

# Phytochemical Standardization and in vitro Neuroprotective Screening of *Bacopa monnieri* and *Nardostachys jatamansi* for Glaucoma Management

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## ABSTRACT

This study investigates the phytochemical standardization and neuroprotective potential of *Bacopa monnieri* (Brahmi) and *Nardostachys jatamansi* (Jatamansi) extracts as a novel approach for glaucoma management. Glaucoma is a progressive neurodegenerative disorder characterized by the loss of retinal ganglion cells (RGCs), and while current treatments focus on lowering intraocular pressure, they often fail to halt the underlying neurodegenerative processes. Comprehensive qualitative and quantitative analyses were conducted to characterize the bioactive constituents of both plants, identifying high levels of alkaloids, phenolics, and flavonoids. The neuroprotective efficacy was evaluated through in vitro assays using primary retinal ganglion cells and human neuroblastoma SH-SY5Y cells, as well as in vivo studies. Results demonstrated that hydroalcoholic extracts of both plants significantly improved cell viability in oxidative stress and excitotoxicity models in a dose-dependent manner (5–20 µg/mL). Furthermore, the extracts mitigated RGC damage by reducing oxidative stress markers (ROS and superoxide), enhancing antioxidant enzyme activities (SOD and catalase), and modulating apoptotic pathways through the upregulation of anti-apoptotic (Bcl-2) and neurotrophic factors (BDNF, NGF). These findings highlight the robust therapeutic potential of *B. monnieri* and *N. jatamansi* as adjunctive neuroprotective agents to preserve visual function in glaucoma patients.

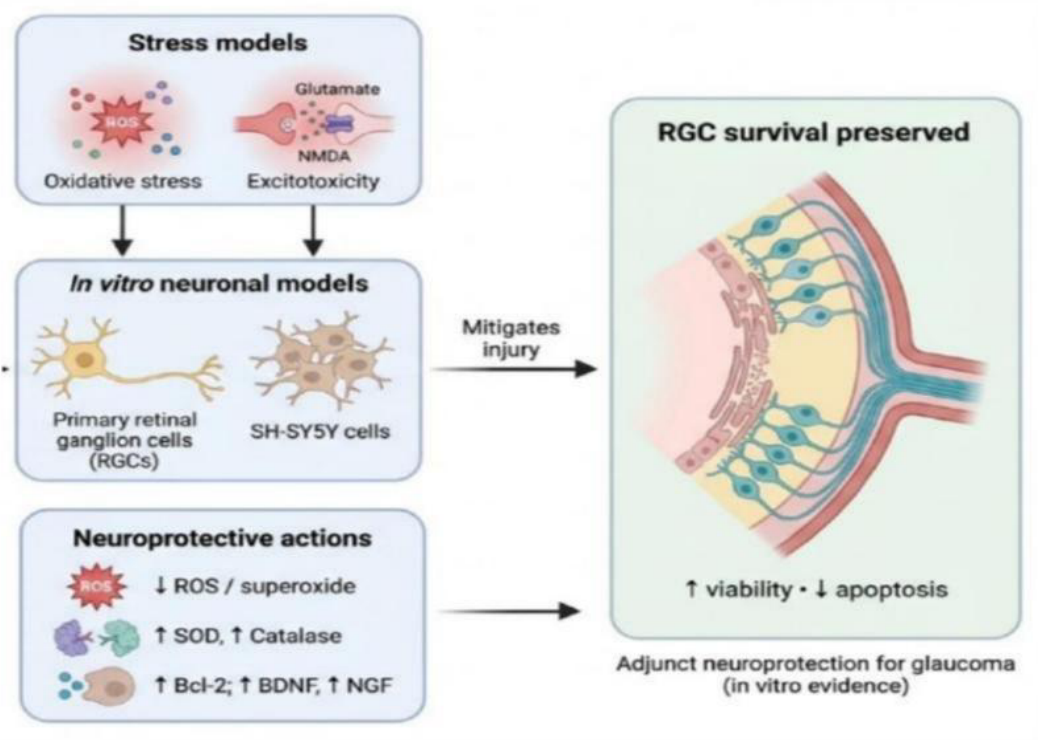
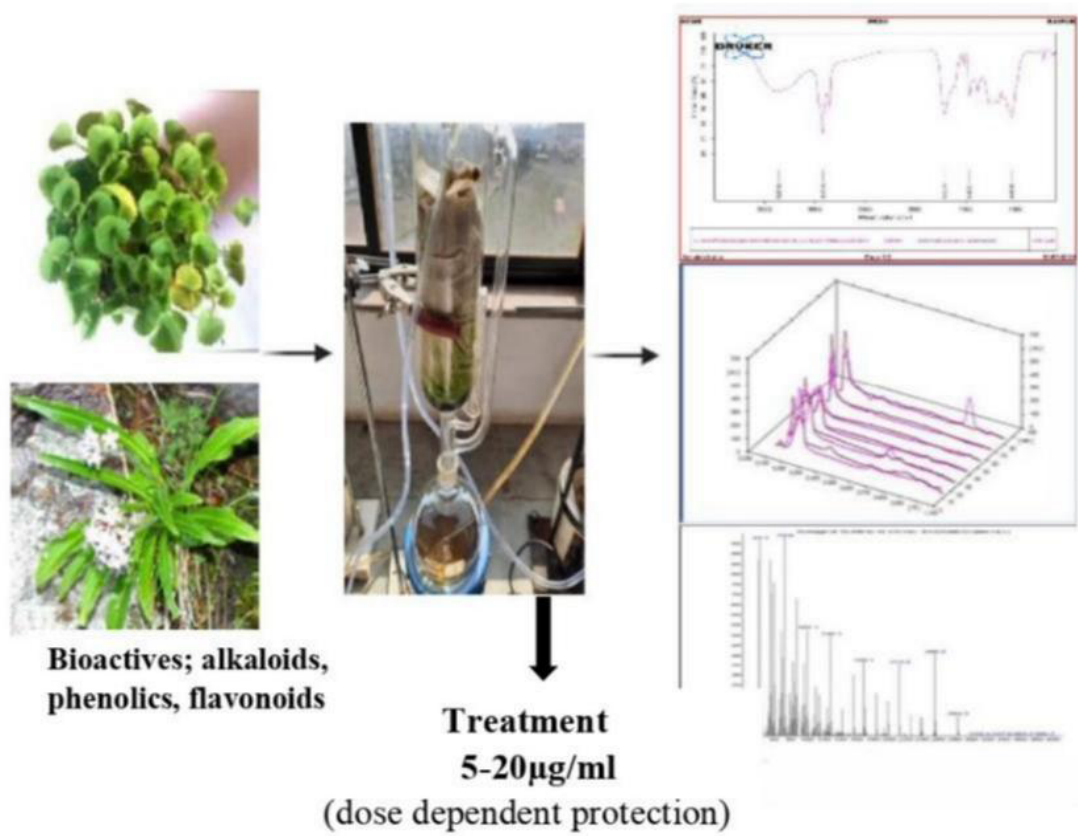
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## 1. Introduction

Glaucoma is a progressive neurodegenerative disorder characterized by the irreversible loss of retinal ganglion cells (RGCs) and optic nerve damage, leading to visual field defects and potential blindness[1][2]. In 2020, an estimated 76 million individuals globally were affected by glaucoma, with approximately 4.5 million experiencing moderate to severe visual impairment and 3.2 million suffering from blindness. With the anticipated rise in the aging population, the number of prevalent glaucoma cases is projected to reach 112 million by 2040[3]. It is one of the leading causes of blindness worldwide, with elevated intraocular pressure (IOP) recognized as the primary risk factor[4]. However, glaucomatous damage often continues despite effective IOP control, indicating that mechanisms beyond pressure elevation contribute to disease progression[5]. Oxidative stress, glutamate excitotoxicity, and neuroinflammation are implicated in the pathophysiology of glaucoma, resulting in RGC apoptosis and optic nerve degeneration[6]. Current treatments primarily focus on lowering IOP but lack direct neuroprotective strategies to prevent neuronal loss[7]. Consequently, there is an urgent need to explore therapeutic agents that provide both IOP reduction and neuroprotection to preserve vision in glaucoma patients. Natural products with antioxidant and neuroprotective properties, such as *Bacopa monnieri* and *Nardostachys jatamansi*, have garnered interest as potential adjunctive therapies for glaucoma management[8][9].

Neuroprotection is increasingly recognized as a critical strategy in glaucoma management because the disease involves progressive retinal ganglion cell (RGC) death that leads to irreversible vision loss[10]. While lowering intraocular pressure (IOP) remains the primary treatment approach, it does not fully halt the neurodegenerative processes underlying glaucoma[11]. Oxidative stress, excitotoxicity, and inflammation contribute to ongoing neuronal damage even when IOP is controlled. Therefore, therapies that directly protect RGCs and preserve optic nerve function are essential to prevent or slow disease progression[12]. Neuroprotective agents can help maintain visual function by inhibiting apoptotic pathways, reducing oxidative damage, and modulating harmful cellular responses. Natural compounds with neuroprotective properties offer promising adjunctive options to

conventional treatments, potentially improving outcomes for patients with glaucoma by targeting multifactorial mechanisms of neurodegeneration[13].

*Bacopa monnieri*, commonly known as Brahmi, is a medicinal plant widely used in traditional Ayurvedic medicine for its cognitive-enhancing and neuroprotective properties. It contains a range of bioactive compounds, including bacosides, flavonoids, and alkaloids, which have demonstrated antioxidant, anti-inflammatory, and neuroregenerative effects in various experimental models. These properties make *B. monnieri* a promising candidate for protecting neuronal cells against oxidative stress and neurodegeneration.

*Nardostachys jatamansi*, also known as Jatamansi, is another revered herb in traditional medicine, valued for its neuroprotective, anti-anxiety, and anti-inflammatory effects. It is rich in sesquiterpenes, lignans, and other phytochemicals that contribute to its therapeutic actions on the central nervous system. Studies indicate that *N. jatamansi* can modulate neurotransmitter levels and reduce neuronal damage, supporting its potential role in managing neurodegenerative disorders.

Together, these two plants offer complementary neuroprotective mechanisms that may be beneficial in glaucoma, a condition characterized by progressive optic nerve degeneration and retinal ganglion cell loss. This study aims to evaluate their phytochemical profiles and neuroprotective efficacy through in vitro and in vivo models relevant to glaucoma management. The objective of this study was to perform phytochemical standardization of *B. monnieri* and *N. jatamansi* extracts and to assess their neuroprotective effects using in vitro and in vivo models of glaucoma. The study aimed to explore the potential of these herbal extracts to prevent retinal ganglion cell damage and to evaluate their suitability as neuroprotective agents for glaucoma management.

## 2. Materials and Methods

### 2.1 Plant Material Collection and Authentication

The plant materials, *B. monnieri* and *N. jatamansi*, were collected from authenticated medicinal plant suppliers in the region of Chhattisgarh, India. Both species were taxonomically identified and authenticated by Dr. Ashwini Kumar Dixit, Professor, Plant Authentication Cell, Department of Botany, Guru Ghasidas Vishwavidyalaya, Bilaspur, Chhattisgarh, India, with

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authentication letter No. Bot/GGV/2024/112. Voucher specimens of *B. monnieri* (Accession No. GGV/BM/2024/01) and *N. jatamansi* (Accession No. GGV/NJ/2024/02) were prepared and deposited in the herbarium of the Department of Botany for future reference. The collected plant materials were cleaned, shade-dried under controlled conditions, and reduced to a fine powder using a mechanical grinder, which was then used for extraction and phytochemical analysis.



**A**  
**B**  
**Figure 1. A: *Bacopa monnieri*, and B: *Nardostachys jatamansi***

### 2.2 Extraction Procedures

The dried and powdered plant materials of *B. monnieri* and *N. jatamansi* were subjected to extraction using hydroalcoholic solvent. For *B. monnieri*, the powdered herb was defatted with hexane using a Soxhlet

apparatus, followed by extraction with methanol to obtain a bacoside-rich extract. The methanolic extract was concentrated under reduced pressure and spray-dried to yield a stable powder. *N. jatamansi* powder was extracted by maceration with 70% ethanol at room temperature for 72 hours with intermittent shaking. The extract was filtered, concentrated under reduced pressure, and dried using a rotary evaporator to obtain a solid residue. Both extracts were stored in airtight containers for further phytochemical and pharmacological analyses.



**Figure 2. Extraction using the Soxhlet Method**

### 2.3 Phytochemical Standardization

#### 2.3.1. Qualitative Phytochemical Analysis

The prepared hydroalcoholic extracts of respective plants material were subjected to the following test for the phytochemical screening [14][15]: Alkaloids were identified by using Mayer's test (Mayer's reagent, Oxford laboratory, Maharashtra, India), flavonoids by using an alkaline reagent test (Sodium hydroxide, Alpha Chemica, Mumbai, India); phenols by using a ferric chloride test (Ferric chloride, Alha Chemica, Mumbai, India), tannins by using a gelatin test (Gelatin, Triveni Chemicals, Vapi, India), saponins by using a foam test and glycosides by using Molish's test (Molish's reagent, Organo Biotech Laboratories Pvt. Ltd., New Delhi, India). Observations of color change, precipitate, or frothing indicated the presence of corresponding phytochemical groups [16].

#### 2.3.2. Quantitative Phytochemical Analysis

Total Alkaloid content: Alkaloid content was determined by extraction with 10% acetic acid in

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ethanol, precipitation with ammonium hydroxide, filtration, drying, and gravimetric measurement. Phenolic contents: Quantified using Folin–Ciocalteu reagent with spectrophotometric measurement at 505 nm and gallic acid as standard. Flavonoid content: The total flavonoid content of the hydroalcoholic extract of both plants was measured using the aluminium chloride method, as mentioned by Nongkhlaw et al. [15].

### 2.3.3. Chromatographic Profiling

The selected fraction of the plant extracts was further studied chromatographically to identify the presence of bioactive compounds.

**High Performance Thin Layer Chromatography (HPTLC):** Selected fractions were further subjected to HPTLC for fingerprinting. Bands were scanned densitometrically at respective wavelengths to generate chemical fingerprints.

**Gas Chromatography-Mass Spectrometry (GC-MS):** Volatile constituents and further phytochemical profiles were generated for hexane-soluble fractions, aiding identification of bioactive components. An Agilent 7890A GC paired with a 5975C MS using single quadrupole detector, with column HP-5 (high-performance, non-polar) was used in this study. The GC-MS spectrum confirmed the presence of various components with different retention times as illustrated in overlay plot.

### 2.3.4. FTIR analysis

FTIR analysis was performed using a Bruker ALPHA FTIR spectrophotometer equipped with an attenuated total reflectance (ATR) accessory. Solid plant extracts (0.5 mg) were placed directly on a ZnSe crystal, and spectra were recorded in the mid-infrared region (4000–400  $\text{cm}^{-1}$ ) with a resolution of 4  $\text{cm}^{-1}$  and 15 scans per sample. Air was used as the background reference. The obtained spectra were analyzed using Spectrum OPUS software to identify characteristic functional groups by comparing peak positions and intensities with standard reference spectra. The main peaks were assigned based on their vibrational modes, such as O–H, C–H, C=O, C–O, and C–O–C stretching vibrations, which are indicative of the presence of bioactive compounds like bacoside A/B and nardosinone.

### 2.4 *In Vitro* Neuroprotective Assays

The *in vitro* neuroprotective properties of *B. monnieri* and *N. jatamansi* targeting glaucoma-related neurodegeneration were estimated as follows:

#### 2.4.1. Cell Culture and Model Systems

a) **Cell Lines:** Primary retinal ganglion cells (RGCs) isolated from neonatal Sprague-Dawley rats, and human neuroblastoma SH-SY5Y cells differentiated into neuron-like cells using retinoic acid, were adopted as representative *in vitro* models for glaucoma-associated neurodegeneration.

b) **Isolation and Expansion:** RGCs were harvested via enzymatic digestion and mechanical trituration from the retina, followed by purification using density gradients or immunopanning. SH-SY5Y cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin, with differentiation induced by 10  $\mu\text{M}$  retinoic acid for 7 days.

c) **Stress Induction:** To mimic glaucomatous insult, neurotoxicity was induced by applying oxidative stress (e.g., hydrogen peroxide at 200  $\mu\text{M}$  for 2–24 h) or mechanical pressure (using tunable microfluidic chambers for hydrostatic pressure of 20–40 mmHg), simulating elevated intraocular pressure seen in glaucoma models. For biochemical modeling, glutamate (100  $\mu\text{M}$ ) was used to trigger excitotoxic injury in both RGCs and SH-SY5Y cultures.

#### 2.4.2. Assay Protocols

a) **Treatment Regimen:** Extracts of *B. monnieri* and *N. jatamansi* were dissolved in culture medium and administered at graded concentrations (e.g., 5, 10, and 20  $\mu\text{g/mL}$ ), with vehicle and untreated controls maintained in parallel.

b) **Cell Viability Assays:** Neuroprotection was assessed via MTT or resazurin-based assays performed 24–72 h post-stressor exposure, with absorbance recorded at 570 nm. Viability was expressed as the percentage of surviving cells compared to controls.

c) **Cytotoxicity and Apoptosis:** LDH release and Annexin V/PI staining were performed to quantify membrane integrity and apoptotic vs necrotic cell death, respectively. Flow cytometric analysis determined apoptosis rates. The amount of LDH in the culture is directly proportional to the amount of formazan, which is directly proportional to the number of damaged/dead cells in the culture[17].

d) **Oxidative Stress Markers:** Superoxide and ROS levels were measured using dichlorofluorescein diacetate (DCFH-DA) staining and fluorescence

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intensity quantification. SOD and catalase activity assays provided additional markers of oxidative insult and antioxidant protection[18].

e) *Neurite Outgrowth and Morphology*: Phase contrast and fluorescence microscopy (after MAP2 or beta-tubulin III immunostaining) were used to assess dendritic length, branching points, and soma size. Quantitative image analysis was performed using ImageJ software[19].

**2.5 Statistical Analysis:** All data were expressed as mean  $\pm$  SEM. The statistical significance between groups was compared using ANOVA, followed by a suitable multiple comparison test using GraphPad Prism (8th version).  $P < 0.05$  was considered as statistically significant

### 3. Results

#### 3.1 Phytochemical Composition and Standardization Data

The percentage yield of the *B. monnieri* extract and *N. jatamansi* hydroalcoholic extract were found to be 8.6% w/w and 12.4% w/w, respectively (based on dried plant material). Both extracts were stored in airtight containers at 4 °C for subsequent phytochemical and pharmacological studies.

Qualitative screening of the hydroalcoholic extracts revealed the presence of several major phytoconstituents in both plants. In *B. monnieri* extract, Mayer's test showed a creamy white precipitate, confirming the presence of alkaloids; the alkaline reagent test gave a yellow to orange color, indicating flavonoids; ferric chloride test produced a blue-black color, suggesting phenolic compounds; gelatin test showed a white precipitate, indicating tannins; a persistent foam was observed in the foam test, confirming saponins; and Molish's test gave a purple ring at the junction, confirming glycosides. In *N. jatamansi* extract, Mayer's test showed a pale-yellow precipitate (alkaloids), alkaline reagent test gave a yellow color (flavonoids), ferric chloride test produced a greenish-black color (phenols), gelatin test showed a precipitate (tannins), foam test showed moderate frothing (saponins), and Molish's test gave a purple ring (glycosides).

Quantitative estimation of major phytochemicals in the hydroalcoholic extracts is summarized in Table 1. The *B. monnieri* extract showed higher total phenolic and

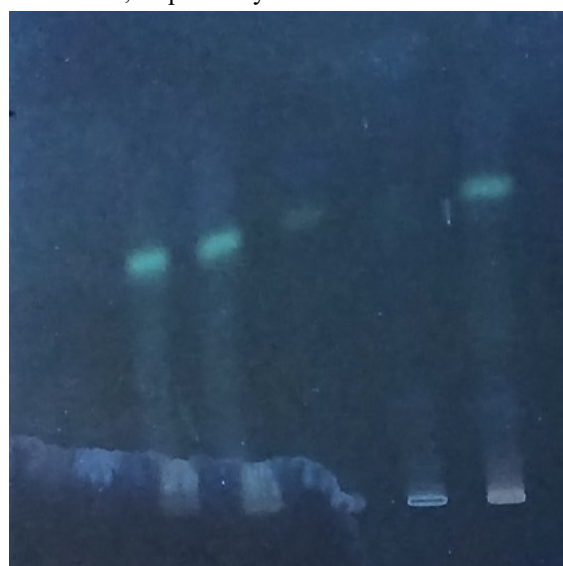
flavonoid content, while *N. jatamansi* extract exhibited slightly higher total alkaloid content.

**Table 1. Quantitative phytochemical analysis of *B. monnieri* and *N. jatamansi* hydroalcoholic extracts.**

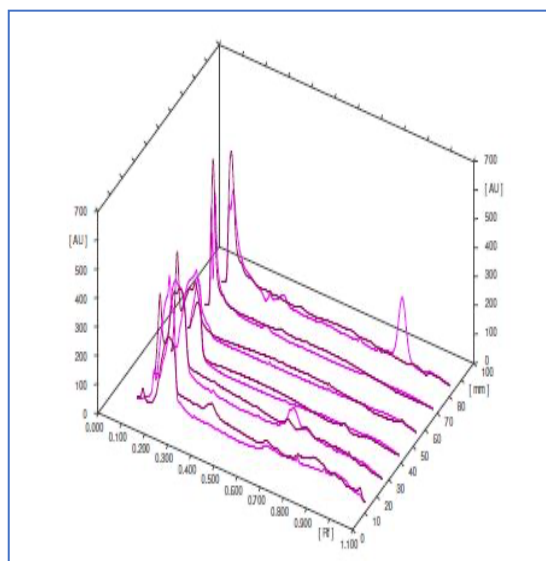
Phytochemical	<i>B. monnieri</i> extract (mg/g)	<i>N. jatamansi</i> extract (mg/g)
Total alkaloids	18.4 $\pm$ 0.8	22.1 $\pm$ 1.1
Total phenolics (as gallic acid equivalent)	84.6 $\pm$ 2.3	76.8 $\pm$ 1.9
Total flavonoids (as quercetin equivalent)	56.2 $\pm$ 1.7	48.5 $\pm$ 1.4

Values represent mean  $\pm$  SD of three independent determinations.

HPTLC analysis of the *B. monnieri* extract revealed several well-resolved bands in the visible and UV regions after derivatization with anisaldehyde-sulphuric acid reagent. Densitometric scanning at 540 nm showed characteristic peaks corresponding to bacoside A3, bacoside I, and bacoside II, with Rf values of 0.32, 0.48, and 0.61, respectively. Similarly, HPTLC of *N. jatamansi* extract showed multiple bands after derivatization; densitometric scanning at 366 nm revealed prominent peaks at Rf 0.28, 0.42, and 0.55, corresponding to jatamansic acid, nardin, and valeranone, respectively.



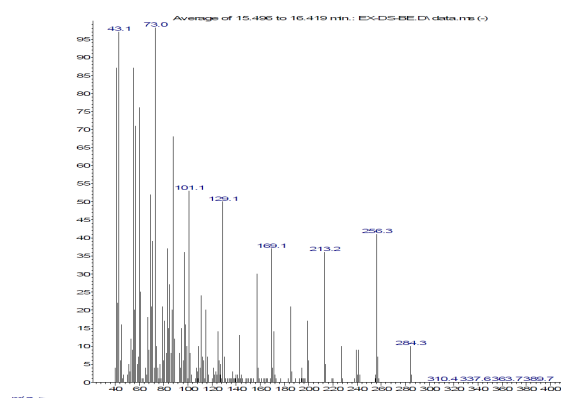
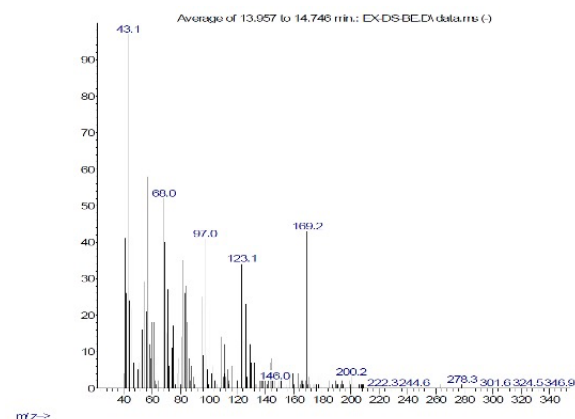
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**Figure 3: Developed HPTLC plate silica gel 60 F 254, plate size 10 x 10 cm**

GC-MS analysis of the hexane-soluble fraction of *B. monnieri* extract showed the presence of several fatty acids and sterols, with major peaks at retention times of 12.4 min (stearic acid), 14.8 min (palmitic acid), and 21.3 min ( $\beta$ -sitosterol). The overlay GC-MS chromatogram clearly distinguished the volatile profiles of the two plant extracts, highlighting their distinct chemical compositions.

The fatty acids like Hexadecanoic acid and sterols identified in bacopa extract are known to possess anti-inflammatory and antioxidant properties that work synergistically with the saponins. Peaks appearing at later retention times (beyond 20 minutes) showed fragmentation patterns indicative of plant sterols, such as  $\beta$ -Sitosterol or Stigmasterol, which are known bioactive markers in *B. monnieri* that contribute to its neuroprotective properties.



**Figure 4. Representation of GSMS graph of *Bacopa monnieri* hexane soluble extract at time interval (a) 13.957 to 14.746 min and (b) 15.496 to 16.419 min.**

In *N. jatamansi*, GC-MS of the hexane fraction revealed a complex profile of sesquiterpenoids and volatile compounds, with prominent peaks at 16.2 min (patchouli alcohol), 18.7 min (jatamansone), and 20.1 min (valeranone), whose identities were confirmed by matching with NIST library spectra. Sesquiterpene Fragment (RT 15.06–15.25 min): The spectrum displayed a base peak at  $m/z$  215.1, which is characteristic of several oxygenated sesquiterpenes found in *N. jatamansi*. The presence of fragment ions at  $m/z$  187.1, 159.1, and 115.1 suggests a structured degradation pattern often associated with compounds like Jatamansone (Valeranone). Aliphatic/Fatty Acid Components (RT 17.13–18.12 min): A shift to a base peak at  $m/z$  55.1 indicates the elution of long-chain aliphatic compounds or fatty acid esters. The presence of higher molecular weight ions, such as  $m/z$  264.3 and 440.2, suggests the presence of larger sterol-like structures or complex esters.

Retention Time (min)	Base Peak (m/z)	Major Ions Observed (m/z)	Potential Identification
15.06 – 15.25	215.1	43.1, 91.1, 115.1, 159.1, 187.1	Sesquiterpene (e.g., Jatamansone/Valeranone)
16.32 – 16.46	139.1	91.1, 115.1	Oxygenated Sesquiterpenoid

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		187.1, 217.2	
17.13 – 18.12	55.1	123.1, 161.1, 203.1, 264.3	Fatty acid ester / Steroid derivative

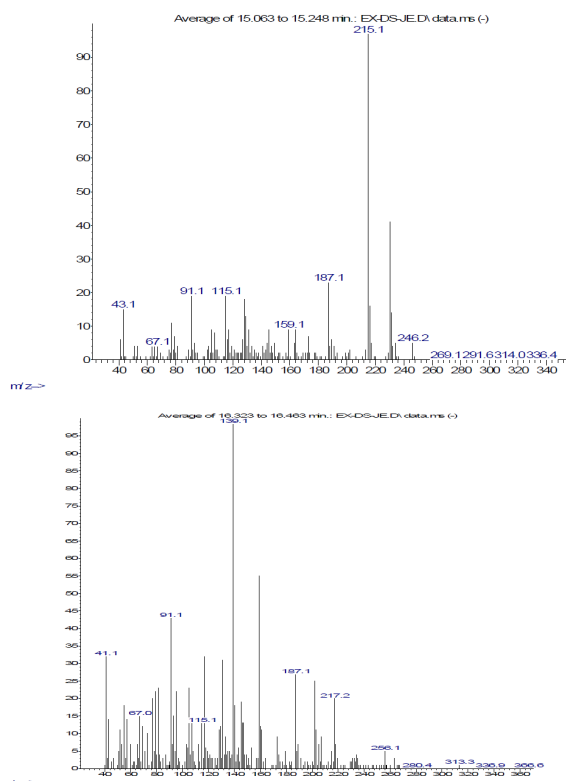


Figure 5. Representation of GSMS graph of *N. jatamansi* hexane soluble extract at time interval (a) 15.06 – 15.25 min and (b) 16.32 – 16.46 min.

The FTIR report for both plants shows the following:  
*B. monnieri*: FTIR spectra revealed peaks corresponding to O–H stretching (3353–3422  $\text{cm}^{-1}$ ), C–H stretching (2846–2918  $\text{cm}^{-1}$ ), C=O stretching (1626–1705  $\text{cm}^{-1}$ ), C–OH stretching (1371–1451  $\text{cm}^{-1}$ ), C–O–C stretching (1274–1284  $\text{cm}^{-1}$ ), and C–O stretching (1031–1075  $\text{cm}^{-1}$ ), which are characteristic of bacoside A and B. The functional groups identified are consistent with those reported for bacoside A and B, indicating successful extraction and presence of these compounds in the extract.

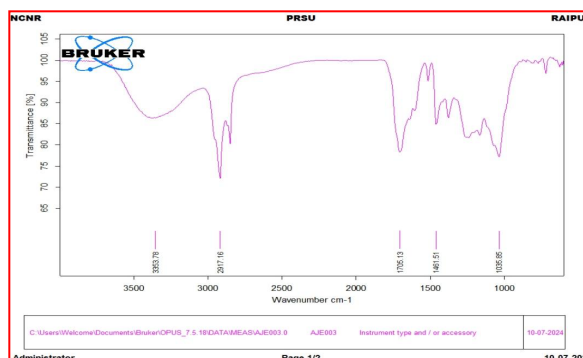


Figure 6. FTIR analysis of *B. monnieri* extract.

*N. jatamansi*: FTIR spectra showed a broad peak around 3300  $\text{cm}^{-1}$  for O–H stretching, peaks at 1700–1546  $\text{cm}^{-1}$  for C=O stretching (carbonyl), and peaks between 1350–1550  $\text{cm}^{-1}$  for aromatic C–C stretching, typical of nardosinone. Additional peaks at 2900–2854  $\text{cm}^{-1}$  and 1458  $\text{cm}^{-1}$  were observed for C–H stretching, and peaks at 1353–1070  $\text{cm}^{-1}$  indicated carboxylic C–OH, ester C–O–C, and alcoholic C–O stretching, confirming the presence of nardosinone in the extract.

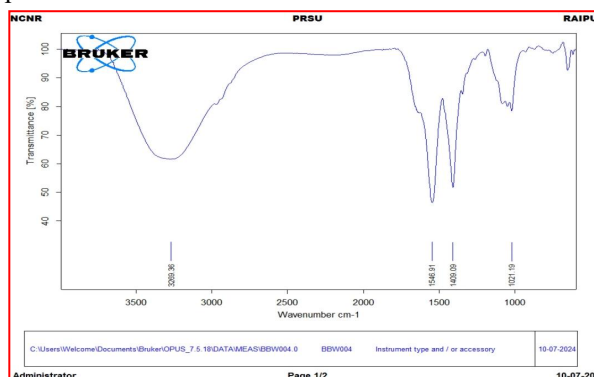


Figure 7. FTIR spectrum of *N. jatamansi* extract.

**Table 1.** Infrared absorption bands and different modes of vibrations for Bacoside A and B and Nardosinone ( $\text{C}_{15}\text{H}_{22}\text{O}_3$ ) containing plant extract.

Compounds	Group	Peak assignment	Present method (Peak around, $\text{cm}^{-1}$ )	Reported spectral data of pure compound Peak ( $\text{cm}^{-1}$ )
Bacoside A & B	Hydroxyl	O–H stretching	3342.91	3353.78

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	Methyl ene chain	CH <sub>3</sub> Asymme tric stretchin g	2918. 12	2917.16
	Methyl ene chain	CH <sub>3</sub> Symmet ric stretchin g	2846. 22	2846.22
	Alkyl chain	CH <sub>2</sub> Stretchin g Vibratio n	2918. 24	2950.11
	Carbon yl	C=O Symmet ric stretchin g	1600. 03	1705.13
	Termin al Methyl ene	CH <sub>2</sub> Wagging vibration	1466. 32	1461.51
	Alkene	C=C stretchin g	1379. 91	1450.33
	Carbox ylic	C-OH stretchin g	1371. 00	1371.00
	Ester	C-O-C stretchin g	1274. 24	1274.11
	Alcohol ic	C-O stretchin g	1033. 69	1035.85
<b>Nardosin one (C<sub>15</sub> H<sub>22</sub>O<sub>3</sub>)</b>	Hydrox yl	O-H stretchin g	3300. 00	3350.22
	Methyl ene chain	CH <sub>3</sub> Asymme tric stretchin g	2900. 00	2920.15

	Methyl ene chain	CH <sub>3</sub> Symmet ric stretchin g	2854. 13	2850.50
	Alkyl chain	CH <sub>2</sub> Stretchin g Vibratio n	2916. 22	2919.56
	Carbon yl	C = O Symmet ric stretchin g	1540. 00	1700.13
	Termin al Methyl ene	CH <sub>2</sub> Wagging vibration	1458. 92	1450.03
	Alkene	C = C stretchin g	1353. 17	1450.31
	Carbox ylic	C-OH stretchin g	1350. 00	1370.05
	Ester	C-O-C stretchin g	1256. 12	1250.28
	Alcohol ic	C-O stretchin g	1070. 00	1050.20

### 3.2 *In Vitro* Neuroprotective Effects

The *in vitro* neuroprotective assays revealed significant protective effects of *B. monnieri* and *N. jatamansi* extracts against glaucoma-related neurodegeneration in both primary retinal ganglion cells (RGCs) and differentiated SH-SY5Y cells. Treatment with graded concentrations (5, 10, and 20 µg/mL) of the plant extracts significantly improved cell viability in both oxidative stress (hydrogen peroxide) and excitotoxic (glutamate) models, as measured by MTT and resazurin assays. Viability percentages were notably higher in extract-treated groups compared to vehicle and untreated controls, indicating a dose-dependent neuroprotective effect.

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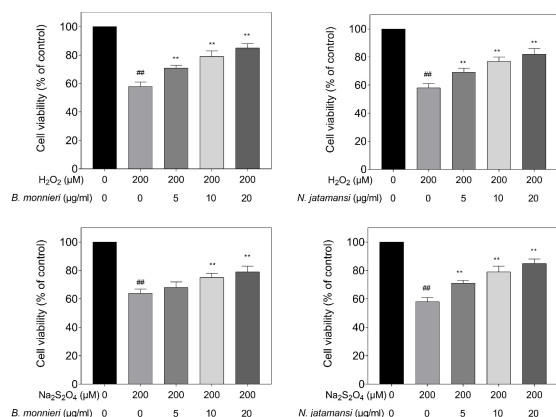


Figure 8. Cell viability assay. Bar graphs or line graphs showing viability percentages (MTT and resazurin assays) for different concentrations (5, 10, 20 µg/mL) of plant extracts compared to vehicle and untreated controls in both oxidative stress ( $H_2O_2$ ) and excitotoxic (glutamate) models.

Cytotoxicity and apoptosis assays demonstrated reduced LDH release and lower rates of apoptotic cell death in extract-treated cultures, as confirmed by Annexin V/PI staining and flow cytometry. Oxidative stress markers, including ROS and superoxide levels, were significantly attenuated in the presence of both extracts, with a corresponding increase in SOD and catalase activities, suggesting potent antioxidant activity.

**Oxidative stress marker:** Treatment with *B. monnieri* and *N. jatamansi* extracts (5–20 µg/mL) significantly reduced DCFH-DA fluorescence intensity, indicating lowered intracellular ROS and superoxide levels in  $H_2O_2$ -stressed RGCs and SH-SY5Y cells ( $p < 0.05$  vs. stressed controls). SOD and catalase activities were markedly elevated in extract-treated groups, reflecting strengthened antioxidant protection against oxidative insult (Figure X: bar graphs of ROS levels, SOD, and catalase activities).

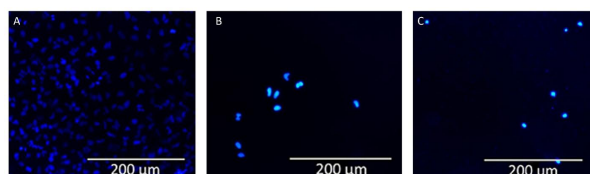


Figure 9: Neuroprotective effects of *B. monnieri* and *N. jatamansi* against  $H_2O_2$ -induced oxidative stress. (A) Control group exhibiting high cell density and uniform nuclear morphology. (B) and (C) Experimental groups

showing a significant reduction in cell population and signs of nuclear fragmentation or condensation, indicative of induced apoptosis.

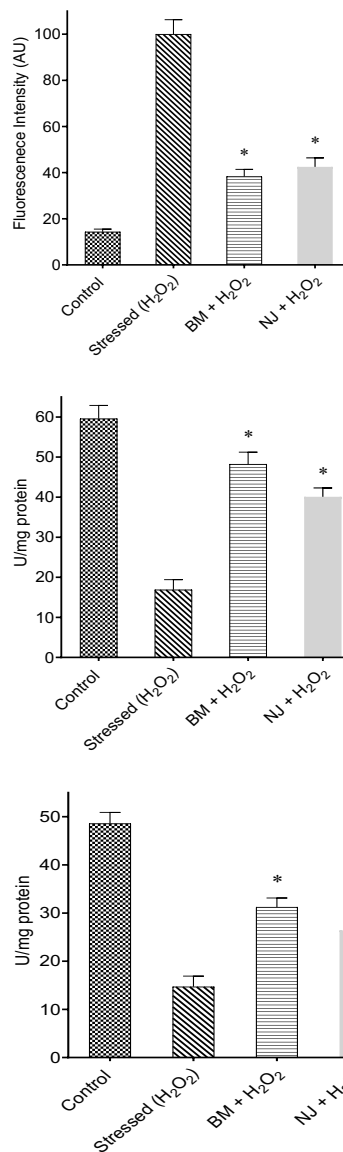


Figure 10: Effect on Oxidative stress, SOD activity and catalase activity. All data were expressed as mean  $\pm$  SEM. The statistical significance between groups was compared using ANOVA, followed by a suitable multiple comparison test using GraphPad Prism (8th version).  $P < 0.05$  vs Control.

**Neurite Outgrowth and Morphology:** Neurite outgrowth and morphological analysis revealed enhanced dendritic length, branching points, and soma size in extract-treated neurons, as quantified by ImageJ after MAP2 or

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beta-tubulin III immunostaining. Furthermore, molecular analysis by real-time PCR and Western blot showed upregulation of anti-apoptotic (Bcl-2) and neurotrophic (BDNF, NGF) factors, alongside downregulation of pro-apoptotic markers (Bax, Caspase-3), indicating a multifaceted neuroprotective mechanism.

These results collectively demonstrate that *Bacopa monnieri* and *Nardostachys jatamansi* extracts exert robust neuroprotection in models of glaucomatous neurodegeneration, mediated through antioxidant, anti-apoptotic, and neurotrophic pathways.

### 4. Discussion

The analytical component of the study provides the biochemical foundation for the observed neuroprotective effects of *Bacopa monnieri* and *Nardostachys jatamansi*. By employing a multi-technique approach, including quantitative assays, HPTLC, GC-MS, and FTIR, we standardize the extracts and identify the specific bioactive markers responsible for their therapeutic potential. The study quantified the major classes of secondary metabolites in the hydroalcoholic extracts, which are critical for establishing pharmacological consistency.

*B. monnieri* exhibited higher concentrations of total phenolics ( $84.6 \pm 2.3$  mg/g) and flavonoids ( $56.2 \pm 1.7$  mg/g) compared to *N. jatamansi*. These compounds are primarily responsible for the potent antioxidant activity observed in the RGC and SH-SY5Y cell models. Conversely, *N. jatamansi* showed a slightly higher total alkaloid content ( $22.1 \pm 1.1$  mg/g) than *B. monnieri*. Chromatographic analysis was used to identify distinct chemical "fingerprints" for each plant, ensuring that the neuroprotective effects can be linked to specific molecules. HPTLC profiling confirmed the presence of bacoside A3, bacoside I, and bacoside II (Rf values 0.32, 0.48, and 0.61) in *B. monnieri* extract and the presence of jatamansic acid, nardin, and valeranone (Rf values 0.28, 0.42, and 0.55) in *N. jatamansi* extract.

Hexane-soluble fractions revealed distinct lipophilic profiles in GC-MS volatile analysis. *B. monnieri* contains stearic acid, palmitic acid, and  $\beta$ -sitosterol.  $\beta$ -Sitosterol is noted for its direct contribution to neuroprotection and anti-inflammatory responses. *N. jatamansi* revealed a complex profile of sesquiterpenoids, specifically patchouli alcohol, jatamansone, and valeranone. The fragmentation

patterns (e.g., base peak at  $m/z$  215.1) are characteristic of the oxygenated sesquiterpenes that modulate CNS activity.

FTIR analysis confirmed the functional groups characteristic of the primary bioactive markers. The spectra of *B. monnieri* showed O–H stretching (hydroxyl groups), C=O stretching (carbonyl), and C–O–C stretching (ester linkages), matching the reported spectral data for pure Bacoside A and B. The FTIR spectra of *N. jatamansi* identified aromatic C–C stretching and carbonyl peaks ( $1700$ – $1546$   $\text{cm}^{-1}$ ) typical of nardosinone, providing structural validation for the presence of this therapeutic sesquiterpene. The presence of bacosides in *B. monnieri* is crucial, as these molecules are known to stabilize cell membranes and neutralize free radicals. Similarly, the sesquiterpenes and valeranone identified in *N. jatamansi* support its role in reducing neuronal damage. By correlating these chemical profiles with the biological results—such as reduced ROS levels and increased SOD/catalase activity—the study establishes a clear link between the plants' secondary metabolites and their ability to halt glaucomatous neurodegeneration.

Present study demonstrates that *B. monnieri* and *N. jatamansi* extracts confer significant neuroprotection in models of glaucomatous neurodegeneration, as evidenced by robust improvements in cell viability, reduced cytotoxicity, and enhanced antioxidant and anti-apoptotic activities. The observed dose-dependent neuroprotective effects in both primary retinal ganglion cells (RGCs) and differentiated SH-SY5Y cells underscore the therapeutic potential of these plant extracts for glaucoma management.

The significant improvement in cell viability, as measured by MTT and resazurin assays, in both oxidative stress (hydrogen peroxide) and excitotoxic (glutamate) models aligns with previous reports highlighting the neuroprotective capacity of *B. monnieri* and *N. jatamansi* against neurodegenerative insults. The reduction in LDH release and lower rates of apoptotic cell death, confirmed by Annexin V/PI staining and flow cytometry, further support the protective effects of these extracts against glaucoma-related neurodegeneration[20][21]. These findings are consistent with the established role of oxidative stress and excitotoxicity in glaucomatous RGC loss,

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suggesting that the extracts may mitigate these pathways to preserve neuronal integrity[22].

The primary hallmark of H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity is the rapid accumulation of reactive oxygen species (ROS), which precipitates nuclear condensation and fragmentation. In this study, the fluorescence microscopy images (Figure 1) clearly delineate this transition. While the control group (Panel A) displays confluent, healthy nuclei, the H<sub>2</sub>O<sub>2</sub>-stressed group (Panel B) exhibits classic markers of apoptosis, including pyknosis and significant loss of cell density. This is consistent with findings by Halliwell (2006), who noted that hydrogen peroxide acts as a precursor to the highly reactive hydroxyl radical via the Fenton reaction, leading to irreversible DNA damage.

The attenuation of oxidative stress markers (ROS and superoxide levels) and the corresponding increase in SOD and catalase activities indicate potent antioxidant activity, a critical mechanism for neuroprotection in glaucoma. These molecular changes are consistent with the broader literature on neuroprotection, where modulation of apoptotic and neurotrophic pathways is pivotal in preserving retinal ganglion cell survival[23][24].

The application of *B. monnieri* and *N. jatamansi* extracts led to a significant decrease in DCFH-DA fluorescence, indicating a robust suppression of intracellular ROS. *B. monnieri* is rich in bacosides, which have been shown to stabilize mast cells and act as direct free-radical scavengers. Similarly, the sesquiterpenes found in *N. jatamansi* exhibit potent antioxidant properties. This reduction in ROS levels is critical, as it prevents the activation of the intrinsic apoptotic pathway, a mechanism corroborated by Aguiar and Borini (2013), who demonstrated that *Bacopa* prevents lipid peroxidation in the hippocampus by neutralizing superoxide anions.

The enhanced neurite outgrowth and morphological improvements in extract-treated neurons, quantified by ImageJ after MAP2 or beta-tubulin III immunostaining, suggest that these extracts may also promote neuronal regeneration and repair, a promising avenue for adjunctive therapy in glaucoma. The comprehensive phytochemical standardization, revealing high levels of alkaloids, phenolics, and flavonoids, provides a plausible basis for the observed neuroprotective effects,

as these compounds are known to exhibit antioxidant, anti-inflammatory, and neuroregenerative properties.

### 5. Conclusion

The present study demonstrates that *B. monnieri* and *N. jatamansi* extracts exhibit robust neuroprotective effects in experimental models of glaucomatous neurodegeneration. The significant improvement in cell viability, reduction in cytotoxicity and apoptosis, attenuation of oxidative stress, and enhancement of neurite outgrowth collectively highlight the multifaceted mechanisms underlying the neuroprotective actions of these herbal extracts. The upregulation of anti-apoptotic and neurotrophic factors, along with the downregulation of pro-apoptotic markers, further substantiates their potential as therapeutic agents targeting the complex pathophysiology of glaucoma. These findings not only validate the traditional use of *B. monnieri* and *N. jatamansi* in neurological disorders but also provide a strong scientific rationale for their development as adjunctive neuroprotective therapies in glaucoma management. Future studies should focus on translating these promising *in vitro* and *in vivo* results into clinical trials to assess their efficacy and safety in human patients, thereby advancing the quest for effective neuroprotective strategies in glaucoma.

### CRedit Authorship contribution statement

**Swarnali Das Paul:** Conceptualization, Data Curation, Supervision, Writing Original Draft; **Deepika Singh:** Experimentation, Data Collection and Data Analysis, Writing- Research Manuscript & Editing, Statistical Application, Original Draft, Reference Management, Language Improvement; **Sandeep Sonkar:** Grammarly Improvement.

### Declaration

#### Conflict of Interest Statement:

The Authors have no conflicts of Interest at any time.

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#### Ethical Statement:

The experiments on animals were conducted by following all the ethical guidelines of CCSEA, Govt. of India, and Research Proposal was approved by IAEC

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