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Metabolomics Profiling of *Boerhavia diffusa* (*Punarnava*): High-Resolution Mass Spectrometry Unveils Hepatoprotective Metabolites

Research Article

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Abstract

Boerhavia diffusa (*Punarnava*), a highly valued medicinal plant in Ayurveda, has been traditionally used for its hepatoprotective properties. Despite its extensive use in treating liver disorders, the specific bioactive compounds and underlying mechanisms responsible for its hepatoprotective effects remain largely undefined. This study utilizes High-Resolution Mass Spectrometry (HRMS) to comprehensively analyse and profile the bioactive compounds in *Boerhavia diffusa*, aiming to uncover its hepatoprotective potential at the molecular level. The HRMS analysis revealed a diverse spectrum of phytochemicals many of which are associated with anti-inflammatory, antioxidant, and liver-regenerative activities. These compounds were further explored for their interactions with key hepatic pathways, particularly in mitigating oxidative stress, inhibiting inflammatory mediators, and regulating enzymes crucial for liver function and detoxification. The study further explores potential mechanisms of action of these phytochemicals through reviews of existing pharmacological data, proposing a multifaceted approach to liver protection that includes scavenging free radicals, reducing lipid peroxidation, and inhibiting pro-inflammatory cytokines. The findings underscore the potential of *Boerhavia diffusa* as a natural therapeutic agent for liver diseases, reinforcing its place in both traditional and modern medicine. These findings could facilitate the development of novel plant-based therapeutics aimed at liver health.

Keywords: High Resolution Mass Spectrometry Analysis, Hepatoprotective Activity, *Boerhavia diffusa*, *Punarnava,* Liver function.

Introduction

The liver is a vital organ essential for maintaining homeostasis, performing critical metabolic and physiological functions such as bile production, energy generation, vitamin storage, and the metabolism of carbohydrates, proteins, and fats. After intestinal absorption, nutrient-rich blood flows to the liver via the portal vein, which also carries various toxic substances, including ethanol, drugs, and toxins. This makes the liver particularly vulnerable to damage and toxicity. Many individuals suffer from liver conditions, including fatty liver, non-alcoholic steatosis, hepatitis A, B, or C, cirrhosis, and hepatocellular carcinoma (one of the leading causes of cancer-related deaths worldwide) (1) As the primary organ responsible for metabolism, the liver is especially prone to damage from drugs and chemicals. The impact of such damage can vary widely, from mild, asymptomatic increases in liver enzymes to severe, life-threatening fulminant hepatic failure (2). Despite significant advancements in hepatology in recent years, liver disorders are increasingly prevalent. Unfortunately, the available drugs for treating liver diseases are limited and often come with serious side effects. Given the adverse effects of synthetic medications, there is a growing interest in exploring the therapeutic potential of medicinal plants through systematic research approaches (3). *Boerhavia diffusa* (*Punarnava*) belongs to the family Nyctaginaceae, is a perennial creeping herb widely used in Ayurveda for its therapeutic properties. Known for its rejuvenating and detoxifying effects, it is primarily valued for its role in supporting liver and kidney health. *Punarnava* exhibits anti-inflammatory, diuretic, antioxidant, hepatoprotective, and immunomodulatory properties. Previous studies have shown that the root of *Boerhavia diffusa* exhibits significant hepatoprotective activity (4). Consequently, an attempt has been made to isolate the bioactive compounds responsible for the hepatoprotective effects of *Boerhavia diffusa* and to explore the mechanisms through which they exert their action.

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Materials and Method

Collection of Boerhavia diffusa

The raw material of *Boerhavia diffusa* used for analysis by High-Resolution Mass Spectrometry (HRMS) was sourced from a local herbal market in Varanasi, Uttar Pradesh. Subsequently, the *Boerhavia*

diffusa sample was authenticated by the Department of Dravyaguna (related to Ayurvedic Pharmacognosy) with the accession number DG/24-25/853. It was also validated by the Department of Botany with the Voucher Specimen No. Nyctagina. 2024/02

The study utilized *Boerhavia diffusa* root powder, methanol, distilled water, and Eppendorf tubes. A High-Resolution Accurate Mass Spectrometry system, specifically the Orbitrap Eclipse Tribrid Mass Spectrometer from Thermo Fisher Scientific, was employed. For the analysis of small molecules, the Dionex UltiMate 3000 RS UHPLC system was employed to conduct detailed phytochemical analysis (5).

Method employed for HRMS analysis

The sample preparation for HR-MS analysis started with the addition of individual optimized sample of *Boerhavia diffusa* [Root powder (100 mg)] with 1.5 ml solvent (Methanol:Water; 80:20) and homogenized using Eppendorf Thermo-mixer at 750 rpm for 30 min at 25°C. Then, the sample was centrifuged (3500 rpm/ 10 min/ 25 °C). The supernatant was filtered with a 0.22 µ PTEF syringe filter and 4 µl of the filtrate was used as injection volume on C18 RP-HPLC column (Hypersil GOLD™: Particle size 1.9µ, 2.1mm × 100mm)

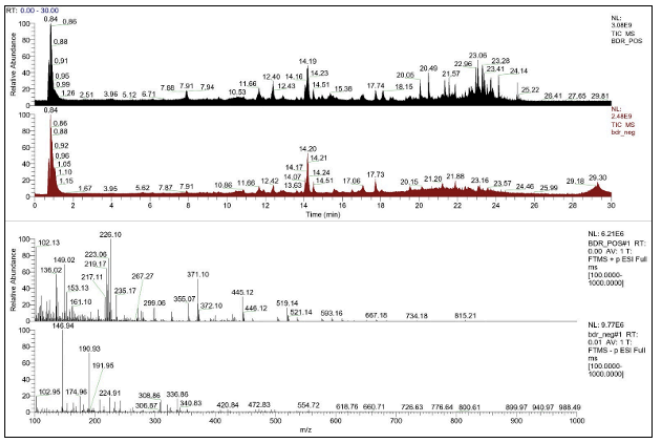
The Reversed-phase chromatographic separation start with a high aqueous phase (+0.1% formic acid) and ends on highly organic phase (MeOH+ 0.1% formic acid) typically 100% aqueous to 100% organic. The LC gradient parameters were 0-6 min 5% MeOH, 6-10 min 30% MeOH, 10-20 min 50% MeOH, 20-25 min 90% MeOH, 25-27 min 90% MeOH, and 27-30 min 5% with flow rate of 300 l/min and column oven temperature 40 °C. The optimised sample of *Boerhavia diffusa* was tested for metabolomics analysis. Thermo Fisher Scientific - High Resolution Accurate Mass Spectrometry System of the model "Orbitrap Eclipse Tribrid Mass Spectrometer coupled with Nano Liquid Chromatography and Ultra High-Pressure Liquid Chromatography" (Diones Ultimate 3000 RSLC) system, Heated Electro Spray Ionisation (HESI) source was used to fed the sample to the mass spectrometer post chromatographic separation. The Orbitrap analyser was utilized at 60,000 resolutions separately for positive/negative polarity with mass range (m/z) 100-1000, 35% RF Lens, 25% Normalized AGC Target keeping 2.0e5 as intensity threshold to perform MS-OT (Master scan). To obtain ddMS2 OT HCD the selection parameters were, Quadrupole isolation mode with 1.5 isolation window (m/z) HCD Activation type, 30, 45, 60HCD collision energy (%), 15000 Orbitrap Resolution, 20% Normalized AGC Target (6).

The raw data obtained from the mass analyser were performed through default parameters of "Compound discoverer 3.3.2.31" using online databases. The chosen workflow was the Natural Product Unknown ID, incorporating both online and local database searches. This untargeted food research ID workflow operates without statistics, focusing on the detection and identification of unknown compounds. It includes retention time alignment, unknown compound detection, and compound grouping across all samples. The workflow predicts elemental compositions for all identified compounds and filters out chemical background using blank samples. Compound identification is achieved through mzCloud (using ddMS2 and/or DIA), ChemSpider (based on exact mass or formula), and local database searches against mass lists (with or without retention time). Additionally, it conducts spectral similarity searches against mzCloud for compounds with ddMS2 and applies spectral distance scoring to matches from ChemSpider and mass lists (7).

Result

A Total Ion Chromatogram (TIC) represents the overall intensity of all ions detected over time and serves as an essential tool for analysing the composition of a sample. By providing a visual representation of the ions detected during chromatographic separation, the TIC allows for a detailed understanding of the various compounds present within the sample. The Total Ion Chromatogram of the components found in *Boerhavia diffusa* is illustrated in Figure 1, offering insights into its chemical profile.

Figure 1: Total ion chromatogram of the *Boerhavia diffusa*. It shows Total ion chromatogram obtained by UHPLC-HRAMS analysis of the *Boerhavia diffusa* (*Punarnava*) sample in positive and negative ion mode



The Standard Ion Chromatogram serves as a reference for the identification and quantification of ions within the tested sample. In the case of Boerhavia diffusa, the identified hepatoprotective metabolites were quantified and characterized according to their retention times and peak intensities, as depicted in Figure 2-7.

Mechanisms of Hepatoprotective action of bioactive metabolites derived from *Boerhavia diffusa*

In a comprehensive analysis utilizing High-Resolution Mass Spectrometry (HRMS), a total of 1,584 metabolites were identified from the *Boerhavia diffusa* sample. Among these, six specific compounds demonstrated notable hepatoprotective activity. The bioactive metabolites identified are as follows: 1) Citral; 2) Choline; 3) Betaine; 4) Itaconic acid; 5) Syringic

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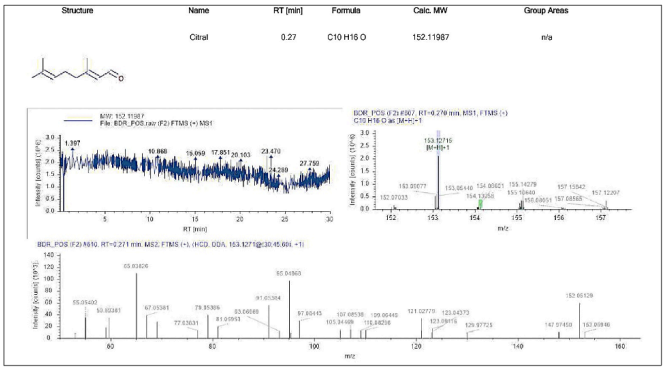
acid; and 6) Catechin. The specific mechanisms through which these metabolites confer liver protection are outlined in the references provided below.

Citral

Previous research investigated the effects of citral on acetaminophen (APAP)-induced liver toxicity in a murine model. To assess citral's hepatoprotective properties, liver function markers such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and gamma-glutamyl transferase (γGT) were measured. Additionally, liver tissues were analysed for myeloperoxidase (MPO) activity, nitric oxide (NO) production, and through histological examination. Citral's impact on leukocyte migration and antioxidant activity was also evaluated in vitro. Pretreatment with citral significantly reduced ALT, AST, ALP, γGT, MPO activity, and NO production. Histological findings showed improvement in liver damage after citral pretreatment. Furthermore, citral inhibited neutrophil migration and demonstrated antioxidant properties, suggesting its protective role against APAP-induced liver toxicity (8). Citral has also demonstrated renoprotective and hepatoprotective properties in mice, attributed to its antioxidative and anti-inflammatory effects (9)(10). NAD(P)H quinone oxidoreductase 1 (NQO1) has been reported to shield cells from oxidative stress caused by reactive and harmful quinines (11). Another study found that rats treated with citral exhibited a significant decrease in hepatic testosterone 6β-hydroxylation and ethoxyresorufin O-de-ethylation activities. Furthermore, citral significantly increased the activity of NAD(P)H quinone oxidoreductase 1 (NQO1). Additionally, treatment with citral led to reduced lipid peroxidation and lower levels of reactive oxygen species in the liver. The study suggested that citral may alter the activities of drug-metabolizing enzymes and alleviate oxidative stress in the liver (12). Similarly, citral was shown to inhibit the expression of pro-inflammatory cytokines such as IL-6 and TNFα in LPS-stimulated hepatocytes by preventing lipopolysaccharide (LPS)-induced phosphorylation and nuclear translocation of NF-κB. Citral may function as a TLR4 antagonist, blocking LPS binding to the receptor and, consequently, counteracting the downstream effects of LPS/TLR4 signalling, including NF-κB activation and its target genes involved in the inflammatory response. Additionally, pretreatment with citral mitigated LPS-induced oxidative stress by reducing reactive oxygen species (ROS) levels. These findings support the hypothesis that citral acts as a TLR4 antagonist by inhibiting the TLR4 signalling cascade, rendering cells unresponsive to LPS-induced oxidative stress. Furthermore, citral was found to counteract the expression of epithelial–mesenchymal transition (EMT) markers in LPS-stimulated hepatocytes, emphasizing its potential to prevent the establishment of a fibrotic environment in the liver (13). The standard ion chromatogram is depicted in Figure 2.

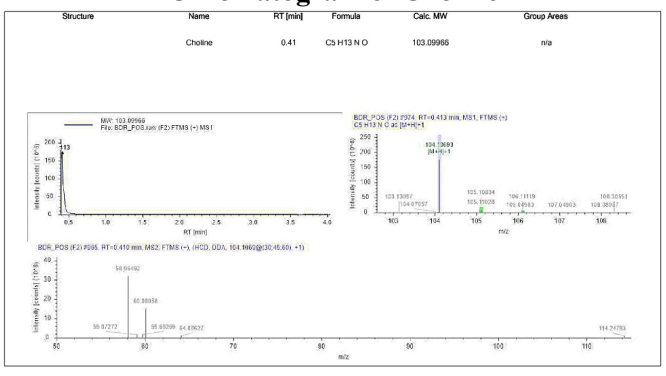
Figure 2: Illustrates the Standard Ion Chromatogram of Citral



Choline

The liver is the central organ for the metabolism of choline, methyl folate, methionine, and S-adenosylmethionine (SAM), and it is where most of the methylation reactions occur. It is particularly sensitive to the availability of dietary methyl donors like choline. When these nutrients are deficient, the liver becomes susceptible to damage, leading to fat accumulation (steatosis), hepatocyte death, fibrosis, and, ultimately, the development of carcinogenic foci. This underscores the critical importance of methyl donors in preserving liver function and preventing serious liver diseases (14). A study demonstrated that choline supplementation effectively prevented weight loss and liver function decline, while also reducing inflammation by normalizing hepatic cholesterol levels and metabolism. Choline supplementation increased hepatic phosphatidylcholine (PC) and phosphatidylethanolamine (PE) levels but did not affect triglyceride (TG) concentrations in phosphatidylethanolamine N-methyltransferase (PEMT)-deficient mice. By restoring cholesterol metabolism, choline supplementation improved liver function and prevented the progression to non-alcoholic steatohepatitis (NASH) and liver failure. Notably, choline supplementation significantly improved liver function by regulating markers related to macrophage activity, oxidative stress, and fibrosis. The findings suggest that choline supports liver health by maintaining cholesterol homeostasis (15). The standard ion chromatogram is depicted in Figure 3.

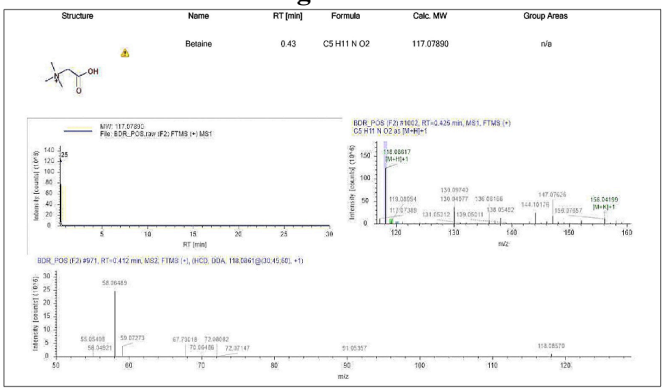
Figure 3: Illustrates the Standard Ion Chromatogram of Choline



Betaine

Betaine is recognised as a vital nutrient with protective properties, particularly for the liver. According to a study by, betaine demonstrated effectiveness in preventing liver damage induced by carbon tetrachloride. As a potent antioxidant, betaine has also been shown to positively influence redox balance during ischemia-reperfusion liver injury in rats. The administration of betaine as an antioxidant demonstrated significant protective effects on the liver, specifically by preventing necrosis. It reduced the production of inflammatory mediators and enhanced tissue repair by mitigating degenerative changes, with optimal protective outcomes observed at doses of 150 and 250 mg/kg (16). Betaine treatment has been reported to significantly reduce oxidative stress, lower cytochrome P450 (CYP450) activity, enhance glutathione transferase (GSH-T) activity, decrease caspase-3 activity, and reduce fibrotic markers, all contributing to improved liver function. Through its antioxidant properties, along with its ability to enhance liver detoxification and reduce apoptosis, betaine may help slow the progression of liver fibrosis and provide a protective effect against radiation-induced liver damage (17). The standard ion chromatogram is depicted in Figure 4.

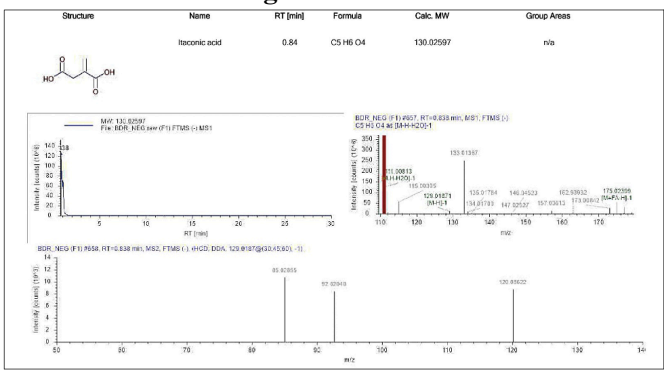
Figure 4: Illustrates the Standard Ion Chromatogram of Betaine



Itaconic acid

Itaconic acid is a metabolite generated by immune cells, particularly macrophages, during inflammation and can aid in reducing inflammation. It is reported that Itaconic acid blocks the activity of the NF-κB pathway, decreases the buildup of the transcription factor P-P65 in the nucleus, and diminishes the expression of inflammatory proteins linked to the downstream effects of the NF-κB pathway. As a result, it helps mitigate liver injury and inflammation during ischemia-reperfusion (18). Nrf2 is a transcription factor involved in intracellular signalling that helps protect organs from oxidative stress. Numerous studies have shown that a reduction in Nrf2 levels makes the liver more vulnerable to toxin-induced damage, providing strong evidence for Nrf2's role in liver protection. Furthermore, it has been established that activating Nrf2 can safeguard the liver from ischemia/reperfusion (I/R) injury in mice. Recent research has also highlighted that itaconate can stimulate Nrf2-driven signalling. Treatment with itaconic acid has been found to enhance the expression and nuclear translocation of Nrf2, as well as increase the activity of its downstream protective pathways (HO-1 and NQO1), in both mouse and primary human hepatocytes thus contributing to its hepatoprotective activity (19). The standard ion chromatogram is depicted in Figure 5.

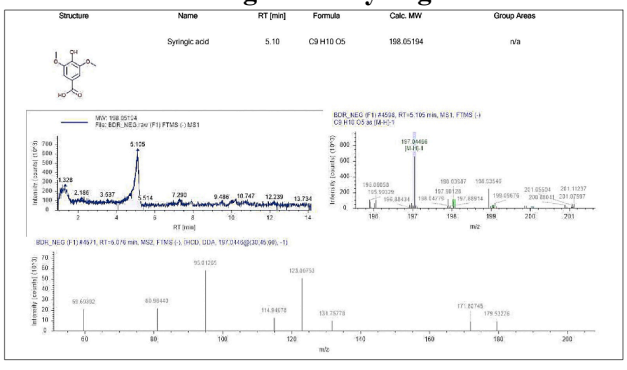
Figure 5: Illustrates the Standard Ion Chromatogram of Itaconic acid



Syringic acid

Syringic acid reduced the levels of transaminases and malondialdehyde in mice treated with CCl4. This compound exhibits 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, suggesting that the reduction of reactive oxygen species (ROS) generation may be responsible for its hepatoprotective effect (20). Syringic acid has been shown to effectively inhibit the activation of cultured hepatic stellate cells, which play a crucial role in liver fibrogenesis. When administered, syringic acid reduced hepatic fibrosis in the context of chronic liver injury. A study examining the effects of syringic acid on acetaminophen (APAP)-induced hepatotoxicity in rats revealed that it significantly lowered markers of lipid peroxidation while increasing the activity of enzymatic antioxidants in the liver. These findings indicate that syringic acid offers substantial protection against APAP-induced liver damage in rats. Additionally, syringic acid demonstrates hepatoprotective effects against hepatic encephalopathy by reducing hepatotoxicity biomarkers. Its antioxidant and anti-inflammatory properties further contribute to its hepatoprotective actions (21). The standard ion chromatogram is depicted in Figure 6.

Figure 6: Illustrates the Standard Ion Chromatogram of Syringic acid



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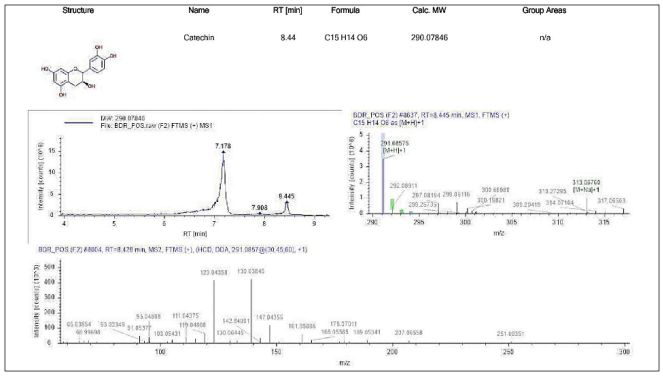
Catechin

Research demonstrated that catechin has a notable protective effect on the liver. This hepatoprotective effect may be attributed to its ability to scavenge free radicals and suppress cytokines. Treatment with catechin led to the restoration of levels of TNF-α, IL-1β, IL-6, and NO. Additionally, catechin effectively shielded hepatocytes from drug induced oxidative damage. It also restored antioxidant enzymes and biochemical markers, while lowering serum cytokine levels (22). The standard ion chromatogram is depicted in Figure 7.

Figure 7: Illustrates the Standard Ion Chromatogram of Catechin



Discussion

Hepatoprotection in Ayurveda refers to the safeguarding and restoration of liver health through the use of specific herbs and treatments. The liver or "*Yakrit*" is seen as an essential organ for digestion, metabolism, and detoxification. Ayurvedic hepatoprotective herbs help detoxify, rejuvenate, and protect the liver from damage caused by toxins, infections, and imbalances in bodily functions. *Punarnava*, botanically known as *Boerhavia diffusa*, belongs to the Nyctaginaceae family. It is renowned as one of the most effective diuretic herbs referenced in Ayurvedic texts. True to its name, which implies a capacity for rejuvenation, *Punarnava* is recognized for its revitalizing properties (23). Research has shown that the alcoholic extract of the *Boerhavia diffusa* plant is a potent and safe antihepatotoxic agent. When administered orally, the alcoholic extract of the entire plant demonstrated hepatoprotective effects against carbon tetrachloride-induced liver toxicity in rats and mice (24). As per *Bhavaprakasha Nighantu, Punarnava* exists in two primary varieties: *Rakta Punarnava* (*Boerhavia diffusa* Linn) and *Shweta Punarnava* (*Boerhavia verticillata* Poir) (25). In this study, the hydroalcoholic extract of the root of Rakta *Punarnava* (*Boerhavia diffusa*) was subjected to High-Resolution Mass Spectrometry (HRMS) analysis in order to obtain its metabolomic profile and unveil its hepatoprotective activity. The HRMS analysis of *Boerhavia diffusa* identified 1,584 bioactive metabolites. Notably, six of these compounds namely Citral, Choline, Betaine, Itaconic acid, Syringic acid, and Catechin have been recognized for their significant hepatoprotective properties.

Citral is reported to reduce myeloperoxidase activity, nitric oxide production, and neutrophil migration, indicating its hepatoprotective and antioxidant properties (8). Citral is also reported to significantly increase the NAD(P)H quinone oxidoreductase 1 (NQO1) activity. Additionally, citral decreased lipid peroxidation and reactive oxygen species levels as well, thus reducing the oxidative stress in the liver (12). Choline supplementation maintains cholesterol homeostasis to support liver health. It normalizes hepatic cholesterol levels and metabolism, reduces inflammation and improves liver function markers (15). Betaine has antioxidant and detoxifying properties. It can also reduce apoptosis. It decreases oxidative stress, lowers cytochrome P450 activity, enhances glutathione transferase activity, decreases caspase-3 activity, and reduces fibrotic markers, improving liver function (17). Itaconic acid is known to inhibit the NF-κB pathway 18 and activate Nrf2-driven signalling (19). Thus, contributing to its hepatoprotective activity. The liver protective effect of Syringic acid are likely due to its ability to lower reactive oxygen species (ROS) levels (20) and hepatotoxicity biomarkers. Its strong antioxidant and anti-inflammatory properties further enhance its liver-protective actions (21). The hepatoprotective effect of catechin may be attributed to its ability to neutralize free radicals and inhibit cytokine production (22). A summarized overview of the metabolites with identified hepatoprotective activity is presented in Table 1.

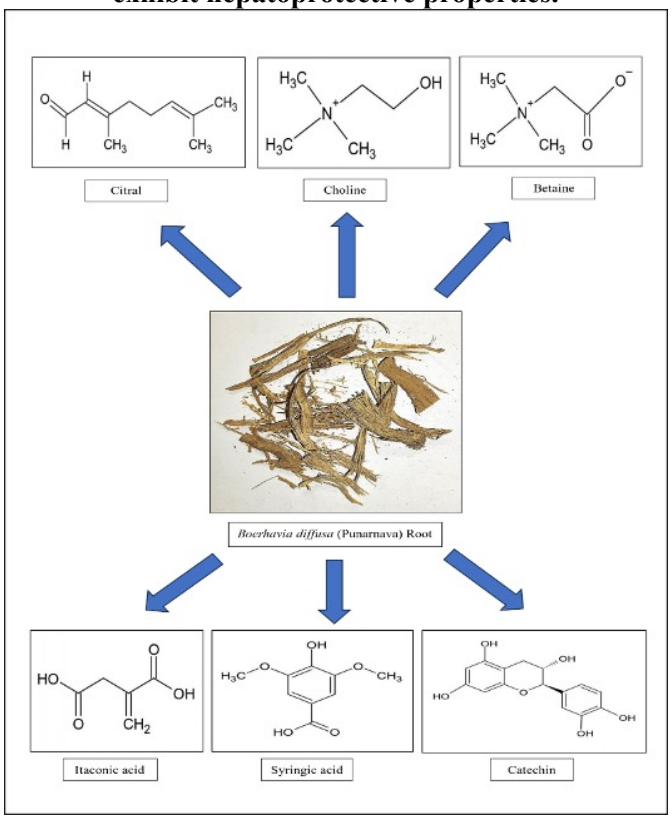
Table 1: Illustrates the details of the Metabolites isolated by HRMS. It shows the Chemical formula, Calculated Molecular Weight (MW), Retention Time (RT) values, Delta Mass and the Peak area under Negative (NEG) / Positive (POS) ion mode of the bioactive metabolites.

Bioactive Metabolite	Formula	Calc. MW	Retention Time (min)	Delta Mass (ppm)	Area NEG	Area POS
Citral	$C_{10}H_{16}O$	152.11987	0.269	-1.58	-	43770954.72
Choline	$C_5H_{13}NO$	103.09966	0.412	-0.55	-	323936836.84
Betaine	$C_5H_{11}NO_2$	117.0789	0.427	-0.7	-	352699468.42
Itaconic acid	$C_5H_6O_4$	130.02597	0.838	-4.92	444518852.76	-
Syringic acid	$C_9H_{10}O_5$	198.05194	5.102	-4.46	14486809.04	-
Catechin	$C_{15}H_{14}O_6$	290.07846	8.444	-2.01	-	19782377.47

The molecular structure of a metabolite is fundamental to its hepatoprotective activity, as it determines the interaction of the metabolite with liver enzymes, cellular membranes, and their key biochemical

pathways. Structural features like functional groups, polarity, and size influence antioxidant, anti-inflammatory, and detoxification effects, which are crucial for protecting liver cells from oxidative stress and inflammation. Additionally, certain structures aid in the regulation of apoptosis and cell regeneration. Synergistic interactions between metabolites, driven by their diverse structures, can enhance overall hepatoprotective efficacy. Therefore, the molecular structures of the active metabolites identified in *Boerhavia diffusa* are illustrated in Figure 8.

Figure 8: Shows the Molecular Structures of bioactive metabolites isolated from *Boerhavia diffusa*. This figure highlights the molecular structures of compounds identified through High-Resolution Mass Spectrometry analysis, which exhibit hepatoprotective properties.



This study primarily focuses on the identification and profiling of bioactive compounds through HRMS, with limited in vivo or clinical validation of the proposed mechanisms of action. While pharmacological data were reviewed, direct experimental evidence linking these phytochemicals to specific hepatic pathways in humans remains to be established. Additionally, variations in phytochemical composition due to environmental, seasonal, and geographical factors were not fully explored. Future studies should include in-depth pharmacokinetic analyses and clinical trials to substantiate these findings and assess the therapeutic efficacy and safety of *Boerhavia diffusa* in the treatment of liver disorders.

Conclusion

This study provides a detailed molecular profile of *Boerhavia diffusa* using High-Resolution Mass Spectrometry, identifying a diverse range of bioactive compounds with strong hepatoprotective potential. Six metabolites with hepatoprotective properties were identified. The hepatoprotective effects of *Boerhavia diffusa* are likely the result of the combined, synergistic actions of these metabolites. These compounds exhibit antioxidant, anti-inflammatory, and liver-regenerative properties, supporting the traditional use of *Boerhavia diffusa* in treating liver disorders. The proposed mechanisms, such as the reduction of oxidative stress, inhibition of pro-inflammatory cytokines, and regulation of liver detoxification pathways, present a multifaceted approach to liver protection. These findings reinforce the relevance of *Boerhavia diffusa* in both Ayurvedic and modern medicine, offering a scientific foundation for its use as a natural therapeutic agent in maintaining hepatic health. This study could serve as a critical step toward the development of plant-based treatments targeting liver health, offering new insights for future pharmacological applications.

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