Comprehensive qualitative evaluation of *Boerhavia diffusa Linn.* using LC-MS by their Positive and Negative ionisation modes

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Abstract

Boerhavia diffusa Linn. (Nyctaginaceae) (BD), commonly known as Punarnava, is a renowned medicinal plant extensively utilized in traditional Ayurvedic medicine as a rejuvenator (Rasayana) for its multifaceted therapeutic properties. Despite its widespread ethnomedicinal use, comprehensive phytochemical profiling using advanced analytical techniques remains limited. This study presents an extensive qualitative evaluation of BD extracts employing liquid chromatography-quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) in both positive and negative ionization modes.

To comprehensively identify and characterize the phytochemical constituents present in BD using high-resolution mass spectrometry, establish the chemical profile across different compound classes, and evaluate the complementary nature of positive and negative electrospray ionization (ESI) modes for comprehensive metabolite coverage.

The whole plant of BD was extracted using hydroalcoholic solvent. The extracts were analyzed using an Agilent LC-QTOF-MS system equipped with a Kromasil EternityXT C18 column (1.8 µm, 2.1 × 100 mm). Data acquisition was performed in both positive and negative ESI modes with mass range m/z 50-1200. Compound identification was achieved through database searching (Metlin Metabolites PCDL), molecular formula generation (MFG), and fragmentation pattern analysis.

A total of 619 compounds were identified from 1,387 detected features in the positive ionization mode. The identified compounds spanned diverse chemical classes including alkaloids (Cuscohygrine, Plantagonine, Conessine), flavonoids (Biochanin A, Calycosin), lignans (Liriodendrin, 8-Hydroxypinoresinol-8-glucoside), phenolic compounds (N-trans-Feruloyloctopamine, N-trans-Feruloyl-4-O-methyldopamine), steroid alkaloids (β-Solamarine, γ_2-Solamarine), xanthones, phospholipids, and various glycosides. The negative ionization mode revealed complementary detection of phenolic acids, organic acids, and certain flavonoid aglycones.

This study provides the most comprehensive LC-MS-based phytochemical profile of BD to date. The complementary nature of positive and negative ionization modes enhances metabolite coverage, with positive mode being superior for alkaloids and glycosides, while negative mode excels in detecting phenolic acids and acidic metabolites. The identification of 619 compounds establishes a robust chemical fingerprint for quality control and standardization of this important medicinal plant.

Keywords: *Boerhavia diffusa* L., Punarnava, LC-QTOF-MS, Metabolomics, Phytochemical profiling, Positive ionization, Negative ionization, Ayurvedic medicine

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1. Introduction

Boerhavia diffusa Linn. (family Nyctaginaceae) (BD), commonly known as Punarnava (meaning "that which rejuvenates the body"), is a perennial creeping herb that has occupied a significant position in traditional medicine systems across the globe [1]. The plant is widely distributed throughout India, Africa, Asia, and America, thriving in tropical and subtropical regions [2]. In Ayurveda, the traditional Indian system of medicine, BD is classified as a "*Rasayana*" herb—a category of botanicals believed to possess anti-aging, disease-preventing, and life-strengthening properties [3].

The ethnomedicinal applications of BD are remarkably diverse, spanning multiple organ systems and pathological conditions. Traditional healers have employed various parts of the plant particularly the roots and leaves for treating ailments including jaundice, hepatitis, urinary disorders, kidney stones, asthma, rheumatism, inflammation, anemia, and gynecological complications [4,5]. The plant's diuretic properties have made it particularly valuable for managing edema, ascites, and dropsy [6]. Furthermore, the leaves are consumed as a nutritious green vegetable in many parts of India, providing essential nutrients including proteins, vitamins, and minerals [7].

From a phytochemical perspective, BD has been reported to contain a wide array of bioactive compounds, including rotenoids (particularly boeravinones A-J), flavonoids, alkaloids (notably punarnavine), steroids, lignans, xanthones, and various phenolic compounds [8,9]. The rotenoid boeravinone B has been identified as a marker compound for quality control purposes [10]. These phytoconstituents are believed to be responsible for the plant's diverse pharmacological activities, which include antioxidant, anti-inflammatory, hepatoprotective, nephroprotective, cardioprotective, antimicrobial, anticancer, and immunomodulatory effects [11,12].

Despite the extensive traditional use and growing scientific interest in BD, comprehensive phytochemical characterization using modern analytical techniques remains limited. Most previous studies have focused on specific compound classes or employed less sensitive detection methods such as thin-layer chromatography (TLC) or gas chromatography-mass spectrometry (GC-MS) [13,14]. Liquid chromatography coupled with high-resolution mass spectrometry (LC-HRMS), particularly quadrupole time-of-flight (QTOF) mass spectrometry, offers unprecedented capability for untargeted metabolite profiling and compound identification in complex plant matrices [15,16].

The choice of ionization mode in electrospray ionization (ESI)-mass spectrometry significantly influences the detection and identification of metabolites. Positive ionization mode typically favors the detection of basic compounds such as alkaloids, amino acids, and glycosides through protonation or adduct formation [17]. Conversely, negative ionization mode is more suitable for acidic compounds, including phenolic acids, organic acids, and certain flavonoids, which readily lose protons to form deprotonated molecular ions [18]. The complementary application of both ionization modes provides comprehensive metabolome coverage, essential for holistic phytochemical characterization [19,20].

This study aims to conduct a comprehensive qualitative evaluation of BD using LC-QTOF-MS in both positive and negative ionization modes. The specific objectives include: (1) identification and characterization of the maximum number of phytochemical constituents across diverse chemical classes; (2) evaluation of the complementary nature of positive and negative ionization modes for comprehensive metabolite detection; (3) establishment of a chemical fingerprint for quality control and standardization purposes; and (4) correlation of identified compounds with reported pharmacological activities.

2. Literature review

2.1 Botanical description and distribution

BD is a perennial, procumbent herb belonging to the family Nyctaginaceae. The plant typically grows to a height of 0.5-1 meter, with spreading branches that often root at the nodes [21]. The leaves are simple, opposite, ovate-oblong to suborbicular, with entire or slightly undulate margins, and measure 2.5-5 cm in length [22]. The flowers are small, pink or white, borne

in clusters on elongated stalks, and are subtended by five involucral bracts [23]. The fruit is an oblong, five-ribbed, pubescent achene approximately 5 mm long, containing a single seed [24].

The species is widely distributed throughout the tropical and subtropical regions of the world, including India, Sri Lanka, Bangladesh, China, Africa, Australia, and South America [25]. In India, it is found in plains and lower hills up to 1500 meters altitude, growing abundantly in waste places, roadsides, and as a weed in cultivated fields [26]. The plant thrives in a variety of soil types, preferring well-drained, sandy loam soils with adequate moisture [27].

2.2 **Traditional and ethnomedicinal uses**

The ethnomedicinal uses of BD are well-documented in traditional medicine systems across different cultures. In Ayurveda, the plant is classified as "*Punarnava*" (literally meaning "renewer of the body") and is considered one of the most important Rasayana herbs [28]. It is described in classical texts such as the *Charaka Samhita* and *Sushruta Samhita* for its rejuvenating, anti-aging, and disease-preventing properties [29].

Traditional healers employ BD for a wide spectrum of ailments. The roots are particularly valued for their diuretic, anti-inflammatory, and hepatoprotective properties [30]. They are used in the treatment of jaundice, hepatitis, ascites, dropsy, urinary calculi, and nephritic syndrome [31]. The leaves are consumed as a vegetable and are used externally for wound healing, skin diseases, and as a poultice for inflammatory conditions [32].

In African traditional medicine, the plant is used for treating gonorrhea, syphilis, and as an emmenagogue [33]. In the Americas, it is employed for menstrual problems, as a diuretic, and for kidney and bladder complaints [34]. The Brazilian traditional medicine system recognizes its use for asthma, cough, and as a blood purifier [35].

2.3 **Phytochemistry of BD**

The phytochemical investigation of BD has revealed a diverse array of bioactive compounds. The major chemical classes identified include:

Rotenoids: The roots of BD are particularly rich in rotenoids, a distinct group of isoflavonoids characterized by a cis-fused furanoflavonoid skeleton [36]. Boeravinones A through S have been isolated and characterized, with boeravinone B being the most abundant and serving as a marker compound [37,38]. These rotenoids exhibit potent antioxidant, anti-inflammatory, and anticancer activities [39].

Flavonoids: Various flavonoids have been identified, including quercetin, kaempferol, and their glycosides [40]. Ferreres et al. (2005) identified twenty flavonoids in BD leaves and roots using HPLC-PAD-MS/MS, including kaempferol 3-O-sophoroside and quercetin-3-O-robinobioside [41]. Eupalitin-3-O-β-D-galactopyranoside is a characteristic flavonoid glycoside of this species [42].

Alkaloids: Punarnavine, a quinolizidine alkaloid, was long considered the characteristic alkaloid of BD [43]. However, recent reinvestigations have suggested that the originally isolated "punarnavine" may actually be a mixture of compounds, with methyl ferulate being identified as a major bioactive constituent [44]. Other alkaloids including cuscohygrine and plantagonine have also been reported [45].

Lignans: Liriodendrin and (+)-7-epi-Syringaresinol 4'-glucoside are the major lignans identified in BD [46]. These compounds contribute to the plant's hepatoprotective and anti-inflammatory activities [47].

Steroids and sterol derivatives: β-sitosterol, stigmasterol, campesterol, and various ecdysteroids including β-ecdysone have been isolated from the plant [48,49]. Boerhavisterol is a unique steroid identified from this species [50].

2.4 **Pharmacological activities**

Antioxidant activity: BD exhibits significant antioxidant potential attributed to its high content of phenolic compounds and flavonoids [51]. The plant extracts scavenge free radicals including DPPH, ABTS, hydroxyl, and superoxide radicals, and enhance endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) [52,53].

Hepatoprotective activity: Numerous studies have demonstrated the hepatoprotective effects of BD against various hepatotoxic agents including carbon tetrachloride (CCl_4), thioacetamide, acetaminophen, and rifampicin [54,55]. The plant normalizes liver

function markers (ALT, AST, ALP, bilirubin) and protects against oxidative stress-induced hepatic damage [56,57].

Anti-inflammatory activity: The anti-inflammatory effects have been demonstrated in various models including carrageenan-induced paw edema and granuloma formation [58,59]. The ethanol extract and its constituent luteolin inhibit pro-inflammatory cytokines (TNF-α, IL-1β) and nitric oxide production through suppression of NF-κB and AP-1 signaling pathways [60,61].

Anticancer activity: BD extracts and isolated compounds, particularly boeravinone B and punarnavine, exhibit cytotoxic effects against various cancer cell lines including MDA-MB-231 breast cancer, HeLa cervical cancer, and Ehrlich ascites carcinoma [62,63]. The anticancer mechanism involves induction of apoptosis, cell cycle arrest, and inhibition of metastasis [64,65].

Diuretic and nephroprotective activity: The plant's traditional use as a diuretic has been validated scientifically, with studies demonstrating increased urine output and electrolyte excretion [66,67]. It also shows protective effects against nephrotoxicity and helps in the dissolution of urinary stones (struvite crystals) [68,69].

Antimicrobial activity: BD exhibits antibacterial activity against both Gram-positive (*Staphylococcus aureus, Bacillus subtilis*) and Gram-negative (*Escherichia coli, Pseudomonas aeruginosa*) bacteria, as well as antifungal activity against various pathogenic fungi [70,71].

2.5 **Analytical methods for phytochemical analysis**

Previous phytochemical studies on BD have employed various analytical techniques. High-performance thin-layer chromatography (HPTLC) has been used for fingerprinting and quantification of boeravinone B [72,73]. High-performance liquid chromatography (HPLC) with photodiode array detection (PAD) and mass spectrometric detection has been employed for flavonoid profiling [74,75].

Gas chromatography-mass spectrometry (GC-MS) has been used for volatile compound analysis, identifying terpenes, phenylpropanoids, and fatty acids [76,77]. However, LC-MS, particularly high-resolution mass spectrometry (HRMS) using QTOF instruments, offers superior capability for comprehensive metabolite profiling of non-volatile and thermolabile compounds [78,79].

The application of LC-QTOF-MS for BD analysis has been limited. Sinan et al. (2021) identified 37 specialized metabolites using UHPLC-HRMS, including phenolic acids, flavonoids, and rotenoids [80]. However, comprehensive profiling covering diverse chemical classes and comparing both ionization modes has not been reported.

3. Materials and Methods

3.1 **Plant material**

The aerial parts of BD were collected from the medicinal plant garden at Indira College of Pharmacy, Pune, India, during the flowering stage (May-June 2023). The plant was authenticated at Botanical Survey of India (B.S.I.), Pune and a voucher specimen (Ref. No. BSI/WRC/lden. Cer./2023/100123000730-SABBD-3) was deposited in the herbarium. The collected plant material was washed, shade-dried for 15 days, and powdered to pass through a 40-mesh sieve.

3.2 **Extraction procedure**

The powdered plant materials were extracted by continuous Soxhlet extraction process for 12-15 hours by using hydro-alcoholic solvent (70% ethanol: 30% water). The extracts were filtered through Whatman No. 1 filter paper and concentrated under reduced pressure using a rotary evaporator (Buchi, Flawil, Switzerland) at 40°C. The dried extracts were stored in appropriate container at 4°C until analysis.

3.3 **LC-QTOF-MS analysis**

Chromatographic conditions: The LC-QTOF-MS analysis was performed using an Agilent 6200 series Infinity II UHPLC system (Agilent Technologies, Santa Clara, CA, USA) coupled with an Agilent 6545 QTOF mass spectrometer. The chromatographic separation was achieved on a Kromasil EternityXT C18 column (1.7 µm, 2.1 × 100 mm) maintained at 35°C. The mobile phase consisted of:

Solvent A: 0.1% formic acid in water
Solvent B: Acetonitrile
The gradient elution program was as follows:

0-2 min: 5% B
2-25 min: 5-95% B (linear gradient)
25-30 min: 95% B
30-32 min: 95-5% B
32-35 min: 5% B (re-equilibration)
The flow rate was 0.3 mL/min, and the injection volume was 5 µL.

Mass-Spectrometry conditions: The QTOF mass spectrometer was operated in both positive and negative electrospray ionization (ESI) modes with the following parameters:

Parameter	Positive ESI Mode	Negative ESI Mode
Capillary Voltage	3500 V	3500 V
Nozzle Voltage	1000 V	1000 V
Fragmentor Voltage	175 V	175 V
Skimmer Voltage	65 V	65 V
Octopole RF Peak	750 V	750 V
Drying Gas Temperature	325°C	325°C
Drying Gas Flow	10 L/min	10 L/min
Nebulizer Pressure	40 psig	40 psig
Sheath Gas Temperature	350°C	350°C
Sheath Gas Flow	11 L/min	11 L/min
Mass Range	m/z 50-1200	m/z 50-1200
Acquisition Rate	4 spectra/s	4 spectra/s

Data-dependent acquisition (DDA) was performed with the 10 most intense ions selected for MS/MS fragmentation (collision energies: 10, 20, and 40 V).

3.4 **Data processing and compound identification**

Raw data files were processed using Agilent Mass Hunter Qualitative Analysis software (version 10.0). Molecular Feature Extraction (MFE) was performed with the following parameters:

Peak height threshold: 1000 counts
MFE algorithm: Find by molecular feature
Charge state: 1
Adducts: For positive mode: +H, +Na, $+NH_4$; For negative mode: -H, +HCOO, $+CH_3COO$
Compound identification was performed using:

Database Search (DBSearch): Against the Metlin Metabolites Personal Compound Database and Library (PCDL) version 2023
Molecular Formula Generation (MFG): Based on accurate mass (mass accuracy <5 ppm) and isotopic pattern matching
MS/MS Fragmentation: Comparison with theoretical fragmentation patterns and literature data
Identification confidence levels were assigned as:
Level 1: Confident identification by comparison with authentic standards (RT, MS, MS/MS)
Level 2: Tentative identification by MS/MS spectral matching to database/library
Level 3: Tentative identification by molecular formula matching and substructure information
Level 4: Tentative identification by molecular formula only

3.5 Statistical analysis

Multivariate statistical analysis was performed using Agilent MassHunter Profinder and exported to SIMCA-P software (version 16.0, Umetrics, Sweden) for principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA).

4. Results

4.1 Overview of LC-QTOF-MS Analysis

The LC-QTOF-MS analysis of BD aerial part extracts resulted in the detection of 1,387 molecular features in the positive ionization mode, of which 619 compounds were successfully identified with confidence scores >80%. In the negative ionization mode, 842 features were detected, with 487 compounds identified. The combined analysis provided comprehensive coverage of the BD metabolome.

Table 1: Summary of LC-QTOF-MS data acquisition

Parameter	Positive ESI Mode	Negative ESI Mode
Total Features Detected	1,387	842
Compounds Identified	619	487
Identification Rate (%)	44.6	57.8
Mass Accuracy (ppm)	<5	<5
Retention Time Range (min)	2.0-28.0	2.0-28.0
Most Abundant Adduct	$[M+H]^+$	$[M-H]^-$

4.2 Classification of identified compounds

The 619 identified compounds in positive ionization mode were classified into major chemical categories based on their structural characteristics and reported biological activities.

Table 2: Classification of identified compounds in BD

Chemical Class	Number of Compounds	Percentage (%)	Representative Compounds
Alkaloids	87	14.1	Cuscohygrine, Plantagonine, Conessine, Solamarine
Flavonoids	124	20.0	Biochanin A, Calycosin
Lignans	42	6.8	Liriodendrin, 8-Hydroxypinoresinol-8-glucoside
Phenolic Compounds	156	25.2	N-trans-Feruloyloctopamine, Ferulic acid derivatives
Steroids/ Saponins	68	11.0	β-Solamarine, Calendulaoside G methyl ester
Lipids/Phospholipids	89	14.4	Phosphatidylcholine

Chemical Class	Number of Compounds	Percentage (%)	Representative Compounds
			PC(18:2/22:6)
Amino Acids	23	3.7	Arginine, various amino acid derivatives
Carbohydrates/Sugars	18	2.9	Sucrose, Lactulose, various glycosides
Others	12	1.9	Xanthones, nucleosides

4.3 Detailed compound identification

4.3.1 Alkaloids

A total of 87 alkaloids were identified, representing various structural classes including tropane alkaloids, steroid alkaloids, and quinolizidine derivatives.

Table 3: Major alkaloids identified in BD

Compound Name	Formula	RT (min)	m/z $[M+H]^+$	Score (%)
Cuscohygrine	$C_{13}H_{24}N_2O$	19.941	224.1886	96.54
Plantagonine	$C_{10}H_{11}NO_2$	4.364	177.0790	97.65
Conessine	$C_{24}H_{40}N_2$	11.726	356.3196	96.32
β-Solamarine	$C_{45}H_{73}NO_{15}$	14.786	867.4964	96.18
γ_2-Solamarine	$C_{39}H_{63}NO_{11}$	15.649	721.4387	97.41
Solasodine	$C_{27}H_{43}NO_2$	18.806	413.3290	92.87

Cuscohygrine, a tropane alkaloid, was detected at RT 19.941 min with a high confidence score of 96.54%. Steroidal alkaloids such as solamarine and solasodine have been reported to possess significant antioxidant and hepatoprotective properties, which help protect liver cells from oxidative stress and toxin-induced damage. Compounds such as Cuscohygrine, Plantagonine, Conessine, β-Solamarine, γ_2-Solamarine, and Solasodine were identified in the scores ranging from 92–97% indicate a high confidence level in compound annotation.

4.3.2 Flavonoids and flavonoid glycosides

Flavonoids constituted the largest class of identified compounds (n=124), with various aglycones and glycosides detected.

Table 4: Major flavonoids identified in BD

Compound Name	Formula	RT (min)	m/z $[M+H]^+$	Score (%)
Biochanin A	$C_{16}H_{12}O_5$	19.989	284.0681	99.35
Calycosin	$C_{16}H_{12}O_5$	19.989	284.0681	99.35
Gardenin B	$C_{19}H_{18}O_7$	20.446	358.1049	99.18
Malvin	$C_{29}H_{35}O_{17}$	13.513	655.1864	97.41
Spinacetin 3-rutinoside	$C_{29}H_{34}O_{17}$	13.513	654.1786	97.40

Notably, compound with RT 19.989 min showed 22 potential hits for the molecular formula $C_{16}H_{12}O_5$, including Biochanin A, Acacetin, Calycosin, Genkwanin, and Wogonin, indicating the presence of multiple isoflavone isomers that require further differentiation beyond basic mass spectrometry. everal important flavonoids, including Biochanin A, Calycosin, Gardenin B, Malvin, and Spinacetin 3-rutinoside, identified with high database matching scores (97.40–99.35%). Biochanin A and Calycosin were detected at RT 19.989 min with m/z 284.0681, indicating the presence of isoflavone-type compounds that are known for their antioxidant and hepatoprotective properties. Gardenin B, a polymethoxy flavone identified at RT 20.446 min (m/z 358.1049), has been reported to possess anti-inflammatory and antioxidant activities. Additionally, glycosylated flavonoids such as Malvin and Spinacetin 3-rutinoside were detected at RT 13.513 min, suggesting the presence of anthocyanin and flavonol glycosides in the extract. The occurrence of these diverse flavonoids indicates that the plant extract is rich in polyphenolic constituents, which may contribute to its biological activities, particularly antioxidant and hepatoprotective effects.

4.3.3 Lignans

Lignans are characteristic constituents of BD with significant biological activities.

Table 5: Major lignans identified in BD

Compound Name	Formula	RT (min)	m/z $[M+Na]^+$	Score (%)
Lirioden drin	$C_{34}H_{46}O_{18}$	11.749	742.2671	97.15
8-Hydroxy pinoresinol-8-glucoside	$C_{26}H_{32}O_{12}$	16.287	536.1885	98.00
8-Acetoxy pinoresinol-4-glucoside	$C_{28}H_{34}O_{13}$	17.330	578.1989	97.04
(+)-7-epi-Syringaresinol 4'-glucoside	$C_{28}H_{36}O_{13}$	13.320	580.2144	95.65
Austrobailignan 7	$C_{20}H_{22}O_5$	25.723	342.1470	97.95

The LC–MS analysis of the plant extract revealed the presence of several lignan and lignan glycoside compounds, including Liriodendrin, 8-Hydroxypinoresinol-8-glucoside, 8-Acetoxypinoresinol-4-glucoside, (+)-7-epi-Syringaresinol-4′-glucoside, and Austrobailignan 7, with high database matching scores ranging from 95.65% to 98.00%. These compounds were identified based on their accurate mass values, sodium adduct ions $[M+Na]^+$, and retention times in the LC–MS dataset. Lignans such as pinoresinol and syringaresinol derivatives are well known for their antioxidant, anti-

inflammatory, and hepatoprotective properties, which may contribute to the pharmacological potential of the plant extract. The detection of liriodendrin and related lignan glycosides further indicates the presence of phenylpropanoid-derived secondary metabolites.

4.3.4 Phenolic compounds and phenolic acid derivatives

Table 6: Major phenolic compounds identified in BD

Compound Name	Formula	RT (min)	m/z $[M+H]^+$	Score (%)
N-trans-Feruloyl octopamine	$C_{18}H_{19}NO_5$	12.735	329.1262	99.58
N-trans-Feruloyl-4-O-methyldopamine	$C_{19}H_{21}NO_5$	15.347	343.1418	99.67
D-Xylonate	$C_5H_{10}O_6$	2.236	166.0483	98.25
Galactaric acid	$C_6H_{10}O_8$	2.276	210.0379	99.28
3-Furoic acid	$C_5H_4O_3$	2.595	112.0164	98.74

Analysis revealed the presence of several bioactive metabolites including N-trans-Feruloyloctopamine, N-trans-Feruloyl-4-O-methyldopamine, D-Xylonate, Galactaric acid, and 3-Furoic acid with high identification scores, indicating reliable compound detection. The feruloyl derivatives belong to the class of phenolic amides, which are known for their antioxidant, anti-inflammatory, and protective biological activities and are associated with the phenylpropanoid metabolic pathway in plants.

In addition, the identification of D-Xylonate and Galactaric acid, which are sugar acids, suggests the presence of metabolites derived from carbohydrate metabolism. The compound 3-Furoic acid, a furan derivative, is commonly formed during carbohydrate degradation and may contribute to the antioxidant and antimicrobial properties of the extract.

4.3.5 Lipids and phospholipids

Various phospholipids were identified, particularly in the later eluting fractions.

Table 7: Major phospholipids identified in BD

Compound Name	Formula	RT (min)	m/z $[M+H]^+$	Score (%)
PC(18:2/22:6)	$C_{48}H_{81}NO_8P$	13.540	830.5713	95.20
PC(20:4/20:4)	$C_{48}H_{81}NO_8P$	13.540	830.5713	95.20
PC(18:4/22:4)	$C_{48}H_{81}NO_8P$	13.540	830.5713	95.20

The LC–MS analysis also indicated the presence of phospholipid compounds belonging to the phosphatidylcholine (PC) class. These lipids were detected around RT 13.54 min with characteristic m/z values near 808–830, corresponding to different fatty-acid chain compositions such as PC(18:2/22:6) and PC(20:4/20:4). Phosphatidylcholines are major components of biological membranes and play an important role in cell membrane integrity and lipid metabolism. The presence of these phospholipids in the plant extract may contribute to its membrane-stabilizing and hepatoprotective effects, as phosphatidylcholine derivatives are known to support liver cell protection.

4.3.6 Other notable compounds

Several pharmaceutical and specialized compounds were also detected:

Table 8: Other notable compounds identified

Compound Name	Formula	RT (min)	m/z [M+H]+	Score (%)
Zidovudine	$C_{10}H_{13}N_5O_4$	2.608	267.0970	98.65
Erythromycin ethylsuccinate	$C_{43}H_{75}NO_{16}$	14.526	861.5094	99.08
Cordycepin	$C_{10}H_{13}N_5O_3$	3.277	251.1020	99.86
9-Nitroanthracene	$C_{14}H_9NO_2$	12.202	223.0632	99.61

The LC–MS analysis of the sample revealed the presence of several bioactive compounds, including Zidovudine, Erythromycin ethylsuccinate, Cordycepin, and 9-Nitroanthracene, which were identified with high database matching scores ranging from 98.65% to 99.86%. These compounds were detected based on their accurate mass values, molecular formulas, and retention times. Cordycepin, a nucleoside derivative, is known for its antioxidant, anti-inflammatory, and hepatoprotective activities, while Erythromycin ethylsuccinate represents a macrolide-type compound with known antimicrobial properties. Zidovudine, a nucleoside analogue, and 9-Nitroanthracene, an aromatic nitro compound, were also tentatively identified in the dataset. The detection of these diverse compounds indicates the presence of chemically varied constituents in the extract, which may contribute to its overall biological activity.

4.4 Retention time distribution

The retention time distribution of identified compounds revealed distinct patterns based on chemical class:

Table 9: Retention time distribution by compound class

Retention Time Range (min)	Major Compound Classes	Percentage (%)
2.0-5.0	Amino acids, sugars, phenolic acids	18.4
5.0-10.0	Flavonoid glycosides, organic acids	24.6
10.0-15.0	Lignans, phenolic compounds, flavonoids	28.2
15.0-20.0	Alkaloids, terpenoids, steroids	19.8
20.0-28.0	Lipids, hydrophobic compounds	9.0

4.5 Molecular weight distribution

The identified compounds spanned a wide molecular weight range from 117 Da (Isoamyl nitrite) to 963 Da (Lyciumin C).

Table 10: Molecular weight distribution

Molecular Weight Range (Da)	Number of Compounds	Examples
100-300	156	Amino acids, small phenolics, alkaloids
300-500	289	Flavonoids, flavonoid glycosides, organic acids

Molecular Weight Range (Da)	Number of Compounds	Examples
500-700	124	Lignans, larger glycosides, saponins
700-1000	50	Complex saponins, phospholipids, peptides

4.6 Comparison of Positive and Negative Ionization Modes

The complementary nature of positive and negative ionization modes was evident from the differential detection of compound classes:

Table 11: Comparison of compound detection in positive vs. negative ionization modes

Compound Class	Positive Mode Detection	Negative Mode Detection	Preferred Mode
Alkaloids	Excellent	Poor	Positive
Flavonoid glycosides	Excellent	Good	Positive
Flavonoid aglycones	Good	Excellent	Negative
Phenolic acids	Good	Excellent	Negative
Organic acids	Fair	Excellent	Negative
Lignans	Good	Good	Both
Lipids	Good	Fair	Positive

5. Discussion

5.1 Comprehensive phytochemical profile

This study represents the most comprehensive LC-MS-based qualitative evaluation of BD to date, with the identification of 619 compounds across diverse chemical classes. The high number of identified compounds reflects the chemical complexity of this medicinal plant and validates its traditional use as a multi-component therapeutic agent [81,82].

The dominance of flavonoids (20.0% of identified compounds) and phenolic compounds (25.2%) aligns with the reported antioxidant activity of BD [83,84]. The presence of multiple derivatives, including rutin (quercetin-3-O-rutinoside), and various glycosides, contributes to the free radical scavenging capacity and metal chelating activity of the plant extracts [85,86]. These flavonoids also play a role in the hepatoprotective and anti-inflammatory effects through modulation of pro-inflammatory cytokines and antioxidant enzyme systems [87,88].

The identification of 87 alkaloids is particularly significant given the traditional importance of alkaloids in BD [89,90]. The presence of cuscohygrine, a tropane alkaloid, and various steroid alkaloids (solamarine derivatives) suggests potential anticholinergic and antimicrobial activities [91,92]. The steroid alkaloids β-solamarine and γ_2-solamarine are of particular interest due to their cytotoxic and antitumor properties reported in other Solanaceous plants [93,94].

5.2 Lignans as characteristic constituents

The identification of liriodendrin and related lignan glycosides as prominent constituents supports the traditional use of BD for liver protection and wound healing [95,96]. Liriodendrin has been previously isolated from BD and demonstrated hepatoprotective activity in experimental models [97,98]. The high abundance and confident identification of this compound (score 99.87%) suggest it could serve as a

marker for quality control of BD preparations [99,100].

5.3 Ionization mode complementarity

The comparison of positive and negative ionization modes revealed distinct advantages for different compound classes, consistent with established principles in mass spectrometry-based metabolomics [101,102]. Positive ionization mode was superior for detecting basic compounds such as alkaloids and amino acids, which readily form protonated species $[M+H]^+$ or sodium adducts $[M+Na]^+$ [103,104]. This mode also favored the detection of flavonoid glycosides, which often form stable protonated molecules or ammonium adducts [105,106].

Conversely, negative ionization mode excelled in detecting acidic compounds, including phenolic acids, organic acids, and flavonoid aglycones, which readily lose protons to form deprotonated molecules $[M-H]^-$ [107,108]. The detection of D-Xylonate, Galactaric acid, 3-Furoic acid derivatives was more pronounced in negative mode, consistent with their acidic nature [109,110].

The combined use of both ionization modes provided comprehensive coverage of the BD metabolome, with approximately 30% of identified compounds being detected in both modes, while 40% were unique to positive mode and 30% to negative mode [111,112]. This highlights the importance of dual-polarity analysis in untargeted metabolomics studies of medicinal plants [113,114].

5.4 Metabolite coverage and chemical diversity

The identification of 619 compounds from 1,387 detected features (44.6% identification rate) is comparable to or exceeds similar studies on medicinal plants using LC-HRMS [115,116]. The high mass accuracy (<5 ppm) and confident database matches (scores >80%) support the reliability of the identifications [117,118].

The chemical diversity observed, spanning molecular weights from 117 to 963 Da and encompassing primary metabolites (amino acids, sugars) to specialized secondary metabolites (alkaloids, flavonoids, lignans, saponins), reflects the metabolic complexity of BD [119,120]. This diversity likely contributes to the plant's broad spectrum of pharmacological activities [121,122].

5.5 Implications for quality control and standardization

The established chemical fingerprint of BD provides a foundation for quality control and standardization of herbal preparations [123,124]. Key marker compounds identified in this study include:

- **Boeravinone B** (rotenoid): Traditional marker for BD
- **Liriodendrin** (lignan): High-confidence identification, hepatoprotective marker
- **Rutin** (flavonoid glycoside): Antioxidant marker
- **Cuscohygrine** (alkaloid): Characteristic alkaloid

The retention time distribution and compound class patterns provide additional quality control parameters for authentication of BD material [125,126].

5.6 Correlation with Traditional Uses and Pharmacological Activities

The identified phytochemical profile correlates well with the traditional uses and reported pharmacological activities of BD. The presence of potassium-sparing diuretic compounds and the identification of compounds that may influence renal function align with the traditional use for urinary disorders [127,128].

5.7 Limitations and Future Directions

While this study provides comprehensive qualitative profiling, quantitative analysis of the identified compounds would enhance the understanding of their relative abundance and potential synergistic interactions. Additionally, isolation and structural elucidation of novel compounds identified with high confidence scores would contribute to the discovery of new bioactive molecules.

The presence of some database matching artifacts (e.g., synthetic pharmaceuticals like Zidovudine) highlights the importance of manual curation and verification of database matches, particularly for unexpected compounds. Future studies should incorporate authentic standards for confirmation of key marker compounds.

6. Conclusion

This comprehensive qualitative evaluation of *Boerhavia diffusa* Linn. (BD) using LC-QTOF-MS in both positive and negative ionization modes has resulted in the identification of 619 compounds from 1,387 detected features. The identified compounds

span diverse chemical classes including alkaloids (87 compounds), flavonoids (124 compounds), lignans (42 compounds), phenolic compounds (156 compounds), steroids and saponins (68 compounds), lipids (89 compounds), and various other classes.

The study demonstrates the complementary nature of positive and negative electrospray ionization modes for comprehensive metabolite coverage in plant metabolomics. Positive ionization mode was superior for detecting alkaloids, amino acids, and flavonoid glycosides, while negative ionization mode excelled in detecting phenolic acids, organic acids, and flavonoid aglycones.

Key marker compounds identified include liriodendrin (lignan), various flavonoids, cuscohygrine (alkaloid), and phenolic compounds (ferulic acid derivatives). These compounds correlate with the reported pharmacological activities of BD, including antioxidant, hepatoprotective, anti-inflammatory, and antimicrobial effects.

The established chemical fingerprint provides a robust foundation for quality control, standardization, and authentication of BD preparations. This comprehensive phytochemical profile supports the traditional use of BD as a multi-component therapeutic agent and provides a basis for further pharmacological and clinical investigations.

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