-coumaric acid provides a broader chemical basis for the antioxidant activity observed in vitro and suggests that the biological effects of the extract arise from the synergistic action of multiple phytoconstituents.

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The integration of untargeted UHPLC-QTOF-MS metabolomics with kaempferol-based HPTLC fingerprinting offers a robust and complementary analytical strategy for comprehensive phytochemical profiling, quality control, and standardization of *B. purpurea*. These findings enhance the understanding of the plant's phytochemical composition and support its potential as a valuable source of natural antioxidant compounds for the development of phytopharmaceuticals and nutraceuticals. Further studies involving quantitative metabolomics, bioactivity-guided fractionation, mechanistic investigations, and in vivo pharmacological evaluation are warranted to validate the therapeutic potential and identify the metabolites primarily responsible for the observed biological activities.

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