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Research Article

BIOLOGICAL ACTIVITIES AND ANNOTATION OF BIOACTIVE PRINCIPLE BY MASS SPECTROMETRY IN THE ROOT EXTRACT OF *Boerhavia diffusa*

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ABSTRACT

Boerhavia diffusa is a well-known plant for ethnomedical uses. The plant is well known for its antioxidant and antidiabetic properties. Therefore, in addition to the antioxidant and antidiabetic properties of this plant, the present study focuses on exploring antibacterial properties, and metabolites present in *Boerhavia diffusa*. The preliminary phytochemistry was studied by the colour differentiation method. The phenolic and flavonoid content of the plant extracts was calculated using the Folin-Ciocalteu phenol and aluminum chloride colorimetry methods. Similarly, to find out the radical scavenging capacity of the available samples, one of the reliable methods (DPPH assay) was conducted. To evaluate the antidiabetic property, α-amylase and α-glucosidase inhibition were conducted by colorimetry methods. Results of phytochemical screening indicate that the plant is rich in metabolites including alkaloids, flavonoids, and terpenoids. Along with this, root extracts were found to be rich in total phenolic and flavonoid content with calculated values of 40.84±1.90 mg GAE/g and 37.01±1.61 mg QE/g, respectively. Similarly, among three methanolic extracts of root, stem, and leaf; the root showed the highest antioxidant activity with IC_{50} value 152.83±4.85 μg/mL. Among different solvent fractions of the stem of the same plant, the most potential antioxidant activity was shown by ethyl acetate fractions with an IC_{50} value of 82.96 ± 2.23 μg/mL. Additionally, no extracts and fractions were significantly active against α-amylase and α-glucosidase inhibition except ethyl acetate fraction. This fraction exhibited the enzyme inhibition property with IC_{50} value of 493.91±2.53 μg/mL for α-amylase and 96.31±10.65 μg/mL for α-glucosidase. To identify the chemical composition, mass spectrometry-based isolation and molecular annotation were carried out and metabolites like 3,9,10-trimethoxy-13-methyl-5,6-dihydroisoquinoline[2,1-b]isoquinolin-7-ium-2-olate, (E)-3-(4-hydroxy-3-methoxyphenyl)-N-[2-(4-hydroxyphenyl) ethyl]prop-2-enamide, 11,12-dihydro-2,3-(methylenedioxy)-7,8-dimethoxybenzo[c]phenanthridine, and (E)-3-(4-hydroxy-3-methoxyphenyl)-N-[2-(4-hydroxy-3-methoxyphenyl)ethyl]prop-2-enamide were reported. These results provide strong support for the ethnomedical uses of *B. diffusa* since the ancient period against diabetes and to cure infections. However, the evaluation of biological activities from the active compounds is not performed in this study.

Keywords: *Boerhavia diffusa*, antioxidant, diabetes, DPPH, secondary metabolites

INTRODUCTION

Diabetes, a global health issue, is considered a 21st-century epidemic, chronic metabolic chaos (Nowicka *et al.*, 2016) caused by complications with insulin production or usage (Diabetes Atlas, 2021). Especially, Type 2 diabetes (T2D) is a global concern whose complexity is related to insulin resistance (Sathiyaseelan *et al.*, 2021). One of the most appropriate methods to manage T2D is lowering the glucose level in the blood which is possible by inactivating the digestive enzymes. More specifically α-glucosidase and α-amylase are two digestive enzymes which on inhibition blood glucose level decrease and also help to control postprandial hyperglycemia (Chiou *et al.*, 2017; Kalita *et al.*, 2018; Lankatillake *et al.*, 2019). The inhibition of these two enzymes can slow the oligosaccharide hydrolytic cleavage and carbohydrate digestion process (Kumar *et al.*, 2011). Various antidiabetic drugs such as acarbose, miglitol, dapagliflozin, and empagliflozin are currently available, and they aid in regulating blood glucose levels through diverse metabolic mechanisms, such as insulin secretion, α-glucosidase inhibition, and SGLT-2 inhibition (Sathiyaseelan *et al.*, 2021). Extended use of antidiabetic drugs can result in toxicity, hypoglycemia (Sathiyaseelan *et al.*, 2021), and gastrointestinal issues (Ardalani *et al.*, 2021). Hence, there is a growing global demand for safe and potent natural-plant-based alternative agents for managing type 2 diabetes (T2D). *Boerhavia diffusa* is a plant that possesses potential medicinal properties for managing diabetes.

The usage of *B. diffusa* in traditional medicine dates back a considerable period. *B. diffusa*, a perennial creeping weed of the Nyctaginaceae family, has tough, tapered roots and spreading branches. The entire plant of *B. diffusa* is utilized globally for medicinal purposes (Nayak & Thirunavoukkarasu, 2016). According to Ayurveda, *B. diffusa* is categorized as a "Rasayana" herb, known for its anti-aging properties, improving brain function, and disease prevention (Mishra *et al.*, 2014).

Additionally, pharmacological research on the same plant has shown that the plant exhibits anti-inflammatory, antibacterial, antidiabetic, and antioxidant activities (Jain & Khanna, 1989; Olukoya *et al*., 1993; Pari & Amarnath Satheesh, 2004). Furthermore, these pharmacological activities of the plant are believed to be due to the presence of potential metabolites that protect and help to cure different disorders and diseases (Chaudhary & Dantu, 2011; Kaur, 2019). Some of the metabolites isolated from *B. diffusa* using different plant parts with their significant properties are shown in Table 1.

Table 1. Some of the secondary metabolites reported from *Boerhavia diffusa* with biological importance

Organic groups	Reported compounds	Reported property	Parts used	References
Isoflavone	2'-O-Methyl abronisoflavone	-	Leaves	(Borrelli *et al*., 2005)
C-methyl flavone	Borhaavone	-	Roots	(Mishra *et al*., 2014)
Phenolic glycoside	Punarnavoside	Antifibrinolytic	Roots	(Jain & Khanna, 1989)
Flavonol	Quercetin, kaempferol	Antinflammatory	Leaves	(Mishra *et al*., 2014)
Lignin	Liriodendrin	Ca^{2+} channel antagonist	Roots	(Ojewole & Adesina, 1985)

The literature review reveals that there has been limited research on the genus currently growing in Nepal. Plant metabolite synthesis heavily relies on geographical conditions and habitat. Therefore, it's crucial to explore the pharmacological significance of this plant, considering its historical use across various regions of the country. Consequently, the current research aims to reveal the antioxidant, antibacterial, and antidiabetic potential of crude methanolic extracts and different solvent fractions of *B. diffusa* growing in Nepal. Additionally, the aim also includes this research helps to global exposure of this plant regarding the richness of phytochemicals and the biological activities.

MATERIALS AND METHODS

Chemicals and Equipment

Acarbose, digestive enzymes (α-amylase and α-glucosidase), CNPG3, *p*-NPG, and DPPH were brought from Sigma-Aldrich (Germany). Gallic acid, quercetin, dimethyl sulphoxide, disodium hydrogen phosphate, sodium dihydrogen orthophosphate, and methanol were of analytical grade and bought from Fisher Scientific.

Plant Collection and Preparation of Extracts

The plant *B. diffusa* was collected from western Nepal. The collected specimens were identified at the National Herbarium and Plant Laboratories Godawari, Nepal, with voucher number 756. The whole plant was washed and air dried for a single day and after that different plant parts were separated as root, stem, and leaves. These separated parts were washed again and dried for a few days at room temperature. Subsequently, 200 g of dried powdered samples of the roots, stems, and leaves were separately extracted with methanol for 3 days. The solvents were filtered, and the extraction process was repeated two more times. After the collection of solvent from the extraction process, it was dried using a rotary evaporator at 39 °C. The crude extract obtained was stored in a refrigerator available at the laboratory and the dry extract was stored at 4 °C till further use.

Fractionation of Crude Extract

Methanolic extract of *B. diffusa* stem part was further used in the solvent-solvent extraction process. The prior methanol crude was treated with hexane, DCM, and ethyl acetate in a separating funnel to obtain a respective semi-purified solvent fraction. Three successive fractionations were carried out using hexane, DCM followed by ethyl acetate. Each solvent fraction was collected in the beaker and the solvent was evaporated and placed in the water bath at 40 °C. After a week of evaporation, solvent-free extracts were obtained which were used to evaluate various biological activities and estimation of some important classes of organic compounds.

Phytochemical Screening

The plant's chemical constitutes analysis was performed by following standard protocol with slight modifications (Sasidharan *et al*., 2010; Yadav *et al*., 2014).

Estimation of Total Phenolic Content (TPC)

The total phenolic content of crude extract of root, stem, and leaves was measured by using the Folin-Ciocalteu phenol reagent with slight modification (Ainsworth & Gillespie, 2007). To calculate the TPC, the first standard gallic acid was diluted and the calibration curve was drawn. The results obtained were expressed as milligrams of gallic acid equivalent per gram (mg GAE/g) of the dry weight of the extract. Initially, in the 96-well plate, 20 µL gallic acid solutions, 500 µg/mL concentration of 20 µL of the different parts of plant samples, were loaded. After that in each well containing standard and sample, 100 µL F-C reagent followed by 80 µL of 1M of Na_2CO_3 solution were added. After completion of the addition of the solution, the 96-well plate was kept in the dark place for

15 minutes, and absorbance was subsequently measured at 765 nm using the microplate reader.

Estimation of Total Flavonoid Content (TFC)

The total flavonoid content of crude extract of root, stem, and leaves was measured by using the aluminum chloride colorimetric method with slight modification (Bag *et al.*, 2015). To calculate the TFC, the first standard quercetin was diluted, and the calibration curve was drawn. The results obtained were expressed as milligrams of quercetin equivalent per gram (mg QE/g) of the dry weight of the extract. Initially, in the 96-well plate, 130 µL quercetin solutions, 500 µg/mL concentration of 20 µL of the different parts of plant samples, were loaded. After that in each well containing the standard and sample, 110 µL distilled water only in the sample, followed by 60 µL of ethanol, 5 µL of aluminum chloride, and 5 µL of potassium acetate were added. After completion of the addition of the solution, the 96-well plate was kept in the dark place for 25 minutes, and absorbance was subsequently measured at 415 nm using the microplate reader.

Evaluation of Antioxidant Potential

Free radical scavenging activity of the crude extracts and different solvent fractions of *B. diffusa* were calculated by using DPPH assay (Sharma & Adhikari, 2023; Sofiane *et al.*, 2017). In this method, initially, the crude extracts and the solvent fractions of concentration 500 µg/mL were screened. For this, in a 96-well plate, 100 µL of the sample was loaded in triplicate, and in each well 100 µL of 0.1 mM DPPH solution was added. After completion of the addition of the solution, the 96-well plate was kept in the dark place for 25 minutes, and absorbance was subsequently measured at 517 nm using the microplate reader. Samples having inhibition above 50% were further tested by diluting through the same process. In this experiment, quercetin served as a positive control, and 50 % DMSO was taken as a negative control. To calculate the percentage inhibition of DPPH radical following equation was used.

$$\%inhibition = \frac{A\ control - A\ sample}{A\ sample}\ x\ 100$$

In the given equation, A control represents the absorbance of control whereas A sample indicates the absorbance of the sample respectively.

Antidiabetic Assay

***In-vitro* α-Amylase Inhibition Assay**

α-Amylase inhibition assay was carried out in 96-well plates by adopting the method used by Senger *et al.*, with slight modifications (Senger *et al.*, 2012). In this assay, 20 µL of plant extracts of concentration 500 µg/mL prepared in 50% DMSO and 80 µL of porcine pancreatic α-amylase of concentration 1.5 U/mL prepared in sodium phosphate buffer of pH 7.0 was loaded in microplate wells and initial absorbance was taken. After that, the plate was incubated at 37 °C for 10 minutes. Then, 100 µL of a substrate of 1 mM $CNPG_3$ was added to start the reaction with incubation for 25 minutes, and the change in absorbance was monitored at 405 nm. 50% DMSO was used as a negative control. During the experiment, substrate 2-chloro-4-nitrophenyl-α-D-maltotrioside ($CNPG_3$) acts upon the α-amylase to release more than 98% of 2-choloro-4-nitrophenyl (CNP), and forms 2-choloro-4-nitrophenyl-α-D-maltoside($CNPG_2$), maltotriose (G_3) and Glucose (G) which is inhibited by the sample extracts. The percentage inhibition of α-amylase enzyme was given by:

$$\%inhibition = \frac{A\ control - (A\ extract + enzyme)}{A\ control}\ x\ 100$$

***In-vitro* α-Glucosidase Inhibition Assay**

α-Glucosidase inhibition assay was carried out in a 96-well plate by adopting the method used by Hugues *et al.*, with slight modification (Fouotsa *et al.*, 2012). In a brief procedure, a 20 µL sample extract, 20 µL of the α-glucosidase enzyme of concentration 0.5 U/ML, and 120 µL of phosphate saline buffer solution of pH 6.8 were loaded in microplate wells, and initial absorbance was taken. Later the plate was incubated at 37 °C for 10 minutes. Then, the substrate of concentration 1.4 mM pNPG was added to start the reaction in the 96-well plate and incubated for 25 minutes, and absorbance was subsequently measured at 400 nm. During the experiment, substrate p-Nitrophenyl-α-D-Glucoside (pNPG) acts upon the α- glucosidase and forms p-nitrophenol and α-D-glucose which is inhibited by the sample extracts. In this assay, 30% DMSO was taken as the negative control and acarbose served as the positive control. The percentage inhibition of α-glucosidase enzyme was given by:

$$\%inhibition = \frac{A\ control - (A\ extract + enzyme)}{A\ control}\ x\ 100$$

Evaluation of Antibacterial Activity

To calculate the antibacterial properties of different parts of a plant extract, the agar well diffusion method was carried out (Bhattarai *et al.*, 2023). For the test of antimicrobial activities, a 50 mg/mL concentration of crude extracts was prepared. Four different bacteria mentioned in Table 2 were cultured in Muller Hinton broth solution and incubated overnight at 37 °C. In a dried MHA plate, the carpet culture of inoculum was prepared. The well of 6 mm diameter was made by using a cork-borer. In a 100 mm plate, 5 wells were prepared. After this, 50 µL of sample extracts were added along with positive and negative control in respective wells. For the antibacterial activity, 50 % DMSO solution was taken as a negative control whereas Neomycin served as a positive control. After completion of the addition of the sample in each well, the plates were incubated for 16 hours. After that with the help of a measuring scale, the zone of inhibition was measured.

Table 2. Bacteria used in the study with reference number

Bacteria	Reference No. and Strains
Staphylococcus aureus	(ATCC – 25293) Gram-positive
Escherichia coli	(ATCC – 25922) Gram-negative
Salmonella typhi	(ATCC – 14028) Gram-negative
Klebsiella pneumonia	(ATCC -13883) Gram-negative

Metabolomics Profiling

High-resolution-mass spectrometry (HRMS) was utilized to analyze and characterize the molecular structures of compounds present in a bioactive crude extract of roots from the plant *B. diffusa*. The molecular formulas of the compounds were determined by using the SIRIUS 4 platform. The sample for HRMS measurements was prepared by dissolving 0.2 milligrams of the methanolic/aqueous root extract in one milliliter HPLC grade methanol. An LC method was applied with water containing 0.1% formic acid and acetonitrile as solvent. A gradient from 10% to 100% over 32 minutes was used at a flow rate of 0.5 mL/min. A capillary voltage of 4.5 kV, nitrogen gas pressure of 2 bar, and an ion source temperature of 200 °C were employed. For MS2 fragmentation, the 4 most intense ions from MS1 were selected, and stepped CID energy was applied. To evaluate the extent of molecular ionization, the data obtained were first analyzed in the Bruker Compass Data Analysis 4.0 software. The adduct ions $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$, $[2M+H]^+$, $[2M+Na]^+$, $[M+2H]^{2+}$, and $[M+2Na]^{2+}$ were set as primary and seed ions to determine the molecular ions using Metaboscape software. The retention time range of 4-32 minutes and the molecular mass range of 200-1500 m/z were considered for bucketing the list of molecular ions based on the observed base peak chromatogram. The HRMS data of the root extract revealed molecular ions, along with their respective retention times, and abundances (relative ion intensities). A list of these molecular ions, sorted by their relative abundances, was exported in the mascot generic format (MGF) files. To confirm metabolites sorted by relative abundance, individual raw MS data underwent CSI: FingerID analysis in the SIRIUS 4 platform. The laboratory work was carried out at the University of Tübingen.

Statistical Analysis

To make the data more accurate all assays and laboratory work were performed in the triplicate and findings obtained from the experiments were expressed in mean ± standard error of the mean. Gen5 microplate data collection and analysis software, GraphPad Prism software version 8, and MS Excel 2007 were used for the calculation of TPC, TFC, and antioxidant activity.

RESULTS AND DISCUSSION

Phytochemical Screening

Different classes of secondary metabolites are found in the three crude extracts of root, stem, and leaves of *B. diffusa* which is shown in Table 3. However, coumarins and reducing sugars were absent in the crude extract of all parts of *B. diffusa* but saponins remain present in the root extract. These results seem supported by the previous research conducted on the same plant. From the previous study conducted on phytochemical screening of *B. diffusa* similar result was obtained (Chinnappan *et al.*, 2012). This correlation supports the reliability and reproducibility of the reported phytoconstituents. However, certain changes can occur because of variations in altitude, climatic status, and way of extraction. It is widely acknowledged that the phytochemical profile of plants can be influenced by these factors, leading to discrepancies in the reported results.

Table 3. The qualitative phytochemical analysis of extracts of *Boerhavia diffusa*

Group of compounds	Root extract	Stem extract	Leaf extract
Basic alkaloids	+	+	+
Flavonoids	+	+	+
Glycosides	+	+	+
Quinones	-	+	+
Polyphenols	+	+	+
Tannins	+	+	+
Terpenoids	+	+	+
Saponins	+	-	-
Reducing sugars	-	-	-
Coumarins	-	-	-

Note: + indicates the presence of compounds whereas, - indicates the absence of compounds.

Total Phenolic and Flavonoid Content

The crude root extract consists of a higher phenolic content as compared to the crude stem and crude leaves extract. The value of total phenolic content of crude root extract was obtained at 40.84 ± 1.90 mg GAE/g while that of stem value was 32.76 ± 2.48 mg GAE/g. This result is better as compared to a previous study conducted by Pallavi *et al.* having higher phenolic

content in leaves, followed by the stem and root, with values of 24.50 ± 1.70, 7.77 ± 0.7, and 0.25 ± 0.24 mg QE/g respectively (Sharma *et al.*, 2014).

Similarly, the crude root extract consists of a higher flavonoid content as compared to the crude stem and crude leaves extract. The value of total flavonoid content of crude root extract was found 37.01 ± 1.61mg QE/g while the stem exhibited a lower total flavonoid content of 7.85 mg QE/g. The calibration curve for the gallic acid and quercetin is shown in Figure 1. The TPC and TFC values of different crude extracts of plant samples are shown in Table 4.

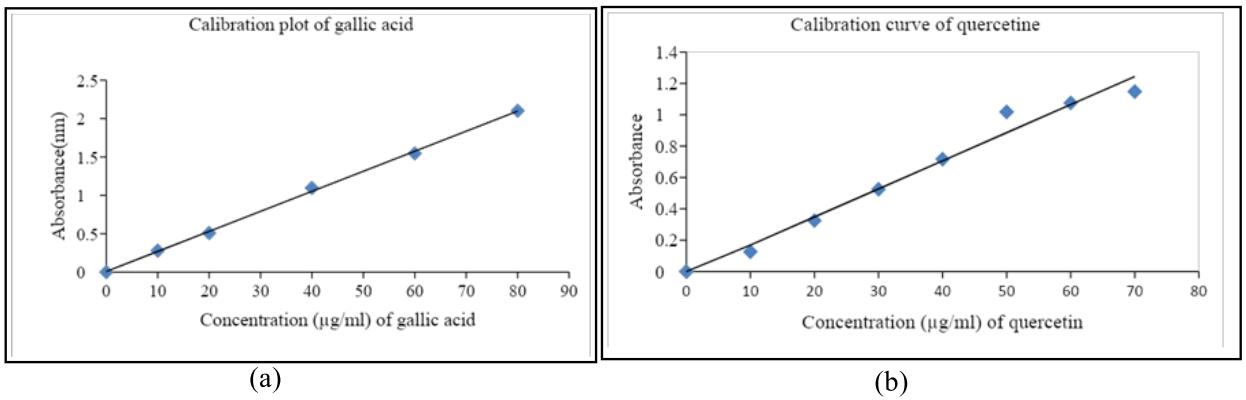

Figure 1. Calibration curve of standard (a) gallic acid with the regression equation y = 0.026 x + 0.0078, R^2 = 0.998 and (b) quercetin with the regression equation y = 0.019x-0.049, R^2 = 0.994

Table 4. Total phenolic and flavonoid content in different extracts of *Boerhavia diffusa*

Crude extracts of the plant	TPC (mg GAE/g)	TFC (mg QE/g)
Root	40.84 ± 1.90	37.01 ± 1.61
Stem	32.76 ± 2.48	7.85 ± 0.43
Leaf	33.28 ± 1.90	9.78 ± 0.64

A previous study (Sirou *et al.*, 2018), reported a TFC value of 6.243 mg QE/100 mg for the dichloromethane extract of the stem and 4.71 mg QE/100 mg for ethanolic extract, showing similar results for the stem extract. In current research roots exhibited a higher content of phenolic and flavonoid than the aerial parts, this can be attributed to the environmental influences such as locality, soil composition, temperature, and the timing of sample collection. These factors influence the distribution of metabolites among the different parts of the plant.

DPPH Radical Scavenging Activity

From the result obtained root exhibited the strong radical scavenging activity among the crude extracts with an IC_{50} value of 152.83 ± 4.85 µg/mL. Similarly, among the solvent fractions, ethyl acetate had the lowest IC_{50} value of 82.96 ± 2.23 µg/mL which is thirteen times more than the standard drug quercetin. All the results of the radical scavenging activity of crude extracts and solvent fractions are presented in Table 5. Previous studies conducted on *B. diffusa* reported that IC_{50} values of methanol extract were 0.240 mg/mL, whereas ethanolic extract had 0.46 mg/mL, and similarly ethyl acetate extract had values of 0.950 mg/mL (Sirou *et al.*, 2018). This finding provides strong evidence of the antioxidant properties of *B. diffusa*.

Table 5. Screening and calculation of IC_{50} value of different extracts of *Boerhavia diffusa*

Plant parts/extracts	Inhibition % at 500 µg/mL	IC_{50} (µg/mL)
Leaf	60.11	349.34 ± 14.06
Stem	63.93	335.13 ± 12.54
Root	79.55	152.83 ± 4.85
Fractions of crude extracts		
Hexane	23.51	-
DCM	74.35	158.82 ± 4.26
Ethyl acetate	91.62	82.96 ± 2.23
Water	31.70	-
Quercetin		6.29 ± 1.02

Antibacterial Property

Four different bacteria were screened using the crude extract of the root, stem, and leaf of *B. diffusa*. Among four different bacteria, three were Gram-negative and one bacterium was Gram-positive. The root extract of *B. diffusa* showed better results against bacteria as compared to the other two different extracts. The zone of inhibition of each bacterium against positive control, negative control, and sample is presented in Table 6 and Figure 2. Previously, screening of leaves of methanolic extract of *B. diffusa* was unable to show inhibition against different bacteria including *E. coli, P. aeruginosa,* and *S. typhi* but showed inhibition against *S. aureus,* and *S. faecalis* (Umamaheswari *et al.*, 2010). Methanolic extract of the aerial part of *B. diffusa* performed moderate inhibition against *S. aureus* whereas ethyl acetate and hexane fraction had no inhibition on two bacteria *S. aureus* and *S. dysenteeiae* at a concentration of 500 µg/disc (Apu *et al.*, 2012).

Table 6. Screening of crude of different parts of *Boerhavia diffusa* against four strains

Bacterial strain	The concentration of the sample (mg/mL)	Sample	ZOI (mm)
Staphylococcus aureus	50	PC	11
	50	CR	10
	50	CS	-
	50	CL	-
	50	NC	-
Escherichia coli	50	PC	18
	50	CR	-
	50	CS	-
	50	CL	-
	50	NC	-
Salmonella typhi	50	PC	18
	50	CR	-
	50	CS	-
	50	CL	-
	50	NC	-
Klebsiella pneumoniae	50	PC	21
	50	CR	-
	50	CS	-
	50	CL	7
	50	NC	-

Note: - not active against the bacteria, PC: positive control, CR: Crude root extract, CS: Crude stem extract, CL: Crude leaf extract, NC: negative control

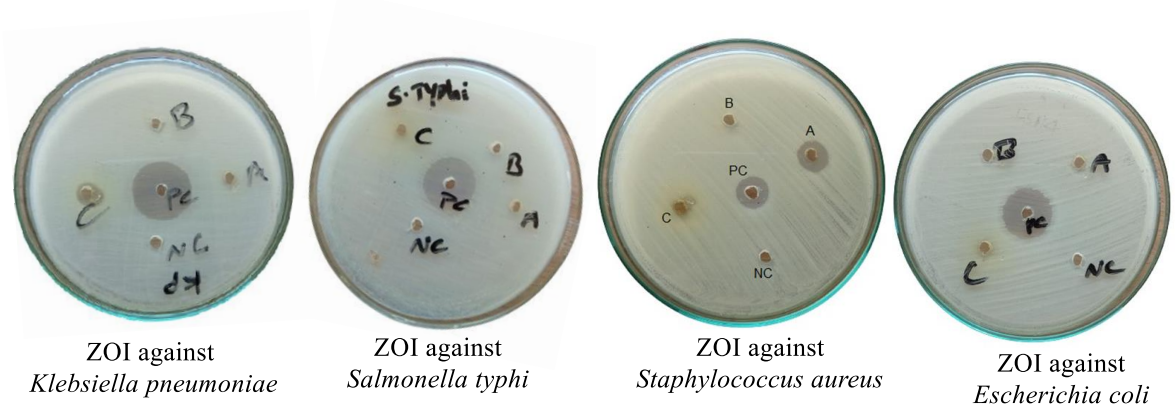

Figure 2. ZOI showed by the plant extracts against different bacteria

Antidiabetic Properties

α-Amylase Inhibition

Before calculating the inhibition on different concentrations, α-amylase inhibition screening was done on the available samples. Results indicated that methanolic crude extracts were worthless for α-amylase inhibition. These crude extracts showed an inhibition percentage less than 10% so they were excluded from the further α-amylase inhibition test. However, among different solvent fractions, only the ethyl acetate fraction showed promising results, with an inhibition percentage of 51.54% at 500 µg/mL concentration. This indicates that ethyl acetate fraction has the potential to inhibit the α-amylase. Therefore, the fraction with higher inhibition was further diluted and subjected to calculate the IC_{50} value. To compare the

potential of a sample, the standard drug acarbose was also used. The screening percentage and calculated IC_{50} values are presented in Table 7. These α-amylase inhibition results of the sample showed that the ethyl acetate fraction can inhibit moderately as compared to the acarbose. To justify the current result, previous research conducted was compared and in the previous study α-amylase inhibition percentage of root extract of *B. diffusa* was 57.24% at 500 µg/mL concentration (Akhter *et al*., 2013). These findings provide evidence that *B. diffusa* may have potential as an antidiabetic agent due to its ability to inhibit α-amylase.

α-Glucosidase Inhibition

Digestive enzyme α-glucosidase is also involved in carbohydrate metabolism. Thus, the inhibition of α-glucosidase is key to controlling diabetes. First of all, all the crude extracts and different solvent fractions at a concentration of 500 µg/mL were screened to test the possibility of α-glucosidase inhibition. The results showed that crude extracts did not show potent activity against glucosidase inhibition, with inhibition ranging from 40% to 44%. Therefore, these crude extracts were not subjected to further tests. However, for solvent fractions, screening of ethyl acetate fraction has inhibition of 80.39% at the same concentration, which is followed by hexane and DCM fractions, 62.73% and 56.66% inhibition, respectively. These three solvent fractions were subjected to IC_{50} value calculation. Among these three solvent fractions, ethyl acetate has the lowest IC_{50} value of 96.31 ± 10.65 µg/mL. The hexane and DCM fractions showed IC_{50} values of 385.20 ± 14.67 and 440.94 ± 18.62 µg/mL, respectively. To compare the potential effectiveness, acarbose was used as a standard drug, that had an IC_{50} value of 344.23 ± 1.04 µg/mL. This result indicated that the ethyl acetate fraction of *B. diffusa* is most effective against α-glucosidase inhibition. The percentage inhibition and IC_{50} values of each crude extract and different solvent fractions against α-glucosidase are shown in Table 8.

Table 7. α-Amylase screening and IC_{50} value of extract from *Boerhavia diffusa*

Plant parts/extracts	Fraction	Inhibition % at 500 µg/mL	IC_{50} (µg/mL)
Root		4.41	
Stem	Ethyl acetate	51.54	493.91 ± 2.53
	Hexane	18.91	-
	DCM	14.22	-
	Water	5.31	
Leaf		7.55	-
Acarbose (standard)			6.02 ± 1.04

Table 8. Screening of α- Glucosidase and IC_{50} value of extract from *Boerhavia diffusa*

Extracts / solvent fractions	%inhibition at 500 µg/mL	IC_{50} (µg/mL)
Root	44.43	-
Stem	40.21	-
Leaf	10.21	-
Ethyl acetate	80.39	96.31 ± 10.65
DCM	56.66	440.94 ± 18.62
Hexane	62.73	385.20 ± 14.67
Water	-	-
Acarbose (standard)		344.23 ± 1.04

Mass Spectrometry and Compounds Annotation

In this study, mass spectrometry was employed to identify and annotate several compounds found in bioactive plant extracts. Through mass measurements and the determination of molecular formulas, the compounds were characterized by using MS2 spectra in the database search where the structures of the compounds were predicted using the Sirius software. This software utilizes advanced algorithms and databases to analyze the mass spectrometry data, both MS1 and MS2. The compounds were selected for the structural annotation prioritized on their abundance (shown in Figure 3) in the crude extract.

At the retention time of 16.98 minutes, a compound with m/z 352.15479 Da was eluted. Its accurate mass measurement was determined 351.14751 Da and its predicted molecular formula is $C_{21}H_{21}NO_4$, From this formula three fragments $C_{20}H_{17}NO_3$, $C_{20}H_{17}NO_4$, and $C_{20}H_{18}NO_4$ were formed by removing the CH_4O^-, CH_4, and CH_3^+ unit respectively. Also, these fragments are shown in the spectrum with a mass of 320.1290 Da, 336.1236 Da, and 337.1309 Da respectively. The first fragment remained stable but the second and third fragments underwent further breaking and formed multiple stable compounds. The fragment $C_{20}H_{17}NO_4$ stopped further fragmentation after the formation of the compound with formula $C_{17}H_{13}NO_2$ while

$C_{20}H_{18}NO_4$ fragmented into multiple stable compounds with formula $C_{19}H_{16}NO_2$, $C_{17}H_{12}NO_3$, and $C_{16}H_{11}NO_2$. After getting this fragmentation tree, the compound was identified through the Sirius and it is 3,9,10-trimethoxy-13-methyl-5,6-dihydroisoquinolino[2,1-b]isoquinolin-7-ium-2-olate. The structure of the compound and its mass spectrum is shown in shown in Figure 4.

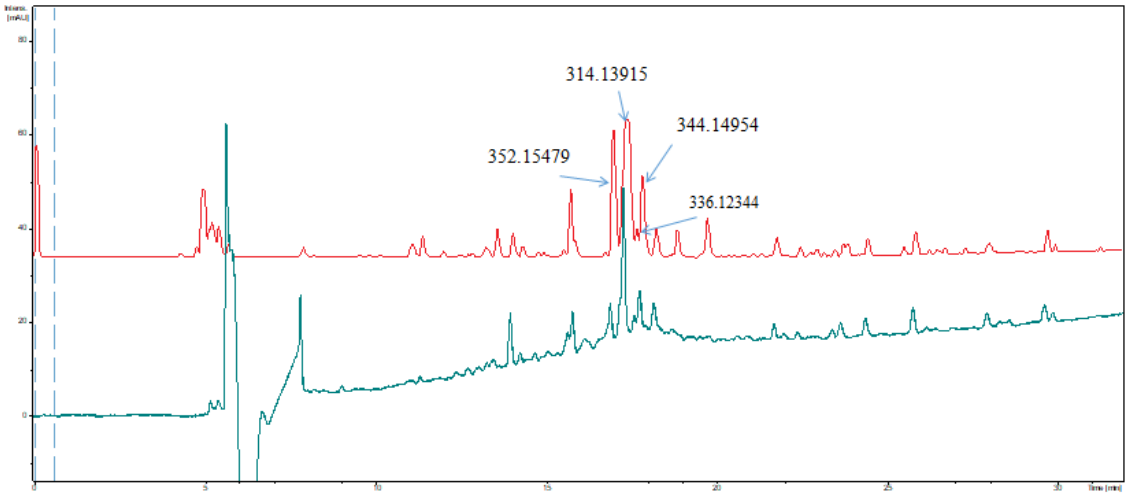

Figure 3. Red chromatogram representing the most intense peak (base peak) observed in the mass spectrum of a *Boerhavia diffusa* root extract while green chromatogram represents UV absorbance at 250 nm

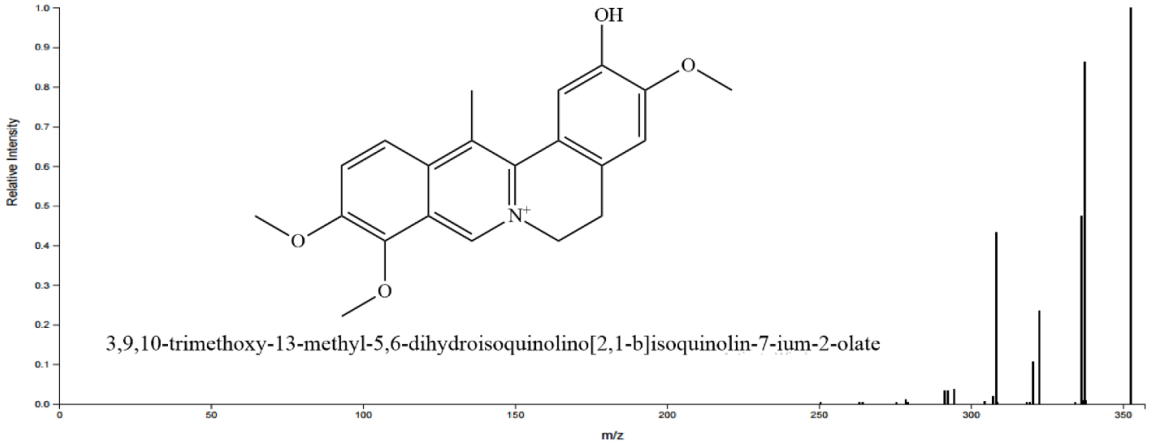

Figure 4. The mass spectrum (MS2) of the annotated compounds

Similarly, another compound eluted at a retention time of 17.24 minutes with a m/z of 314.13915 Da. Its accurate mass measurement was determined to be 313.13191 Da. This compound has molecular formula $C_{18}H_{19}NO_4$. When the $C_6H_6O^-$ group was removed from this fragment another fragment $C_{12}H_{13}NO_3$ formed with a mass of 220.0978 Da. Furthermore, on subsequent removing the two units C_2H_2 and NH_3 two fragments $C_{10}H_{11}NO_3$ and $C_{10}H_8O_3$ were formed. These fragments are also shown in the mass spectrum. On further breaking three stable compounds C_8H_4O, C_7H_8, and C_8H_6 were formed. On analyzing this fragmentation, the identified compound is (E)-3-(4-hydroxy-3-methoxyphenyl)-N-[2-(4-hydroxyphenyl)ethyl]prop-2-enamide. The structure of the compound and its mass spectrum are shown in Figure 5.

At the retention time of 17.37 minutes, a compound with an m/z value of 336.12344 Da was eluted. Its actual mass measurement was 335.11616 Da. The predicted molecular formula for this eluted compound was $C_{20}H_{17}NO_4$. This compound underwent the elimination of small molecules CH_3^+, CH_4, and CH_4O to form $C_{19}H_{14}NO_4$, $C_{19}H_{13}NO_4$, and $C_{19}H_{13}NO_3$ respectively. These three fragments have a mass of 321.0993 Da, 320.0918 Da, and 304.0973 Da respectively. The third fragment was a stable one which did not proceed further with fragmentation while the first and second molecules underwent fragmentation and formed multiple stable units. The first fragment formed $C_{17}H_{11}NO_3$, $C_{17}H_{10}NO_3$, $C_{18}H_{12}NO_2$ and $C_{17}H_{12}NO_2$ units. While the initial second fragment formed $C_{17}H_{11}NO_2$ and $C_{18}H_{13}NO_3$ units. By analyzing these patterns from Sirius, the identified compound is 11,12-dihydro-2,3-(methylenedioxy)-7,8-dimethoxybenzo[c]phenanthri -dine. The structure of the compound and its mass spectrum is shown in Figure 6.

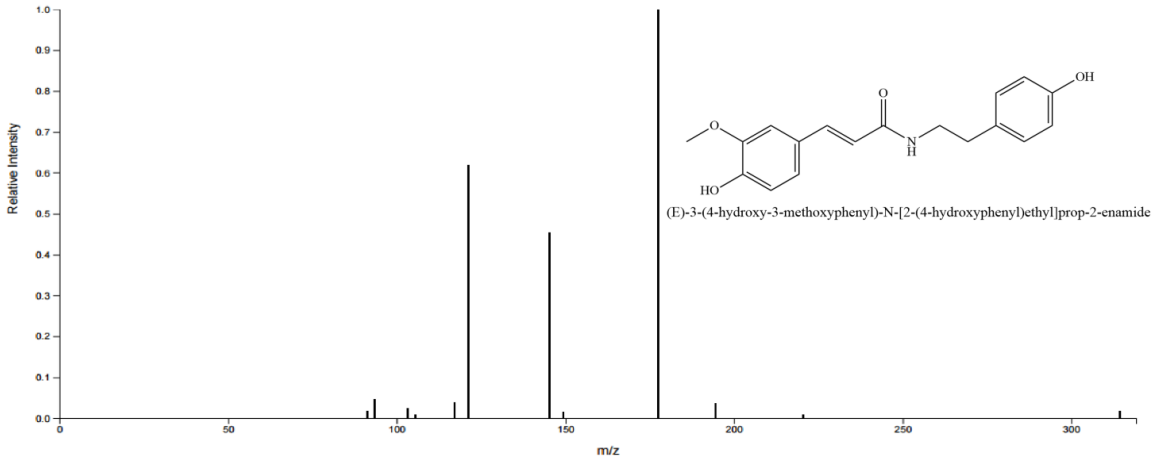

Figure 5. The mass spectrum (MS2) of the annotated compounds

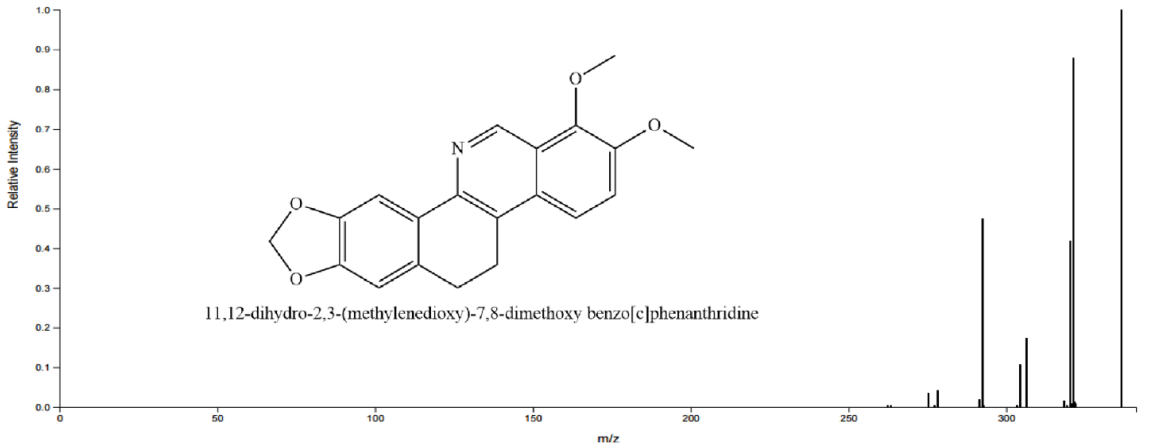

Figure 6. The mass spectrum (MS2) of the annotated compounds

The last annotated compound was eluted at 17.83 minutes. This compound exhibited an m/z of 344.14954 Da and a measured mass of 343.14228 Da. The predicted molecular formula for this compound was $C_{19}H_{21}NO_5$ which underwent multiple fragmentations of $C_{10}H_8O_3$ and $C_9H_{10}O_2$. These two fragments have mass of 177.0551 Da and 151.0758 Da which peaks are also seen in the mass spectrum. These two fragments underwent further fragmentation and formed three stable units $C_9H_8O_2$, C_8H_4O, and C_7H_6. From this fragmentation tree, the identified compound was (E)-3-(4-hydroxy-3-methoxyphenyl)-N-[2-(4-hydroxy-3-methoxyphenyl)ethyl]prop-2-enam ide. The structure of the compound and its mass spectrum is shown in Figure 7.

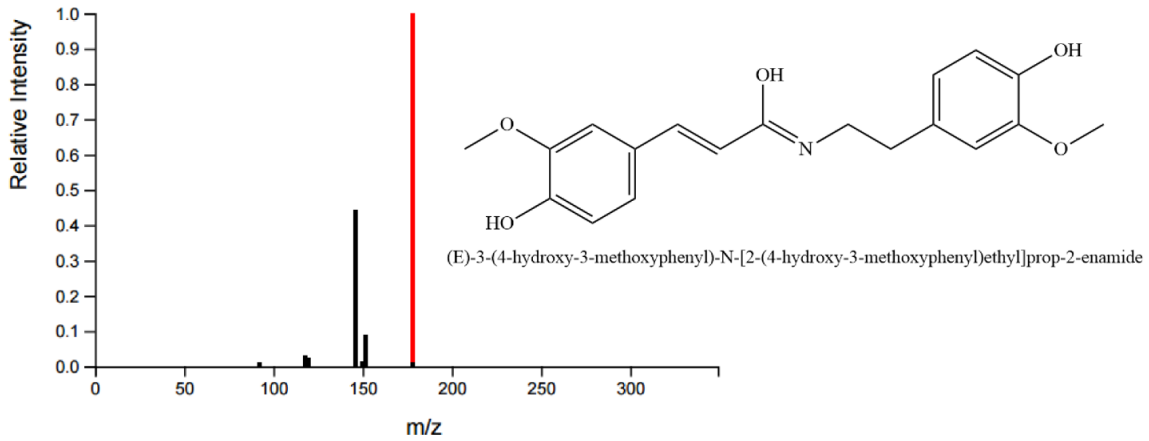

Figure 7. The mass spectrum (MS2) of the annotated compounds

From the previous research conducted on *B. diffusa*, the compound (E)-3-(4-hydroxy-3-methoxyphenyl)-N-[2-(4-hydroxyphenyl)ethyl]prop-2-enamide(Moupinamide) was isolated and identified (Balkrishna *et al.*, 2020). In this research, aqueous extract of the plant was used to identify various metabolites through liquid chromatography. This discovery provides additional evidence that supports the current findings. However, the other identified compounds were not reported properly. But the complex metabolites like N-(4-{1-[4-(4-Acetylaminophenoxy)-3-methoxyphenyl]-2-[(4-acetyl amino-phenyl)methylamino]ethoxy}phenyl)acetamide, Benzenee thanamine, N-[(pentafluorophenyl) methylene]-.beta.,3,4-tris[(trimethylsilyl)oxy]- and 5,12-d-Ethano(furo[2,3,4-mn]oxepino[2,3,4-ed]anthracen-9-ol-2-one),6-methyl-12acetoxy-2a,3,4,4a,5,7,8a-octahydr o- were reported previously (Kaviya *et al.*, 2022). So, the non-identified metabolites from the *B. diffusa* need further structure elucidation using analytical tools like IR and NMR. The characterization of compounds, particularly in terms of molecular formula, m/z, and retention time, as shown in Table 9 provides essential information for further studies. The identification of these compounds offers insights into their potential pharmacological importance and broadens our understanding of their roles in natural product drug discovery and drug development.

Table 9. Annotated compounds with their m/z and RT.

S.N.	Name of compound	Molecular formula	m/z	Mass measurement	RT (min.)	CSI: Finger ID (Score)	Mass error in ppm	Reference
1	3,9,10-Trimethoxy-13-methyl-5,6-dihydroisoquinolino[2,1-b]isoquinolin-7-ium-2-olate	$C_{21}H_{21}NO_4$	352.155	351.148	16.98	77.41	1.287	(PubChem, 2023-a)
2	(E)-3-(4-hydroxy-3-methoxyphenyl)-N-[2-(4-hydroxyphenyl)ethyl]prop-2-enamide	$C_{18}H_{19}NO_4$	314.139	313.132	17.24	82.61	1.603	(PubChem, 2023-b)
3	11,12-dihydro-2,3-(methylenedioxy)-7,8-dimethoxybenzo[c]phenanthridine	$C_{20}H_{17}NO_4$	336.123	335.116	17.37	78.71	1.199	(PubChem, 2023-c)
4	(E)-3-(4-hydroxy-3-methoxyphenyl)-N-[2-(4-hydroxy-3-methoxyphenyl)ethyl]prop-2-enamide	$C_{19}H_{21}NO_5$	344.149	343.142	17.83	64.72	0.895	(PubChem, 2023-d)

CONCLUSIONS

Boerhavia diffusa, an esteemed medicinal plant with a rich historical background, has been the subject of extensive research due to its diverse range of biological activities. The plant's leaves stems, and roots were meticulously examined to unravel their chemical composition, leading to the detection of crucial metabolites such as alkaloids, glycosides, tannins, and terpenoids. Remarkably, the results obtained demonstrated that the root comprised the highest values of phenolic content, flavonoid content, and the lowest antioxidant IC_{50} value as compared to other crude extracts. Similarly, the ethyl acetate fraction exhibited a strong antioxidant potential as compared to other tested samples which highlights its potent inhibitory effect.

Regarding the antibacterial activity of plants, no exciting results were found against *E. coli*, but promising inhibition against *Staphylococcus aureus* focuses on its antibacterial potential. Furthermore, the ethyl acetate fraction seems potent against the α-amylase and α-glucosidase enzyme inhibition. It exhibited moderate IC_{50} as compared to quercetin, and lower IC_{50} as compared to acarbose for α-amylase and α-glucosidase inhibition respectively. Additionally, the presence of alkaloids highlights the promising activity of *B. diffusa* in managing diabetes. In conclusion, this study focused on different bioactivity of *B. diffusa*, including antioxidant, antibacterial, and antidiabetic. In the future, the isolation of bioactive compounds and their structure elucidation using tools like HPLC and NMR is highly recommended. Additionally, conducting comparative studies encompassing various regions and species within the same genus would unlock the full potential of *B. diffusa*.

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AUTHOR CONTRIBUTIONS

KRS prepared the final manuscript, supervised the work, and analyzed the data, and KB and IP performed the laboratory work, preparation of the draft, and analyzed the data.

CONFLICTS OF INTEREST

All authors declared no conflicts of interest while publishing this paper.

DATA AVAILABILITY

All data of this research are with the corresponding author and if needed, these could be provided.

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