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Original Article

Phytochemical profiling, antioxidant activity and multivariate analysis of *Boerhavia diffusa*

Muhammad Talha Khalil[1], Muhammad Akram[1*], Abid Rashid[2]
[1]Department of Eastern Medicine, Government College University, Faisalabad, Pakistan
[2]Faculty of Medical Sciences, Government College University, Faisalabad, Pakistan

**Corresponding author's email:* muhammadakram@gcuf.edu.pk
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Abstract

Chronic kidney disease (CKD) is a rising health crisis in the world resulting in progressive renal impairment due to oxidative stress and inflammation. This research has undertaken the phytochemical and bioactivity description of *Boerhavia diffusa* L. (punarnava), an Ayurvedic herb used in renal and hepatic rejuvenation. Maximum concentrations of the bioactive constituents were obtained in the 70% ethanolic extract which had a total phenolic content (TPC) of 34 mg GAE/g and total flavonoid content (TFC) of 60.52 ug CE/mL. As the major flavonoid, rutin (0.42 mg/g) and quercetin (0.31 mg/g) and the presence of significant amounts of phenolic acids such as chlorogenic (0.25 mg/g), gallic (0.18 mg/g), and caffeic (0.12 mg/g) acids were determined by high-performance liquid chromatography (HPLC) quantification. Assessment of antioxidants through DPPH assay indicated a high radical scavenging potential (24.31% inhibition) in the 70% ethanol extract that is similar to a standardized phenolic mixture (23.85). Multivariate statistical analysis involving correlation heat maps, hierarchical clustering and principal component analysis (PCA) showed that the capacity of antioxidants was highly linked to individual flavonoids but not the total phenolic content, and that compound specific bioactivity was important. Moreover, non-linear, synergistic relationships between phytochemical pools were estimated by regression modeling, highlighting the complexity of whole-plant therapeutic actions. The presence of a large number of alkaloids, flavonoids, saponins, phenols, and glycosides was confirmed by qualitative screening; no tannins or steroids were detected, which is also consistent with the historical safety of the plant in chronic preparations. These results provide a phytochemical and in vitro antioxidant characterization of *B. diffusa* and place its traditional use in a phytochemical context.

Keywords: Chronic kidney disease (CKD), Phytochemical profiling, Antioxidant activity, Flavonoids, Rutin, Quercetin oxidative stress, Ayurvedic herb

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Introduction

Chronic kidney disease (CKD) is proportionally growing in global health that is afflicting close to a billion people and causing immense socioeconomic and healthcare challenges on the global population. Although pharmacotherapy improves CKD treatment, the optimal efficacy, side effects, and the absence of a curative agent have limited the current CKD treatment status, and thus, there is continued interest in identifying phytochemical sources associated with renal health in traditional medicine (Biglari et al., 2025).

Boerhavia diffusa (Punarnava) has been used in Ayurveda, Unani, and Siddha medicine traditionally to treat renal and hepatic disease, rejuvenate skin and anti-age effects (Mondal et al., 2024). It has been used to treat edema, urinary diseases, and liver dysfunction, and is traditionally used in formulations associated with urinary and hepatic health, which is ethnopharmacologically supported and clinically applied and serves as a diuretic, hepatoprotective, and nephroprotective agent (Kumari et al., 2024) The roots and leaves of *B. diffusa* are considered especially popular in terms of their therapeutic diversification in Asia, Africa, and the Americas (Mishra et al., 2014).

The outstanding pharmacological potential of *B. diffusa* is due to its abundant and diverse phytochemistry. However, the most important bioactive constituents are alkaloids (e.g., punarnavine), flavonoids (e.g., quercetin and rutin), saponins, phenolic acids, rotenoids (e.g., boeravinones), and other secondary metabolites. These compound classes have been reported in the literature to be associated with antioxidant and anti-inflammatory mechanisms and the alleviation of oxidative stress- a core contributor to the development of CKD. Nevertheless, bioavailability and pharmacokinetics of these phytochemicals are not fully understood yet and the clinical applicability of therapies based on extracts of *B. diffusa* is hampered by the variability in extractions, absence of compound-specific standardization, and the inadequate combination of phytochemical and bioactivity information (Patel et al., 2025; Das et al., 2025) and despite promising in vitro and in vivo evidence, critical research gaps persist. Few studies have reported integrated analyses combining phytochemical profiles with in vitro antioxidant data using multivariate approaches. Standardization of extraction protocols and compound-specific profiling are the keys to ensure reproducibility and therapeutic consistency (Baratta et al., 2024).

Therefore, this study performed qualitative and quantitative phytochemical profiling of key phenolics, flavonoids and in vitro antioxidant potential via DPPH assay. Multivariate statistical analyses were employed to elucidate the complex relationships between the phytochemical profile and antioxidant activity. Overall, the study applies contemporary analytical methods to examine the phytochemical and antioxidant profile of *B. diffusa* within the context of its traditional use.

Material and Methods

Plant material

Fresh specimens of *Boerhavia diffusa w*ere collected from Faisalabad, Pakistan. Taxonomic identification was performed for each sample. Plant material was cleaned, shade-dried and pulverized into a fine powder for further analysis. The Plants were collected at vegetative stage from an area near by Gutwala Forest Park, Faisalabad, Punjab Pakistan (31°28′41″N, 73°12′30″E). To ensure traceability and reproducibility, voucher specimens were submitted to the Herbarium, Department of Eastern Medicine, Government College University Faisalabad (GCUF), and assigned an official voucher specimen number (GCUF-EM-202). The plant materials (vegetative stage) for other species (*Galium aparine*, *Centarium erythrea*) were obtained from already stored samples collected from the same vicinity.

Extraction procedures

Aqueous extraction

Powdered plant material (5 g) was dissolved in 200 mL of distilled water and incubated at 40°C in an oven for 30 min. The mixture was filtered through Whatman No. 1 filter paper and aqueous extract was stored at 4°C for further analysis (Yadav et al., 2025).

Ethanol extraction

The Soxhlet method was used for the extraction of phytochemicals from crude plant samples. Five grams (5 g) of powdered plant sample was placed in the thimble and subjected to 250 ml of 70 % or 50% alcohol for dissolution. The extraction process was completed in 24 hours as during this time the solvent in the siphon tube turned clear. The mixture was then transferred to a beaker and boiled till all the solvents

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were evaporated after preheating the oven at 30 to 40°C. The dry extract was stored at 4 °C in the refrigerator for phytochemical analysis (Gaião et al., 2023).

Phytochemical screening

Preliminary qualitative analysis was performed according to standard protocols (Mudau et al., 2022). Tests included Alkaloids (Wagner's, Mayer's, Dragendorff's, and Hager's tests), Flavonoids (Shinoda test, alkaline reagent test, lead acetate test), Phenols (Ferric chloride test, lead acetate test), Saponins (Frothing test), Glycosides (Keller-Kilani test, Legal's test), Terpenoids (Salkowski test), Steroids (Liebermann–Burchard test), Tannins (Gelatin test), Carbohydrates (Molisch's, Benedict's, and Fehling's tests), and Proteins/Amino acids (Ninhydrin test, xanthoproteic test). Reaction intensities were graded as +++ (strong), ++ (moderate) and + (weak), − (negative).

Quantitative phytochemical analysis

Total phenolic content (TPC)

The TPC was determined using the Folin-Ciocalteu method (Singleton et al., 1999). Briefly, 0.5 mL of extract (1 mg/mL) was mixed with 2.5 mL of 10% Folin-Ciocalteu reagent and 2 mL of 7.5% sodium carbonate. After 30 min incubation in the dark, absorbance was measured at 765 nm. TPC was expressed as mg gallic acid equivalent per gram of extract (mg GAE/g).

Total flavonoid content (TFC)

TFC was measured using the aluminum chloride colorimetric method (Zhishen et al., 1999). Extract (0.5 mL, 1 mg/mL) was mixed with 1.5 mL methanol, 0.1 mL 10% aluminum chloride, 0.1 mL 1 M potassium acetate, and 2.8 mL distilled water. After 30 min, absorbance was read at 415 nm. Results were expressed as µg catechin equivalent per mL of extract (µg CE/mL).

High-performance liquid chromatography (HPLC) analysis

Quantification of specific phenolic acids (chlorogenic, gallic, caffeic) and flavonoids (quercetin, rutin) was performed using HPLC system (Agilent LC/MSD iQ). The mobile phase consisted of 0.1% formic acid in water (A) and acetonitrile (B) in a gradient elution: 0–10 min, 10–25% B; 10–20 min, 25–40% B; 20–30 min, 40–60% B; flow rate 1.0 mL/min; detection at 280 nm and 360 nm for phenolic acids and flavonoids respectively. Identification of compounds was based on comparison of retention times and UV spectra with certified reference standards (Sigma-Aldrich). Quantification was performed using external calibration with the corresponding standards for comparative analysis (Kumar et al., 2023).

Antioxidant activity assay

DPPH radical scavenging activity was determined according to Brand-Williams et al. (1995). Extract (0.1 mL) at various concentrations was added to 3.9 mL of 0.1 mM DPPH methanolic solution. After 30 min in the dark, absorbance was measured at 517 nm. Percentage inhibition was calculated as:

$$\textbf{Inhibition}\ (\%) = \frac{(\boldsymbol{A}\textbf{control} - \boldsymbol{A}\textbf{sample})}{\boldsymbol{A}\textbf{control}} \times 100$$

where A_{control} is the absorbance of DPPH solution without extract, and A_{sample} is the absorbance with extract.

Statistical analysis

All experiments were conducted in three independent biological replications (n = 3). Technical triplicates were used for spectrophotometric assays (TPC, TFC, DPPH) and the statistical studies done on mean values. Biological replicate means were analyzed and modeled in multivariate analyses and regression to ensure that pseudo-replication did not occur. The data were subjected to various statistical analyses. The Pearson's Correlation analysis was performed to assess interaction between phytochemical parameters and antioxidant activity. The data were subjected to minimum-maximum normalization for subsequent use in multivariate analysis. Hierarchical clustering analysis (HCA) and principal component analysis (PCA) were conducted using normalized data with Euclidean distance and Ward's linkage method. Multiple linear regression was used to model DPPH activity as a function of TPC and TFC. All analyses were performed in R-Project(v4.2.1) (FactoMineR, and corrplot) (Farajpour et al., 2024).

Results

Qualitative phytochemical screening

A preliminary qualitative phytochemical screening of extracts of *Boerhavia diffusa* was performed in order

to find the major classes of bioactive compounds. The results uncovered a rich and diverse phytochemical profile (Table 1). Alkaloids, flavonoids, phenols, saponins and glycosides were detected with high intensity (+ +), indicating their abundance. Terpenoids and carbohydrates were found in moderate quantities (+ to ++) while coumarins were found in trace quantities (+). The tannins and steroids were not observed which aligns with its historical use in chronic formulations and indicates that tannins and steroids were not detected under the qualitative conditions employed.

Table-1. Qualitative phytochemical screening of *B. diffusa* extracts.

Phytochemical Class	Qualitative Result	Intensity
Alkaloids	Positive	+++
Flavonoids	Positive	+++
Phenols	Positive	+++
Saponins	Positive	+++
Glycosides	Positive	+++
Terpenoids	Positive	++
Carbohydrates	Positive	++
Coumarins	Positive	+
Tannins	Negative	−
Steroids	Negative	−

Quantitative phytochemical profiling and antioxidant capacity

Quantitative analysis of extracts from the *B. diffusa* and comparative species given a detailed composition profile (Table 2). Total Phenolic Content (TPC: 34 mg GAE/g) and Total Flavonoid Content (TFC: 60.52 ug CE/mL) were high in 70% ethanolic extract as compared to the 50% ethanolic or aqueous extracts as well as the extracts of other plant species. The HPLC quantification also proved the presence of important phenolic acids (chlorogenic, gallic, caffeic) and flavonoids quercetin and rutin, the most abundant flavonoid being rutin (0.42 mg/g). The better extraction efficiency of ethanol highlights its suitability for optimum polyphenol extraction which is relevant for phytochemical characterization under the tested extraction conditions.

Antioxidant activity was measured by DPPH radical scavenging assay showed a significant variation in samples. *C. erythraea* showed the best DPPH inhibition result (37.02%), although it showed low TPC, suggesting the role of non-phenolic antioxidants. *B. diffusa* 70% ethanol extract showed a good antioxidant power (24.31%) which is similar to that of the StandardMix (23.85%), enhancing its role as a potent free radical scavenger. The aqueous extract had low levels of activity (10.22%) and is correlated to the low polyphenolic content. These results show the solvent dependency of the bioactivity of *B. diffusa* and its potential exploitation as a source of natural antioxidants, as an indicator of chemical radical scavenging capacity.

Correlation analysis and heatmap visualization

Correlation matrix was calculated to understand the relationships between the phytochemicals content and the antioxidant activity. The results are represented as a heatmap (Figure 1). A strong positive correlation was observed between TPC and TFC ($r = 0.955$). The separate phenolic compounds like chlorogenic acid, gallic acid, caffeic acid, quercetin and rutin also showed strong positive correlation among themselves ($r > 0.94$). The correlation between DPPH activity and TPC was weak ($r = 0.008$), while moderate with chlorogenic acid ($r = 0.692$) and quercetin ($r = 0.661$). This indicates the contribution of specific flavonoids to the antioxidant capacity of these extracts. The compound-specific activity describes the importance of detailed phytochemical profiling to predict therapeutic potential of studied materials.

Table-2. Quantitative phytochemical and antioxidant profile of plant extracts.

Sample_ID	TPC (mg GAE/g)	TFC (µg CE/mL)	Chlorogenic (mg/g)	Gallic (mg/g)	Caffeic (mg/g)	Quercetin (mg/g)	Rutin (mg/g)	DPPH (%)
B_diffusa_70E	34.00	60.52	0.25	0.18	0.12	0.31	0.42	24.31
B_diffusa_50E	29.80	55.23	0.22	0.16	0.11	0.28	0.38	21.45
B_diffusa_water	12.30	28.45	0.08	0.05	0.04	0.12	0.15	10.22
G_aparine	10.20	31.31	0.18	0.12	0.09	0.25	0.38	12.88
C_erythraea	5.60	32.10	0.22	0.15	0.10	0.28	0.35	37.02
Standard_Mix	27.50	58.90	0.24	0.17	0.11	0.30	0.41	23.85

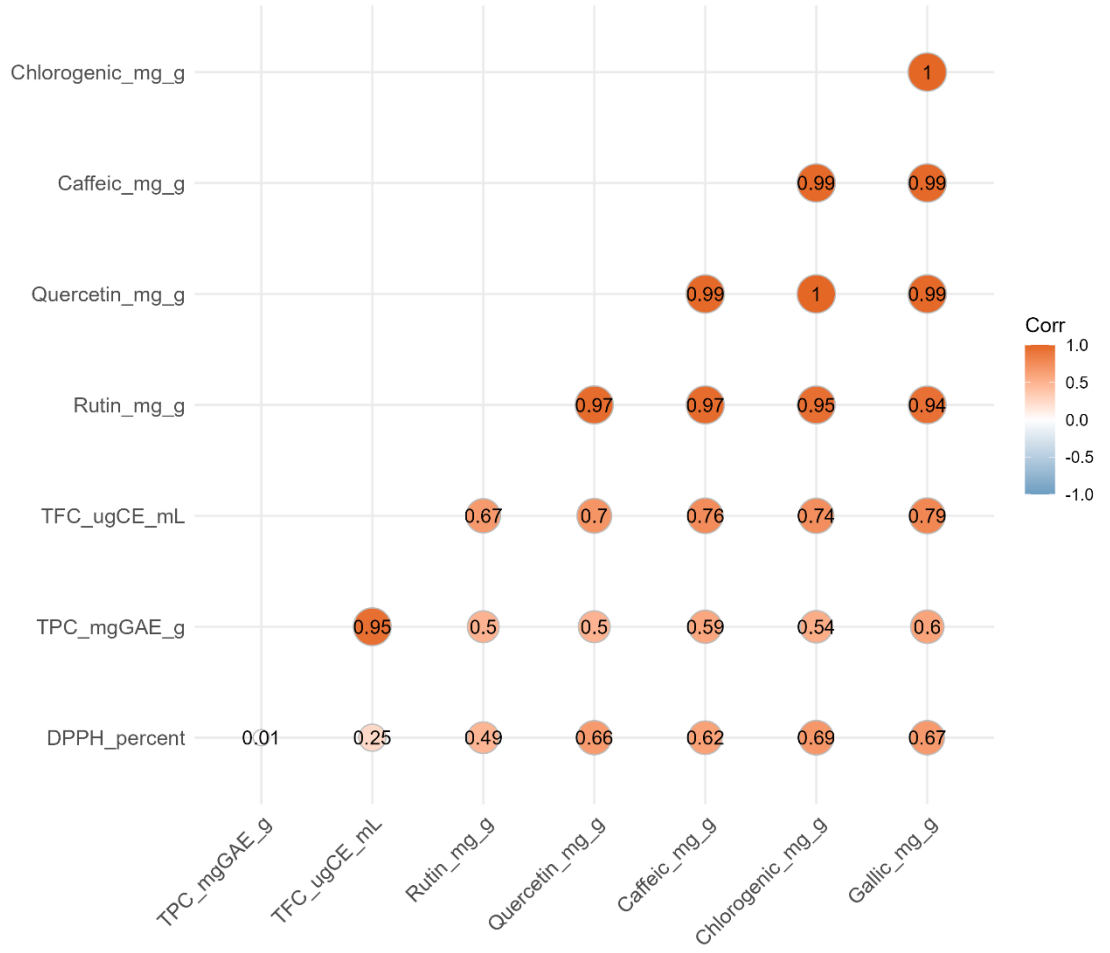

Figure-1. Correlation matrix heatmap showing relationships between phytochemical parameters and DPPH antioxidant activity.

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Hierarchical clustering and dendrogram analysis

Hierarchical clustering analysis showed grouping of the samples on the basis of their phytochemical similarity, represented in the form of dendrogram and integrated heatmap (Figure 2). The result of the analysis showed two main clusters of *B. diffusa* ethanolic extracts, *B. diffusa* aqueous extract, *G. aparine* and *C. erythraea* forming a cluster with the Standard Mix. The close clustering of ethanolic extracts to the Standard Mix proves that the extracts are compositionally rich and balanced. The *C. erythraea* is subclustered due to its unique antioxidant profile in association with its lower TPC. This clustering pattern highlights the importance of extraction solvent on phytochemical yield and demonstrates the unique phytochemical composition of each species which may be reflected on the differential therapeutic efficacy.

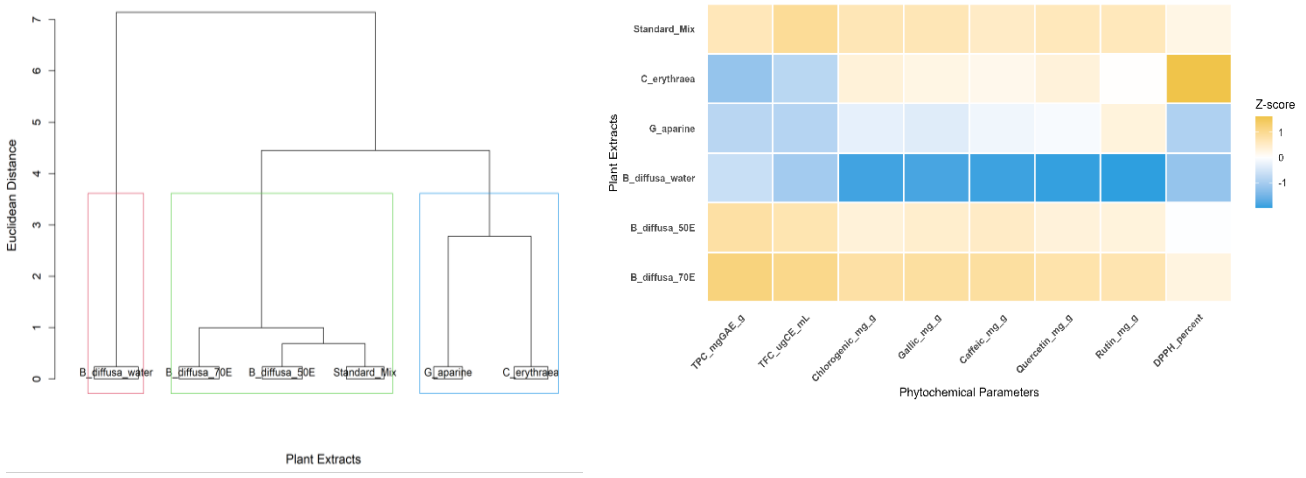

Figure-2. Hierarchical clustering dendrogram with heatmap shows phytochemical similarity among plant extracts. Samples are grouped based on Euclidean distance and Ward's linkage method.

Principal component analysis (PCA) and biplot

A Principal Component Analysis (PCA) was also performed to decrease the data dimensions and visualization of sample distribution according to phytochemical variables. The resulting biplot (Figure 3) accounted for 88.7% of the overall variance present among the first two principal components (PC1: 72.3%, PC2: 16.4%). *B. diffusa* ethanolic extracts and the StandardMix were located on the positive quadrant of PC1, close to high TPC, TFC and individual phenolics. *C. erythraea* was separated in the direction of the vector PC2; it strongly correlated with DPPH activity. This spatial separation implies that even though *B.diffusa* is marked by high polyphenolic content, *C. erythraea* can have distinctive antioxidant compounds, responsible for the higher radical scavenging activity.

Regression analysis for antioxidant activity prediction

A multiple linear regression model was developed to predict DPPH activity according to TPC and TFC (Table 3). The model was not statistically significant ($p > 0.05$) for both the predictors (TPC: $p = 0.107$, TFC: $p = 0.096$). Coefficient estimates are therefore reported descriptively only and are not interpreted mechanistically or causally. These results highlight the limitations of using aggregate phytochemical indices alone to predict antioxidant activity and support the need for compound-level analyses.

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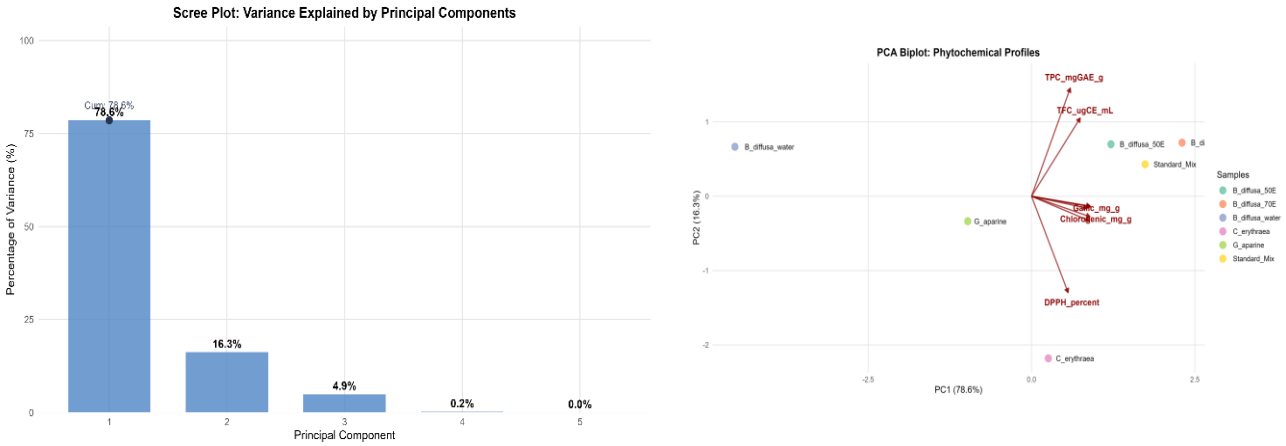

Figure-3. PCA biplot displaying sample distribution and variable loading across the first two principal components. Vectors indicate the contribution of each phytochemical parameter to the principal components.

Table-3. Multiple linear regression results for DPPH prediction.

Parameter	Coefficient	Std. Error	P-Value
(Intercept)	-12.81	15.59	0.471
TPC_mgGAE_g	-2.08	0.912	0.107
TFC_ugCE_mL	1.71	0.713	0.096

Comparative bioactivity profiling

Figure 4 illustrates the concentrations of individual phenolic acids and flavonoids across all plant extracts, as quantified by HPLC-DAD analysis. The grouped bar chart shows that rutin was the most abundant flavonoid in *B. diffusa* ethanol extracts, with concentrations of 0.42 mg/g and 0.38 mg/g in the 70% and 50% ethanol extracts, respectively. Quercetin also showed high concentration in the 70% ethanol extract (0.31 mg/g). Among phenolic acids, chlorogenic acid was present at 0.25 mg/g in the 70% ethanol extract, followed by gallic acid (0.18 mg/g) and caffeic acid (0.12 mg/g). Aqueous extracts of *B. diffusa* showed comparatively lower levels of all compounds, with rutin and quercetin at 0.15 mg/g and 0.12 mg/g, respectively. The comparative species, *C. erythraea* contained moderate levels of chlorogenic acid (0.22 mg/g) and rutin (0.35 mg/g). *G. aparine* exhibited lower but consistent concentrations across all measured compounds. The Standard Mix displayed a profile similar to that of the *B. diffusa* ethanol extracts, with rutin at 0.41 mg/g and quercetin at 0.30 mg/g.

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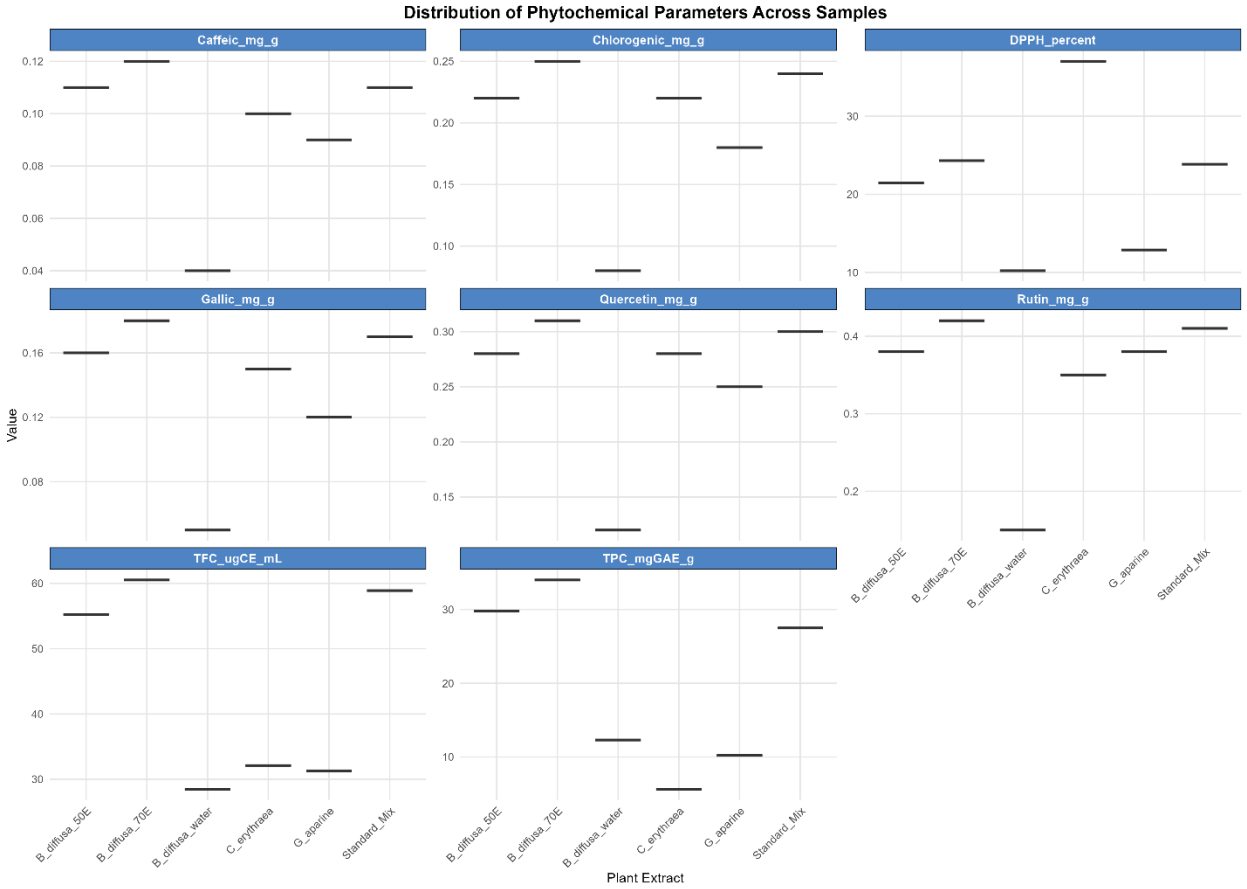

Figure-4. Grouped bar chart showing concentrations of individual phenolic acids and flavonoids in each extract.

Discussion

The current study successfully demonstrated phytochemical and bioactivity profile providing an insight into the phytochemical composition and in vitro antioxidant properties of *Boerhavia diffusa* L. (Punarnava). The qualitative profiling revealed the abundance of alkaloids, flavonoids, phenols, saponins and glycosides, while tannins and steroids were absent. These findings indicate a balanced combination of metabolites identified under the present analytical conditions (Seetharaman et al., 2024). Notably, saponins identified in this study have been associated in literature with diuretic effects (Diniz et al., 2012).

The quantitative profiling revealed that 70% ethanolic extract showed higher extraction efficiency for polyphenols with the highest polyphenolics of 34 mg GAE/g and flavanones of 60.52 ug CE/mL. This clearly shows the efficacy of ethanol in solubilizing medium polarity phenolics and flavonoids that are not easily accessible to aqueous extraction (Patel et al., 2025). The predominant flavonoids were found to be rutin (0.42 mg/g) and quercetin (0.31 mg/g) as found by quantification using high-performance liquid chromatography (HPLC). Both compounds are well documented in the literature for antioxidant and anti-inflammatory activities - rutin (Al-Kuraishy et al., 2022; Li et al., 2022). Their abundance provides a phytochemical reference relevant to antioxidant-related literature (Dutta et al., 2021).

Results of DPPH radical scavenging showed that *C. erythraea* performed better than *B.diffusa* despite the low TPC levels, showing the limitation of TPCs as only bioactivity predictors. This is in line with current understanding in phytopharmacology that it is not the total phenolic pools that handle biological effects, but rather specific metabolites (Upadhya et al., 2014). The moderate correlation of DPPH with chlorogenic acid ($r = 0.692$) and quercetin ($r = 0.661$), but not with TPC ($r = 0.008$) strengthens this idea and highlights the

flavonoid-specific redox chemistry (Kumar et al., 2023).

Multivariate analyses contributed more dimensionality to the data. Hierarchical clustering of the ethanolic extracts of *B. diffusa* with the Standard Mix resulted in confirmation of their chemical richness and their potential as quality standards. PCA explained 88.7% of variance where PC1 (72.3%) correlated with the phenolic abundance and PC2 (16.4%) correlated with the antioxidant activity visually discriminating the *B. diffusa* (high phenolics) and *C. erythraea* (high antioxidant activity). This separation highlights the metabolic uniqueness of each species and the multi-dimensionality of phytochemical activity (Tugizimana et al., 2018).

The non-significant multiple linear regression model that predicts DPPH from TPC and TFC ($p > 0.05$) reflects the complex and non-linear interactions existing within the matrices of phytochemicals. The negative coefficient for TPC (-2.08) shows a potential pro-oxidant or antagonistic effect of some phenolics, whereas the positive coefficient for TFC (1.71) indicates a more direct, although still non-linear, contribution of flavonoids to the antioxidant capacity. This complexity is similar to those in other medicinal plants in which between alkaloids, flavonoids, and terpenoids synergistic interactions occur and generate emergent bioactivities beyond those that are predictable from the compounds alone (Yang et al., 2014).

The non-linear relationships revealed by the chemometric analyses suggest the presence of synergistic or antagonistic interactions within the phytochemical matrix of *B. diffusa*. For instance, certain compounds may enhance the solubility or stability of others, or they may target complementary nodes in the oxidative stress cascade (e.g., radical scavenging, enzyme induction, metal chelation) (Yang et al., 2014). This complexity reflects the multifactorial nature of phytochemical interactions within whole-plant extracts.

The significance of the rutin and quercetin is especially relevant for nephroprotection. The high concentration of rutin is noteworthy because this compound has been reported in the literature to inhibit renal fibrosis via modulation of pathways such as PI3K/Akt/mTOR (Dong et al., 2023). Furthermore, punarnavine - an alkaloid marker of *B. diffusa* exerts reno-protective effects through angiotensin-converting enzyme inhibition and downregulation of pro-fibrotic cytokines such as TNF-a and IL-6. These mechanisms are in line with the traditional use of the plant in the management of edema and renal inflammation (Tugizimana et al., 2018).

Saponins in *B diffusa* have an action in addition to diuretic activity; they have a membranolytic action which can be related to the inhibition of calcium oxalate crystallization which further relates to the anti-urolithiatic potential of the plant (Talware et al., 2025). This highlights a holistic approach to urinary tract health.

Oxidative stress as well as inflammation are interrelated and causative factors in the progression of CKD. Flavonoid-rich profile of *B. diffusa* acts in both pathways. Quercetin suppresses the activation of the inflammasome, a component of the innate immune system, in the renal macrophage cells, while rutin upregulates antioxidant enzymes such as catalase and superoxide dismutase in the renal tissue. This multi-target action is pharmacologically beneficial over the single pathway inhibitors, which is aligned with the poly-pharmacology paradigm, as practiced in herbal medicine (Talware et al., 2025).

The lack of tannins is considered clinically beneficial as tannins have the ability to precipitate proteins and may worsen renal tubular toxicity in the case of compromised kidneys. This may be the reason for the historical reputation of the plant in chronic use for safety. Moreover, the absence of steroids eliminates the risk of unintentional endocrine modulation which is a common concern with prolonged phytotherapy (Mishra et al., 2014). However, the absence of tannins and steroids in qualitative screening cannot lead to safety conclusions without toxicological evaluation.

Overall, this study establishes a detailed phytochemical and in vitro antioxidant profile of *Boerhavia diffusa* that can be used for future biological investigations. The identification of ethanol as the best extractant, rutin and quercetin as important bioactive flavonoids, and proof of complex phytochemical synergy offer a good basis for standardization. Further studies may explore *in vivo* dose–response relationships and pharmacokinetics. By linking the knowledge of Ayurveda with contemporary phytochemistry, this work has supported the creation of evidence-based, quality-controlled Punarnava formulations for renal health.

Conclusion

This study provides a detailed phytochemical and bioactivity profile of *Boerhavia diffusa*. The 70%

ethanolic extract was richest in polyphenols, with rutin and quercetin identified as major flavonoids, and exhibited significant in vitro antioxidant activity. Multivariate analysis revealed that this activity was linked to specific flavonoids rather than total phenolic content, highlighting the importance of compound-specific standardization. While the absence of tannins and steroids is consistent with historical safety records, formal toxicological studies are warranted. Collectively, these findings provide a phytochemical and antioxidant framework for future biological investigations of *B. diffusa* and establish a clear foundation for standardizing its extracts based on key flavonoid markers for future biological evaluation.

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Contribution of Authors

Khalil MT & Akram M: Conceived idea, planned the experiments and edited manuscript.
Khalil MT: Conducted the experiments, collected data, prepared the initial draft and edited manuscript.
Rashid A: Managed the essential materials, reagents, analysis tools and reviewed manuscript.
Akram M: Assisted in data analysis and reviewed manuscript.

All authors read and approved the final draft of the manuscript.

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