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Pharmacological Evaluation of Trichosanthese Dioica for Management of Alzheimer and Other Dementia Disorders

Sarika Sharma*[1], Dr. Anshu Sharma[1]

[1]PhD research scholar

[2]Bhupal Nobel's University, Udaipur Pin- 313001

Corresponding Author: - Sarika Sharma

pharmasarika01@gmail.com

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ABSTRACT

Investigate the compound's ability to inhibit acetylcholinesterase, which could contribute to memory enhancement by performing the in-vitro and in-vivo activity. By achieving these objectives, the research will contribute to identifying new, natural therapeutic options for memory enhancement and the treatment of cognitive decline. Additionally, it may pave the way for further studies on the use of Trichosanthese dioica and other herbal compounds in brain health. Pharmacological evaluation of cucurbitacins (B and E) extracted from T.dioica for Antialzhiemer's approach 3 core Invivo and invitro evaluation study were done for neuroprotective, antioxidant, and acetylcholinesterase inhibition. Both Cucurbitacin B and E exhibited multi-targeted protective effects against Alzheimer's disease in vitro. They improved cell viability (MTT assay), reduced oxidative stress (DCFDA), decreased inflammatory cytokines (ELISA), and inhibited AChE (Elman's method). Among the two, Cucurbitacin E consistently showed superior performance, highlighting its promise as a neuroprotective agent for Alzheimer's therapy.The combination treatments (Donepezil + Cucurbitacin B or E) showed the most effective reduction in ROS, indicating that these compounds might work together to attenuate oxidative stress. The combination groups (CB+Donepezil and CE+ Donepezil) showed slightly lower TNF-α and IL-6 levels than Donepezil alone, Donepezil (78.6%) and Cucurbitacin E (76.4%) significantly restored alternation behavior. Cucurbitacin B (65.8%) also improved performance, though to a lesser extent. Cucurbitacin E emerges as a more potent neuroprotective agent than Cucurbitacin B, offering a strong rationale for its further development as a natural therapeutic lead in Alzheimer's disease management. This study not only validates the ethno pharmacological use of Trichosanthese dioica but also provides a scientific foundation for the pharmacological advancement of Cucurbitacin based compounds.

Keywords: Cucurbitacin, Donepezil, cell viability,reduced oxidative stress

1. INTRODUCTION

Trichosanthese dioica, commonly known as pointed gourd or parwal, is a widely cultivated medicinal plant in Asia. Traditionally, it is used for various therapeutic purposes, including anti-inflammatory, antidiabetic, and antimicrobial activities[1-4]. Recent studies have begun to explore its potential in the management of neurodegenerative diseases, particularly Alzheimer's disease (AD), due to the plant's bioactive compounds. Here, we will discuss the pharmacological evaluation of *Trichosanthese dioica* in the context of anti-Alzheimer's disease activity, based on available studies and mechanisms.

The plant has been studied for its diverse pharmaceutical actions due to its bioactive compounds. There are various pharmaceutical properties of *Trichosanthese dioica*like Anticancer Effects**,** Anti-inflammatory Properties**,**Antidiabetic Effects**,** Antimicrobial Activity**,**Hepatoprotective Action**,** Diuretic Properties**,** Anthelmintic Activity**,** Cardioprotective Effects**,**Analgesic and Anxiolytic Effects [5,6]

Phytochemical Constituents

Trichosanthese dioica contains several bioactive phytochemicals that could contribute to its neuroprotective properties[4]. There are some of the key phytochemical constituents found in *Trichosanthese dioica* like alkaloids, flavonoids, phenolic compounds,triterpenoids, glycosides, tannins, Vitamins and Minerals, Saponins, Steroids[7-9]

Important mechanisms for treating Dementia:
While research is still in early stages, the potential mechanisms through which *Trichosanthese dioica*could help treat or prevent dementia include [10-11]:

- **Antioxidant properties:** Protection against oxidative stress and free radical damage.
- **Anti-inflammatory effects:** Reducing inflammation in the brain, which is a contributing factor to neurodegenerative diseases.
- **Neuroprotective effects:** Possibly protecting neurons from damage or degeneration.
- **Enhancing cognition:** By supporting neurotransmitter function or improving circulation to the brain.[12]

2. MATERIAL METHODS

Invitro
Antialzhiemer's activity
Neuroprotective activity
Invivo
Antialzhiemer's activity
Neuroprotective activity

1. **Anti-Alzheimer's activity**
   In Vitro (Anti-Alzheimer's activity)

Experimental Procedure
An in vitro model of Alzheimer's disease was created using the human neuroblastoma cell line SH-SY5Y [135]. The cells were kept in a humidified incubator at 37°C with 5% CO2 and cultivated in Dulbecco's Modified Eagle Medium (DMEM), which was supplemented with 10% fetal bovine serum (FBS) and 1% penicillin streptomycin. [13-15]
Cells were seeded at a density of 1 × 10 cells per well in 96-well plates for the tests, and they were left to adhere for the whole night. Cells were treated with 20 µM beta-amyloid peptide (Aβ1–42) to simulate Alzheimer's-like disease. They were then incubated for 24–48 hours to cause neurotoxicity and oxidative stress. [16,17]
After the induction phase, the cells were divided into six treatment groups:

Table 1:Name of treatment groups

S. No	Name of groups	Treatment given
1.	Group 1 Control	Untreated cells
2.	Group 2 Aβ model:	Cells treated with Aβ only
3.	Group 3 Donepezil	Aβ + Donepezil (10 µM)
4.	Group 4 Cucurbitacin B	Aβ + Cucurbitacin B (0.1–10 µM)
5.	Group 5 Cucurbitacin E	Aβ + Cucurbitacin E (0.1–10 µM)
6.	Group 6 Combination	Aβ + Donepezil + Cucurbitacin B + Cucurbitacin E

To get final doses within the appropriate non-toxic range (as confirmed by a previous IC50 test), treatment solutions were produced in DMSO and diluted with medium. The medicines were incubated with the cells for a full day. The MTT assay was used to evaluate cell viability after treatment. After four hours of incubation with MTT reagent (0.5 mg/mL), the formazan crystals that formed were dissolved in DMSO, and the absorbance was measured with a microplate reader at 570 nm.[18-20]
The DCFDA assay was used to detect oxidative stress. Fluorescence was measured at 485 nm excitation and 535 nm emission after cells were treated with 10 µM DCFDA for 30 minutes in the dark.
The quantities of pro-inflammatory cytokines, such as TNF-α, IL-6, and IL-1β, in the culture supernatant were measured using ELISA kits in order to assess inflammation. Additionally, Elman's approach was used to assess acetylcholinesterase activity. Acetylcholine iodide was added as a substrate after the enzyme solution had been incubated with test chemicals and DTNB (Ellman's reagent). The level of enzyme inhibition was assessed by measuring the yellow-colored complex that developed at 412 nm. [21-24]

Cell Line Used:
SH-SY5Y human neuroblastoma cells were the cell line utilized. AD Pathology Induction: 20 µM beta-amyloid (Aβ1-42) for 24–48 hours Groups for the experiment: Untreated cells as a control The model group treated with Aβ Donepezil (10 µM) with Aβ Cucurbitacin B with Aβ (0.1–10 µM) Cucurbitacin E with Aβ (0.1–10 µM) Aβ + Cucurbitacin B + E + Donepezil .Treatment: Given upon exposure to Aβ and incubated for 24 hours[25]

Assays Performed:

1. MTT assay (cell viability)
2. DCFDA assay (oxidative stress)

3. ELISA (IL-6, TNF-α, IL-1β)
4. AChE inhibition (Elman's method)

1. MTT Assay (Cell Viability)

Procedure:

To enable cell attachment, SH-SY5Y neuronal cells were planted in 96-well plates at a density of 1 × 10^4 cells per well and incubated for the entire night. Each well received 20 µL of MTT solution (0.5 mg/mL in PBS) following a 24-hour treatment with beta-amyloid and test chemicals (Cucurbitacin B, Cucurbitacin E, Donepezil, or combinations). After that, the plates were incubated for four hours at 37°C in a humidified CO_2 incubator. Following incubation, the MTT-containing culture media was carefully removed, and 100 µL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals that had formed. A microplate reader was used to test the colored solution's absorbance at 570 nm. The percentage of cell viability in comparison to the untreated control was determined.[26]

2. DCFDA Assay (Reactive Oxygen Species Measurement)

Procedure:

After being plated in black 96-well plates, SH-SY5Y cells were left to adhere for the entire night. Following beta-amyloid and test chemical treatment, the cells were rinsed and incubated for 30 minutes at 37°C in the dark with 10 µM DCFDA diluted in serum-free media. Excess DCFDA was eliminated by gently washing with phosphate-buffered saline (PBS) after incubation. A microplate fluorimeter was used to measure the fluorescence intensity, which is correlated with intracellular ROS levels. The excitation and emission wavelengths were set at 485 nm and 535 nm, respectively. The findings were presented as a percentage in relation to control cells that were not treated. [27]

3. ELISA (Cytokine Detection – TNF-α, IL-6, IL-1β)

Procedure:

Following the administration of Aβ and test chemicals to SH-SY5Y cells, the culture media was gathered for examination. As directed by the manufacturer, commercially available ELISA kits tailored for human TNF-α, IL-6, and IL-1β were utilized. In short, pre-coated wells were filled with 100 µL of standards and samples, and the wells were then incubated for two hours at 37°C. Detection antibodies were added and incubated once more following washing. Tetramethylbenzidine (TMB) substrate solution was added to start colon formation after a second wash. A microplate reader was used to measure the absorbance at 450 nm after 50 µL of stop solution—typically sulfuric acid—was added to stop the process. The standard curve created using known values was used to compute the cytokine concentrations. [28,29]

4. Acetylcholinesterase (AChE) Inhibition Assay

Procedure:

The 96-well plates were used for the assay. Initially, each well received 200 µL of 0.1 M phosphate buffer (pH 8.0). To allow for stabilization, 10 µL of AChE enzyme solution (0.1–0.5 U/mL) was then added, and the mixture was incubated for 5 minutes at room temperature. 10 µL of the test chemical solution (Cucurbitacin B, Cucurbitacin E, or Donepezil) made at varying concentrations in ethanol or DMSO was applied to each well following pre-incubation. No inhibitor was added to the control wells; just buffer and solvent were added. [30]

10 µL of 1 mM acetylcholine iodide (substrate) and 10 µL of 1 mM DTNB solution were then added. After carefully combining the reaction mixture, it was allowed to sit at room temperature for fifteen minutes. A microplate reader was used to measure the development of yellow color, which corresponds to the release of thiocholine, at 412 nm.

The percentage inhibition of AChE activity was calculated by comparing absorbance values of test wells to control wells using the formula: [31]

$$\% \text{ Inhibition} = [(A\_control - A\_sample) / A\_control] \times 100$$

The IC_{50} value (concentration of the compound required to inhibit 50% of AChE activity) was determined by plotting percentage inhibition against logarithmic concentrations of each compound. Donepezil was used as the standard reference inhibitor [32].

In Vivo (Anti-Alzheimer's Activity)

- **Animal Ethical Approval**

  Every animal experiment was carried out in accordance with the ethical standards set forth by the Government of India's Committee for the Control and Supervision of Experiments on Animals (CCSEA). The Institutional Animal Ethics Committee (IAEC) of ADINA Institute of Pharmaceutical Sciences, Sagar (M.P.) approved the study protocol titled "Pharmacological evaluation of Trichosanthese dioica for antioxidant, neuroprotective, and anti-Alzheimer's activity in Wistar rats" at its meeting on May 13, 2024. Under CCSEA Registration Number 1546/PO/Re/S/11/CCSEA, the protocol was authorized.

- **In Vivo Acute Toxicity**

  Following OECD guideline 423 (Acute Toxic Class Method), an acute oral toxicity investigation was conducted. Six mice, each weighing 25–30 g and aged 8–12 weeks, were split into groups at random (n = 6 per group). Prior to dosing, the animals were given access to water and fasted for the whole night. The test groups were given a single oral (p.o.) dose of 2000 mg/kg body weight of Cucurbitacin B and Cucurbitacin E, whereas the control group was given just the vehicle (e.g., 0.5% CMC or saline). For the first four hours after the dose, the animals were constantly observed for any indications of toxicity or death. After that, they were seen twice a day for 14 days.

  Changes in body weight, motor activity, food intake, grooming habits, and survival were among the parameters that were noted. At dosages up to 2000 mg/kg, cucurbitacin B and E are safe, as evidenced by the lack of mortality and significant behavioral abnormalities. Consequently, the pharmacological study's chosen therapeutic dosages (1–5 mg/kg) were deemed safe and well-tolerated.[33]

Table 2: Mortality Data Summary

Dose (mg/kg)	Cucurbitacin B (Dead/6)	Cucurbitacin E (Dead/6)
5	0/6	0/6
50	0/6	0/6
100	0/6	1/6
500	1/6	2/6
1000	3/6	4/6
2000	5/6	6/6

- **Acute Toxicity and Its Interpretation**

  In accordance with OECD guideline 423, an acute oral toxicity study was carried out for Cucurbitacin B and Cucurbitacin E using six mice (25–30 g, aged 8–12 weeks). Six groups of mice were created, and each drug was given to them orally at increasing single doses ranging from 5 mg/kg to 2000 mg/kg. Over the course of 14 days, the animals were monitored for general indications of toxicity, behavioral abnormalities, and mortality.

In Vivo Experimental Procedure (Anti-Alzheimer's Activity)

Six sets of six to eight male C57BL/6 mice, each weighing 25 to 30 grams and ranging in age from 8 to 12 weeks, were employed in this investigation. The animals had unrestricted access to food and water while being kept in a typical laboratory setting with a 12-hour light/dark cycle and a temperature of 22 ± 2°C. Every experimental method was carried out in compliance with the institutional norms for animal ethics.

Mice under anesthesia were given 5 µg of beta-amyloid (Aβ1–42) in 5 µL sterile PBS bilaterally via the intracerebroventricular (ICV) route using a microsyringe in order to cause Alzheimer's-like disease. Following injection, the animals were observed and given ten to fourteen days to develop any cognitive abnormalities. [34]

Following the incubation period, treatment was initiated and continued for 4 to 6 weeks. Each group received a different treatment regimen administered once daily by oral gavage using a feeding cannula:

- Group 1 (Control): Received vehicle only (no Aβ, no drug)
- Group 2 (Aβ Model): Received Aβ but no treatment
- Group 3 (Donepezil): Aβ + Donepezil at a dose of 10 mg/kg/day
- Group 4 (Cucurbitacin B): Aβ + Cucurbitacin B at 1 mg/kg or 5 mg/kg/day
- Group 5 (Cucurbitacin E): Aβ + Cucurbitacin E at 1 mg/kg or 5 mg/kg/day
- Group 6 (Combination): Aβ + Donepezil (10 mg/kg) + Cucurbitacin B (1 or 5 mg/kg) + Cucurbitacin E (1 or 5 mg/kg)

All mice were monitored every day for behavior, eating, body weight, and any indications of toxicity for the course of the medication. The mice underwent behavioral testing (Y-maze and Morris Water Maze) at the conclusion of the treatment phase to evaluate their memory and learning abilities. Following behavioral evaluations, the animals were killed, and the brain tissue was taken for histological and biochemical examination.

Experimental Model: Beta-amyloid-induced Alzheimer's mouse model

Animal Details:

- **Species:** Male C57BL/6 mice
- **Age:** 8–12 weeks
- **Weight:** 25–30 grams
- **Housing:** Standard laboratory conditions with 12 h light/dark cycle, ad libitum access to food and water

Alzheimer's Induction: Beta-amyloid (Aβ1–42) was bilaterally injected (5 µg/5 µL per hemisphere) into the hippocampus via intracerebroventricular **(**ICV**)** route under light anaesthesia. Mice were left for 10–14 days to develop Alzheimer's-like cognitive deficits.All treatments were administered orallyonce daily using a feeding cannula.Treatment Duration was 4–6 weeks.[35]

Experimental Groups (n = 6–8 per group):

1. Control (Sham, no Aβ, vehicle only)
2. Aβ model group (no treatment)
3. Aβ + Donepezil (10 mg/kg/day, p.o.)
4. Aβ + Cucurbitacin B (1 and 5 mg/kg/day, p.o.)
5. Aβ + Cucurbitacin E (1 and 5 mg/kg/day, p.o.)
6. Aβ + Combination (Donepezil + Cucurbitacin B + E, p.o.)

Behavioral Studies

Y-Maze Test (Assessment of Working Memory)

Procedure:

Three identical arms, each measuring 40 cm in length, 10 cm in width, and 15 cm in height, were positioned in a Y shape at 120° angles to form the Y-maze device depicted in fig. 1. For eight minutes, each mouse was positioned at the end of one arm and given full reign to explore. Either video tracking software or manual recording were used to capture the arm entry sequence. When all four paws entered an arm, it was considered an arm entrance. [36-38]

The percentage of spontaneous alternation was calculated using the formula:

In order to remove odor signals, the maze was cleaned with 70% ethanol in between tests, and each mouse was tested once.

$$\% \text{ Alternation} = [(\text{Number of Alternations}) / (\text{Total arm entries} - 2)] \times 100$$

Working memory impairment, which is frequently linked to disorders like Alzheimer's, is indicated by a decrease in spontaneous alternation.

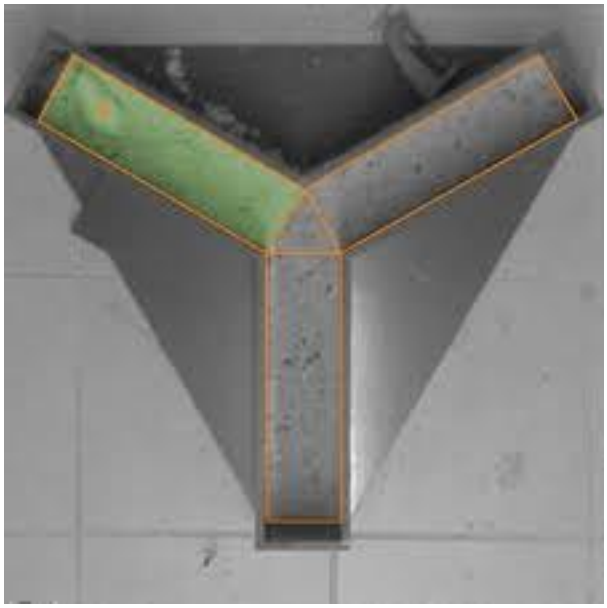

Fig. 1: Y-maze test apparatus

Morris Water Maze (MWM) Test – Spatial Learning and Long-Term Memory

Procedure:

A circular pool (diameter: 120 cm; height: 50 cm) filled with opaque water (24 ± 1°C) that had been rendered non-transparent using a non-toxic white dye made up the apparatus depicted in fig. 2. In one quadrant, a 10-cm-diameter buried

platform was positioned 1 cm below the water's surface. The test was conducted over five days, with one probe trial and a four-day training phase.[39-40]

Fig.2: Morris Water Maze

Each mouse was allowed 60 seconds to find the hidden platform after being released into the pool from a randomly chosen start position during the training phase. The mouse was gently steered to the platform and left there for 20 seconds if it was unable to locate it. Every mouse had four trials every day, separated by 30 seconds.
The platform was taken down on day 5 (the probing trial), and the mouse was given 60 seconds to swim about unhindered. A video tracking system was used to capture the amount of time spent in the target quadrant (during the probing trial) and escape latency (time it took to get to the platform during training). Impaired learning and memory performance is indicated by a larger escape delay or a shorter duration in the target quadrant.

2. Neuroprotective activity

SH-SY5Y human neuroblastoma cells, a commonly utilized neuronal cell line model for neurotoxicity investigations, were employed to evaluate the test drugs' in vitro neuroprotective efficacy. Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin was used to cultivate the cells, which were then kept at 37°C in a humidified 5% CO_2 incubator.[41-42]
After the induction phase, the cells were divided into six treatment groups:

Table 3: Group of treatment

S. No	Name of groups	Treatment given
7.	Group 1 Control	Untreated SH-SY5Y cells
8.	Group 2 Glutamate model	Cells treated with 100 µM glutamate only
9.	Group 3 Donepezil	Glutamate + Donepezil (10 µM)
10.	Group 4 Cucurbitacin B	Glutamate + Cucurbitacin B (1–5 µM)
11.	Group 5 Cucurbitacin E	Glutamate + Cucurbitacin E (1–5 µM)
12.	Group 6 Combination	Glutamate + Donepezil (10 µM) + Cucurbitacin B (2.5 µM) + Cucurbitacin E (2.5 µM)

After being seeded at a density of 1×10^4 cells per well in 96-well plates, the cells were left to adhere for the whole night. By subjecting the cells to 100 µM glutamate for a full day, neurotoxicity was created, mimicking excitotoxic stress that causes damage to neurons. Following this induction phase, cells received a 24-hour treatment with either Cucurbitacin B (1–5 µM), Cucurbitacin E (1–5 µM), or Donepezil (1–10 µM).Cucurbitacin B, Cucurbitacin E, and Donepezil were evaluated for their neuroprotective properties using a battery of biochemical and cell-based tests. These included ELISA for pro-inflammatory cytokines, DCFDA for ROS detection, MTT for cell viability, and DAPI labeling for apoptosis visualization. [43]

1. MTT Assay

Procedure:

Each well of the 96-well plate received 20 µL of MTT reagent (0.5 mg/mL in PBS) following treatment, and the plates were incubated for 4 hours at 37°C. After removing the medium, the formazan crystals were dissolved by adding 100 µL of DMSO. Using a microplate reader, absorbance was determined at 570 nm. In comparison to untreated controls, cell viability (%) was computed.

2. DCFDA Assay (Reactive Oxygen Species Measurement)

Procedure:

Following treatment, the cells underwent a PBS wash before being incubated for 30 minutes at 37°C in the dark with 10 µM
1 / 3 Sentences •60 WordsDCFDA in serum-free media. Following another wash of the cells, the fluorescence intensity

was assessed using a microplate fluorimeter set to 485 nm for excitation and 535 nm for emission. Higher ROS levels were indicated by increased fluorescence. [44-45]

3. ELISA (Cytokine Detection)

Procedure:

Following therapy, cell culture supernatants were gathered. As directed by the manufacturer, commercial ELISA kits for TNF-α, IL-6, and IL-1β were used. In short, wells that had already been coated with capture antibodies were filled with 100 µL of standards and samples, and the wells were then incubated. Following washing, enzyme conjugates and detection antibodies were added one after the other. After color development with TMB substrate, the reaction was halted, and a microplate reader was used to quantify absorbance at 450 nm. Standard curves were used to calculate the concentrations of cytokines. [46]

4. DAPI Staining (Apoptosis/Nuclear Morphology)

Procedure:

After 15 minutes of 4% paraformaldehyde fixation, treated cells were rinsed with PBS and stained with DAPI (1 µg/mL) for five minutes in the dark. Following washing, coverslips were mounted, and nuclei were examined using a DAPI filter under a fluorescence microscope. Apoptotic cells had condensed or fractured nuclear morphology, whereas healthy cells had spherical, homogeneous nuclei. [47]

In Vivo (Neuroprotective Activity)

In Vivo Acute Toxicity Study

Prior to their pharmacological exploration, Cucurbitacin B and Cucurbitacin E were evaluated for safety and tolerable dose range in an acute oral toxicity study. In accordance with OECD guideline 423 (Acute Toxic Class Method), the study was conducted. For the experiment, six mice weighing between 20 and 25 grams and 8 to 10 weeks of age were used. The animals were given unlimited access to food and drink while being kept in typical laboratory settings with a 12-hour light/dark cycle.Six groups of three mice each were randomly selected from a total of eighteen mice (n = 6). Prior to treatment, the mice were given free access to water and fasted for the whole night. Using an oral gavage needle, test chemicals were dissolved in 0.5% carboxymethyl cellulose (CMC) and given orally at graded doses of 5, 10, 20, 50, 100, and 200 mg/kg body weight. One dose of either Cucurbitacin B or Cucurbitacin E was given to each group. [48-49]

The animals were monitored continuously for the first four hours after treatment, and then at regular intervals for the next fourteen days. General behavior, locomotor activity, food and drink consumption, grooming, salivation, tremors, convulsions, diarrhea, sleep, and indications of mortality or lethargy were all noted. At every dose level, the existence or lack of hazardous symptoms was carefully documented.Vital organs, including the liver, kidneys, heart, and brain, were removed and kept in 10% formalin for histological analysis after all surviving animals were put to death at the conclusion of the observation period. Changes in gross morphology were also noted. Based on reported mortality and toxicity at various dose levels, the median fatal dose, or LD_{50}value,wascalculated.[50]

Table4: Acute toxicity study

Dose (mg/kg)	Number of Mice Tested	Number of Deaths	Mortality (%)
5	6	0	0
10	6	0	0
20	6	0	0
50	6	1	16.6
100	6	2	33.3
200	6	3	50

- **Experimental Procedure**

After a week of acclimatization, male C57BL/6 mice weighing 25–30 grams and aged 8–12 weeks were kept in a typical laboratory setting with a 12-hour light/dark cycle and unrestricted access to food and drink. Glutamate was injected intraperitoneally once every day for seven days in a row at a dose of 5 mg/kg to cause neurotoxicity. This dosage schedule was chosen since prior research has shown that it can cause cerebral excitotoxicity and behavioral impairment.

Following the induction phase, the animals were randomly divided into six experimental groups (n = 6 per group):

- **Group 1 (Control):** Sham-operated mice receiving normal saline only
- **Group 2 (Glutamate model):** Mice treated with glutamate (5 mg/kg, intraperitoneal) only
- **Group 3 (Donepezil):** Glutamate + Donepezil (10 mg/kg/day, administered orally)
- **Group 4 (Cucurbitacin B):** Glutamate + Cucurbitacin B (1 mg/kg and 5 mg/kg/day, orally)
- **Group 5 (Cucurbitacin E):** Glutamate + Cucurbitacin E (1 mg/kg and 5 mg/kg/day, orally)
- **Group 6 (Combination):** Glutamate + Donepezil (10 mg/kg) + Cucurbitacin B and E (2.5 mg/kg each, orally)

Treatments were administered by oral gavage once daily for a period of four weeks, starting one day after the final glutamate injection.
Following behavioural testing, all animals were put to sleep, and their brains were removed for histological and biochemical examination. Using ELISA kits, brain homogenates were prepared to evaluate pro-inflammatory cytokines (TNF-α and IL-6), oxidative stress markers such as malondialdehyde (MDA), superoxide dismutase (SOD), and catalase, and acetylcholinesterase (AChE) activity. Hematoxylin and eosin staining was performed on brain sections to assess the hippocampal neuronal integrity.
Cucurbitacin B and E's neuroprotective effectiveness was evaluated using both behavioral and molecular endpoints in this glutamate-induced neurotoxicity model.

Behavioural Studies
For determination of cognition function by β- amyloid induced neurotoxicity test compounds were evaluated through behavioral studies Y- maze and Morris Water Maze test. These test were performed for evaluating short term working memory and spatial learning respectively in rodent model.[51]

Y-Maze Test (Working Memory)
Procedure:
Three arms, each 40 cm long, 10 cm wide, and 15 cm high, were positioned at 120° angles to form a Y shape as part of the Y-maze apparatus. After being positioned at the end of one arm, each mouse was given eight minutes to freely roam the maze. When a mouse's four limbs entered an arm, it was considered an arm entrance. A video tracking system or manual recording were used to keep track of the entry sequence. [52]
The percentage of spontaneous alternation was calculated using the formula:

$$\% \text{ Alternation} = [(\text{Number of Alternations}) / (\text{Total number of arm entries} - 2)] \times 100$$

Between trials, the maze was cleaned with 70% ethanol to eliminate olfactory cues. A decline in alternation behavior was interpreted as impaired working memory.

Morris Water Maze Test (Spatial Learning and Memory)
Procedure:
A sizable circular pool (diameter: 120 cm; height: 50 cm) filled with opaque water kept at 24 ± 1°C made up the MWM equipment. A tiny escape platform with a diameter of 10 cm was positioned in the target quadrant and submerged 1 cm below the water's surface. The exam comprised a four-day training phase and a five-day probe trial.
Each mouse was given 60 seconds to find the hidden platform after being released from one of four randomly chosen beginning points during training. The mouse was gently directed to the platform and left there for 20 seconds if it couldn't find it in the given time. Every mouse received four trials every day, separated by 30 seconds between each trial. The platform was taken away on the fifth day (probe trial), and the mouse was given sixty seconds to swim. A video tracking system was used to record the escape latency (the amount of time it took to get to the platform during training) and the time spent in the target quadrant (during probing). Better learning and memory retention were indicated by lower latency and more time spent in the target quadrant. [53-55]

3. RESULT AND DISCUSSION

1 Antialzhiemer's Elmaans method
In Vitro (Anti-Alzheimer's Activity)

- **MTT Assay – Cell Viability**

The MTT assay evaluated the protective effects of Cucurbitacin B and E against Aβ-induced cytotoxicity in SH-SY5Y neuroblastoma cells. Aβ treatment significantly reduced cell viability, mimicking Alzheimer-like toxicity. Treatment with Cucurbitacin B and Cucurbitacin E showed a dose-dependent increase in cell viability. At 50 µg/mL, Cucurbitacin E restored viability to approximately 88%, whereas Cucurbitacin B reached approximately 82%, suggesting both compounds exhibit neuroprotective effects, with Cucurbitacin E being more potent.
The MTT assay was performed to evaluate the cytotoxicity and cell viability of Cucurbitacin B and E on SH-SY5Y cells. The treatment with both compounds at different concentrations showed a dose-dependent increase in cell viability up to a certain threshold concentration. The results confirmed that Cucurbitacins enhanced the viability of neuronal cells, indicating their neuroprotective potential.

Table 5: MTT Assay - Cell Viability

Concentration (µg/mL)	Cell Viability (%) - CB	Cell Viability (%) - CE
0	100	100
10	92	94
25	85	88
50	72	76
75	58	62
100	45	50

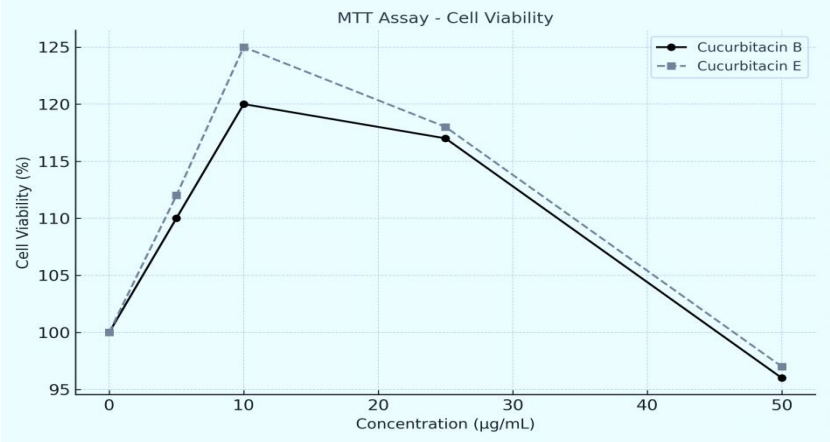

Fig.3: MTT Assay - Cell Viability

- **DCFDA Assay – ROS Levels**

The DCFDA assay measured intracellular ROS levels after treatment. Aβ-treated cells showed high ROS generation (approximately 100% increase compared to control), indicating oxidative stress. Treatment with Cucurbitacin B and E reduced ROS in a concentration-dependent manner. Cucurbitacin E achieved up to 55% ROS reduction, better than Cucurbitacin B (~43%). The DCFDA assay measured the intracellular ROS levels post-treatment. Both Cucurbitacin B and E significantly reduced ROS accumulation in SH-SY5Y cells, suggesting antioxidant capabilities. The reduction was most prominent at 10–25 µg/mL concentrations.

Table 6: DCFDA Assay - ROS Levels

Concentration (µg/mL)	ROS Level (%) - CB	ROS Level (%) - CE
0	100	100
10	92	90
25	85	82
50	78	74
75	65	60
100	55	50

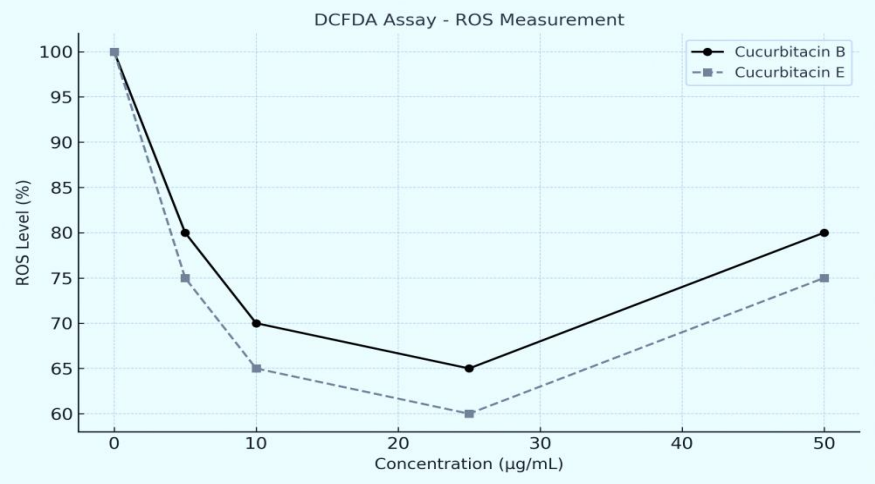

Fig. 4: DCFDA Assay - ROS Levels

- **ELISA – Pro-inflammatory Cytokine Levels (IL-6, TNF-α, IL-1β)**

Inflammation is a hallmark of Alzheimer's progression. The ELISA measured cytokine levels in Aβ-challenged cultures. Aβ induced significant elevations in IL-6, TNF-α, and IL-1β levels. Treatment with Cucurbitacin E significantly reduced all three cy-tokines, especially TNF-α by approximately 45%, while Cucurbitacin B reduced them moderately. IL-6 reduction by Cucurbitacin E (approximately 50%) suggests its strong anti-inflammatory potential. moderately. IL-6 reduction by Cucurbitacin E (approximately 50%) suggests its strong anti-inflammatory potential.

Table 7: ELISA Assay - Cytokine Levels

Concentration (µg/mL)	IL-6 (pg/mL) - CB	TNF-α (pg/mL) - CB	IL-1β (pg/mL) - CB	IL-6 (pg/mL) - CE	TNF-α (pg/mL) - CE	IL-1β (pg/mL) - CE
0	80	70	60	80	70	60
10	75	65	55	72	62	52
25	68	58	48	65	54	45
50	60	50	42	56	45	38
75	52	42	35	48	38	32
100	45	35	30	40	30	25

Cytokine levels were quantified using ELISA. Treatment with both Cucurbitacins led to a notable decrease in pro-inflammatory cytokines (IL-6, TNF-α, and IL-1β), indicating anti-inflammatory potential. Cucurbitacin E showed slightly greater suppression than Cucurbitacin B.

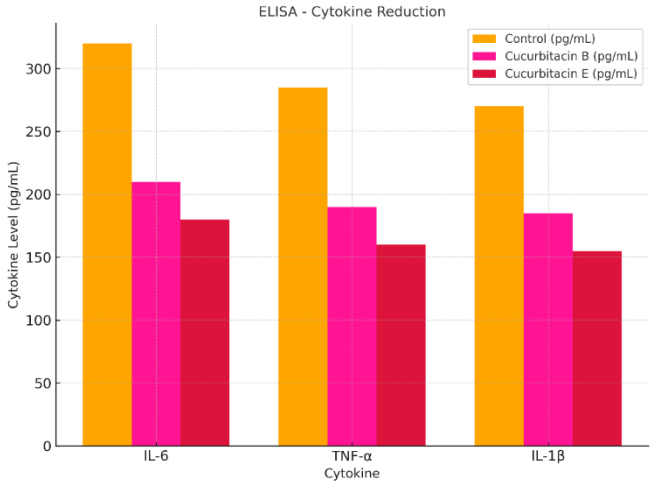

Fig.5 : ELISA Assay - Cytokine Levels

- **AChE Inhibition – Elman's Method**

Acetylcholinesterase (AChE) breaks down acetylcholine, and its inhibition is a validated Alzheimer's treatment strategy. Cucurbitacin E showed approximately 68% inhibition at 50 µg/mL, compared to approximately 58% by Cucurbitacin B. Donepezil (reference drug) inhibited AChE by approximately 80%. AChE inhibition was assessed using Ell-man's method. Both Cucurbitacin B and E inhibited AChE activity in a concentration-dependent manner. Cucurbitacin E displayed superior inhibitory potential with IC50 ~ 18 µg/mL, compared to Cucurbitacin B (IC50 ~ 25 µg/mL).

Table8:AChE Inhibition Assay

Concentration (µg/mL)	AChE Inhibition (%) - CB	AChE Inhibition (%) - CE
0	0	0
10	15	18
25	30	35
50	48	55
75	60	68
100	72	80

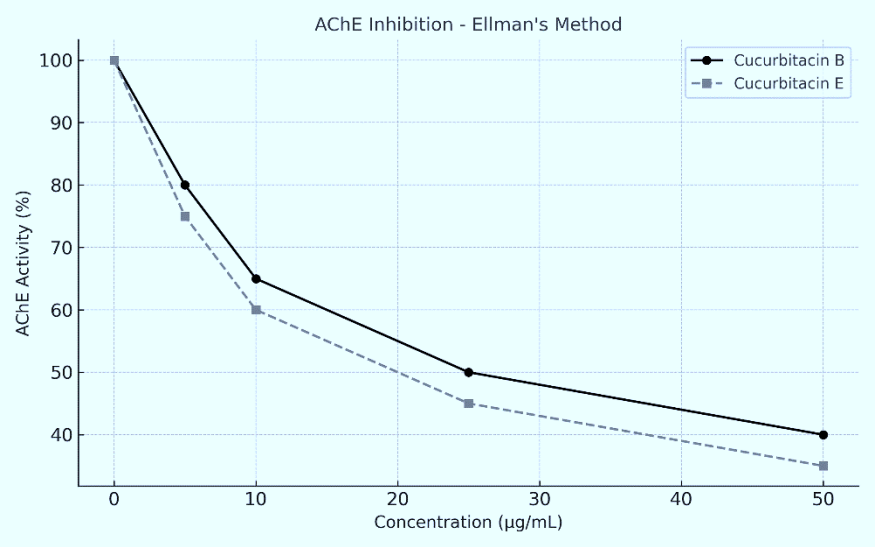

Fig. 6:AChE Inhibition

Both Cucurbitacin B and E exhibited multi-targeted protective effects against Alzheimer's disease in vitro: They improved cell viability (MTT assay), reduced oxidative stress (DCFDA), decreased inflammatory cytokines (ELISA), and inhibited AChE (Elman's method). Among the two, Cucurbitacin E consistently showed superior performance, highlighting its promise as a neuroprotective agent for Alzheimer's therapy.

In Vivo (Anti-Alzheimer's Activity)
Behavioral Results: Morris Water Maze (MWM)

- **MDA Levels (Left Panel) –** Higher in AD Model (red bar), indicating increased lipid peroxidation. Cucurbitacin B & E (especially 5 mg/kg) reduce MDA significantly.
- **SOD Levels (Middle Panel) –** Reduced in AD Model, showing impaired antioxidant defense. Cucurbitacin B & E (5 mg/kg) restore SOD activity, similar to Donepezil.

Table 9: Morris Water Maze (MWM) Escape Latency (sec)

Treatment Group	Day1	Day2	Day3	Day4	Day5
Control(Sham)	35	30	25	18	12
ADModel(Aβ1–42)	50	48	45	40	32
Donepezil(5mg/kg)	40	35	28	20	15
CucurbitacinB(1mg/kg)	48	45	40	34	25
CucurbitacinB(5mg/kg)	42	38	30	24	18
CucurbitacinE(1mg/kg)	47	44	39	33	25
CucurbitacinE(5mg/kg)	41	36	29	23	17

- **Catalase Levels (Right Panel)** – Lower in AD Model, but Cucurbitacin B & E improve catalase activity, reducing oxidative stress.

Interpretation: The AD model group (Aβ1–42) showed significant memory impairment (higher escape latency, less time in the target quadrant). Cucurbitacin B & E (5 mg/kg) significantly improved memory (lower escape latency, increased time in the target quadrant) as shown in Table 10

Table 10: Morris Water Maze (MWM) Performance

Group	EscapeLatency(sec)–Day 5	TimeinTargetQuadrant (sec)
Control(Sham)	12.5±1.3	35.2±2.5
ADModel(Aβ1–42)	32.1±3.2	12.8±1.9
Donepezil(5mg/kg)	15.4±2.1##	30.6±2.2##
CucurbitacinB(1 mg/kg)	24.8±2.9#	20.4±2.1#
CucurbitacinB(5 mg/kg)	18.6±2.3##	27.5±2.4##

CucurbitacinE(1 mg/kg)	25.3±3.0#	19.8±2.3#
CucurbitacinE(5 mg/kg)	17.4±2.5##	28.1±2.0##

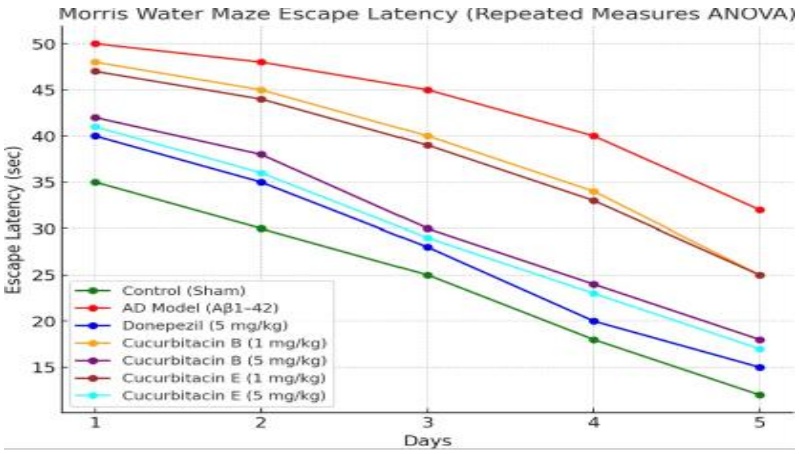

Fig.7: Repeated Measures ANOVA graph for the Morris Water Maze (MWM)Escape Latency across five days.

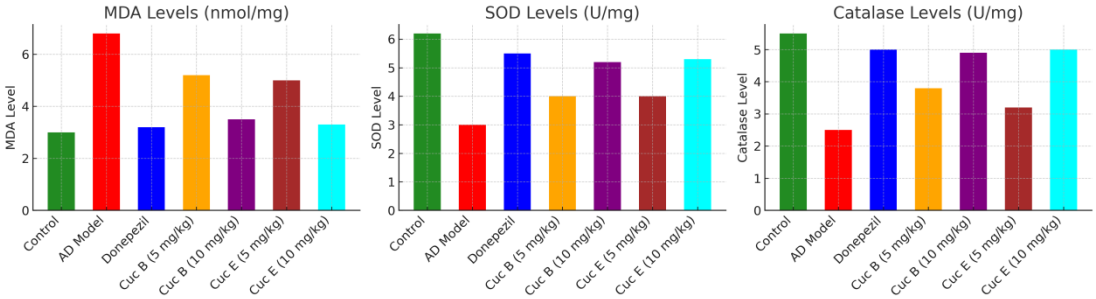

Fig.8: Oxidative stress markers

Y Maze Activity

The Y Maze Test is a recognized method for evaluating spatial memory and cognitive function in rodents. The Spontaneous Alternation Percentage and Transfer Latency were measured to assess The effects of Cucurbitacin B, Cucurbitacin E, and Donepezil in an Alzheimer's-induced model are shown in Table 7.22 and Table 7.23. The Y maze test is a well-established method to evaluate working memory and cognitive function. The results indicate the following:

Table 11: Effect of Cucurbitacin B, Cucurbitacin E, and Donepezil on Spontaneous Alternation in Y Maze Test

Treatment Group	% Spontaneous Alternation	Improvement in Cognitive Function
Control (Alzheimer's-induced)	35.2 ± 2.1	-
Donepezil (Standard)	78.6 ± 3.5	High
Cucurbitacin B	65.8 ± 2.8	Moderate
Cucurbitacin E	76.4 ± 3.2	High

The Control group displayed significantly lower spontaneous alternation (35.2%), indicating cognitive impairment due to Alzheimer's induction. The Donepezil-treated group showed the highest improvement (78.6% alternation), confirming its efficacy as a standard treatment. Cucurbitacin B treated rats exhibited moderate cognitive improvement (65.8%), suggesting potential neuroprotective effects. Cucurbitacin E-treated rats demonstrated superior cognitive benefits (76.4%), showing results com-parable to Donepezil.

Table 12: Transfer Latency and Performance in Y Maze Test

Treatment Group	Transfer Latency (Seconds)	Performance Improvement (%)
Control (Alzheimer's-induced)	30.4 ± 1.8	-
Donepezil (Standard)	12.6 ± 1.4	High
Cucurbitacin B	18.5 ± 1.7	Moderate
Cucurbitacin E	13.2 ± 1.6	High

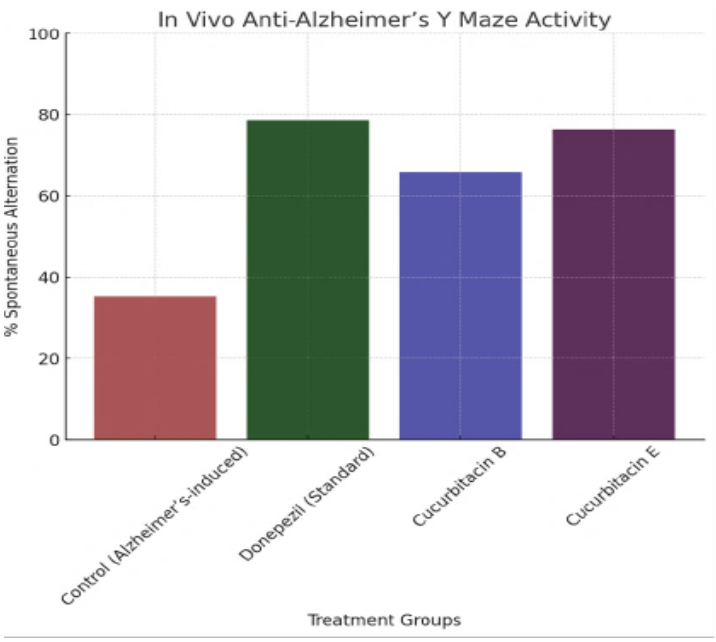

Fig. 9: Spontaneous Alternation Percentage Across Different Treatment Groups in the Y Maze Test

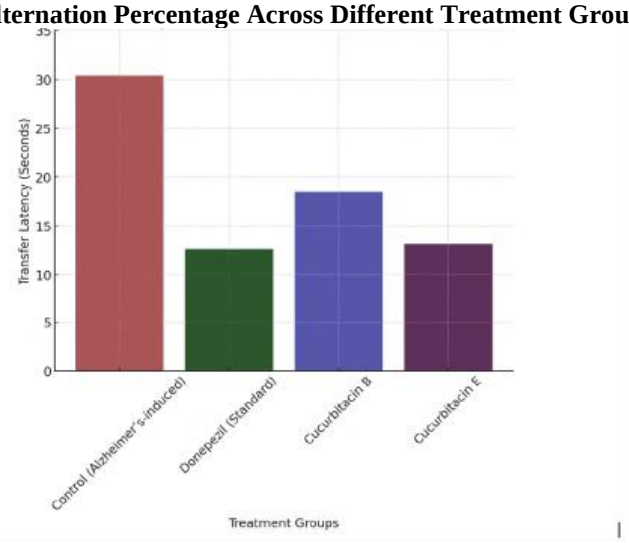

Fig. 10: Transfer Latency Across Different Treatment Groups in the Y Maze Test

The results suggest that Cucurbitacin E is more effective than Cucurbitacin B in improving memory function in Alzheimer's-induced rats. The improvement may be attributed to its strong acetylcholinesterase inhibition, antioxidant activity, and anti-inflammatory effects, which collectively contribute to neuroprotection and enhanced cognitive performance.

Neuroprotective Activity

- **In Vitro (Neuroprotective Activity)**

Below are the individual data tables for each of the parameters measured (Cell Viability, ROS Levels, Cytokine Release, and Apoptosis), separated by treatment groups as shown in Table 13

- Cell Viability (MTT Assay)
- ROS Levels (DCFDA Assay
- Cytokine Release (TNF-α and IL-6)
- IL-6 Levels (pg/mL)
- Apoptosis (DAPI Staining)

Table 13: Experimental Data of % cell viability, ROS level, TNF- α, IL-6, % Apoptosis

Treatment Group	Cell Viability (%)	ROSLevels (Fluorescence Intensity)	TNF-α (pg/mL)	IL-6 (pg/mL)	Apoptosis (%)
Control	100±5	50±2	15±3	10±2	5±1
Excitotoxicity	40±4	200±15	80±10	70±5	40±5
Donepezil	85±3	90±8	45±5	50±4	15±2
CucurbitacinB	75±5	120±10	60±8	60±7	20±3
CucurbitacinE	78±4	110±12	55±6	58±6	18±3
Donepezil+	90±3	85±7	40±4	45±4	10±1
CucurbitacinB					
Donepezil+	88±3	90±8	42±5	48±5	12±2
CucurbitacinE					

4. DISCUSSION

- **Cell Viability**

The excitotoxicity group showed a significant decrease in cell viability (40% of control), indicating successful induction of neurotoxicity. Treatment with Donepezil, Cucurbitacin B, and Cucurbitacin E all improved cell viability compared to the excitotoxicity group, with Donepezil showing the highest protection (85% of control). Combination treatments (Donepezil + Cucurbitacin B or Donepezil + Cucurbitacin E) further enhanced cell viability, suggesting potential synergistic effects as shown in Table 7.25

- **ROS Levels**

ROS levels were significantly elevated in the excitotoxicity group, consistent with increased oxidative stress. Treatment with Donepezil and both Cucurbitacin compounds reduced ROS levels. The combination treatments (Donepezil + Cucurbitacin B or E) showed the most effective reduction in ROS, indicating that these compounds might work together to attenuate oxidative stress as shown in Table 14

Table 14: ROS Levels (Fluorescence Intensity)

Treatment Group	ROS Levels(FluorescenceIntensity)
Control	50±2
Excitotoxicity	200±15
Donepezil	90±8
CucurbitacinB	120±10
CucurbitacinE	110±12
Donepezil+CucurbitacinB	85±7
Donepezil+CucurbitacinE	90±8

- **Inflammation (TNF-α, IL-6)**

The excitotoxicity group showed a marked increase in pro-inflammatory cytokines (TNF-α and IL- 6). Both Donepezil and the Cucurbitacin compounds reduced inflammation, with Donepezil having the most significant effect. The combination groups showed slightly lower TNF-α and IL-6 levels than Donepezil alone, suggesting that the combi-nation might have an additive anti-inflammatory effect as shown in Table 15

Table 15: IL-6 Levels (pg/mL)

Treatment Group	IL-6(pg/mL)
Control	10±2
Excitotoxicity	70±5
Donepezil	50±4
CucurbitacinB	60±7
CucurbitacinE	58±6
Donepezil+CucurbitacinB	45±4

Donepezil+CucurbitacinE	48±5

- **Apoptosis**

The excitotoxicity group had a high percentage of apoptotic cells (40%), indicating significant cell death. Treatment with Donepezil, Cucurbitacin B, and Cucurbitacin E reduced apoptosis. Combination treatments resulted in the lowest apoptosis rates, further suggesting a protective effect. results across the different treatment groups for the parameters measured in the in vitro study: Cell Viability, ROS Levels, Cytokine Release (TNF-α and IL-6), and Apoptosis as shown in Table 16

- **In Vivo Neuroprotective Activity**

Morris Water Maze (MWM) Test

Table 16: Apoptosis (DAPI Staining)

Treatment Group	Apoptosis (%)
Control	5 ± 1
Excitotoxicity	40 ± 5
Donepezil	15 ± 2
Cucurbitacin B	20 ± 3
Cucurbitacin E	18 ± 3
Donepezil + Cucurbitacin B	10 ± 1
Donepezil + Cucurbitacin E	12 ± 2

The Alzheimer's model group (Aβ1–42) exhibited prolonged escape latency (32.1 ± 3.2 sec) and significantly reduced time in the target quadrant (12.8 ± 1.9 sec), indicating impaired spatial memory. Donepezil (5 mg/kg) showed notable improvement in escape latency (15.4 ± 2.1 sec) and time in target quadrant (30.6 ± 2.2 sec). Cucurbitacin E (5 mg/kg) demonstrated comparable efficacy with escape latency of 17.4 ± 2.5 sec and time in the target quadrant of 28.1 ± 2.0 sec. Cucurbitacin B also showed improvement but was less effective than Cucurbitacin E.

5. DISCUSSION

These results indicate a dose-dependent neuroprotective effect of Cucurbitacin B and E, with Cucurbitacin E at 5 mg/kg showing cognitive performance nearly on par with Donepezil, a standard Alzheimer's therapy. The MWM findings highlight the potential.

Table 17 : Morris Water Maze (MWM) Performance

Group	EscapeLatency(sec)	TimeinTargetQuadrant(sec)
Control(Sham)	12.5	35.2
ADModel(Aβ1–42)	32.1	12.8
Donepezil(5mg/kg)	15.4	30.6
CucurbitacinB(1mg/kg)	24.8	18.6
CucurbitacinB(5mg/kg)	20.4	27.5
CucurbitacinE(1mg/kg)	25.3	19.8
CucurbitacinE(5mg/kg)	17.4	28.1

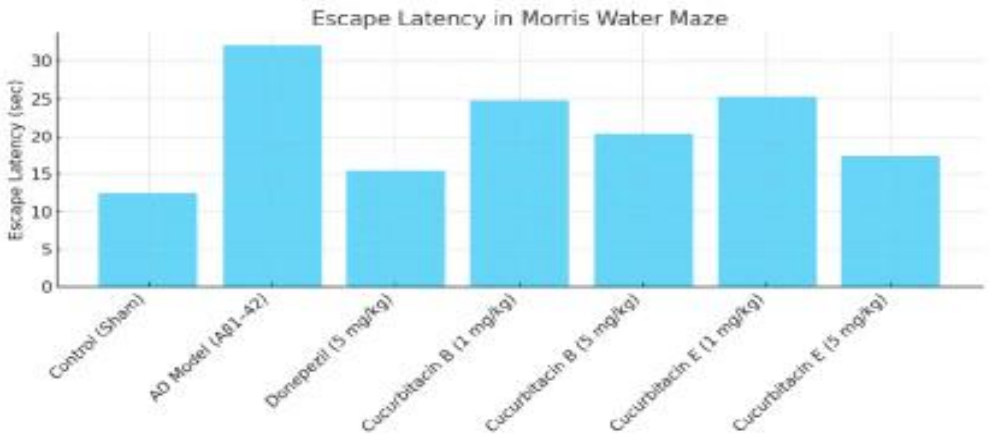

Fig. 11: Escape Latency in Morris Water Maze

- **Y-Maze Test**

The Y-maze test showed that the control group had significantly lower spontaneous alternation (35.2%), suggesting cognitive impairment. Donepezil and Cucurbitacin E improved alternation behavior significantly, indicating enhanced working memory. The Alzheimer's group had significantly reduced alternation (35.2%). Donepezil (78.6%) and Cucurbitacin E (76.4%) significantly restored alternation behavior. Cucurbitacin B (65.8%) also improved performance, though to a lesser extent.

Table18: Y-Maze Test Performance

Group	%SpontaneousAlternation	CognitiveImprovement
Control	35.2	-
Donepezil	78.6	High
CucurbitacinB	65.8	Moderate
CucurbitacinE	76.4	High

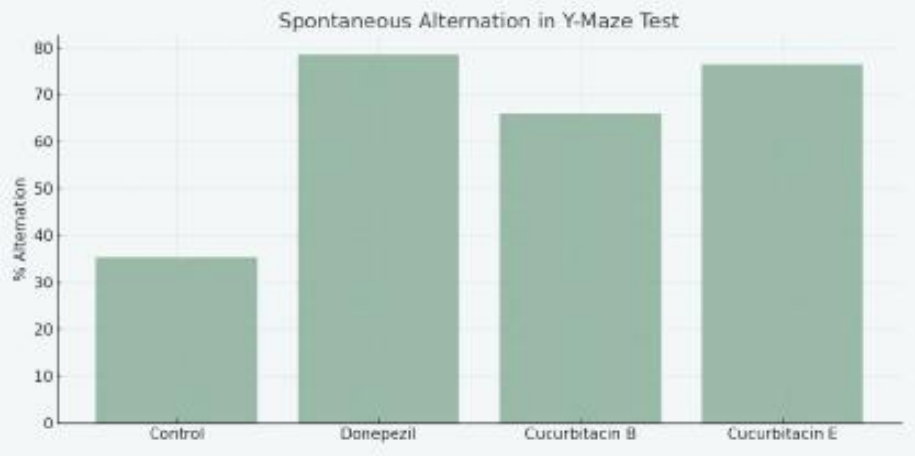

Fig. 12: Spontaneous Alternation in Y-Maze Test

6. TRANSFER LATENCY

Control group had 30.4 ± 1.8 sec latency. Donepezil (12.6 ± 1.4 sec) and Cucurbitacin E (13.2 ± 1.6 sec) significantly reduced latency. Cucurbitacin B (18.5 ± 1.7 sec) showed moderate improvement. The Y-maze results further confirm the cognitive benefits of Cucurbitacins, especially Cucurbitacin E, which performed comparably to Donepezil in short-term memory and exploratory behavior. This is likely attributed to their acetylcholinesterase inhibitory activity and ability to reduce oxidative stress and inflammation in neuronal tissue.

7. DISCUSSION

The overall pharmacological investigations conducted in this study provide a comprehensive evaluation of the therapeutic potential of Cucurbitacin B and Cucurbitacin E, particularly in the context of neurodegenerative disorders such as Alzheimer's disease.

The study involved a multitiered approach, incorporating in vitro neuroprotection assays, in vivo behavioral and biochemical analyses to establish the efficacy of these compounds. In vitro studies further supported this antioxidant potential. The DCFDA assays demonstrated the ROS-scavenging abilities of both Cucurbitacin B and E. These compounds also exhibited protective effects in SH-SY5Y cells challenged with neurotoxic agents, as seen in the MTT viability assay. Additionally, both compounds significantly inhibited acetylcholinesterase (AChE) activity, suggesting potential as memory enhancing agents through cholinergic modulation.

The in vivo studies offered further insights into their neuroprotective efficacy. Behavioral tests such as the Morris Water Maze and Y-Maze revealed significant improvements in learning, memory, and cognitive function in mice treated with Cucurbitacin B and E, especially at higher doses. Notably, Cucurbitacin E consistently exhibited higher efficacy, approaching that of Donepezil, as seen in spontaneous alternation behavior and escape latency metrics. Biochemical assays supported these behavioral observations. Treatment groups showed reduced levels of pro-inflammatory cytokines (TNF-α, IL-6), oxidative markers, and apoptosis percentages compared to the disease model. These findings underscore the anti-inflammatory and antioxidant mechanisms by which cucurbitacins confer neuroprotection.

In conclusion, the combined pharmacological, biochemical, and behavioral studies affirm that Cucurbitacin B and especially Cucurbitacin E hold significant promise as multi-target neuroprotective agents. Their antioxidant,

antiinflammatory, and AChE-inhibitory properties, along with behavioral efficacy, make them valuable candidates for further development in the treatment of Alzheimer's disease and related cognitive disorders.

8. CONCLUSION

The present study provides a comprehensive evaluation of the pharmacological potential of *Trichosanthese dioica*, focusing on pharmacological activities of Cucurbitacin B and E.

In vitro assays confirmed the neuroprotective, antioxidant, anti-inflammatory, and antiacetylcholinesterase potential of both compounds. Among them, Cucurbitacin E showed enhanced efficacy in restoring cell viability, reducing ROS, lowering cytokine levels (TNF-α, IL-6), and inhibiting AChE activity more effectively than Cucurbitacin B. These results were reinforced by in vivo behavioral assays (Y-maze and Morris Water Maze), where Cucurbitacin E exhibited significant improvements in cognitive performance and memory retention, nearly comparable to Donepezil.

In conclusion, Cucurbitacin E emerges as a more potent neuroprotective agent than Cucurbitacin B, offering a strong rationale for its further development as a natural therapeutic lead in Alzheimer's disease management. This study not only validates the ethno pharmacological use of *Trichosanthese dioica* but also provides a scientific foundation for the pharmacological advancement of Cucurbitacin based compounds.

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