

NEUROPROTECTIVE EFFECT OF ANDROGRAPHOLIDE IN COMBINATION WITH PIPERINE AGAINST STREPTOZOTOCIN-INDUCED ALZHEIMER'S DISEASE

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ABSTRACT

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by cognitive decline, amyloid-beta accumulation, oxidative stress, and neuroinflammation. This study evaluates the synergistic neuroprotective effects of andrographolide, a diterpenoid having antioxidant and anti-inflammatory properties, with piperine, a bioavailability enhancer, in streptozotocin-induced, AD-like pathology in Wistar rats by an in-vivo study. Rats received intracerebroventricular streptozotocin (3mg/kg) followed by oral treatment with andrographolide (15, 30, 60 mg/kg) and its combination with piperine (2.5 mg/kg). Behavioral (actophotometer, morris water maze, elevated plus maze), biochemical (catalase, nitrite, MDA, GSH), and histological analyzes revealed that the combination significantly improved cognition, reduced oxidative stress and neuroinflammation (inflammatory cytokines), and restored neurotransmitter balance. The findings suggest that andrographolide-piperine holds promise as a therapeutic strategy for Alzheimer's, warranting further clinical investigation.

KEYWORDS: Alzheimer's disease, andrographolide, streptozotocin, piperine, neuro-inflammation

INTRODUCTION

Alzheimer's disease (AD) is defined as a progressive neurodegenerative disorder marked by memory impairment, cognitive decline, and neuronal loss, leading to severe dysfunction in daily life [1]. Globally, it accounts for 60–80% of dementia cases in elderly individuals, affecting an estimated 35 million people [2]. The complex pathophysiology of AD involves oxidative stress, neuroinflammation, mitochondrial dysfunction, and accumulated amyloid- beta plaques, which ultimately contribute to neuronal degeneration [3]. Amyloid-beta (A β) have an essential role in Alzheimer's disease by promoting plaque formation, synaptic dysfunction, and neuronal death. Additionally, excessive oxidative stress and chronic neuroinflammation triggered by A β aggregation further accelerate mitochondrial dysfunction and cognitive decline. Despite extensive research, current therapeutic strategies remain largely symptomatic; underscoring the urgent need for novel neuroprotective agents with disease- modifying potential. Natural compounds have garnered significant attention for their therapeutic potential in neurodegenerative diseases due to their anti-inflammatory, neuroprotective, and antioxidant properties [4]. Among these, andrographolide, a bioactive diterpenoid from *Andrographis paniculata* (Burm. F.) wall. ex-Nees and family is Acanthaceae, which is mainly obtained from the aerial parts of the plant. It has been reported to exert potent anti-inflammatory, antioxidant, and cognitive-enhancing effects [5]. Additionally, piperine, an alkaloid derived from the fruits of *Piper nigrum*, family Piperaceae, is known to enhance the bioavailability and pharmacological efficacy of various compounds while exhibiting neuroprotective properties of its own [6]. Additionally, both andrographolide and piperine have been widely reported to exhibit multiple pharmacological activities, including antidiabetic, immunomodulatory, and neuroprotective effects. The study investigates the neuroprotective effect of andrographolide in combination with piperine in an intracerebroventricular (i.c.v.)-streptozotocin -induced rat model of AD. Streptozotocin (STZ), a glucosamine-nitrosourea compound, impairs insulin receptor function in the brain, disrupts glucose metabolism, and causes cognitive decline along with AD-like symptoms [7]. The i.c.v.-streptozotocin model replicates key AD pathological features, including amyloid-beta accumulation, oxidative stress, and cognitive deficits

1. MATERIALS AND METHODS

1.1 Experimental animals

In this study, adult male Wistar rats, each weighing between 250 and 280 g, were obtained from the Central Animal House of the ISF College of Pharmacy, Moga (Punjab) The rats were housed in polyacrylic cages under standard laboratory conditions, with a temperature maintained at $22 \pm 2^\circ\text{C}$, relative humidity levels between 55% and 60%, and a 12-hour light/dark cycle. They were provided with a commercial pellet diet and had free access to water. Behavioral evaluations were performed during two specific periods: from 9:00 to 11:00 a.m. and from 2:00 to 5:00 p.m., to maintain consistency in testing. All experimental protocols received approval from the Institutional Animal Ethical Committee (IAEC) under the designated protocol numbers ISFCP/IAEC/CPCSEA/Meeting No. 29 /2025/ Protocol No.431 at 11:00 AM, and animals were kept in the animal house for the next 6 months from the approval date. The study adhered to the guidelines established by the Committee for the Control and Supervision of Experiments on Animals (CCSEA), Government of India.

1.2 Drugs and chemicals

The test compound, andrographolide, was sourced from Sigma Aldrich (St. Louis, MO, USA), with 98% purity and CAS number 5508-58-7 and piperine also from Sigma Aldrich with 97% purity and CAS number 94-62-2, while ELISA kits for interleukin-1 β (IL-1 β), interleukin-6 (IL-6), and tumor necrosis factor-alpha were acquired from Krishgen Biosystems, India, and Bio. Sys (Whittier, CA, USA). Piperine and streptozotocin were also obtained from Sigma-Aldrich. Throughout the study, high-purity analytical-grade reagents were employed. To maintain stability and effectiveness, all drug solutions and reagents were freshly prepared before use.

2.3 Surgical procedure for intracerebroventricular administration of streptozotocin For accurate intracerebroventricular delivery, rats were anesthetized with ketamine hydrochloride (70 mg/kg, intraperitoneal). The anesthetized rats were then placed securely on a stereotactic frame, and a midline sagittal incision was made on the skull. Two bilateral holes were drilled into the scalp, targeting the lateral ventricles with the following stereotactic coordinates: 0.8 mm posterior to Bregma, 1.5 mm lateral to the sagittal suture, and 3.6 mm dorsal depth into the brain. A freshly prepared streptozotocin solution (3 mg/kg body weight), dissolved in sterile saline, was injected directly into both brain hemispheres (20 μ l) through these openings. To aid recovery after surgery, the animals were given sweetened milk (1.5 ml, orally) via an intragastric tube for four consecutive days until they resumed normal feeding [8].

1.3 Experimental procedures

The animals were randomly assigned to eight groups (n = 6 per group). All rats underwent behavioral training for seven consecutive days using the actophotometer, morris water maze (MWM), and elevated plus maze (EPM) apparatus to familiarize them with the experimental setup. After the training period, the disease model group received two i.c.v. injections of streptozotocin (3 mg/kg body weight) on Day 1 and Day 3 to induce cognitive dysfunction. The treatment groups were given andrographolide (15, 30, and 60 mg/kg, orally), the combination group received andrographolide (15 mg/kg, orally) + (2.5 mg/kg, orally), and the standard group was administered Donepezil (5 mg/kg, orally) daily for the next 21 days. Behavioural assessments (actophotometer, EPM tests) were conducted on day 21, and MWM was conducted from days 17 to 21 to evaluate cognitive and motor functions. On Day 21, following the completion of behavioural assessments, the animals were humanely sacrificed, and the brains were carefully isolated for further analysis. The study focused on estimating biochemical markers: malondialdehyde, nitrite, glutathione, superoxide dismutase, catalase, and acetylcholinesterase, neuroinflammatory mediators: interleukin 1 β , interleukin 6 and tumour necrosis factor-alpha, and neurochemical parameters: GABA, glutamate, and histological assessment.

Experimental schedule of the study.

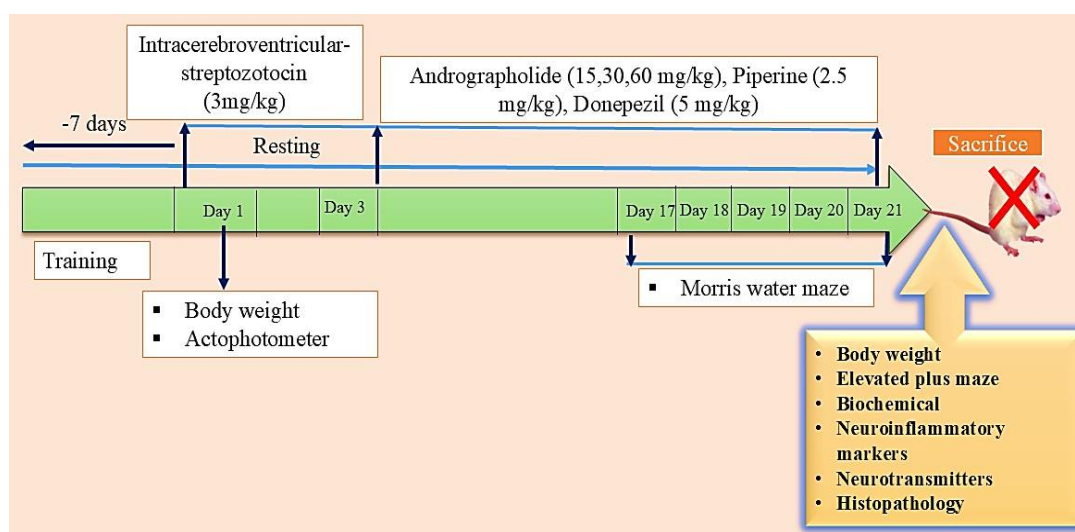


Figure: 1

The experimental design of the study involved taking an adult animal and training it for 7 days. After 7 days of training, i.c.v.-streptozotocin was given at 3 mg/kg. From days 1 to 3, rats were given rest. The treatment started according to group, and all parameters were observed. After day 21, animals were sacrificed, and all behaviour and histological parameters were assessed.

Table Num: 1 Experimental Group

S. No.	Experimental Groups	Animal Species	No. of Animals
1	Normal Control	Adult Wistar Rats	6
2	Piperine per se (2.5 mg/kg/p.o.)	Adult Wistar Rats	6
3	ICV-STZ (3 mg/kg/i.c.v.)	Adult Wistar Rats	8
4	STZ + ANDRO (15 mg/kg/p.o.)	Adult Wistar Rats	8
5	STZ + ANDRO (30 mg/kg/p.o.)	Adult Wistar Rats	8

6	STZ + ANDRO (60 mg/kg/p.o.)	Adult Wistar Rats	8
7	STZ + ANDRO (15 mg/kg/p.o.) + Piperine (2.5 mg/kg/p.o.)	Adult Wistar Rats	8
8	STZ + Donepezil (5 mg/kg/p.o.)	Adult Wistar Rats	8
Total			60

Assessment of body weight

Body weight was measured and recorded at the beginning (Day 1) and end (Day 21) of the experimental schedule to monitor any changes associated with disease progression and treatment effects.

Behavioural assessment

Actophotometer assessment

Locomotor activity was assessed on Day 1 and Day 21 to evaluate spontaneous movement in each animal. Ambulatory activity was recorded using a computerized actophotometer (INCO, India). Before testing, animals were individually placed in the actophotometer chamber for a brief acclimatisation period. Locomotor activity was then measured for 5 minutes using an infrared-sensitive photocell system, and the results were expressed as movement counts per minute [9].

Elevated plus maze assessment

The EPM was used to evaluate learning and memory in rodents on Day 21. The apparatus comprised two open arms (16 × 5 cm) and two closed arms (16 × 5 × 40 cm) extending from a central platform (10 × 10 cm), elevated 25 cm above the floor. During the test, each animal was initially placed at the end of an open arm, facing the central platform. The transfer latency (TL) {Initial Transfer Latency (ITL) and Retention Transfer Latency (RTL)} was recorded as the time taken for the animal to move entirely into a closed arm with all four legs. If the rat failed to enter the closed arm within 90 seconds, it was gently guided, and a maximum TL of 90 seconds was assigned. After reaching the closed arm, the animal was allowed to explore the maze freely for an additional 2 minutes before being returned to its home cage. This test served as a measure of spatial learning and memory, with reduced transfer latency indicating improved cognitive function [10].

Morris water maze assessment

The Morris water maze assessment was used to evaluate spatial learning and memory in rodents. The apparatus consisted of a circular tank (60 cm diameter, 25 cm height) filled with water (20 cm depth) made opaque using a non-toxic white dye. The water temperature was maintained at 25°C. The tank was divided into four quadrants using two threads intersecting at a 90° angle, and a hidden platform (6 × 6 cm, white-colored) was placed 1 cm below the water surface in the target quadrant. The platform location remained unchanged throughout the training period. Each rat underwent four consecutive training trials per day for four days (Days 17–20), with a 5-minute inter-trial interval. During training, animals were gently placed in different quadrants, facing the pool centre. Each rat had 120 seconds to locate the hidden platform. If unsuccessful, it was guided to the platform and allowed to remain there for 30 seconds before the subsequent trial. Escape latency (time taken to find the hidden platform) was recorded. On Day 21, a probe trial was conducted by removing the platform. The animals were allowed to swim freely for the same duration as in training, and the time spent in the target quadrant was recorded. This parameter reflected memory retention and the effectiveness of learning during the training phase [11].

Biochemical analysis

Dissection and homogenization for biochemical estimation On day 21, rats were sacrificed by cervical dislocation, and their brains were carefully removed and preserved at –80°C in an ultra-low temperature freezer. On the day of the experiment, the brains were thawed, and the cortex and hippocampus were isolated, weighed, and homogenized in 0.1 M phosphate buffer (pH 7.4) containing: 1 mM ethylene diamine-tetra-acetic acid (EDTA), 0.25 M Sucrose, 10 mM potassium chloride (KCl), and 1 mM phenyl methyl sulfonyl fluoride (PMSF). The homogenized brain tissue was then centrifuged at 10,000 g for 15 minutes, and the supernatant was collected for further analysis. Various biochemical and neurochemical estimations were performed, including: acetylcholinesterase (AChE) activity, amyloid beta (1-42) levels, oxidative stress markers, neuroinflammatory mediators, and neurotransmitter assessments. This comprehensive analysis helped evaluate the neuropathological and neurochemical alterations associated with the experimental conditions.

Protein estimation

Protein concentration in rat brain samples was determined using the Lowry method, with bovine serum albumin (BSA) (1 mg/ml) as the standard. This method provided a reliable quantification of protein levels, essential for biochemical and neurochemical assessments.

Estimation of malondialdehyde

Lipid peroxidation in cortical and hippocampal tissue homogenates was quantified by Measuring malondialdehyde (MDA), a key end-product of oxidative stress. The malondialdehyde levels were determined through their reaction with thiobarbituric acid (TBA), and the absorbance was recorded at 532 nm using an ELISA reader (Model 680 Microplate Reader, Bio-Rad, Japan). The malondialdehyde concentration was calculated from a standard curve and expressed as nmol/mg protein [12, 13].

Estimation of nitrite

The production of nitric oxide (NO) was assessed by measuring nitrite accumulation in the supernatant using a colorimetric Griess reagent assay. The Griess reagent contained N-(1-naphthyl) ethylenediamine dihydrochloride (0.1%), sulfanilamide (1%), and phosphoric acid (2.5%). Equal volumes of the sample supernatant and Griess reagent were mixed and incubated for 10 minutes at room temperature in the dark, and the absorbance was recorded at 540 nm using a Perkin Elmer Lambda 20 spectrophotometer. The nitrite concentration was determined from a sodium nitrite standard curve and expressed as $\mu\text{mol/mg protein}$ [14].

Estimation of catalase

Catalase activity was determined following the methods of Aebi et al. (1984). The assay mixture contained 0.2 ml of tissue homogenate, 1.2 ml of phosphate buffer (0.05 M, pH 7.0), and 1.0 ml of hydrogen peroxide (H_2O_2 , 0.03 M) to initiate the reaction. The decrease in absorbance was recorded at 240 nm for 3 minutes using a spectrophotometer. An enzyme blank was prepared by replacing hydrogen peroxide with distilled water. The catalase activity was quantified as micromoles of H_2O_2 decomposed per minute per milligram of protein and expressed in $\mu\text{mol/mg protein}$ [9, 15].

Estimation of reduced superoxide dismutase

The SOD activity was measured using the method described by Del-Maestro et al. (1987). This assay is based on the ability of superoxide dismutase to scavenge superoxide anion radicals (O_2^-), thereby reducing reaction chains and decreasing the overall rate of pyrogallol autoxidation. The superoxide dismutase activity was quantified and expressed as $\mu\text{mol/mg protein}$ [16].

Estimation of reduced glutathione

The reduced glutathione (GSH) levels in the cortical and hippocampal homogenate were estimated following Ellman's method (1959). First, 1 ml of supernatant was mixed with 1 ml of 4% sulfosalicylic acid and incubated at 4°C for 1 hour for precipitation. The mixture was then centrifuged at $1200\times g$ for 15 minutes, and the supernatant was collected. To 1 ml of the supernatant, 2.7 ml of 0.1 M phosphate buffer (pH 8) and 0.2 ml of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) were added. The formation of a yellow colour was immediately measured at 412 nm using an ELISA reader (Model 680 Microplate Reader, Bio-Rad, Japan). The concentration of glutathione was determined using a standard curve and expressed as $\mu\text{mol/mg protein}$ [17].

Acetylcholinesterase assay

Acetylcholinesterase activity in rat brain tissue was measured using Ellman's method (1961). The assay mixture comprised 0.05 ml of supernatant, 3 ml of 0.1 M sodium phosphate buffer (pH 8.0), 0.10 ml of acetylthiocholine iodide, and 0.10 ml of DTNB (Ellman reagent). The change in absorbance was recorded immediately at 412 nm using an ELISA reader (Model 680 Microplate Reader, Bio-Rad, Japan). The enzymatic activity was expressed as $\text{nmol/min/mg protein}$ [18].

Estimation of amyloid beta (1-42) level

The levels of amyloid beta (1-42) were quantified using a rat amyloid beta (1-42) ELISA kit following the solid-phase sandwich ELISA principle. The assay was performed using a microtiter plate reader at 450 nm, and the amyloid beta (1-42) concentrations were determined from a standard curve [9].

Assessment of neuroinflammatory cytokine levels

The levels of interleukin-6 (IL-6), tumour necrosis factor- α (TNF- α), and interleukin-1 beta (IL-1 β) were quantified using respective rat immunoassay kits (KRISHGEN BioSystem, USA). The assays followed the solid-phase sandwich ELISA principle, and absorbance was recorded at 450 nm using a microtiter plate reader. The concentrations of interleukin-6, tumour necrosis factor- α , and interleukin-1 beta were determined from the plotted standard curve and expressed as pg/mg protein [19].

Neurotransmitter analysis

GABA and glutamate estimation by HPLC-ECD Neurotransmitter levels, including GABA and glutamate, were quantified using a Waters HPLC system equipped with an electrochemical detector (ECD). The system comprised a high-pressure isocratic pump, a 20 μl manual injector, and a C18 reverse-phase column. The mobile phase consisted of 100 mM disodium hydrogen phosphate (anhydrous), 25 mM EDTA, and 22% methanol, adjusted to pH 6.5. The electrochemical detector was set at +0.65 V with a sensitivity range of 5 to 50 nA. Separation was performed at a flow rate of 1.2 ml/min, while the column was maintained at 40°C . On the day of the experiment, frozen brain samples were thawed and homogenized in 0.2 M perchloric acid, then centrifuged at $12,000 g$ for 15 minutes. The supernatant was derivatized using OPA/ β -mercaptoethanol and filtered through a 0.22 μm nylon filter before being manually injected (20 μl) into the HPLC system via a Rheodyne valve injector. Data were recorded and analyzed with Breeze software, and neurotransmitter concentrations were determined from a standard curve prepared with standards ranging from 10 to 100 ng/ml, with results expressed relative to the normal control group [20].

Pre-column derivatization procedure

Proteins were quantified using the OPA/ β -ME derivatization method described by Donzanti and Yamamoto (1988) [20]. First, a 1 mg/ml amino acid stock solution was prepared. The OPA/ β -ME stock solution was then made by dissolving 27 mg of OPA in 1 ml of methanol, followed by the addition of 5 μl of β -mercaptoethanol and 9 ml of 0.1 M sodium

tetraborate buffer (pH 10.3). This stock solution remains stable for up to 5 days when protected from light. For immediate use, a working derivatising reagent was freshly prepared by mixing 2.5 ml of the OPA/ β -ME stock with 7.5 ml of tetraborate buffer. Finally, sample derivatization was carried out by combining the sample with the working reagent in a 1:1.5 ratio (sample: reagent).

Statistical analysis

Statistical analyses were performed by using GraphPad Prism version 9.0 (San Diego, CA, USA). Data are expressed as mean $\pm$ SD. Behavioural data were analysed using two-way ANOVA, followed by Bonferroni's post hoc test for multiple comparisons, with a p-value < 0.05 considered statistically significant. For biochemical and neuroinflammatory parameters, one-way ANOVA followed by Tukey's post hoc test was used, with $p < 0.05$ set as the threshold for statistical significance.

RESULTS

3.1. Effect of andrographolide and piperine treatment on body weight in i.c.v.- streptozotocin-treated rats

Normal control and piperine-treated animals do not significantly change body weight throughout the study on day 1 and day 21. In contrast, i.c.v.-streptozotocin-treated animals exhibited a significant reduction in body weight by day 21 compared to the normal controls. Treatment with andrographolide (15, 30, and 60 mg/kg/day, orally) effectively restored body weight in these animals. Notably, the combination of piperine (2.5 mg/kg/day, orally) with a lower dose of andrographolide (15 mg/kg/day, orally) resulted in a more pronounced improvement in body weight than treatment with andrographolide (60 mg/kg, orally) alone at a higher dose. However, on day 21, donepezil oral administration of 5 mg/kg significantly improved body weight as compared to the combination group.

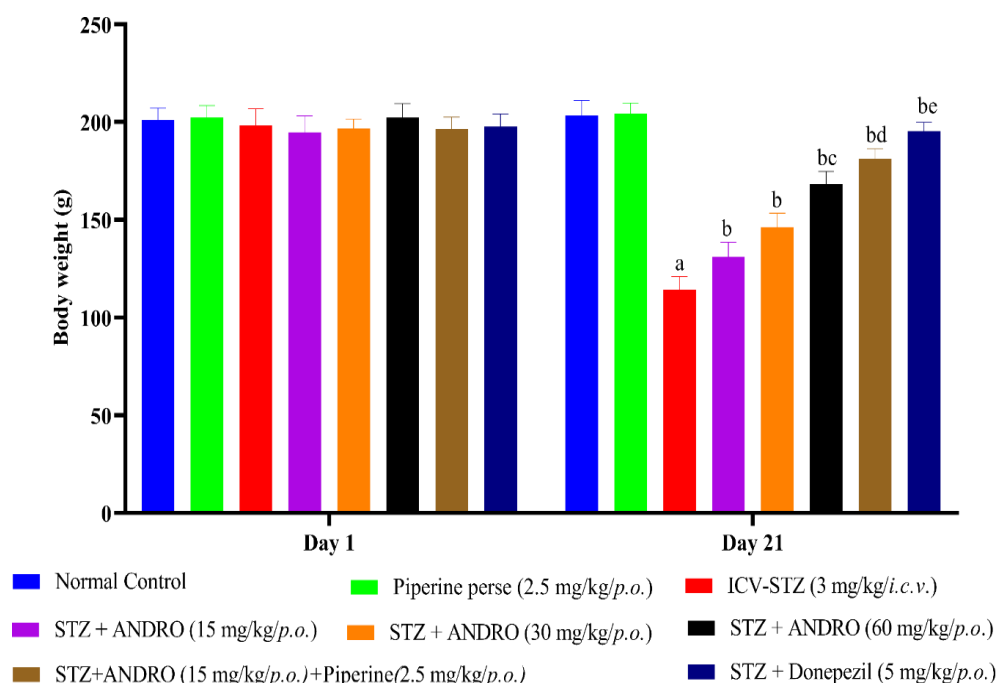


Figure 2: Effect of andrographolide and piperine treatment on body weight in i.c.v.-streptozotocin-treated rats.

Data is expressed as Mean $\pm$ S.D. with $p < 0.05$, which was considered statistically significant. $a p < 0.001$ vs. Normal control, $b p < 0.01$ vs. ICV-STZ, $c p < 0.05$ vs. ICV-STZ+ANDRO (15 and 30 mg/kg), $d p < 0.05$ vs. ICV-STZ+ANDRO (60mg/kg), $e p < 0.05$ vs. ICV-STZ+ANDRO (15 mg/kg) + Piperine (2.5 mg/kg). Data was analyzed using two-way ANOVA followed by Bonferroni's multiple comparisons test. (ICV-STZ: i.c.v.-streptozotocin, ANDRO: andrographolide, PIP: piperine).

3.2. Effect of andrographolide and piperine treatment on the actophotometer in i.c.v.- streptozotocin-treated rats

Normal control and piperine-treated animals did not significantly change in the number of beams crossed on the Actophotometer throughout the study on day 1 and day 21. In contrast, i.c.v.-streptozotocin-treated animals exhibited a significantly reduced number of beams crossed on Day 21 compared to the normal controls. Treatment with andrographolide (15, 30, and 60 mg/kg/day, orally) effectively restored the number of beams crossed in these animals. Notably, the combination of Piperine (2.5 mg/kg/day, orally) with a lower dose of andrographolide (15 mg/kg/day, orally) resulted in a more pronounced improvement in the number of beams crossed than treatment with andrographolide (60 mg/kg, orally) alone at a higher dose. However, on day 21, donepezil oral administration of 5 mg/kg significantly improved the number of beams crossed as compared to the combination group.

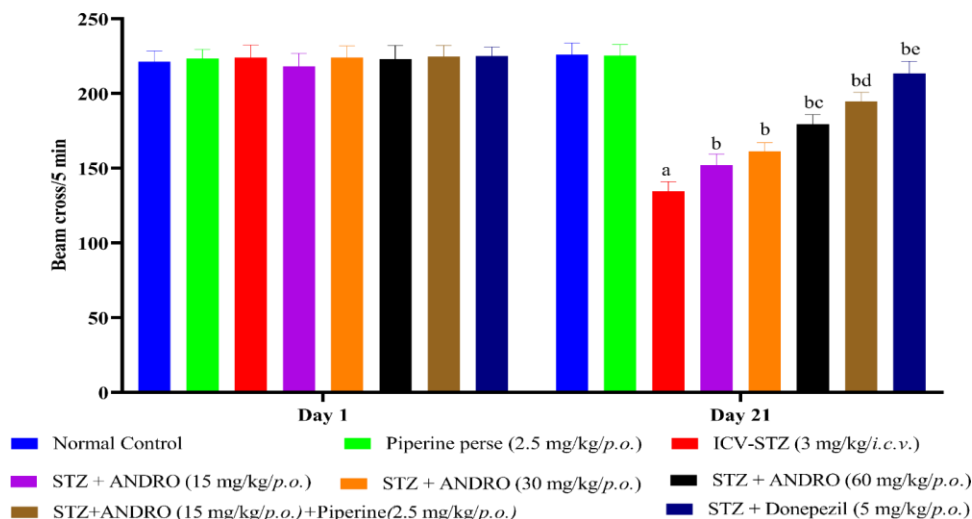


Figure 3: Effect of andrographolide and piperine treatment on the actophotometer in i.c.v.-streptozotocin-treated rats.

Data is expressed as Mean $\pm$ S.D. with $p < 0.05$, which was considered statistically significant. $ap < 0.001$ vs. Normal control, $bp < 0.01$ vs. ICV-STZ, $cp < 0.05$ vs. ICV-STZ+ANDRO (15 and 30 mg/kg), $dp < 0.05$ vs. ICV-STZ+ANDRO (60mg/kg), $ep < 0.05$ vs. ICV-STZ+ANDRO (15 mg/kg) + Piperine (2.5 mg/kg). Data was analyzed using two-way ANOVA followed by Bonferroni's multiple comparisons test. (ICV-STZ: i.c.v.-streptozotocin, ANDRO: andrographolide, PIP: piperine).

3.3. Effect of andrographolide and piperine treatment on transfer latency on elevated plus maze in i.c.v.-streptozotocin-treated rats

Normal control and piperine-treated animals did not significant change in the transfer latency on the EPM throughout the study on day 21. However, in i.c.v.-streptozotocin-induced AD rats had significantly higher transfer latency time in sec [initial acquisition latency (IAL) and retention transfer latency (RTL)] as compared to the normal control. Oral andrographolide treatment at doses (15, 30, and 60 mg/kg, orally) dose-dependently decreased latency time as compared to the i.c.v.-streptozotocin group on day 21. However, on day 21, donepezil oral administration of 5 mg/kg significantly improved transfer latency as compared to the combination group. Moreover, the combination of Piperine (2.5 mg/kg/day, orally) with a lower dose of andrographolide (15 mg/kg/day, orally) decreased transfer latency time than treatment with andrographolide (60 mg/kg, orally) alone at a higher dose.

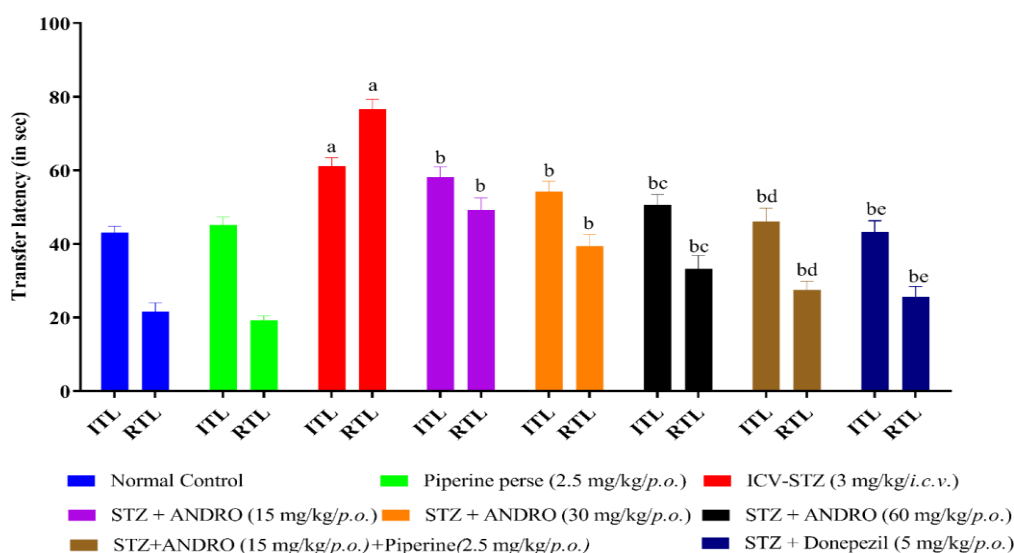


Figure 4: Effect of andrographolide and piperine treatment on Transfer latency on EPM in i.c.v.-streptozotocin-treated rats.

Data is expressed as Mean $\pm$ S.D. with $p < 0.05$, which was considered statistically significant. $ap < 0.001$ vs. Normal control, $bp < 0.01$ vs. ICV-STZ, $cp < 0.05$ vs. ICV-STZ+ANDRO (15 and 30 mg/kg), $dp < 0.05$ vs. ICV-STZ+ANDRO (60mg/kg), $ep < 0.05$ vs. ICV-STZ+ANDRO (15 mg/kg) + Piperine (2.5 mg/kg). Data was analyzed using two-way ANOVA followed by Bonferroni's multiple comparisons test. (ICV-STZ: i.c.v.-streptozotocin, ANDRO: andrographolide, PIP: piperine).

3.4. Effect of andrographolide and piperine treatment on escape latency in morris water maze in i.c.v.-streptozotocin-treated rats

Normal control and piperine-treated animals did not significantly change in the escape latency on the morris water maze throughout the study on day 20. However, in i.c.v.-streptozotocin- induced AD rats had significantly higher escape latency time in seconds as compared to the normal control. Oral andrographolide treatment at doses (15, 30, and 60 mg/kg/orally) dose- dependently decreased escape latency time as compared to the i.c.v.-streptozotocin group on day 20. However, on day 20, donepezil oral administration of 5 mg/kg significantly improved escape latency time as compared to the combination group. Moreover, the combination of piperine (2.5 mg/kg/day, orally) with a lower dose of andrographolide (15 mg/kg/day, orally) decreased escape latency time than treatment with andrographolide (60 mg/kg, orally) alone higher dose.

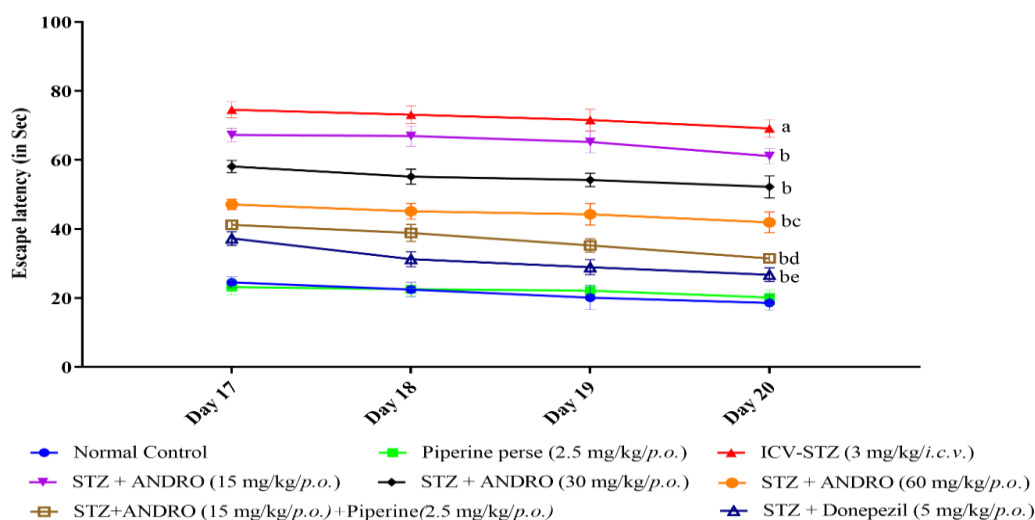


Figure 5: Effect of andrographolide and piperine treatment on escape latency on MWM in i.c.v.-streptozotocin-treated rats.

Data is expressed as Mean $\pm$ S.D. with $p < 0.05$, which was considered statistically significant. $ap < 0.001$ vs. Normal control, $bp < 0.01$ vs. ICV-STZ, $cp < 0.05$ vs. ICV-STZ+ANDRO (15 and 30 mg/kg), $dp < 0.05$ vs. ICV-STZ+ANDRO (60mg/kg), $ep < 0.05$ vs. ICV-STZ+ANDRO (15 mg/kg) + Piperine (2.5 mg/kg). Data was analyzed using two-way ANOVA followed by Bonferroni's multiple comparisons test. (ICV-STZ: i.c.v.-streptozotocin, ANDRO: andrographolide, PIP: piperine).

3.5. Effect of andrographolide and piperine treatment on the percentage of time spent in the target quadrant on morris water maze in i.c.v.-streptozotocin-treated rats

Normal control and piperine-treated animals did not significantly change in the time spent in the target quadrant on the morris water maze throughout the study on day 21. However, mg/kg significantly improved the time spent in the target quadrant as compared to the combination group. Moreover, the combination of Piperine (2.5 mg/kg/day, orally) with a lower i.c.v.- streptozotocin-induced AD rats significantly decreased the time spent in the target quadrant as compared to the normal control. Oral andrographolide treatment at doses (15, 30, and 60 mg/kg, orally) dose-dependently increased the time spent in the target quadrant as compared to the i.c.v.-streptozotocin group on day 21. However, on day 21, donepezil oral administration of dose of andrographolide (15 mg/kg/day, orally) increased the time spent in the target quadrant than treatment with andrographolide (60 mg/kg, orally) alone at a higher dose. (Figure 7)

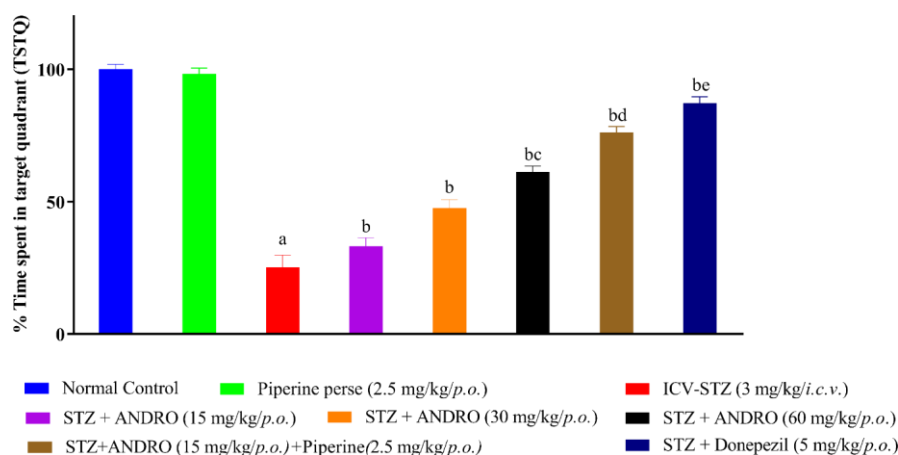


Figure 6: Effect of andrographolide and piperine treatment on percentage of time spent in the target quadrant on MWM in i.c.v.-streptozotocin-treated rats.

Data is expressed as Mean $\pm$ S.D. with $p < 0.05$, which was considered statistically significant. $ap < 0.001$ vs. Normal control, $bp < 0.01$ vs. ICV-STZ, $cp < 0.05$ vs. ICV-STZ+ANDRO (15 and 30 mg/kg), $dp < 0.05$ vs. ICV-STZ+ANDRO (60mg/kg), $ep < 0.05$ vs. ICV-STZ+ANDRO (15 mg/kg) + Piperine (2.5 mg/kg). Data was analyzed using two-way ANOVA followed by Bonferroni's multiple comparisons test. (ICV-STZ: i.c.v.-streptozotocin, ANDRO: andrographolide, PIP: piperine).

3.6. Effect of andrographolide and piperine treatment on MDA, nitrite, superoxidedismutase, glutathione, catalase, and acetylcholinesterase activity levels in streptozotocin-treated rats

Normal control and piperine-treated animals did not significantly change MDA, nitrite, superoxide dismutase, glutathione, catalase, and Acetylcholinesterase activity levels in brain homogenate. However, i.c.v.-streptozotocin-induced AD rats significantly increased the level of MDA, nitrite, and acetylcholinesterase activity and reduced catalase, superoxide dismutase, and glutathione in brain homogenate as compared to the normal control group animals. Treatment with oral andrographolide at doses (15, 30, and 60 mg/kg, orally) dose-dependently improved catalase, superoxide dismutase, and glutathione levels and reduced oxidant levels like MDA, nitrite, and acetylcholinesterase activity as compared to i.c.v.-streptozotocin group on day 21. However, on day 21, donepezil's oral administration of 5 mg/kg significantly restored catalase, superoxide dismutase, and glutathione and reduced oxidant levels like MDA, nitrite, and acetylcholinesterase activity as compared to the combination group. Moreover, the combination of piperine (2.5 mg/kg/day) with a lower dose of andrographolide (15 mg/kg/day, orally) increased catalase, superoxide dismutase, and glutathione and lowered oxidant levels like MDA, nitrite, and acetylcholinesterase activity than treatment with andrographolide (60 mg/kg, orally) alone at a higher dose. (Table 2)

Table Num: 2 Effect of andrographolide and piperine treatment on MDA, nitrite, superoxide dismutase, glutathione, catalase, and acetylcholinesterase activity levels

Sl. No.	Group	MDA (nmol/mg protein)	Nitrite (μ mol/mg protein)	Catalase (μ mol/mg protein)	SOD (μ mol/mg)	GSH (μ mol/mg protein)	Acetylcholinesterase (μ mol/mg protein)
01	Normal Control	3.59 $\pm$ 0.35	2.3 $\pm$ 0.44	24.1 $\pm$ 1.3	8.4 $\pm$ 2.9	0.78 $\pm$ 0.64	0.13 $\pm$ 0.012
02	Piperine per se (2.5 mg/kg/p.o.)	3.56 $\pm$ 0.29	2.1 $\pm$ 0.37	25.12 $\pm$ 1.63	8.7 $\pm$ 0.34	0.79 $\pm$ 0.21	0.12 $\pm$ 0.193
03	ICV-STZ (3 mg/kg)	10.12 $\pm$ 0.76 ^a	8.12 $\pm$ 0.22 ^a	6.42 $\pm$ 0.84 ^a	2.4 $\pm$ 0.45 ^a	0.11 $\pm$ 0.13 ^a	0.86 $\pm$ 0.007 ^a
04	STZ + ANDRO (15 mg/kg/p.o.)	8.64 $\pm$ 0.39 ^b	7.19 $\pm$ 0.93 ^b	11.4 $\pm$ 0.36 ^b	3.95 $\pm$ 0.68 ^b	0.23 $\pm$ 0.63 ^b	0.58 $\pm$ 0.015 ^b
05	STZ + ANDRO (30 mg/kg/p.o.)	6.86 $\pm$ 0.58 ^b	5.96 $\pm$ 0.81 ^b	17.8 $\pm$ 0.45 ^b	5.92 $\pm$ 0.73 ^b	0.41 $\pm$ 0.96 ^b	0.46 $\pm$ 0.043 ^b
06	STZ + ANDRO (60 mg/kg/p.o.)	4.59 $\pm$ 0.87 ^{bc}	4.17 $\pm$ 0.63 ^{bc}	20.26 $\pm$ 0.76 ^{bc}	6.54 $\pm$ 0.68 ^{bc}	0.61 $\pm$ 0.05 ^{bc}	0.31 $\pm$ 0.069 ^{bc}
07	STZ + ANDRO (15 mg/kg/p.o.) + Piperine (2.5 mg/kg/p.o.)	4.13 $\pm$ 0.49 ^{bd}	3.94 $\pm$ 0.36 ^{bd}	22.98 $\pm$ 0.57 ^{bd}	7.24 $\pm$ 0.86 ^{bd}	0.67 $\pm$ 0.04 ^{bd}	0.21 $\pm$ 0.074 ^{bd}

3.7. Effect of andrographolide and piperine treatment on amyloid Beta (1-42) level in streptozotocin-treated rats

Normal control and piperine-treated animals did not significantly change in the amyloid beta (1-42) level throughout the study on day 21 in the brain homogenate. However, in i.c.v.- streptozotocin-induced AD rats, significantly higher A β (1-42) levels were observed as compared to the normal control. Oral andrographolide treatment at doses (15, 30, and 60 mg/kg, orally) dose-dependently decreased A β (1-42) levels as compared to the i.c.v.- streptozotocin group on day 21. However, on day 21, donepezil oral administration of 5 mg/kg significantly improved A β (1-42) levels as compared to the combination group. Moreover, the combination of Piperine (2.5 mg/kg/day) with a lower dose of andrographolide (15 mg/kg/day, orally) decreased A β (1-42) levels than treatment with andrographolide (60 mg/kg, orally) alone at a higher dose.

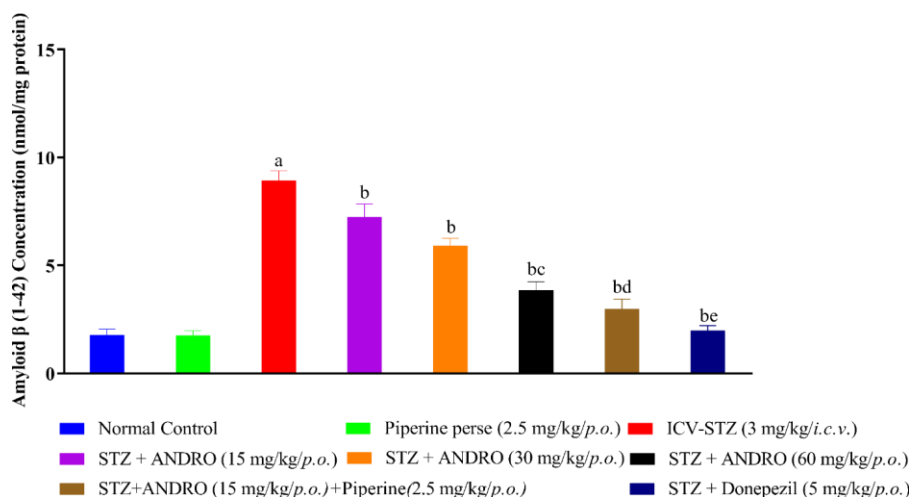


Figure 7: Effect of andrographolide and piperine treatment on amyloid Beta (1-42) ($A\beta$ 1-42) level in streptozotocin-treated rats.

Data is expressed as Mean $\pm$ S.D. with $p < 0.05$, which was considered statistically significant. $a_{p < 0.001}$ vs. Normal control, $b_{p < 0.01}$ vs. ICV-STZ, $c_{p < 0.05}$ vs. ICV-STZ+ANDRO (15 and 30 mg/kg), $d_{p < 0.05}$ vs. ICV-STZ+ANDRO (60mg/kg), $e_{p < 0.05}$ vs. ICV-STZ+ANDRO (15 mg/kg) + Piperine (2.5 mg/kg). Data was analyzed by two-way ANOVA followed by Bonferroni's multiple comparisons test. (ICV-STZ: i.c.v.-streptozotocin, ANDRO: andrographolide, PIP: piperine).

3.8. Effect of andrographolide and piperine treatment on neuroinflammatory cytokine levels in streptozotocin-treated rats

Normal control and piperine-treated animals did not significant change in the cytokines (TNF- α , IL-1 β , IL-6) levels in brain homogenate. However, in i.c.v.-streptozotocin-induced AD rats, significantly higher inflammatory cytokines (TNF- α , IL-1 β , IL-6) levels were observed as compared to the normal control. Oral andrographolide treatment at doses (15, 30, and 60 mg/kg, orally) dose-dependently decreased inflammatory cytokines (TNF- α , IL-1 β , IL-6) as compared to the i.c.v.-streptozotocin group on day 21. However, on day 21, donepezil oral administration of 5 mg/kg significantly improved inflammatory cytokines (TNF- α , IL-1 β , IL-6) as compared to the combination group. Moreover, the combination of piperine (2.5 mg/kg/day) with a lower dose of andrographolide (15 mg/kg/day, orally) decreased inflammatory cytokines (TNF- α , IL-1 β , IL-6) than treatment with andrographolide (60 mg/kg, orally) alone at a higher dose. (Figure 9)

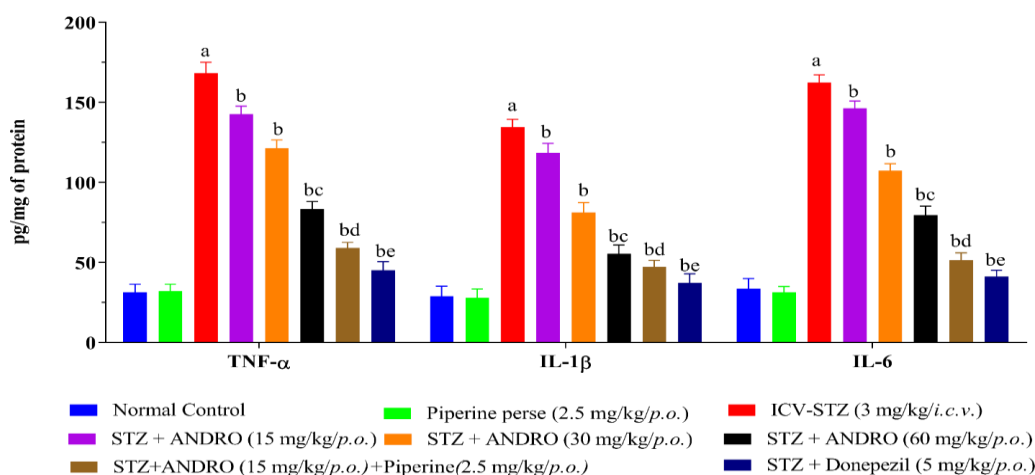


Figure 8: Effect of andrographolide and piperine treatment on Neuroinflammatory Cytokines (TNF- α , IL-1 β , IL-6) levels in streptozotocin-treated rats.

Data is expressed as Mean $\pm$ S.D. with $p < 0.05$, which was considered statistically significant. $a_{p < 0.001}$ vs. Normal control, $b_{p < 0.01}$ vs. ICV-STZ, $c_{p < 0.05}$ vs. ICV-STZ+ANDRO (15 and 30 mg/kg), $d_{p < 0.05}$ vs. ICV-STZ+ANDRO (60mg/kg), $e_{p < 0.05}$ vs. ICV-STZ+ANDRO (15 mg/kg) + Piperine (2.5 mg/kg). Data was analyzed by two-way ANOVA followed by Bonferroni's multiple comparisons test. (ICV-STZ: i.c.v.-streptozotocin, ANDRO: andrographolide, PIP: piperine).

3.9. Effect of andrographolide and piperine treatment on GABA and glutamate levels in streptozotocin-treated rats

Normal control and piperine-treated animals did not significantly change the glutamate and GABA levels in brain homogenate. However, in i.c.v.-streptozotocin-induced AD rats, significantly higher glutamate levels and reduced GABA levels were observed as compared to the normal control. Oral andrographolide treatment at doses (15, 30, and 60

mg/kg, orally) dose-dependently decreased glutamate and restored GABA levels as compared to the i.c.v.-streptozotocin group on day 21. However, on day 21, donepezil oral administration of 5 mg/kg significantly improved glutamate and GABA levels as compared to the combination group. Moreover, the combination of piperine (2.5 mg/kg/day) with a lower dose of andrographolide (15 mg/kg/day, orally) decreased glutamate levels and elevated GABA levels than treatment with andrographolide (60 mg/kg, orally) alone at a higher dose.

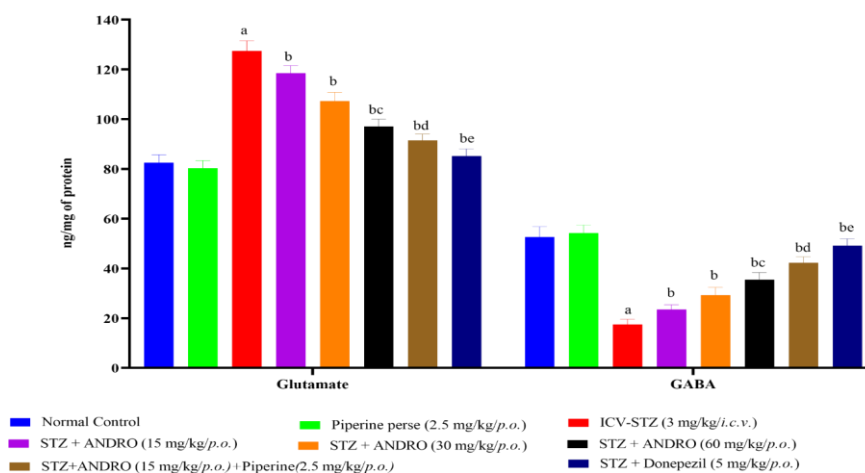


Figure 9: Effect of andrographolide and piperine treatment on GABA and Glutamate levels in streptozotocin-treated rats.

Data is expressed as Mean $\pm$ S.D. with $p < 0.05$, which was considered statistically significant. $a_{p < 0.001}$ vs. Normal control, $b_{p < 0.01}$ vs. ICV-STZ, $c_{p < 0.05}$ vs. ICV-STZ+ANDRO (15 and 30 mg/kg), $d_{p < 0.05}$ vs. ICV-STZ+ANDRO (60mg/kg), $e_{p < 0.05}$ vs. ICV-STZ+ANDRO (15 mg/kg) + Piperine (2.5 mg/kg). Data was analyzed by two-way ANOVA followed by Bonferroni's multiple comparisons test. (ICV-STZ: i.c.v.-streptozotocin, ANDRO: andrographolide, PIP: piperine).

DISCUSSION

In our current study, we highlight the promising neuroprotective potential of andrographolide combined with piperine in a rat model of Alzheimer's disease induced by i.c.v. streptozotocin. In this model, streptozotocin administration resulted in hallmark Alzheimer-like pathology, including significant cognitive impairments, disrupted neurotransmitter balance, oxidative stress, and neuroinflammation. Behavioural assessments revealed that streptozotocin-treated animals exhibited marked deficits in memory and learning, as demonstrated by decreased performance in tests evaluating spontaneous locomotor activity, spatial navigation, and anxiety-related behaviours [21]. Specifically, animals showed reduced beam crossing in actophotometer tests, increased transfer latencies in the elevated plus maze, and prolonged escape latencies with less time spent in the target quadrant during morris water maze assessments. Treatment with andrographolide, administered in a dose-dependent manner, significantly ameliorated these pathological changes. Andrographolide combination with piperine, improved cognitive functions by significant alteration of behavioural assessments. Biochemical analyzes further confirmed the detrimental impact of streptozotocin. However, streptozotocin also induced severe oxidative stress, as indicated by increased levels of MDA, nitrite, and acetylcholine esterase activity, along with a concurrent depletion of vital antioxidants such as reduced glutathione, superoxide dismutase, and catalase [22]. There was a notable elevation in amyloid-beta (1-42) levels in the hippocampus, which is closely linked with the progression of neurodegeneration [23]. Additionally, neurotransmitter imbalances were evident, with significant reductions in GABA and an increase in glutamate levels, disrupting the critical excitatory-inhibitory balance essential for normal cognitive function [24]. This imbalance, coupled with increased acetylcholinesterase (AChE) activity, further exacerbated the cholinergic deficits associated with Alzheimer's disease [25]. The pro-inflammatory environment was confirmed by elevated levels of cytokines, including TNF- α , IL-1 β , and IL-6, which contribute to further neuronal damage and cognitive decline. However, andrographolide in combination with piperine restored neurotransmitter levels, reduced the deposition of amyloid-beta, normalised AChE activity, and re-established antioxidant defense by boosting glutathione, superoxide dismutase, and catalase levels while reducing oxidative markers. Notably, the co-administration of piperine further enhanced these neuroprotective effects by likely increasing the bioavailability of andrographolide and amplifying its therapeutic efficacy.

Overall, these results suggest that the combination of andrographolide with Piperine offers a robust, multi-targeted approach to counteract the cognitive deficits and neuropathological alterations characteristic of Alzheimer's disease, positioning this strategy as a promising candidate for further therapeutic development.

CONCLUSION

In conclusion, this study shows that andrographolide and piperine have new synergistic neuroprotective effects against Alzheimer's disease in rats that have been induced by streptozotocin. The combination enhanced cognition, corrected the neurotransmitter imbalance and decreased amyloid beta, oxidative stress and neuroinflammation. The novelty is the

bioenhancing role played by piperine in augmenting the efficacy of andrographolides. Limitations include the fact that this is a preclinical study and there were no pharmacokinetic and molecular analyses, which in the future should be addressed using advanced mechanistic and clinical approaches. Experimental difficulties, e.g. the precise administration of the streptozotocin, were overcome by standardisation of procedures. Future research should investigate molecular targets and safety profiles in addition to validating these findings in transgenic models.

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Declarations:

Ethical Approval

Animals are approved by IAEC under protocol ISFCP/IAEC/CPCSEA/Meeting No. 29 /2025/ Protocol No.431, ISF College of Pharmacy, Moga (Punjab)

Author contribution

Royal Patel authored the manuscript, organised the data, produced the figures, and performed the study. Pradeep Kumar Mohanty conceived and developed the initial idea and concept for the paper and finalized the text.

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Statements and declarations

Competing interests All authors declared the absence of any financial or personal conflicts about this work

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