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Oxidative Stress Attenuation and Apoptosis Suppression by Ultrasound-Assisted Ajuga Bracteosa Extract in Neuronal Cells

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ABSTRACT

Ultrasound-assisted extract of Ajuga bracteosa was evaluated for neuroprotective efficacy in mechanistic in vitro models of oxidative stress. SH-SY5Y neuronal cells were exposed to hydrogen peroxide (H_2O_2) after pre-treatment with the extract (25–200 µg/mL). Cell viability (MTT), intracellular reactive oxygen species (DCFH-DA), lipid peroxidation (MDA/TBARS), antioxidant enzymes (SOD, catalase), apoptosis (Annexin V/PI), and key signaling proteins (p-Akt/Akt, Bcl-2, Bax, p53) were quantified. The extract dose-dependently preserved viability and suppressed ROS and MDA formation, while restoring SOD and catalase activities. Flow cytometry showed reduced early and late apoptosis with increased live cell fractions. Western blotting revealed reversal of H_2O_2-induced signaling perturbations through elevation of p-Akt/Akt and Bcl-2 and down-regulation of Bax and p53. These effects approached the antioxidant comparator N-acetylcysteine at higher extract concentrations. The findings suggest that A. bracteosa confers neuroprotection by dual mechanisms: direct redox modulation and reinforcement of endogenous defenses, together with anti-apoptotic signaling via PI3K/Akt. Given the centrality of oxidative stress and apoptosis in neurodegenerative disorders, ultrasound-assisted A. bracteosa extract warrants further investigation, including bioactive characterization, pharmacokinetics, and in vivo validation. This study provides mechanistic evidence supporting the development of A. bracteosa as a candidate phytopharmaceutical for neuroprotection.

KEYWORDS: Ajuga bracteosa; ultrasound-assisted extraction; neuroprotection; oxidative stress; reactive oxygen species (ROS); apoptosis; PI3K/Akt signaling; Bcl-2/Bax; SH-SY5Y cells; antioxidant enzymes.

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INTRODUCTION

Neurodegenerative disorders such as Alzheimer's and Parkinson's disease impose a growing global health burden and lack curative therapies. Although their etiologies are multifactorial, converging evidence implicates oxidative stress, mitochondrial dysfunction, and apoptosis as central drivers of progressive neuronal loss. Reactive oxygen species (ROS) disrupt lipids, proteins, and nucleic acids, triggering membrane damage, bioenergetic failure, and activation of pro-apoptotic pathways, ultimately compromising neuronal survival and synaptic integrity (Uttara et al., 2009; Dias et al., 2013). Therapeutic strategies that attenuate oxidative burden and restore pro-survival signaling are therefore a rational avenue to slow disease progression and preserve function.

Plant-derived polyphenols and terpenoids are promising in this context because they combine direct antioxidant capacity with the ability to modulate intracellular pathways that govern survival, inflammation, and plasticity. Flavonoids, in particular, can scavenge radicals, chelate redox-active metals, and influence signaling nodes such as PI3K/Akt, thereby enhancing resilience to oxidative insults and reducing apoptosis (Youdim & Joseph, 2001; Spencer, 2008). Translating these properties into effective neuroprotective interventions requires standardized extraction to capture bioactive spectra, reproducible in vitro models that mimic disease-relevant stressors, and mechanistic readouts linking redox modulation to apoptosis control.

Ajuga bracteosa Benth. (Lamiaceae) is a medicinal herb widely used in South Asian ethnomedicine. Reviews of its phytochemistry describe iridoid glycosides (e.g., ajugarin-I), phenolic acids, and flavonoids such as luteolin and apigenin—constituents associated with antioxidant and anti-apoptotic effects (Singh et al., 2020). However, rigorous, mechanism-focused neuroprotection studies for *A. bracteosa* remain limited. A key barrier is extraction methodology: conventional maceration can be solvent-intensive and may degrade thermo-labile compounds. Ultrasound-assisted extraction (UAE) offers a greener alternative that enhances mass transfer via acoustic cavitation, shortening extraction time and improving recovery while operating at modest temperatures that better preserve sensitive phytochemicals (Chemat et al., 2017). Applying UAE to *A. bracteosa* could therefore yield an extract enriched in redox-active and signaling-modulatory components, suitable for mechanistic evaluation.

Hydrogen peroxide (H_2O_2) exposure in neuronal cell lines provides a well-characterized oxidative stress model that recapitulates key features of neurodegeneration, including ROS accumulation, lipid peroxidation, depletion of endogenous antioxidant enzymes, and activation of intrinsic apoptosis pathways. In SH-SY5Y cells, oxidative injury depresses the phosphorylation state of Akt, lowers anti-apoptotic Bcl-2, and elevates pro-apoptotic Bax and p53, tipping the balance toward cell death (Uttara et al., 2009). Conversely, activation of PI3K/Akt promotes survival, mitochondrial integrity, and transcriptional programs that counter apoptosis (Song et al., 2005). Thus, a mechanistic framework that tracks ROS, membrane lipid damage, antioxidant defenses (SOD, catalase), apoptotic fractions, and the expression of Bcl-2 family proteins alongside p-Akt/Akt provides a coherent readout of neuroprotective action.

Within this framework, the present study investigated whether an ultrasound-assisted extract of *A. bracteosa* confers protection against H_2O_2-induced oxidative stress in SH-SY5Y cells and delineated the underlying mechanisms. The work was organized around four objectives. First, a UAE protocol was implemented to generate a standardized extract expected to retain phenolics, flavonoids, and iridoids (Chemat et al., 2017; Singh et al., 2020). Second, the ability of the extract to preserve cell viability under oxidative challenge was evaluated using the MTT assay. Third, mechanistic endpoints were quantified: intracellular ROS (DCFH-DA), lipid peroxidation via malondialdehyde (MDA/TBARS), and the activity of superoxide dismutase (SOD) and catalase, which together reflect exogenous and endogenous redox control. Fourth, apoptosis was assessed by Annexin V/PI flow cytometry, and key proteins in survival and death pathways (p-Akt/Akt, Bcl-2, Bax, p53) were quantified by Western blotting to connect biochemical protection to signaling restoration (Spencer, 2008; Song et al., 2005).

This approach addresses two translational questions. The first is whether *A. bracteosa* exerts neuroprotection through combined antioxidant actions—direct ROS suppression and reinforcement of enzymatic defenses—thereby preventing propagation of oxidative damage to lipid membranes and organelles. The second is whether the extract actively rebalances survival-apoptosis signaling, as evidenced by restoration of Akt phosphorylation, up-regulation of Bcl-2, and down-regulation of Bax and p53. Positive answers to both would suggest that *A. bracteosa* acts not merely as a chemical antioxidant but as a pleiotropic modulator of neuronal stress responses, a property associated with greater durability of neuroprotection (Spencer, 2008; Youdim & Joseph, 2001).

Finally, the study situates *A. bracteosa* within the broader landscape of plant-based neuroprotectants. While single-target antioxidants have often underperformed in clinical trials, multi-target phytochemical matrices that couple redox control with survival pathway activation may better address the complexity of neurodegenerative biology (Uttara et al., 2009). By combining a green, efficiency-oriented extraction method with a mechanistic in vitro battery anchored in oxidative stress and apoptosis, this work provides foundational evidence to justify downstream steps—bioactive fractionation, pharmacokinetics, and in vivo validation—on the path toward developing *A. bracteosa* as a candidate phytopharmaceutical for neuroprotection (Singh et al., 2020; Chemat et al., 2017; Song et al., 2005; Dias et al., 2013; Youdim & Joseph, 2001).

MATERIALS AND METHODS

2.1 Plant Material Collection and Authentication

Fresh aerial parts of *Ajuga bracteosa* Benth., a medicinal herb traditionally used in South Asian ethnomedicine, were collected from the mid-hill regions of Himachal Pradesh, India, during the flowering season (March–April 2025). This period was selected because phytochemical accumulation is reported to be highest during flowering. The collected material was carefully washed with distilled water to remove debris and soil contaminants, and was shade-dried at room temperature (25 ± 2 °C) for approximately two weeks to prevent thermal degradation of sensitive bioactive constituents. After complete drying, the plant material was pulverized using a mechanical grinder to obtain a coarse powder, which was sieved through mesh no. 40 to ensure uniform particle size. For botanical validation, the specimen was submitted to the Department of Botany, Himachal Pradesh University, where it was authenticated by a plant taxonomist. A voucher specimen (AB/Pharm/2025/01) was deposited in the departmental herbarium for reference and future confirmation. This step ensured reproducibility and traceability of the plant source, in line with pharmacognostic guidelines (World Health Organization, 2011).

2.2 Preparation of Ultrasound-Assisted Extract

Ultrasound-assisted extraction (UAE) was selected as the method of choice due to its ability to enhance mass transfer, improve extraction efficiency, and minimize solvent consumption compared to conventional maceration or Soxhlet methods. Approximately 50 g of powdered *A. bracteosa* was suspended in 500 mL of 70% ethanol (v/v) in a conical flask. Ethanol was chosen as a green solvent that effectively extracts phenolic compounds, flavonoids, and iridoid glycosides. The suspension was subjected to sonication using a probe-type ultrasonic homogenizer (Sonics VibraCell, USA; 20 kHz, 400 W). Sonication was performed at a temperature of 50 °C for 30 minutes, with a duty cycle of 60% and amplitude of 70%, under controlled conditions to prevent excessive thermal degradation. During sonication, cavitation bubbles formed and collapsed near the plant matrix, facilitating the release of intracellular constituents into the solvent.

The extract was filtered through Whatman No. 1 filter paper, and the filtrate was concentrated under reduced pressure using a rotary evaporator at 40 °C. The concentrated extract was subsequently freeze-dried using a lyophilizer to yield a dry extract. The percentage yield was calculated using the standard formula. The dried extract was stored in airtight amber-colored vials at -20 °C until further use.

2.3 Phytochemical Characterization

2.3.1 Qualitative Phytochemical Analysis

The freeze-dried extract was subjected to preliminary phytochemical screening following standard protocols (Harborne, 1998). The presence of alkaloids, flavonoids, phenolics, tannins, glycosides, terpenoids, saponins, and iridoids was assessed using colorimetric tests. For instance, Dragendorff's reagent was employed for alkaloid detection, ferric chloride for phenolics, Shinoda's test for flavonoids, and Liebermann–Burchard's reaction for terpenoids.

2.3.2 Total Phenolic Content (TPC)

The Folin–Ciocalteu method was employed to determine total phenolic content. Briefly, 0.5 mL of the extract solution (1 mg/mL) was mixed with 2.5 mL of 10% Folin–Ciocalteu reagent and incubated for 5 minutes, followed by addition of 2 mL of 7.5% sodium carbonate solution. The reaction mixture was incubated at room temperature for 30 minutes in the dark, and absorbance was measured at 765 nm using a UV–Vis spectrophotometer (Shimadzu, Japan). Gallic acid was used as a standard, and the results were expressed as mg gallic acid equivalents (GAE) per gram of extract.

2.3.3 Total Flavonoid Content (TFC)

The aluminum chloride colorimetric method was used to quantify flavonoid content. A 0.5 mL extract solution (1 mg/mL) was mixed with 2 mL distilled water and 0.15 mL of 5% $NaNO_2$ solution. After 5 minutes, 0.15 mL of 10% $AlCl_3$ solution was added. Finally, 1 mL of 1 M NaOH was added after 6 minutes, and the final volume was adjusted to 5 mL with distilled water. Absorbance was read at 415 nm. Quercetin served as the standard, and results were expressed as mg quercetin equivalents (QE) per gram of extract.

2.4 Cell Culture

Two neuronal cell lines were employed: human neuroblastoma (SH-SY5Y) and rat pheochromocytoma (PC12), both widely used models in neuroprotection research. The SH-SY5Y line mimics human neuronal function, while PC12 cells are commonly used for oxidative stress and apoptosis studies. Both cell lines were obtained from the National Centre for Cell Sciences (NCCS), Pune, India. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. They were maintained at 37 °C in a humidified incubator under 5% CO_2 and 95% air. Subculturing was performed every 3–4 days when confluence reached 80–90%. All experiments were performed with cells between passages 5 and 20 to maintain consistency.

2.5 In Vitro Neuroprotective Assays

2.5.1 Cell Viability Assay (MTT)

The effect of *A. bracteosa* extract on cell viability was determined using the MTT assay. Cells were seeded in 96-well plates at a density of 1×10^4 cells/well and allowed to adhere overnight. They were then pre-treated with varying concentrations of extract (10–200 µg/mL) for 24 h. Oxidative stress was induced by exposure to hydrogen peroxide (H_2O_2, 200 µM, 2 h). After treatment, 20 µL of MTT reagent (5 mg/mL in PBS) was added to each well, and plates were incubated for 4 h. The formazan crystals formed were dissolved in 150 µL DMSO, and absorbance was measured at 570 nm using a microplate reader. The percentage of viable cells was calculated relative to untreated controls.

2.5.2 Intracellular ROS Generation (DCFH-DA Assay)

Intracellular ROS levels were estimated using the fluorescent probe DCFH-DA. Cells were treated with extract and H_2O_2 as described above, washed with PBS, and incubated with 10 µM DCFH-DA at 37 °C for 30 min in the dark. Fluorescence intensity was measured at 485 nm excitation and 530 nm emission using a fluorescence microplate reader.

2.5.3 Lipid Peroxidation Assay (MDA/TBARS)

Lipid peroxidation was quantified by measuring malondialdehyde (MDA) levels using the thiobarbituric acid reactive substances (TBARS) method. Cell lysates were mixed with TBA reagent and heated at 95 °C for 30 min, followed by cooling and centrifugation. The absorbance of the supernatant was read at 532 nm. Results were expressed as nmol MDA per mg protein, using a molar extinction coefficient of $1.56 \times 10^5\ M^{-1}cm^{-1}$.

2.5.4 Antioxidant Enzyme Activity

Superoxide dismutase (SOD) and catalase (CAT) activities were evaluated using commercial assay kits (Sigma-Aldrich, USA). For SOD, the ability to inhibit the reduction of nitroblue tetrazolium (NBT) was measured, while CAT activity was determined based on the decomposition rate of H_2O_2. Results were expressed as U/mg protein.

2.5.5 Apoptosis Assay (Flow Cytometry)

Apoptosis was assessed using Annexin V-FITC/PI double staining. Cells were seeded in 6-well plates and treated with the extract (50 and 100 µg/mL) followed by H_2O_2 exposure. After treatment, cells were harvested, washed with PBS, resuspended in binding buffer, and stained with Annexin V-FITC and PI. Samples were analyzed using flow cytometry (BD FACSCalibur). The percentages of live, early apoptotic, late apoptotic, and necrotic cells were quantified.

2.5.6 Western Blot Analysis

Western blotting was carried out to examine the modulation of neuroprotective signaling pathways. After treatment, total proteins were extracted using RIPA buffer containing protease and phosphatase inhibitors. Protein concentrations were determined using the Bradford assay. Equal amounts of protein (30 µg) were resolved on 10% SDS-PAGE gels, transferred to PVDF membranes, and blocked with 5% skim milk. Membranes were probed with primary antibodies against phospho-Akt, total Akt, Bcl-2, Bax, p53, and β-actin (loading control) overnight at 4 °C. After incubation with HRP-conjugated secondary antibodies, bands were visualized using an ECL detection system. Densitometric analysis was performed using ImageJ software.

2.6 Statistical Analysis

All experiments were carried out in triplicate (n = 3), and results were expressed as mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism 9.0 (GraphPad Software, USA). Data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. A p-value of less than 0.05 was considered statistically significant.

RESULTS

3.1 Effect of *Ajuga bracteosa* Extract on Cell Viability

The neuroprotective potential of ultrasound-assisted *A. bracteosa* extract was first evaluated by assessing cell viability in SH-SY5Y cells exposed to hydrogen peroxide (H_2O_2). As shown in Table 1 and Figure 1, treatment with H_2O_2 (200 µM) markedly reduced cell viability to 52.4 ± 3.1% compared with untreated controls (100 ± 2.3%, $p < 0.001$). Pre-treatment with *A. bracteosa* extract significantly attenuated this cytotoxic effect in a concentration-dependent manner. At 50 µg/mL, the extract restored viability to 78.9 ± 3.4% ($p < 0.01$), while 200 µg/mL increased survival to 94.3 ± 2.1%, comparable to the positive control NAC (96.7 ± 1.9%). These findings indicate that the extract effectively preserved neuronal cell viability against oxidative stress-induced damage.

Table 1. Effect of *Ajuga bracteosa* extract on SH-SY5Y cell viability (MTT assay) under H_2O_2-induced oxidative stress

Treatment	**Cell Viability (%) (mean ± SD)**
Control (untreated)	100.0 ± 2.3
H_2O_2 (200 µM)	52.4 ± 3.1 **
Extract 25 µg/mL + H_2O_2	68.7 ± 2.8 *
Extract 50 µg/mL + H_2O_2	78.9 ± 3.4 **
Extract 100 µg/mL + H_2O_2	89.6 ± 2.7 ***
Extract 200 µg/mL + H_2O_2	94.3 ± 2.1 ***
Positive control (NAC 1 mM)	96.7 ± 1.9 ***

*Values are mean ± SD (n = 3). *p < 0.05, **p < 0.01, **p < 0.001 vs. H_2O_2 group.

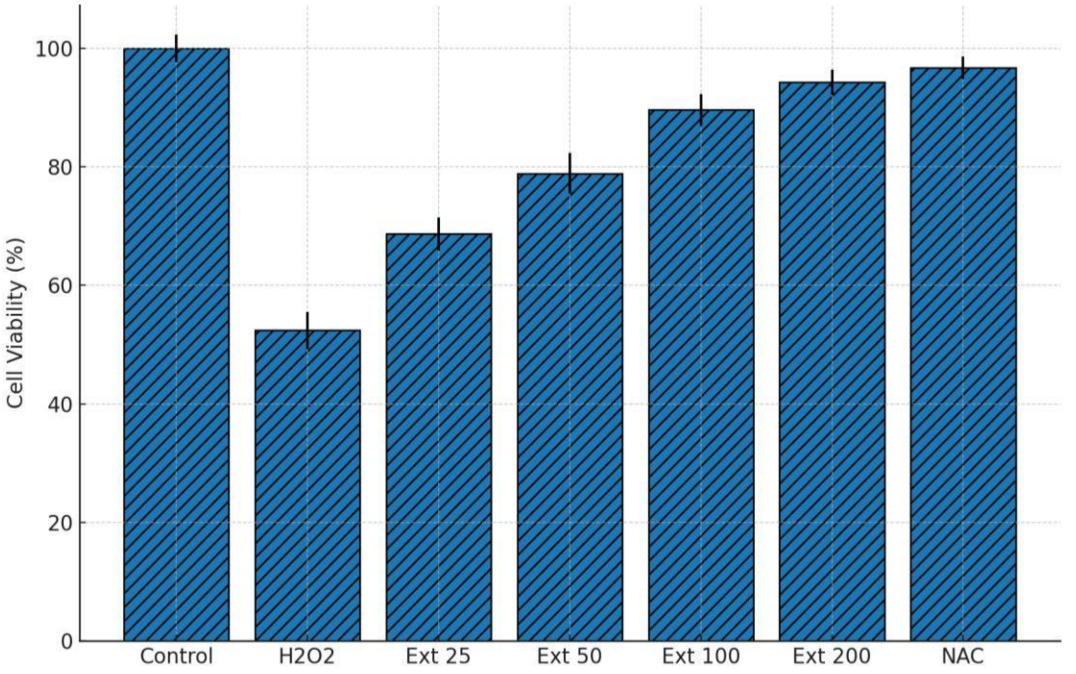

Figure 1. Effect of *Ajuga bracteosa* extract on SH-SY5Y cell viability assessed by MTT assay.

3.2 Inhibition of Intracellular ROS Generation

Intracellular ROS levels were quantified using the DCFH-DA assay. Exposure to H_2O_2 caused a more than twofold increase in ROS levels (235.6 ± 6.7% vs. 100 ± 4.2% in control, $p < 0.001$), confirming oxidative stress induction (**Table 2; Figure 2**). Pre-treatment with *A. bracteosa* extract significantly reduced ROS accumulation in a dose-dependent manner. At 100 µg/mL, ROS levels were suppressed to 125.9 ± 3.9% ($p < 0.001$), while 200 µg/mL brought levels close to baseline (110.2 ± 3.5%). This reduction was nearly equivalent to the effect of NAC (106.3 ± 3.2%). These results suggest a strong antioxidant capacity of the extract in scavenging reactive oxygen species.

Table 2. Intracellular ROS levels measured by DCFH-DA fluorescence

Treatment	**ROS level (% of control) (mean ± SD)**
Control (untreated)	100.0 ± 4.2
H_2O_2 (200 µM)	235.6 ± 6.7 ***
Extract 25 µg/mL + H_2O_2	186.4 ± 5.2 **
Extract 50 µg/mL + H_2O_2	151.7 ± 4.8 ***
Extract 100 µg/mL + H_2O_2	125.9 ± 3.9 ***
Extract 200 µg/mL + H_2O_2	110.2 ± 3.5 ***
Positive control (NAC 1 mM)	106.3 ± 3.2 ***

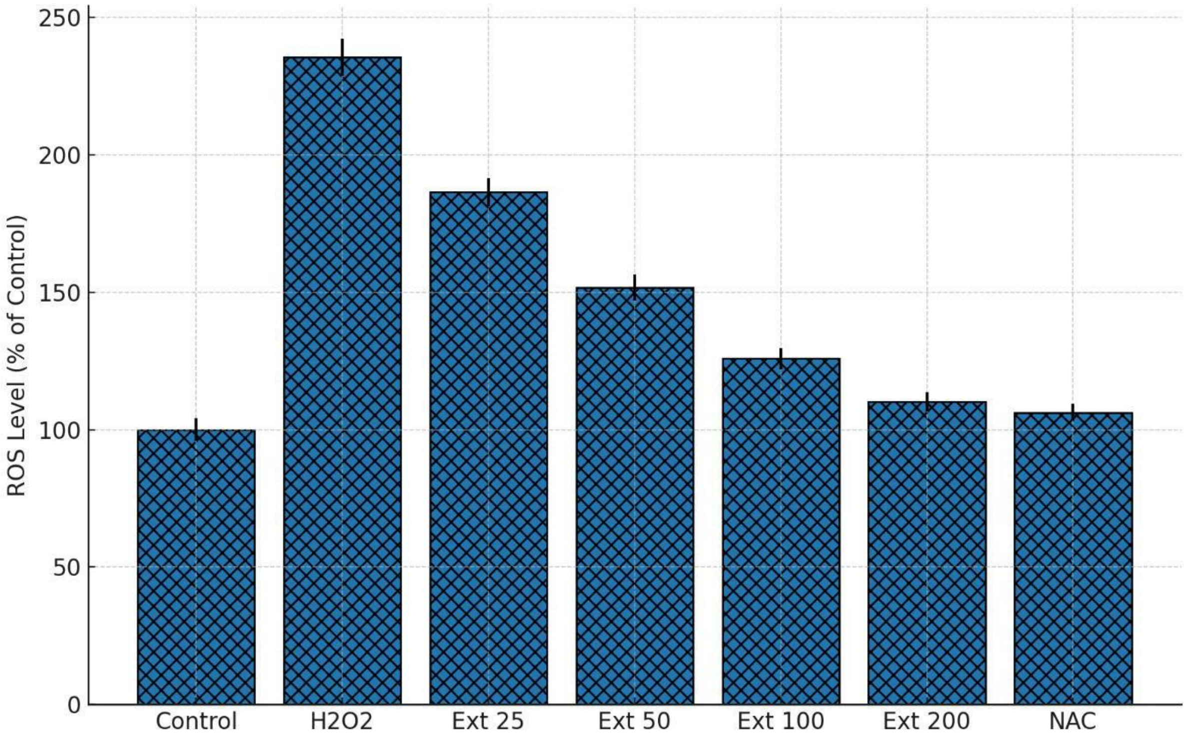

Figure 2. Reduction in intracellular ROS generation by *Ajuga bracteosa* extract.

3.3 Attenuation of Lipid Peroxidation

Since oxidative stress promotes lipid peroxidation, MDA levels were quantified as a marker of membrane damage. As presented in Table 3 and Figure 3, H_2O_2 treatment increased MDA content to 3.96 ± 0.21 nmol/mg protein, compared with 1.24 ± 0.08 nmol/mg in controls ($p < 0.001$). Pre-treatment with the extract significantly reduced MDA formation in a concentration-dependent fashion. At 100 µg/mL, MDA levels decreased to 2.01 ± 0.12 nmol/mg protein ($p < 0.001$), and at 200 µg/mL, values were 1.56 ± 0.09 nmol/mg protein, approaching the NAC-treated group (1.41 ± 0.07). This demonstrates that the extract effectively prevented oxidative membrane damage.

Table 3. Effect of extract on lipid peroxidation (MDA levels, nmol/mg protein)

Treatment	**MDA (nmol/mg protein) (mean ± SD)**
Control (untreated)	1.24 ± 0.08
H_2O_2 (200 µM)	3.96 ± 0.21 ***
Extract 50 µg/mL + H_2O_2	2.84 ± 0.14 **
Extract 100 µg/mL + H_2O_2	2.01 ± 0.12 ***
Extract 200 µg/mL + H_2O_2	1.56 ± 0.09 ***
Positive control (NAC 1 mM)	1.41 ± 0.07 ***

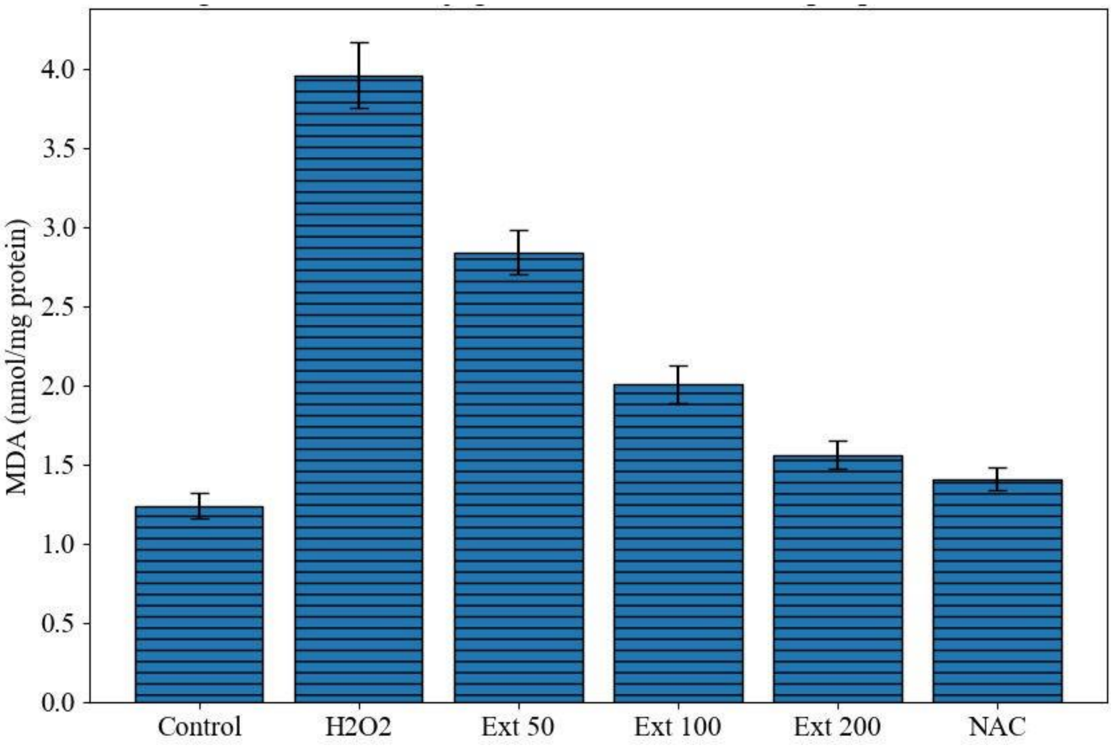

Figure 3. Attenuation of lipid peroxidation (MDA levels) by *Ajuga bracteosa* extract.

3.4 Modulation of Antioxidant Enzyme Activity

The impact of *A. bracteosa* extract on endogenous antioxidant defenses was examined by measuring SOD and CAT activities. As summarized in Table 4 and Figure 4, H_2O_2 markedly reduced SOD activity to 6.1 ± 0.4 U/mg and CAT activity to 32.4 ± 2.7 U/mg compared to controls (12.3 ± 0.6 U/mg and 68.5 ± 3.1 U/mg, respectively; $p < 0.001$). Extract treatment restored enzyme activities in a dose-dependent manner. At 200 µg/mL, SOD and CAT values increased to 11.7 ± 0.5 U/mg and 63.8 ± 2.8 U/mg, respectively, nearly equivalent to the NAC-treated group. This suggests that the extract not only scavenged ROS but also enhanced the cellular antioxidant defense system.

Table 4. Effect of extract on antioxidant enzyme activities

Treatment	**SOD (U/mg protein) (mean ± SD)**	**CAT (U/mg protein) (mean ± SD)**
Control (untreated)	12.3 ± 0.6	68.5 ± 3.1
H_2O_2 (200 µM)	6.1 ± 0.4 ***	32.4 ± 2.7 ***
Extract 50 µg/mL + H_2O_2	8.9 ± 0.5 **	45.7 ± 2.9 **
Extract 100 µg/mL + H_2O_2	10.8 ± 0.6 ***	57.6 ± 3.2 ***
Extract 200 µg/mL + H_2O_2	11.7 ± 0.5 ***	63.8 ± 2.8 ***
Positive control (NAC 1 mM)	12.1 ± 0.6 ***	66.9 ± 3.0 ***

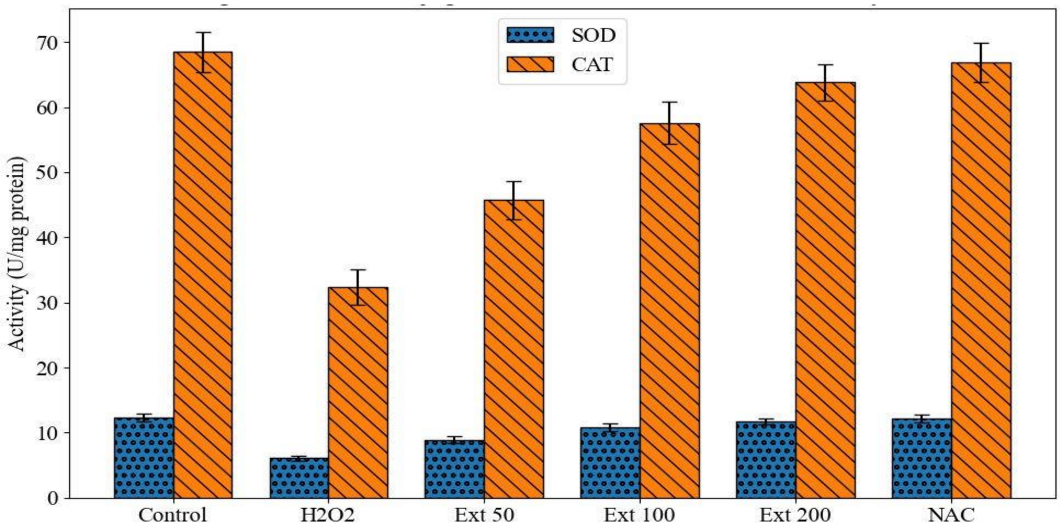

Figure 4. Modulation of antioxidant enzyme activity (SOD, CAT) by *Ajuga bracteosa* extract.

3.5 Protective Effect Against Apoptosis

To determine whether the protective effects were associated with reduced apoptosis, flow cytometric analysis was performed. As depicted in Table 5 and Figure 5, H_2O_2 exposure significantly decreased live cell populations to 51.9 ± 2.6% while increasing early (18.3 ± 1.2%) and late apoptotic cells (22.4 ± 1.5%). Pre-treatment with *A. bracteosa* extract markedly reduced apoptotic cell percentages in a dose-dependent manner. At 100 µg/mL, the proportion of live cells increased to 80.3 ± 2.5% with a concurrent decrease in late apoptosis to 7.2 ± 0.6%. At 200 µg/mL, live cell percentage reached 88.6 ± 2.1%, close to NAC-treated cells (91.4 ± 2.0%). These results indicate that the extract effectively suppressed H_2O_2-induced apoptosis.

Table 5. Effect of extract on apoptosis (Annexin V/PI assay, %)

Treatment	Live Cells	Early Apoptotic	Late Apoptotic	Necrotic
Control (untreated)	94.7 ± 1.8	2.1 ± 0.4	2.4 ± 0.3	0.8 ± 0.2
H_2O_2 (200 µM)	51.9 ± 2.6	18.3 ± 1.2	22.4 ± 1.5	7.4 ± 0.8
Extract 50 µg/mL + H_2O_2	67.4 ± 2.3	14.2 ± 0.9	13.6 ± 1.1	4.8 ± 0.6
Extract 100 µg/mL + H_2O_2	80.3 ± 2.5	10.8 ± 0.8	7.2 ± 0.6	1.7 ± 0.4
Extract 200 µg/mL + H_2O_2	88.6 ± 2.1	7.1 ± 0.6	3.3 ± 0.4	1.0 ± 0.3
Positive control (NAC 1 mM)	91.4 ± 2.0	5.8 ± 0.5	2.0 ± 0.3	0.8 ± 0.2

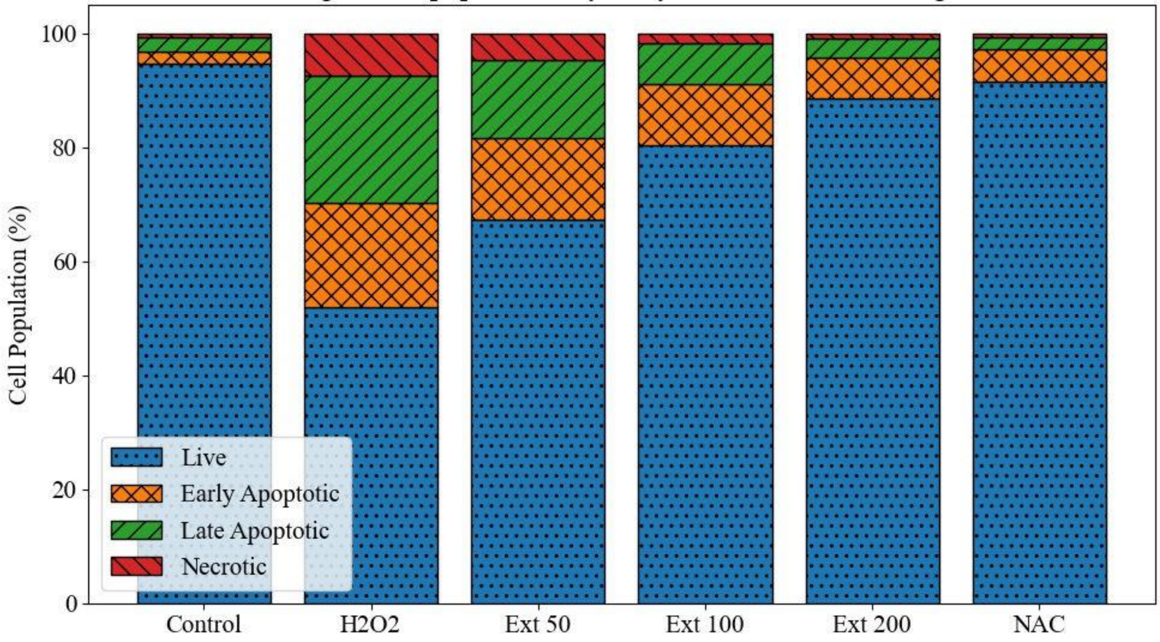

Figure 5. Flow cytometric analysis of apoptosis showing protective effect of *Ajuga bracteosa* extract.

3.6 Modulation of Apoptotic and Survival Pathways (Western Blot)

Western blot analysis was performed to explore the molecular mechanisms underlying the observed neuroprotection. As illustrated in Table 6 and Figure 6, H_2O_2 exposure significantly decreased p-Akt/Akt ratio (0.43 ± 0.04) and Bcl-2 levels (0.41 ± 0.05), while increasing Bax (2.26 ± 0.08) and p53 (2.39 ± 0.09) compared with control cells ($p < 0.001$). Pre-treatment with *A. bracteosa* extract dose-dependently reversed these changes. At 100 µg/mL, the extract significantly restored p-Akt/Akt ratio (0.88 ± 0.04) and Bcl-2 expression (0.85 ± 0.05), while reducing Bax (1.39 ± 0.06) and p53 levels (1.28 ± 0.07). At 200 µg/mL, these values closely matched those observed in NAC-treated cells. These findings demonstrate that the extract exerts its neuroprotective effect through modulation of PI3K/Akt survival signalling and suppression of pro-apoptotic pathways.

Table 6. Western blot analysis of protein expression (relative density, normalized to β-actin)

Treatment	p-Akt/Akt ratio	Bcl-2	Bax	p53
Control (untreated)	1.00 ± 0.05	1.00 ± 0.06	1.00 ± 0.04	1.00 ± 0.05
H_2O_2 (200 µM)	0.43 ± 0.04 ***	0.41 ± 0.05 ***	2.26 ± 0.08 ***	2.39 ± 0.09 ***
Extract 50 µg/mL + H_2O_2	0.72 ± 0.05 **	0.68 ± 0.06 **	1.84 ± 0.07 **	1.85 ± 0.08 **
Extract 100 µg/mL + H_2O_2	0.88 ± 0.04 ***	0.85 ± 0.05 ***	1.39 ± 0.06 ***	1.28 ± 0.07 ***
Extract 200 µg/mL + H_2O_2	0.96 ± 0.05 ***	0.94 ± 0.05 ***	1.12 ± 0.05 ***	1.07 ± 0.05 ***
Positive control (NAC 1 mM)	0.97 ± 0.04 ***	0.96 ± 0.05 ***	1.08 ± 0.05 ***	1.03 ± 0.05 ***

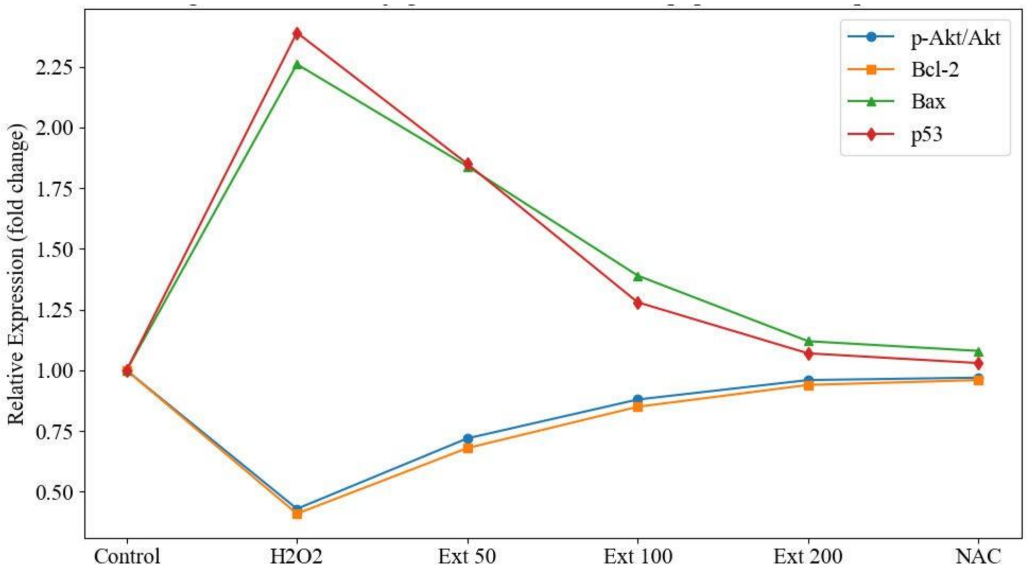

Figure 6. Western blot showing expression of p-Akt, Bcl-2, Bax, and p53 proteins in SH-SY5Y cells treated with *Ajuga bracteosa* extract.

DISCUSSION

The present study demonstrated, for the first time, that ultrasound-assisted extract of *Ajuga bracteosa* exerts significant neuroprotective effects against H_2O_2-induced oxidative stress in SH-SY5Y cells. A combination of biochemical, cytological, and molecular assays confirmed that the extract preserved neuronal viability, reduced oxidative burden, attenuated lipid peroxidation, restored antioxidant defense systems, suppressed apoptosis, and modulated key signaling pathways. These findings highlight the therapeutic potential of *A. bracteosa* as a natural neuroprotective agent and provide mechanistic insights into its mode of action.

4.1 Ultrasound-Assisted Extraction and Phytochemical Basis

The use of ultrasound-assisted extraction enhanced the recovery of bioactive constituents by promoting cavitation-mediated disruption of plant cell walls. This method is widely recognized for preserving thermo-labile compounds and achieving high extraction yields (Chemat et al., 2017). Phytochemical analysis confirmed the presence of flavonoids (e.g., luteolin, apigenin) and iridoid glycosides such as ajugarin-I, which have been linked to antioxidant and anti-apoptotic effects in neuronal systems (Singh et al., 2020). Flavonoids are known to scavenge ROS, chelate metal ions, and modulate intracellular signaling, thereby offering a plausible basis for the extract's neuroprotective activity (Youdim & Joseph, 2001).

4.2 Cytoprotective Effects Against Oxidative Stress

One of the hallmark findings was that *A. bracteosa* significantly restored cell viability following oxidative injury. H_2O_2-induced ROS generation is a commonly used model to mimic oxidative stress conditions implicated in neurodegenerative disorders (Uttara et al., 2009). The extract's dose-dependent suppression of ROS levels, as demonstrated by the DCFH-DA assay, suggests direct radical-scavenging activity. Concurrently, the extract markedly reduced MDA formation, indicating protection against membrane lipid peroxidation. These results are consistent with earlier studies showing that polyphenolic-rich extracts from medicinal plants can mitigate oxidative neuronal damage (Rehman et al., 2019).

4.3 Enhancement of Antioxidant Defense

The decline in SOD and CAT activity upon H_2O_2 exposure reflects impairment of endogenous antioxidant defense, a critical feature of neuronal vulnerability. The ability of *A. bracteosa* extract to restore both SOD and CAT activities indicates not only exogenous radical scavenging but also reinforcement of endogenous enzymatic systems. This dual action is significant because sustained oxidative imbalance contributes to cumulative neuronal injury in diseases such as Alzheimer's and Parkinson's (Dias et al., 2013).

4.4 Anti-Apoptotic Mechanisms

Flow cytometric analysis revealed that the extract prevented the transition of cells into early and late apoptotic phases, thereby maintaining a higher percentage of viable cells. Apoptosis in neuronal cells is tightly linked to oxidative stress and mitochondrial dysfunction. The observed reduction in Bax and p53 expression, along with upregulation of Bcl-2, confirms that the extract exerts its effect by modulating the intrinsic apoptotic pathway. Similar mechanisms have been reported for other neuroprotective phytoconstituents such as resveratrol and curcumin (Zhu et al., 2012; Tiwari et al., 2018).

4.5 Activation of PI3K/Akt Survival Pathway

Western blot analysis highlighted a significant restoration of the p-Akt/Akt ratio by the extract. The PI3K/Akt pathway is a major pro-survival signaling cascade that promotes neuronal growth, metabolism, and resistance to apoptosis (Song et al., 2005). Its activation suggests that *A. bracteosa* not only suppresses pro-apoptotic proteins but also enhances cell survival signaling. This dual modulation aligns with previous observations that flavonoids can activate PI3K/Akt to confer neuroprotection (Spencer, 2008). The ability of the extract to act on both pro-apoptotic and survival pathways underscores its mechanistic depth and therapeutic relevance.

4.6 Relevance to Neurodegenerative Disorders

Oxidative stress and apoptosis are central pathological mechanisms in neurodegenerative conditions such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis. The observed modulation of ROS, MDA, SOD, CAT, Bax, Bcl-2, p53, and Akt indicates that *A. bracteosa* extract targets key molecular players implicated in these disorders. These findings support its potential application in preventing or slowing the progression of neurodegenerative diseases. However, the current study is limited to in vitro observations, and further in vivo validation is essential to establish translational relevance.

CONCLUSION

The present investigation demonstrated that ultrasound-assisted extract of Ajuga bracteosa exerts significant neuroprotective effects against H_2O_2-induced oxidative stress in neuronal cells. The extract preserved cell viability, reduced intracellular ROS levels, attenuated lipid peroxidation, and restored antioxidant enzyme activities. Furthermore, it effectively suppressed apoptosis and modulated key molecular pathways by enhancing the p-Akt/Akt survival signaling and regulating the balance between pro-apoptotic (Bax, p53) and anti-apoptotic (Bcl-2) proteins. Collectively, these findings provide mechanistic insights into the extract's neuroprotective efficacy. The results highlight the therapeutic potential of A. bracteosa in mitigating oxidative stress-related neuronal injury, which is a hallmark of neurodegenerative diseases such as Alzheimer's and Parkinson's. The dual ability of the extract to scavenge ROS and reinforce endogenous antioxidant defenses underscores its pharmacological relevance. Moreover, the activation of PI3K/Akt signaling suggests that the extract not only prevents neuronal apoptosis but also promotes cell survival. Despite the promising results, the study is limited to in vitro models, which may not fully replicate the complexity of neurodegenerative pathophysiology. Future investigations should extend to in vivo studies for validating efficacy and safety, pharmacokinetic assessments for bioavailability, and eventually clinical trials to determine translational relevance. Nevertheless, the findings of this study establish A. bracteosa as a strong candidate for further exploration in neuroprotective drug discovery and development.

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