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# *Gentiana lutea* extract ameliorates scopolamine-induced amnesia in mice *via* cholinergic modulation and inhibition of Aβ/p-tau protein accumulation

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## ABSTRACT

**Objective:** To investigate the potential of hydro-alcoholic extract of *Gentiana lutea* roots (GLE) in scopolamine-induced amnesia model in mice.

**Methods:** The active chemical constituents were determined by GC-MS analysis. *In vitro* antioxidant activity was performed by the DPPH free radical scavenging method. *Ex vivo* anti-acetylcholinesterase assay was conducted to investigate the effect on the cholinergic system. A scopolamine-induced memory impairment model was used. The levels of beta-amyloid (Aβ) and tau protein were measured. The behavioral studies were performed through Morris water maze and passive avoidance learning tests, followed by estimation of biochemical markers (GSH and MDA), immunohistochemistry, and histopathological studies on isolated brain tissues.

**Results:** GLE exhibited DPPH free radical scavenging activity with an $IC_{50}$ value of (76.68±2.28) μg/mL. GLE also manifested inhibitory effect on acetylcholinesterase [$IC_{50}$ (36.58±0.73) μg/mL] to upregulate the cholinergic system. GLE at 200 mg/kg and 400 mg/kg significantly restored the memory impairment induced by scopolamine. GLE at 200 mg/kg and 400 mg/kg significantly reduced brain oxidative stress ($P$<0.001). Immunohistochemistry investigation showed a significant reduction in Aβ deposition and p-tau protein expression in the GLE treatment groups ($P$<0.001). Administration of GLE effectively reduced scopolamine-induced neuronal damage in a dose-dependent manner.

**Conclusions:** The study demonstrates that GLE ameliorates scopolamine-induced memory impairment by alleviating Aβ/p-tau protein accumulation and upregulation in the cholinergic system to improve cognitive dysfunction and behavioral problems.

**KEYWORDS:** Amnesia; Beta amyloid; Scopolamine; Alzheimer's disease; Acetylcholine; p-tau protein; Acetylcholinesterase; *Gentiana lutea*

## 1. Introduction

Amnesia, also known as amnestic syndrome, is characterized by a cluster of symptoms and signs such as cognitive dysfunction, language impairment, behavioral problems, and impairments in daily activities[1]. Alzheimer's disease (AD) is the leading cause of amnesia that accounts for approximately 70% of dementia cases[2]. Amnesia is a type of memory loss that is usually temporary, while dementia is progressive and irreversible[3].

AD is a multifactorial neurodegenerative disorder with complicated pathological aspects, including cholinergic dysfunction, beta-amyloid (Aβ) accumulation in the brain, formation of neurofibril tangles in the nerve due to hyperphosphorylation of tau protein, oxidative stress, and neuroinflammation[4].

**Summary**

**Question:** Does *Gentiana lutea* extract ameliorate scopolamine-induced amnesia in mice?

**Findings:** In the present study, the *Gentiana lutea* extract significantly attenuated scopolamine-induced amnesia by inhibiting acetylcholinesterase activity, enhancing the antioxidant defense system, and reducing Aβ/p-tau protein accumulation.

**Meaning:** *Gentiana lutea* extract shows the potential to improve scopolamine-induced amnesia in mice.

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Cholinergic neurons' neurotransmitter acetylcholine (ACh) is critically essential for attention, learning, memory, stress response, wakefulness, and sleep[5]. Studies suggested that the damage to the cholinergic neurons in the cerebral cortex is the pathogenic evidence related to cognitive impairment[6]. Acetylcholinesterase (AChE) inhibitors AchI such as donepezil and rivastigmine, improve the cognitive impairment symptomatically but can not inhibit the disease progression[7].

It is postulated that Aβ deposits and neurofibril tangles induce neuronal impairment by precipitating synaptic dysfunction, promoting neuronal demise through calcium influx, and activating cellular death pathways such as necrosis and apoptosis[8]. Oxidative stress and pro-inflammatory cytokines have the potential to disrupt the functionality of nerve terminals, leading to synaptic loss, which is closely associated with cognitive decline[9].

Scopolamine has been widely used to induce memory and cognition impairment in animal models for the investigation of various pathological and therapeutic targets for AD. It is a muscarinic ACh receptor antagonist involved in memory and cognition[10]. Administration of scopolamine induces AD-like pathological changes such as increased oxidative damage, neuroinflammation, and AChE activity. Besides these, it also increases the accumulation of Aβ through the sequential cleavage of amyloid precursor protein (APP) by β and γ secretases along with tau kinase that phosphorylates the tau protein, leading to the production of neurofibril tangles[11].

It has been reported that herbal intervention addresses a multifaceted mechanism of action, such as neuroprotection, inhibition of AChE, anti-inflammatory properties, antioxidant effect, and modulation in the accumulation of Aβ and phosphorylated tau (p-tau) protein. It is now widely recognized that bioactive compounds derived from natural sources can exert a neuroprotective effect[12].

*Gentiana lutea* (*G. lutea*), commonly known as yellow gentian, classified within the family Gentianaceae, encompasses approximately 400 species belonging to the genus *Gentiana*[13]. It is mainly distributed in Southeast Asia, North America, and Europe. More than 500 chemical constituents have been isolated from the genus *Gentiana*. These are rich in chemical compounds such as iridoids, triterpenoids, flavonoids, phenols, and glycoside alkaloids[14]. The *Gentiana* genus possesses various pharmacological activities such as neuritogenic, hepatoprotective, anti-inflammatory, antimicrobial, antioxidant, cholinesterase inhibition, antiatherosclerotic, immunomodulatory, and gastroprotective effects[15].

Some species of *Gentiana* exhibited anticholinesterase activity and inhibition of Aβ deposition that could be useful in the treatment of AD[16,17]. Nastasijević *et al*. reported the antioxidant and inhibitory potential of myeloperoxidase of *G. lutea*, which may show the potential role in the treatment of AD[18]. While previous studies have examined cognitive and anti-amyloid-β (Aβ) effects of various *Gentiana* species, including reports on neuroprotective properties, few studies have specifically investigated the effects of *G. lutea* on scopolamine-induced amnesia *via* cholinergic and amyloidogenic pathways using a standardized hydro-alcoholic extract of roots with a particular dosing regimen. Hence, the present study aimed to investigate the potential of hydro-alcoholic extract of *G. lutea* roots (GLE) in scopolamine-induced memory and cognition impairment, exploring underlying mechanisms such as cholinergic enzyme regulation, inhibition of oxidative stress, and Aβ/p-tau pathways.

## 2. Materials and methods

### 2.1. Extracts

GLE was purchased (voucher specimen/ Batch number HN/YDE/202401012) from Harbo Nutra Extract Private Limited, Greater Noida, India. The extract was obtained by the maceration process using the solvent mixture (50%:50% *v/v*) of ethanol and water. Various physicochemical parameters of GLE were reported in certificate of analysis (Doc No. HNEPL/F/QC/09), such as brown, having the characteristic odour and bitter taste, solubility in water and alcohol, extractive value 18.5%, moisture content 10.0% *w/w*, total ash 5.0% *w/w*, and acid insoluble ash 2.4% *w/w*.

### 2.2. Drugs and chemicals

All chemicals and reagents used in the study were of analytical grade and procured from CDH India, including gallic acid, quercetin, Folin-Ciocalteu Reagent, ethanol, aluminum chloride, sodium acetate, DPPH, ascorbic acid, acetyl thiocholine, 5,5-dithiobis (2-nitrobenzoic acid), reduced glutathione, 1,1,3,3-tetraethoxypropane, thiobarbituric acid, acetylthiocholine iodide, scopolamine hydrobromide, and donepezil.

### 2.3. Preliminary phytochemical screening

Phytochemical screening was conducted to assess the qualitative analysis to identify the presence and absence of different chemical phytochemical classes/groups in GLE. Various reagents were prepared for the specific chemical constituents tests[19].

### 2.4. Estimation of total phenolic content

The total phenolic content in the GLE was estimated by the Folin-Ciocalteu Reagent method. A mixture was prepared by adding 1 mL of different concentrations of GLE, 5 mL of 10% Folin-Ciocalteu reagent, and 4 mL of sodium carbonate. The mixture was incubated

at 40 ℃ for 30 min, and the absorbance was measured at 760 nm against a blank. A standard curve of gallic acid was plotted using 10-100 μg/mL. The total phenolic content in the extract was estimated using the regression equation and expressed as μg of gallic acid equivalent per mg of GLE (μg/mg)[20].

### 2.5. Estimation of total flavonoid content

The total flavonoid content in GLE was determined by the aluminum chloride colorimetry method. An amount of 1 mL of different concentrations of the GLE was mixed with 1.5 mL of 95% ethanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M sodium acetate, and diluted with 2.8 mL of distilled water. This mixture was allowed to incubate for 30 min at room temperature. The absorbance was measured at 420 nm. A standard curve of quercetin was obtained using a range of 10-100 μg/mL. The total flavonoid content in the extract was expressed as μg of quercetin equivalent per mg of GLE[21].

### 2.6. Gas chromatography-mass spectroscopy (GC-MS) profiling

The identification of active chemical constituents in GLE was confirmed by GC-MS analysis using GC-MS-TQ8040, Shimadzu, with an AOC-20i autoinjector. The ion source temperature was 230 ℃, and the injector temperature was 250 ℃. The column oven was preheated to 50 ℃ for 2 min, then to 310 ℃ for 25 min. The total running time for GC-MS was fixed at 53 min. The collected mass spectra of compounds were described and identified with the help of the NIST library[22].

### 2.7. In vitro antioxidant activity

DPPH free radical scavenging assay is used to evaluate the *in vitro* antioxidant potential of the GLE. DPPH 0.1 mM solution was prepared by dissolving 3.9 mg of DPPH in 100 mL of 99% methanol and stored in a cool & dark place. Then, 3 mL of 0.1 mM DPPH was added to 1 mL of different concentrations of GLE and ascorbic acid (standard), and the volume was brought to 10 mL with methanol and incubated for half an hour in a dark place. After incubation, absorbance was taken at 517 nm using a control (0.1 mM DPPH) against a blank (methanol). The percentage inhibition of DPPH was calculated by using the equation:

$$\% \text{ inhibition} = (Ac - As)/Ac \times 100$$

Ac: absorbance of control (DPPH solution), As: absorbance of sample/standard. $IC_{50}$ was calculated by plotting the graph between DPPH % inhibition and concentrations of *G. lutea* extract and standard[23].

### 2.8. Ex vivo AChE inhibition assay

The AChE inhibitory activity was performed according to the Ellman method. Mice brains were homogenized in 0.1 M phosphate buffer (pH 7.4) containing 1% Triton X-100 and then centrifuged at 15 000 *g* at 4 ℃ for 20 min. The collected supernatant was used as an enzyme source. The protein content of the tissue homogenate was estimated by Lowry's method. The hydrolysis rate of AChE was monitored spectrophotometrically. The enzyme solution 50 μL was added with 3 μL of the GLE and Physostigmine as a standard in a microplate and incubated for 30 min at 37 ℃. After incubation, 20 μL of 10 mM DTNB, 20 μL of 30 mM acetylthiocholine, and 160 μL of phosphate buffer were added, and immediately taken at 405 nm. The percentage inhibition of AChE was calculated using the formula:

$$\% \text{ Inhibition} = (Ac - As)/Ac \times 100$$

Ac: absorbance of control, As: absorbance of sample/standard. $IC_{50}$ was calculated by plotting the graph between enzyme % inhibition and concentrations of GLE and standard[24].

### 2.9. Experimental animals

The Swiss albino male mice weighing 20-30 g were obtained from the CPCSEA approved animal house of Raj Kumar Goel Institute of Technology (Pharmacy). Male mice were used to minimize the hormonal fluctuation associated with the oestrous cycle, which can affect the neuro-behavioural outcomes. These animals were housed in controlled conditions of temperature (22±3) ℃, relative humidity between (55±10)%, and a 12 h light/dark cycle with free access to water and a standard laboratory diet pellet. The animals were taken care of as per the CPCSEA guidelines.

### 2.10. Ethical approval

The study protocol was duly approved by the Institutional Animal Ethical Committee (IAEC) of Raj Kumar Goel Institute of Technology (Pharmacy), under protocol number RKGIT/IAEC/2022/02, in December 2022.

### 2.11. In vivo studies

#### 2.11.1. Acute oral toxicity study

The acute oral toxicity of GLE was performed using the limit test as per OECD guidelines 425. A total of five female mice were used in the toxicity study. Initially, one animal is administered a single dose of GLE 2 000 mg/kg orally and observed for 48 h. In the absence of mortality, the same dose was administered to other animals. All the animals were monitored for any clinical signs, body weight changes, and survival for a 14-day observation period[25,26].

### 2.11.2. Experiment protocol for scopolamine–induced memory impairment in the mouse model

After one week of acclimatization, animals were divided into 6 groups, with each group consisting of 6 animals. Freshly prepared scopolamine 2 mg/kg in saline was administered intraperitoneally daily for four weeks to induce amnesia in all groups except the control group.

Group Ⅰ (Control group): Mice treated with normal saline 10 mL/kg *p.o.*; Group Ⅱ (Scopolamine group): Mice treated with scopolamine 2 mg/kg *i.p.*; Group Ⅲ (Donepezil as a standard): Mice treated with donepezil 5 mg/kg *p.o.*, followed by administration of scopolamine 2 mg/kg *i.p.* after 30 min; Group Ⅳ (GLE 100 mg/kg): Mice treated with GLE 100 mg/kg *p.o.*, followed by administration of scopolamine 2 mg/kg *i.p.* after 30 min; Group Ⅴ (GLE 200 mg/kg): Mice treated with GLE 200 mg/kg *p.o.*, followed by administration of scopolamine 2 mg/kg *i.p.* after 30 min; Group Ⅵ (GLE 400 mg/kg): Mice treated with GLE 400 mg/kg *p.o.*, followed by administration of scopolamine 2 mg/kg *i.p.* after 30 min; GLE and donepezil were administered orally 30 minutes before scopolamine injection for four weeks.

In the last week of treatment, the behavioral parameters were assessed after 30 minutes of scopolamine administration through the Morris water maze (MWM) test and passive avoidance learning (PAL) test. At the end of the treatments, animals were euthanized as per CPCSEA guidelines, and the brain sample was collected for biochemical, immunohistochemistry, and histopathological studies (Figure 1).

### 2.11.3. Behavioral study

The remarkable features of AD are the deterioration of learning, memory, and cognitive function. In the present study, the MWM test and PAL test were conducted for the assessment of learning and memory.

#### 2.11.3.1. MWM test

MWM is used to evaluate spatial learning and memory. The MWM is a black, circular pool made of PVC, with a diameter of 180 cm and a height of 50 cm. The circular pool was filled with water at (25±3)℃ to a 30 cm height. The pool was divided into four quadrants (Q1, Q2, Q3, Q4). One quadrant was fixed as the target quadrant, in which a 12 cm square (escape platform) was kept 1-2 cm below the water level throughout the experiment. The experiment was performed in two stages: acquisition and probe test. The animals were habituated to the environment one day before the acquisition phase. An acquisition test was performed for four consecutive days. Each animal underwent four trials per day from a randomized start position for 120 s to search for the hidden escape platform.

If the animal was unable to search, it was directed towards the platform and permitted to remain on it for 25 s to observe the surroundings. The escape latency time (ELT) was recorded and learning index was evaluated based on the progressive reduction in ELT over the successive days. On the 5th day, a probe test was conducted where the platform was removed and the water was made opaque by adding milk. Animals were allowed for 120 s to locate it, and the time spent in the target quadrant (TSTQ) was recorded as the index of retrieval memory[27].

#### 2.11.3.2. PAL test

PAL test was used to assess the learning and memory of the animal to avoid the aversive stimulus (electric shock). The PAL test was carried out in an apparatus that consisted of light and dark chambers separated by a guillotine door, and a 1 mA electric shock was supplied in the dark chamber.

Firstly, all experimental groups were habituated with the apparatus before the experiment. On the first day, two trials of habituation were conducted to familiarize animals with the apparatus. On the second day acquisition phase was conducted by placing the animal in the illuminated chamber and opening the doors for 30 s. After the animals entered the dark chamber doors were closed and an electric shock (1 mA ) was supplied for 10 s. After that, the animals were placed back in their home cages.

Finally, on the third day, the retention trial was conducted the same as the acquisition trial, but without supplying an electric shock. The maximum time allocated was 300 s (60-300 s), and mice that did not enter the dark field within this time were recorded to have latencies of 300 s. Step-through latency time (sTL) for both acquisition and retention was recorded[28].

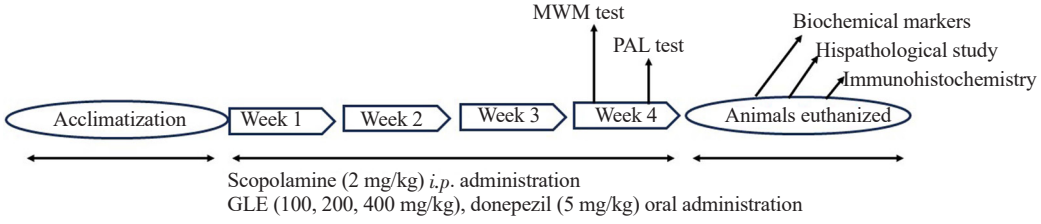


**Figure 1.** Schematic presentation of animal experimental design. MWM: Morris water maze; PAL: passive avoidance learning; GLE: hydro-alcoholic extract of *Gentiana lutea* roots.

#### 2.11.4. Biochemical estimation

Mice were euthanized following CPCSEA guidelines, after which the brain was immediately removed and washed with normal saline. Biochemical parameters were assessed utilizing the supernatant of tissue homogenate prepared in 0.1 M phosphate buffer, pH 7.4.

For the histopathological examination brain hippocampus was preserved in 10% neutral formalin solution[29].

#### 2.11.5. Lipid peroxidation assay

Lipid peroxidation is an indicative biomarker of oxidative stress. The product of lipid peroxidation, malondialdehyde (MDA), was estimated using the TBARS assay as described by the Ohkawa method 1979. In 0.2 mL tissue homogenate, 0.2 mL of 8.1% sodium dodecyl sulphate was added, followed by 1.5 mL of 8% thiobarbituric acid, 1.5 mL of 20% glacial acetic acid and volume was adjusted up to 4 mL with distilled water. The resultant mixture was incubated at 95 ℃ for 1 h. After incubation, the mixture was allowed to cool, after which 5 mL of *n*-butanol:pyridine (15:1) was added. The mixture was centrifuged at 3 000 rpm for 10 min, and the absorbance of the separated pink layer was measured at 532 nm. 1,1,3,3-tetramethoxypropane is used as a standard reference, and lipid peroxidation is expressed as nM of MDA/mg of protein[30].

#### 2.11.6. Estimation of reduced glutathione (GSH) level

The GSH estimation was conducted by the method described by Ellman. The tissue homogenate was centrifuged at 2 000 rpm for 10 min with an equal volume of 10% trichloroacetic acid. Subsequently, to the 0.2 mL of the obtained supernatant, 2 mL of 0.3 M disodium hydrogen phosphate and 0.25 mL of 0.001 M freshly prepared DTNB were incorporated. DTNB was freshly prepared in 1% sodium citrate. At 412 nm absorbance of the mixture was recorded. The standard curve of reduced GSH was plotted, and results were expressed as nM/mg of tissue[31].

#### 2.11.7. Immunohistochemical staining

The protein expression of Aβ and p-tau in the hippocampus of mouse brains was detected by immunohistochemical staining. It was performed on paraformaldehyde-fixed, cryoprotected brain sections. These sections underwent antigen retrieval in citrate buffer at 95 ℃ for 15 min and incubated with primary antibody, anti-beta amyloid antibodies, catalog number BSB-3444 (dilution 1:150), and anti-p-tau antibodies, catalog number BSB-3807-01(dilution 1:100), at 4 ℃ overnight, followed by HRP-labeled secondary antibodies. After that, DAB staining was performed, and sections were counterstained with hematoxylin. The accumulation of Aβ and p-tau was quantified using ImageJ software[32].

#### 2.11.8. Histopathology of the mouse brain

The animal brains were isolated and fixed in 10% neutral buffer formalin for histopathological examination. These were embedded in paraffin blocks and cut into 5 μm sections. Then these sections were stained with hematoxylin and eosin (H&E) and observed under the microscope (400×)[33].

### 2.12. Statistical analysis

All the data were expressed as mean±SEM ($n$=6). Statistical analysis was performed using GraphPad Prism 10.4.1. The data were analyzed by one-way analysis of variance (ANOVA) and two-way ANOVA, followed by Tukey's multiple comparison *post-hoc* test. The threshold for statistical significance was set as $P$<0.05.

## 3. Results

### 3.1. Phytochemical screening

The GLE showed the presence of phytochemical constituents such as carbohydrates, alkaloids, glycosides, phenolic compounds, flavonoids, tannins, and saponins.

### 3.2. Total phenolic and flavonoid contents

The phenolic content in GLE was calculated in terms of gallic acid equivalents using the equation y=0.007 3χ+0.092 8, $R^2$=0.994 2, obtained by plotting the calibration curve for gallic acid as a standard. The flavonoid content was calculated in terms of quercetin equivalent using the equation y=0.005 2χ+0.146 7, $R^2$=0.993 7, obtained by plotting the calibration curve for quercetin as a standard. The total phenolic and flavonoid contents were found to be (47.05±1.64) μg/mg as gallic acid equivalent and (37.17±2.18) μg/mg as equivalent to quercetin, respectively.

### 3.3. GC–MS analysis of GLE

The GC-MS analysis was performed to identify the bioactive chemical constituents in the GLE. The GC-MS spectrum shown in Figure 2 indicates the presence of a total of 25 phytochemicals, which were identified based on the retention time and the NIST library. Among these compounds, three compounds named as mollugin trimethylsilyl ether, 1,2,3-benzenetriol, and 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl, as shown in Table 1, have previously been reported to possess neuroprotective and antioxidant potential. The first compound was mollugin, trimethylsilyl ether, which was tested at a retention time of 13.848 min. Its molecular formula was $C_{20}H_{24}O_4Si$, its molecular weight was 356, and its mass [$M^+$] of 298. The characteristic fragments were *m/z* 73, 167, 237, and 341.

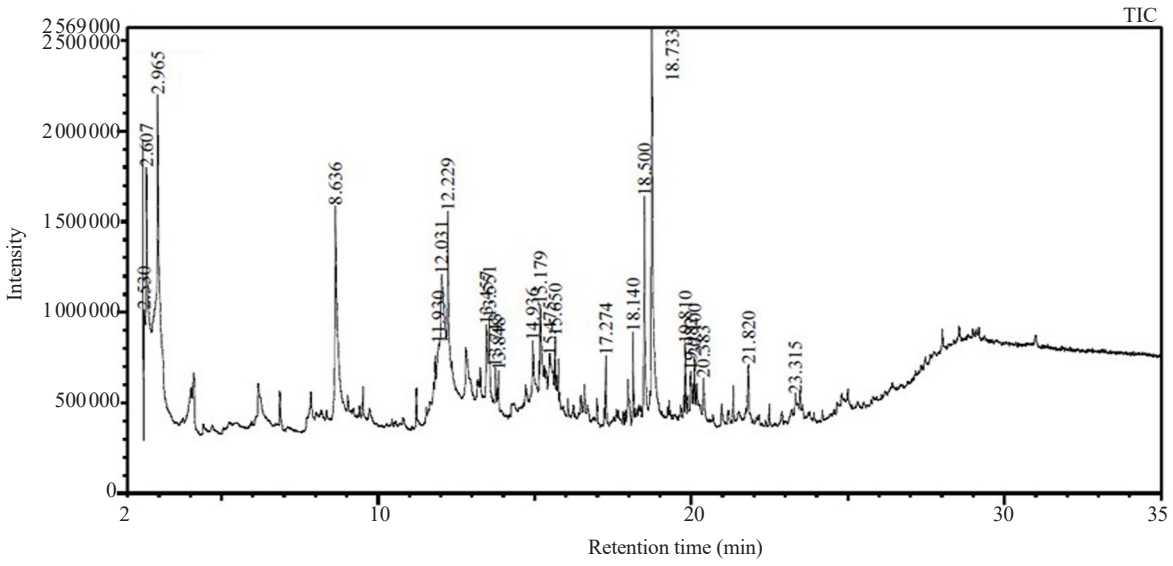


**Figure 2.** GC-MS spectra showing the peaks of chemical constituents present in hydro-alcoholic extract of *Gentiana lutea* (GLE).

**Table 1.** GC-MS analysis of hydro-alcoholic extract of *Gentiana lutea* showing the presence of three bioactive compounds.

| S. No. | Name of compounds | Molecular formula | Molecular weight | Retention time (min) | Area | Area % | Height |
|---|---|---|---|---|---|---|---|
| 1. | Mollugin, trimethylsilyl ether | $C_{20}H_{24}O_4Si$ | 356 | 13.848 | 517763 | 1.17 | 210664 |
| 2. | 1,2,3-Benzenetriol (Pyrogallol) | $C_6H_6O_3$ | 126 | 12.031 | 4514712 | 10.23 | 606230 |
| 3. | 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl (DDMP) | $C_6H_8O_4$ | 144 | 8.636 | 4943824 | 11.20 | 1062080 |

The second compound was 1,2,3-benzenetriol, which is known as pyrogallol, with the molecular formula of $C_6H_6O_3$, a molecular weight of 126, and a retention time of 12.031 min. The peak at 12.031 min was associated with a mass [$M^+$] of 219.

The third compound was 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl, which has a molecular formula $C_6H_8O_4$ and is also known as DDMP, having a molecular weight of 144, and a retention time of 8.636 min. The peak at 8.636 min had a mass [$M^+$] of 289. The daughter ion spectra of these compounds (inserts) revealed the characteristic fragments at *m/z* 43, 55, 72, 101, and 144.

### 3.4. In vitro antioxidant activity of GLE

The GLE exhibited a significant DPPH radical scavenging activity (Figure 3A). The $IC_{50}$ value was calculated as 76.68 μg/mL for GLE and 56.26 μg/mL for ascorbic acid, indicating that GLE possesses less antioxidant activity than ascorbic acid but has a significant antioxidant potential.

### 3.5. Effect of GLE on AChE inhibition

The findings suggested that GLE exhibited a dose-dependent inhibition of AChE activity. The $IC_{50}$ value of GLE and physostigmine was found to be 36.58 μg/mL and 9.45 μg/mL, respectively (Figure 3B).

### 3.6. Acute oral toxicity study

No mortality and toxicity symptoms were observed on administration of GLE at 2000 mg/kg. Based on these findings, doses ranging from 1/20th to 1/5th of the limit dose were selected to evaluate the dose-dependent therapeutic potential of the GLE.

### 3.7. Effect of GLE on spatial learning ability in the MWM

On the first day of the trial, all the groups had time to locate the hidden platform, and no significant difference was observed among the groups. In the subsequent trials, a significant reduction in ELT ($P$<0.05) was observed in all groups except the scopolamine group on different days. Except for the GLE 100 mg/kg group, the ELT was significantly shortened in the treatment groups compared with the scopolamine group ($P$<0.001) (Figure 4A).

A probe trial was conducted on the 5th day of the trial to assess the spatial memory. During this session, TSTQ and the number of crossings across the platform were recorded. Except for the GLE 100 mg/kg group, TSTQ was significantly increased in the treatment groups compared with the scopolamine group ($P$<0.05) (Figure 4B).

The number of crossings was significantly reduced ($P$<0.001) in the scopolamine group than in the control group. Except for the GLE 100 mg/kg group, groups treated with donepezil ($P$<0.001) and GLE ($P$<0.05) showed a significant increase compared to the scopolamine group (Figure 4C).

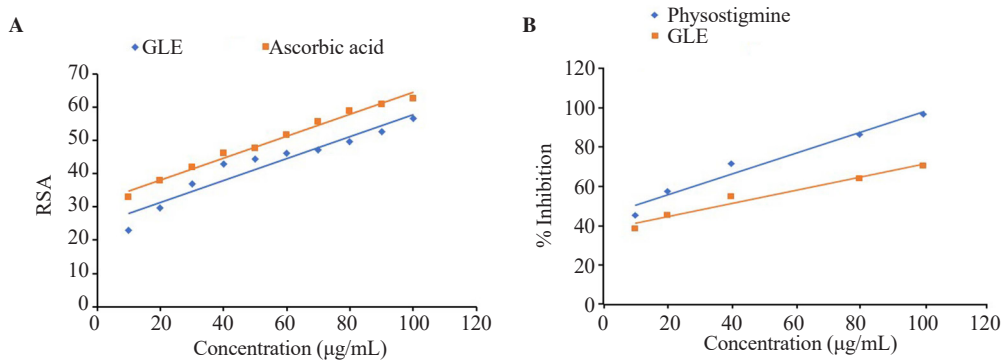


**Figure 3.** (A) *In vitro* antioxidant property. (B) *Ex vivo* acetylcholinesterase inhibition. RSA: radical scavenging activity.

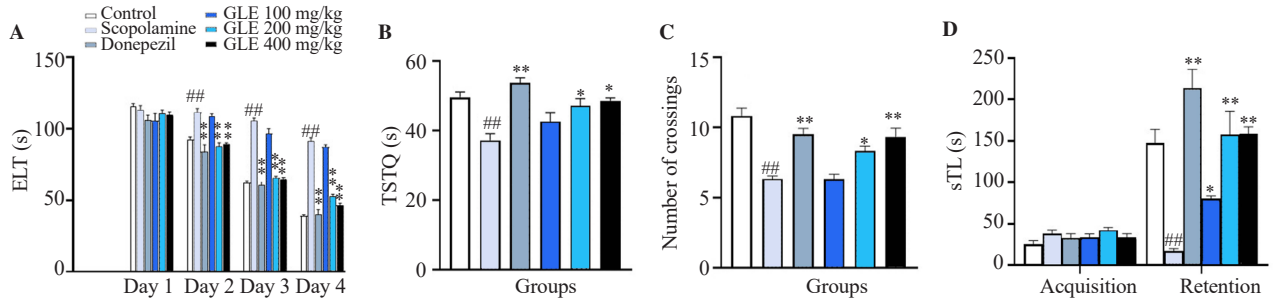


**Figure 4.** Effect of GLE on behavioral studies in scopolamine-induced memory impairment model. (A) Escape latency time (ELT), (B) time spent in the target quadrant (TSTQ), (C) number of crossing, (D) step-through latency time (sTL). All values are expressed as mean ± SEM ($n$=6) and analyzed by one-way and two-way ANOVA followed by Tukey's multiple comparison *post-hoc* test. $^{*}P$<0.05, $^{**}P$<0.001 compared with the scopolamine group. $^{\#\#}P$<0.001 compared with the control group.

### *3.8. Effect of GLE on the ability of learning and memory using PAL test*

Learning and memory were evaluated by comparing the sTL time on acquisition and retention trials among the groups (Figure 4D). Results showed no significant difference in the sTL time of the acquisition trial among all the different groups. Nevertheless, on the retention trial, a highly significant latency time reduction was observed in the scopolamine group ($P$<0.001) compared to the control group. However, the latency time was significantly increased ($P$<0.001) in the groups treated with GLE and donepezil compared to the scopolamine group.

### *3.9. Effect of GLE on brain GSH level*

The data indicated that GSH significantly declined in the scopolamine-treated group as compared to the control ($P$<0.001). The level of GSH was improved in the groups treated with donepezil ($P$<0.001), GLE 200 mg/kg, and GLE 400 mg/kg ($P$<0.05) in comparison to the scopolamine-treated group (Figure 5A). GLE 100 mg/kg reduced the GSH level, but this difference was not significant.

### *3.10. Effect of GLE on brain MDA level*

The administration of scopolamine resulted in a significant increase in the MDA levels of brain tissue compared to the control group ($P$<0.05). In the group treated with donepezil as well as those treated with GLE 200 mg/kg and GLE 400 mg/kg, the MDA levels were significantly reduced ($P$<0.001) in comparison with the scopolamine-treated group (Figure 5B). GLE 100 mg/kg decreased the MDA levels, however, this difference was not significant.

### *3.11. Effect of GLE on Aβ deposition and p-tau expression*

Excessive accumulation of Aβ deposition and p-tau overexpression play a crucial role in the pathology of AD. To quantify the Aβ plaque load and expression, an IHC assay was performed. Scopolamine administration significantly increased Aβ deposition and p-tau protein expression as compared to the control group ($P$<0.001) (Figure 6). Furthermore, GLE 200 mg/kg and GLE 400 mg/kg significantly ($P$<0.001) reduced the % area of Aβ overload and p-tau expression in scopolamine-treated animals.

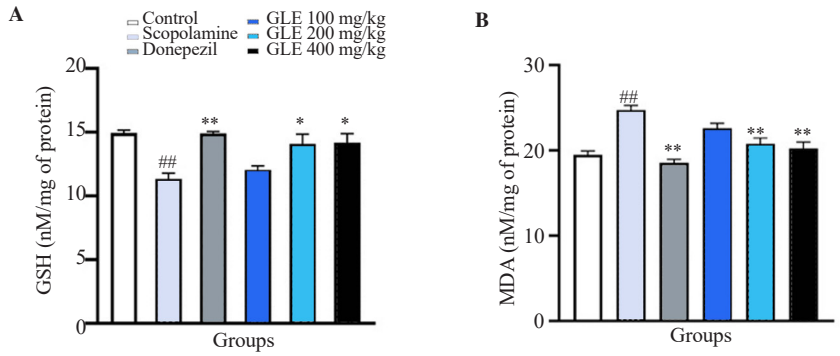


**Figure 5.** Effect of GLE on biochemical markers in scopolamine-induced memory impairment model. (A) Reduced glutathione (GSH), (B) malondialdehyde (MDA). All values are expressed as mean±SEM ($n$=6) and analyzed by one-way ANOVA followed by Tukey's multiple comparison *post–hoc* test. $^{*}P<0.05$, $^{**}P<0.001$ compared with the scopolamine group. $^{\#\#}P<0.001$ compared with the control group.

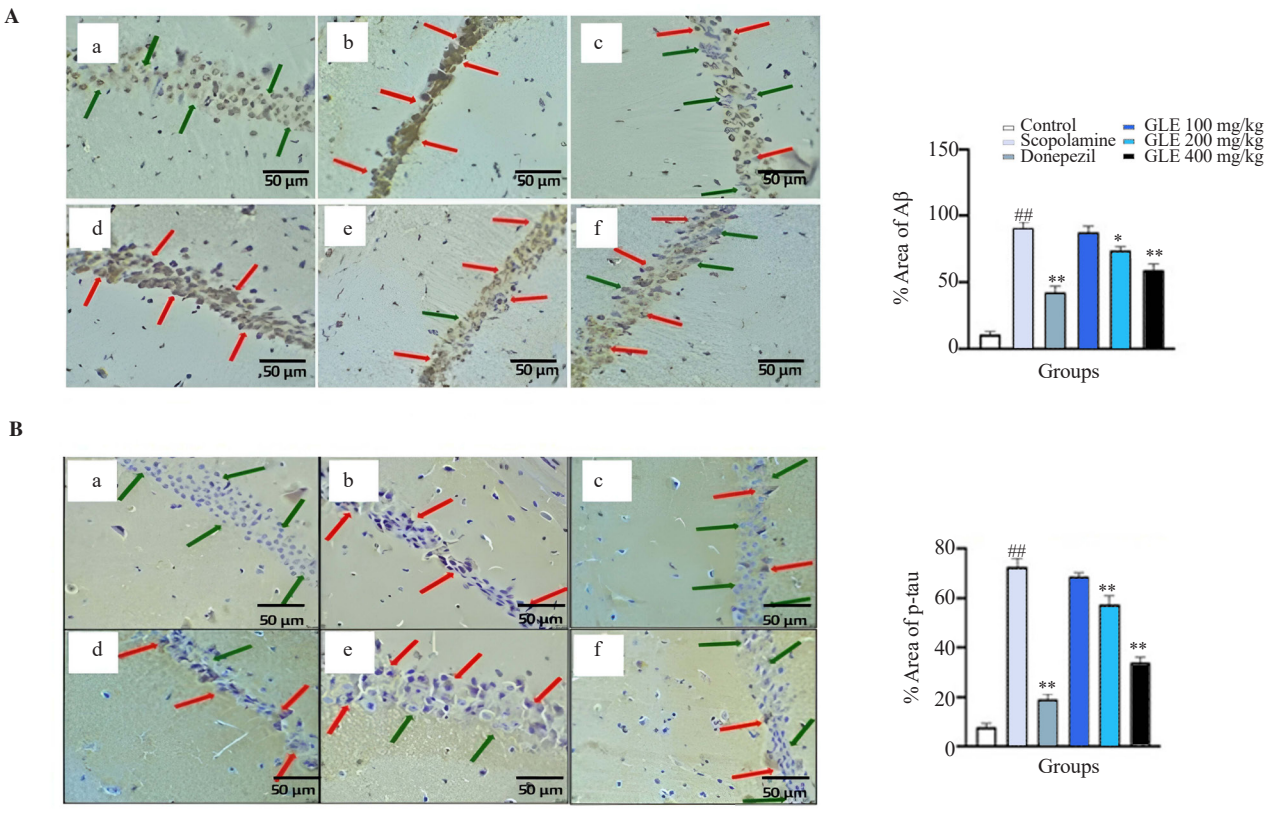


**Figure 6.** Images of immunohistochemistry. (A) Aβ deposition and (B) p-tau protein expression. (a): Control, (b): Scopolamine, (c): Donepezil, (d): GLE 100 mg/kg, (e): GLE 200 mg/kg, (f): GLE 400 mg/kg. $^{*}P<0.05$, $^{**}P<0.001$ compared with the scopolamine group. $^{\#\#}P<0.001$ compared with the control group. Red arrow: Aβ/p-tau accumulation, green arrow: no Aβ and p-tau deposition.

### *3.12. Effect of GLE on the CA1 region of the hippocampus*

Histopathological alterations in the CA1 region of the mice hippocampus were evaluated using hematoxylin and eosin stain. The control group showed normal histological features. However, the scopolamine-treated group exhibited significant neuronal damage, pyknosis, and atopic and apoptotic changes. However, GLE 200 mg/kg and 400 mg/kg significantly reduced the scopolamine-induced neuronal damage (Figure 7).

## 4. Discussion

Memory and cognition deficits are the signature symptoms of AD. The present study aimed to investigate the potential of GLE in scopolamine-induced memory and cognition impairment. GLE was

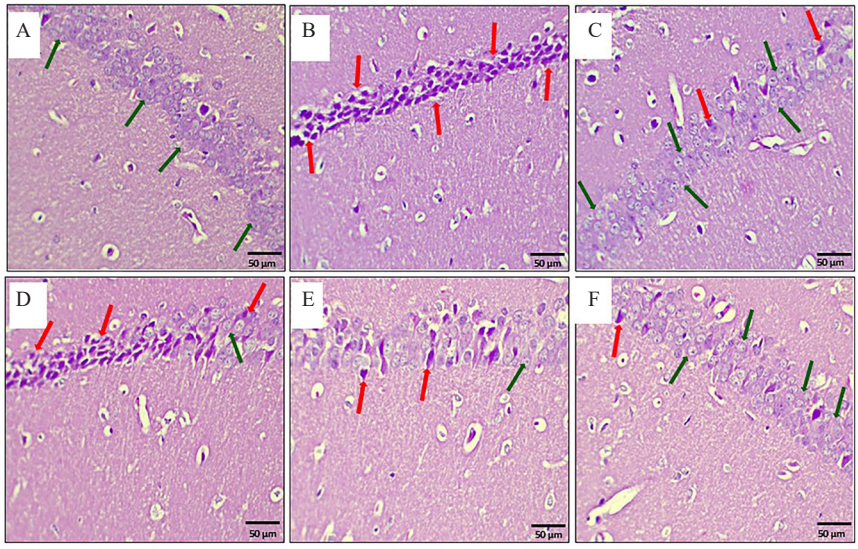


**Figure 7.** Images showing the histopathology of the hippocampus in the CA1 region by H & E staining (scale bar 50 µm, magnification 400×). (A) Control, (B) Scopolamine, (C) Donepezil, (D) GLE 100 mg/kg, (E) GLE 200 mg/kg, (F) GLE 400 mg/kg. The scopolamine group exhibits significant neuronal damage. GLE at 200 mg/kg and 400 mg/kg significantly improves scopolamine-induced neuronal damage. Red arrow: neuronal damage with pyknotic nucleus, green arrow: healthy neurons.

subjected to preliminary phytochemical screening, and the findings showed the presence of flavonoids, phenolic compounds, glycosides, tannins, and alkaloids as reported earlier[34].

As reported in the literature, phenolic compounds and flavonoids exhibit a wide range of pharmacological properties, including strong antioxidant activity, free radical scavenging potential, neuroprotection, anticholinesterase effects, anti-amyloidogenic effects, and inflammation suppression[35]. In the study, the flavonoid and phenolic contents were estimated quantitatively and found in substantial amounts, expressed in the form of quercetin and gallic acid equivalents, respectively.

GC-MS analysis confirmed the presence of three bioactive molecules, mollugin, pyrogallol, and DDMP. It was previously investigated that mollugin could improve cognitive dysfunction in type 2 diabetes mellitus mice by activating the GLP-1R/cAMP/PKA signal pathway[36].

Pyrogallol was reported as an effective inhibitor for AChE in previous research and may ameliorate the pathological changes due to AChE inhibition[37]. The presence of DDMP in GLE may show the potential effect in AD due to the free radical scavenging activity of DDMP, which is also reported earlier in another research work[38].

GLE exhibited DPPH radical scavenging activity of 42.89%, which indicates its ability to neutralize free radicals, though this was lower than that of ascorbic acid. Despite this, GLE still displayed notable antioxidant activity, suggesting the presence of bioactive compounds capable of mitigating oxidative stress, which is important in preventing cellular damage associated with neurodegeneration.

In terms of potency, the $IC_{50}$ value of GLE was estimated as 76.68 µg/mL, which was higher than the $IC_{50}$ of ascorbic acid (56.26 µg/mL), meaning that a higher concentration of GLE was required to achieve 50% radical scavenging. These results indicate that the extract is less potent than ascorbic acid, but it still demonstrates a significant antioxidant activity, which may be attributed to the synergistic effects of the various compounds present in the extract. This suggests that the extract contains bioactive molecules with antioxidant potential that could contribute to overall health benefits. Overall, these results highlight the extract's promising antioxidant activity, which could serve as a valuable source of natural antioxidants.

ACh is a key neurotransmitter that is responsible for signal transduction and neuronal communication concerning memory and learning capabilities. Hyperactivity of AChE depletes the level of Ach, resulting in Ach deficiency that leads to cholinergic disruption and impairment in cognition. The *ex vivo* efficacy of GLE was evaluated, revealing a significant inhibitory effect on AChE with an $IC_{50}$ of (36.58±0.73) µg/mL, thereby enhancing cholinergic communication, which is integral to memory and learning[39].

In the current investigation, scopolamine was used as an inducing agent for memory and learning impairment. It is well documented that scopolamine triggers significant pathological changes in animal models, including increased AChE activity, elevated oxidative stress, neurodegeneration in the brain, and accumulation of Aβ and neurofibril tangle, all of which contribute to memory and cognition impairment[40].

This research explored the potential of GLE and targeted scopolamine-induced pathological changes, such as behavioral changes due to cholinergic disturbance, oxidative stress, and histopathological alternations. The administration of GLE extract at 200 and 400 mg/kg body weight attenuated the scopolamine-induced

memory and cognition impairment in animals. To investigate the neuroprotective effect of GLE on memory and learning MWM test was performed, which assessed the spatial learning capability. GLE significantly improved the scopolamine-induced memory and learning impairment, as evidenced by reducing the ELT and increasing the TSTQ and the number of crossings within the targeted quadrant. These findings suggest that GLE administration improves spatial learning and memory ability. Additionally, a PAL test was also performed to assess the impact of GLE treatment on learning and memory ability. The group treated with GLE showed an elevation in sTL in the retention trial in scopolamine-induced learning and memory deficit.

Several investigations have suggested that increased oxidative stress in the brain may constitute a significant risk factor for AD[41]. Scopolamine induces oxidative stress by enhancing MDA levels, generated from lipid peroxidation, and impairing the antioxidant defense system, such as reduced GSH. Therefore, MDA and GSH levels were measured to assess the oxidative stress in the brain. The current investigation showed a significant rise in MDA level and a reduction in reduced GSH in the scopolamine-treated group. The treatment with GLE (200 and 400 mg/kg) significantly reduced oxidative stress by reducing the brain MDA level and improving the reduced GSH level. Hence, findings provide evidence that the neuroprotective effect of GLE may be linked with its antioxidant action[42].

The hallmark pathological features of AD include the accumulation of Aβ plaque and the formation of neurofibril tangles composed of hyperphosphorylated tau protein[43]. As reported, these abnormal aggregates contribute to neuronal damage, and progressive cognitive decline plays a crucial role in the pathogenesis of AD. Aβ peptide is the metabolic byproduct produced by the APP cleavage. The cleavage of APP by α secretase produces nonamyloidogenic Aβ, while cleavage through β secretase (BACE1) and γ secretase activates the amyloidogenic pathway and generates neurotoxic amyloidogenic Aβ[44]. Aβ accumulation not only contributes to neuronal toxicity but also plays a crucial role in triggering the hyperphosphorylation of tau, which is a microtubule-associated protein essential for maintaining neuronal structure and functions. Normally, tau stabilizes microtubules and intracellular transport. However, insoluble hyperphosphorylated tau, referred to as neurofibrillary tangles, disrupts neuronal cytoskeleton and impairs neuronal communication; consequently, these pathological events progressively decline cognition and memory associated with AD. Scopolamine increases the level of Aβ along with the overexpression of APP mRNA. Additionally, scopolamine upregulates the glycogen synthase kinase 3β, a tau kinase that accelerates the hyperphosphorylation of tau protein, and inhibits ACh synthesis.

In the present study, IHC was performed to find out the Aβ plaque and p-tau expression, and it was observed that the scopolamine-treated group overexpressed the Aβ plaque and p-tau. However, GLE 200 mg/kg and GLE 400 mg/kg notably attenuated the scopolamine-induced Aβ plaque and p-tau expression.

In the present study, histopathological examination revealed substantial neuronal damage in the CA1 region of the hippocampus following scopolamine administration, consistent with previous research[45]. Notably, the findings suggest that GLE 400 mg/kg treatment may exert a neuroprotective effect by enhancing neuronal repair in scopolamine-induced neurotoxicity.

The limitations of the present study are the lack of molecular investigation to elucidate the precise mechanism. Specifically, the study did not include the evaluation of the amyloidogenic enzyme BACE1 and its associated signaling pathways using molecular validation techniques, such as Western blotting. Although the behavioral study and IHC staining findings suggest the neuroprotective potential of GLE, the lack of direct molecular evidence limits the ability to fully elucidate its mechanism in modulating amyloidogenic and tau pathological pathways. Future studies should investigate how GLE affects key proteins like BACE1, APP, and p-tau using molecular techniques to better understand the mechanistic approach for its neuroprotective actions.

In the present study, we explored the effect of GLE on scopolamine-induced amnesia. The treatment of GLE showed a significant reduction of oxidative stress and inhibition of AChE. It also reduced the accumulation of Aβ and p-tau protein. These findings of the study suggest that GLE could be a useful alternative neuroprotective agent for the management of amnesia.

## Conflict of interest statement

The authors declare that there is no conflict of interest.

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## Data availability statement

The data supporting the findings of this study are available from the corresponding author upon request.

## Authors' contributions

TR conceptualized the study, conducted the experimental work, and data analysis, and drafted the manuscript. SM arranged all the necessary resources, supervised the investigation, and reviewed the manuscript. KN critically reviewed and edited the manuscript. All the authors reviewed and approved the final version of the manuscript.

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