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Original Article

Neuroprotective Effect of IQUP-B against Scopolamine-Induced Cognitive Impairment in Wistar Rats

Kezia Mangalampalli[1,#], Aravinda Sai Kolusu[1,#], Hrushik Kowtinya Ramineni[1], Harshitha Mudigonda[1], Surya Devarakonda[2], Pavan Kumar Samudrala[1,*]

[1]Department of Pharmacology, Shri Vishnu College of Pharmacy-Vishnupur, Bhimavaram, West Godavari, Andhra Pradesh, INDIA.
[2]Department of Pharmacology, Mallareddy College of Pharmacy-Dhulapally, Hyderabad, Telangana, INDIA.
[#]*Authors with equal contribution*

ABSTRACT

Background: Cholinergic neurons in the brain and hippocampus appear to be most affected in the early stages of Alzheimer's Disease (AD). Cognitive impairment has been induced in rodents for many years with the muscarinic receptor antagonist scopolamine. In this work, we assessed the polyherbal formulation extract's ability to protect against scopolamine induced cognitive impairment and oxidative stress. **Materials and Methods:** Scopolamine (3 mg/kg, i.p.) was given to rats 1 hr after pre-treatment with IQUP-B (400 mg/kg, p.o.,). The NORT, Y-maze, and Morris water maze tests were used to assess memory performance after 30 min of the scopolamine injection. On the sixteenth day of the study, the rats were sacrificed, and the hippocampus was isolated to estimate the levels of cholinergic enzymes and oxidative stress markers. **Results and Discussion:** Memory impairment was indicated by a deficit in percentage alternation behaviour in Y-maze ($p<0.05$), discrimination index, escape latency and overactive cholinergic enzymes. Furthermore, the pre-treatment with polyherbal formulation improved decline and suppressed the cholinergic enzyme activity. Additionally, polyherbal formulation mitigated the effects of scopolamine injection i.e., a rise in lipid peroxidation, nitrite production, and a decrease in glutathione and superoxide dismutase levels. **Conclusion:** These results demonstrated that polyherbal formulations can be used as an adjuvant therapy against Alzheimer's complications by protecting the cholinergic neurons and maintaining antioxidant defence.

Keywords Scopolamine, Cognitive impairment, Antioxidant defence, Polyherbal formulation.

Correspondence:
Dr. Pavan Kumar Samudrala
Professor, Department of Pharmacology, Shri Vishnu College of Pharmacy, Vishnupur, Bhimavaram, West Godavari-534202, Andhra Pradesh, INDIA.
Email: pavanniper199@gmail.com, pavankumar.s@svcp.edu.in

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INTRODUCTION

Alzheimer's Disease (AD) is a neurological illness characterised by cognitive impairment, aberrant behaviour, memory loss and cholinergic neuronal loss (Selkoe, 2001). Despite being one of the most common neurodegenerative disorders, even after all these years the core etiology of the disease remains enigmatic in nature with multiple pathological indices. As per the World Health Organization, about 50 million people are suffering from dementia in the world. Furthermore, the global incidence of dementia is expected to rise to 82 million by 2030, with AD accounting for about 60-70% of the increase in cases (Qiu *et al.*, 2009).

Amyloid Beta (Aβ) plaques and neurofibrillary tangles are the main contributors to cognitive decline and neuronal cell death (Braak and Braak, 1998; Ju and Tam, 2022). Aβ and tangles are most commonly get accumulated in regions of the brain involved in learning and memory such as hippocampus (Ju and Tam, 2022; Wenk, 2003).

The original source for the formation of amyloid plaques starts at APP (Amyloid Precursor Protein). The normal metabolism of APP involves it getting cleaved by alpha secretase but in the case of AD pathogenesis, the irregular cleaving of the APP with beta secretase and gamma-secretase is the reason for the formation of amyloid plaques (Mattson, 2004).

The pathogenesis of AD involves many variables with the most common being the cholinergic system where the levels of AChE (Acetylcholinesterase) are crucial for carrying out the synaptic functions properly. Nonetheless, the progression of the disease is associated with diminished levels of AChE (Braak and Braak, 1996; Dickson, 1997; Gahtan and Overmier, 1999; Van Gassen and Annaert, 2003).

As per the amyloid hypothesis which is cemented for over 25 years the main factor contributing for the pathogenesis of the disease is the Aβ plaques. It is associated with the formation of Reactive Oxygen Species (ROS) and leading to the progression

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of AD (Hampel *et al.*, 2018; LIN *et al.*, 2001). Oxidative stress contributes to the progression of Alzheimer's disease further by upregulating the Aβ and p-tau formation (Gordon *et al.*, 2016).

Oxidative stress is often associated with the triggering of neuronal inflammation as evidenced by elevated levels of inflammatory mediators like Nuclear Factor kappa (NF-κB). It is a key transcription factor for further downstream regulation of proinflammatory cytokines such as Interleukin-1 (IL-1), Interleukin-6 (IL-6), Tumour Necrosis Factor-α (TNF-α) in the brain (Aliev *et al.*, 2002; Ramassamy *et al.*, 1999).

The current therapies for the management of AD include AChE inhibitors which are most typically used for the management of symptoms of AD. Drugs such as donepezil and rivastigmine are routinely used for the management of the disease (Aisa *et al.*, 2007). However, these drugs do not tackle the root cause of the problem and only provide temporary alleviation of the symptoms. Moreover, the use of these drugs always comes with a cost, such as hepatotoxicity, nausea, diarrhoea, and other side effects may arise along the therapy (Balazs and Leon, 1994).

As a result, there is an urgent need for novel formulations and medications that are more efficient and safer to use. The illness's intricacy makes it more challenging to develop medications that are both safe and effective in suppressing the pathological abnormalities of Alzheimer's disease (Hippius and Neundörfer, 2003).

Plants and herbs have been used for centuries in traditional therapies to treat a variety of human diseases. Traditional medicine is defined by the World Health Organization as "knowledge, skills, and practices based on different cultures' theories, beliefs, and experiences used to maintain health and prevent, diagnose, improve, or treat physical and mental illness" (Fatima *et al.*, 2022a; Rajendran *et al.*, 2020). In the current study, we used a polyherbal formulation with the composition of Brahmi (enhancement of memory), Shankhapushpi, green tea (immunity booster enhancement of metabolic rate), Ashwagandha (anxiolytic, antihypertensive), Misreya, Atasi (anti-inflammatory, antioxidant), Haldi (anti-inflammatory, immunity booster), Tulasi (antioxidant, immunity booster) and Lavanga (immunity booster). All the mentioned ingredients possess some degree of antioxidant and anti-inflammatory activity which ultimately contribute to countering the pathogenesis of AD.

This formulation is well combined for maintenance and promotion for growth and development. In large-scale research and clinical studies, the Bacopa monnieri (Brahmi) extract has been reported for its nootropic, antioxidant, anti-microbial, and analgesic activity (Fatima *et al.*, 2022b).

Scopolamine, a muscarinic cholinergic receptor antagonist causes the downregulation of cholinergic system and is widely used to assess cognitive deficits in experimental animals. Indeed, after scopolamine administration, cholinergic neurotransmission was blocked, resulting in impaired cognitive performance and cholinergic dysfunction in animals (Flood and Cherkin, 1986). Recently, it was reported that scopolamine administration caused cognitive impairment in rats, upregulation of amyloid plaques which was associated with altered oxidative stress status (Khakpai *et al.*, 2012).

In this present study we aim to investigate behavioral conservation and recovery in rodents along with the improved status of cholinergic transmission and suppression of inflammatory mediators.

MATERIALS AND METHODS

Chemicals

Scopolamine and Donepezil were purchased from yarrow chemicals (Mumbai). Polyherbal formulation – IQUP-B, Acetylthiocholine Iodide (ACTCI), 2,2-Diphenyl-2-Picrylhydazyl (DPPH), 5,5'-Dithiobis-2-Nitrobenzoic acid (DTNB), bovine serum albumin, Bradford's reagent etc., were purchased from sigma Aldrich.

Collection and preparation of polyherbal formulation

The formulation is composed of Brahmi, shankhapushpi, green tea, ashwagandha, misery, Atasi, Tulasi, Haldi, Lavanga, sugar and permitted levels of preservatives and excipients. The final powdered form of plant products was used to prepare the required concentrations of the formulation. This polyherbal formulation was developed and named as IQUP-B by Herbindo Labs Pvt. Ltd., Hyderabad.

Animal housing

Adult male wistar rats weighing between 220-250 g were procured from Vyas Laboratories, Hyderabad. The animals were acclimatised for one week in the animal house facility of Shri Vishnu College of Pharmacy in Bhimavaram (439/PO/S/01/CPCSEA). All the animals were maintained under the standard husbandry conditions mentioned in CPCSEA. After one week, the animals were divided into four groups and each group containing 6 animals. The protocol for the study was approved by the Institutional Animal Ethics Committee (04/IAEC/SVCP/2022).

Experimental design

The animals were randomly divided into four groups namely Group A (normal control group treated with 1% CMC, p.o.), Group B (disease control group treated only with scopolamine 3 mg/kg/day, i.p.), Group C (standard group which receives Donepezil 3mg/kg/day, p.o. and scopolamine 3 mg/kg/day, i.p.), and Group D (treatment group which receives IQUP-B 400 mg/kg/day, p.o. and scopolamine 3 mg/kg/day, i.p.).

The total study lasted for 16 days. Group 3 and 4 was pre-treated with donepezil and IQUP-B for 16 days, respectively. From day 8 to 15, behavioural tests were carried out by giving scopolamine 30 min before the study and the polyherbal formulation was given 1 hr before the scopolamine injection. On the 16th day after completing all the behavioural tests, rats were euthanized under ketamine anaesthesia and hippocampus was isolated and homogenised to determine biochemical parameters such as BDNF expression, amyloid-β, acetylcholinesterase, and oxidative stress biomarkers like SOD, MDA, GSH, NO.

Behavioural tests

Novel Objective Recognition (NORT) Test

The NORT maze is made up of black acrylic plastic. This test consists of three phases namely habituation phase, training phase and testing phase. In the habituation phase, animals were permitted to explore the maze for 10 min to minimize the anxiety of the animals. In the training phase, two identical objects with the same dimensions were placed at opposite corners from 10 cm away from the wall and allowed to explore both the objects for 5 min. After 30-min of intersection interval, testing phase was carried out by replacing one of the old objects with a novel object and observing for 5 min. In the testing phase, recorded the time spent by each animal with old and new objects and calculated the Discrimination Index (DI). DI is useful to evaluate the cognitive ability of each animal, and it is calculated by using the formula (DI) is equal to B-A/B+A. Here, A is the time-spent by the animal with old object and B is the time spent by the animal with novel object (Grayson *et al*., 2015; Zhang *et al*., 2012).

Y-maze test

The Y-maze test is used to examine short-term spatial memory of the animal. In this test, memory was evaluated by using spontaneous alternations. Spontaneous alteration is defined as consecutive entry of the animal into all three arms (i.e., BCA, BAC etc.,). Testing takes place in a Y-shaped maze with three arms (A, B, C) with an angle of 120^{o}C each. The animal is in the centre arm (D) and permitted to explore the maze for 8 min. During this time, the number of arm entries and percentage spontaneous alternations of the animal were measured (Kraeuter *et al*., 2019; Ma *et al*., 2007).

Morris Water Maze (MWM) test

MWM is used to evaluate the long-term memory and learning of the animals. It is a round shaped maze with six feet diameter and three feet in height. The maze is virtually divided into four quadrants, and each quadrant is specifically identified by placing the visual cues on the inner wall of the maze. In this test, throughout the study a hidden platform is positioned in a fixed location. During the training phase, the platform must be visible and one inch above the water surface. Each animal is trained for four trials at four different starting locations and allows them to locate the platform by using visual cues. Animals, which were incapable of tracing the platform at the end of training session were excluded from this study. Evaluation phase is conducted for three consecutive days i.e., from day 12 to 14. In this phase, water in the maze was made opaque with milk powder and the platform was submerged 1cm under the water. All the habituated animals were allowed to identify the hidden platform within 60 sec at four distinct drop locations. On day 15th of the experiment, probe test was executed by take out the platform. In this test, time spent by the animal in target quadrant was measured (Mendez *et al*., 2008; Nunez, 2008).

Biochemical parameter estimation

Brain homogenate and sample preparation

After completion of all the behavioural tests, blood was collected by retro orbital method on day 16th of the experiment. Then animals were decapitated by cervical dislocation under ketamine anaesthesia. Brain tissue was harvested from the animals and then hippocampus was isolated. Further, it was homogenised and centrifuged for 20 min at 6000 RPM at 4^{o}C. Then supernatant was collected and stored at -20^{o}C for the estimation of biochemical parameters.

Estimation of acetylcholinesterase activity

Activity of Acetylcholinesterase (AChE) was measured using acetylthiocholine iodide with minor adjustments. In brief 140 µL of PBS, 40 µL of the tissue homogenate, and 10 µL of DTNB was added to the 96 well plate. Then, it is incubated for 10 min at 37^{o}C and 10 µL of starter reagent (ACTCI) was supplemented to the plate. AChE present in the sample cleaves the acetylthiocholine iodide into acetate and thiocholine iodide. It reacts with DTNB and produces the colour. The intensity of the yellow colour was measured at 405 nm using microplate reader (Human Reader HS) for 30 min at every 10 min interval (Ahmed and Gilani, 2009; Pohanka *et al*., 2011; Rinne *et al*., 2003; Wenk *et al*., 2000).

Estimation of nitric oxide

The Griess reagent method was used to measure the nitric oxide levels. In this method, colour was developed after adding the griess reagent to the sample. The intensity of the colour was measured at 540 nm (Malinski, 2007; Togo *et al*., 2004).

Estimation of Superoxide Dismutase (SOD)

SOD activity in hippocampus was quantified by using the pyrogallol auto-oxidation method. This process involves the self-oxidation of pyrogallol in alkaline solutions to produce superoxide anion radicals which are then reduced by SOD. In brief, 50 mM Tris-cacodylate buffer with pH 8.2, 40 µg catalase, 1 mM EDTA, 100 µL of a sample, and the required amount of distilled water were added to make the 2 mL solution. From this reaction mixture 250 µL was transferred to 96 well plate in sample/blank wells. Pyrogallol (2.6 mM) was added just before

measuring the absorbance. Absorbance was measured for 4 min at every 30 sec intervals by using microplate reader at 450 nm. The unit of measurement for SOD activity is U mg-1 of protein (Gsell *et al.*, 1995; Zemlan *et al.*, 1989).

Estimation of GSH

One of the most important antioxidants in cells, glutathione which involved in oxidative free radical detoxification and inhibition of lipid peroxidation. GSH was measured by using Ellman's reagent. The glutathione standards were prepared by using phosphate buffer pH 7.4 to get the final concentrations of 0, 0.5, 1, 2, 4, 8, 12, and 16 mM. From each concentration 80 µL of standard/sample mixed with 60 µL of DTNB and 60 µL of PBS. The intensity of yellow colour produced in standard/samples were measured spectrophotometrically at 412 nm (Barai *et al.*, 2019; Saharan and Mandal, 2014).

Estimation of Malondialdehyde (MDA)

MDA is the end product of lipid peroxidation. Quantification of MDA can be a good measure for understanding the extent of lipid peroxidation in brain or hippocampus. Okawa *et al.*, established a methodology for measuring MDA levels using tetra methoxy propane as standard. In brief, 100 µL of the standard/sample, 750 µL of 0.8% TBA, 750µL of 20% glacial acetic acid, and 100 µL of sodium dodecyl sulphate were combined (8.1%). The resulting reaction mixture was incubated at 95°C for 1 hr. After incubation, samples were cooled and subjected to centrifugation at room temperature for 5 min. 300 µL of supernatant was taken into 96 well plate and absorbance was measured at 532 nm (Aybek *et al.*, 2007; Dib2002, n.d.).

Statistical analysis

Statistical analysis was performed by using Graph pad prism software version - 8.4.2. All the values were expressed as Mean±S.E.M, n=6. Statistical difference between and within the groups was analysed by using one-way followed by Dunnett's *post-hoc* test. (p<0.05) was considered statistically significant. *p<0.05, **p<0.01 and ***p<0.001 versus normal control group. ###p<0.001, ##p<0.01, #p<0.05 versus scopolamine treated group.

RESULTS

Effect of IQUP-B on discrimination index

The NORT was used to calculate cognitive ability of the animal. When compared to Group A, the rats received scopolamine injection showed a significant decrease in the discrimination index (p<0.01). The IQUP-B pre-treatment group differentiated between unfamiliar and novel objects and displayed a greater discrimination index (p<0.05) when compared to group B (Figure 1A).

Effect of IQUP-B on short-term memory

Short-term working memory is evaluated using the Y-maze task. % Spontaneous changes were compared between the groups (Figures 1B, 1C). Spontaneous alternations were significantly decreased in group B when compared to group A (p<0.001). Whereas the animals treated with IQUP-B had shown significant increase in spontaneous alterations when compared to group B (### p<0.001).

Effect of IQUP-B on long-term memory

In this test escape latency is recorded as an index for long term memory. From day one, scopolamine administration significantly reduced its ability to learn (day 1, *p 0.05) and it increased on day 3, ***p<0.001 when compared to group A. IQUP-B attenuates the scopolamine induced memory significantly (p<0.05, ## p<0.01, ### p<0.001). The interday variations across the groups demonstrated a significant increase in escape latency time between day-1 and day-2 ($^{@}$ p<0.05), day-1and day-3 ($^{\$\$\$}$ p<0.001) (Figure 2A). In probe analysis, scopolamine significantly diminishes the long-term memory evidenced by decrease in time spent in

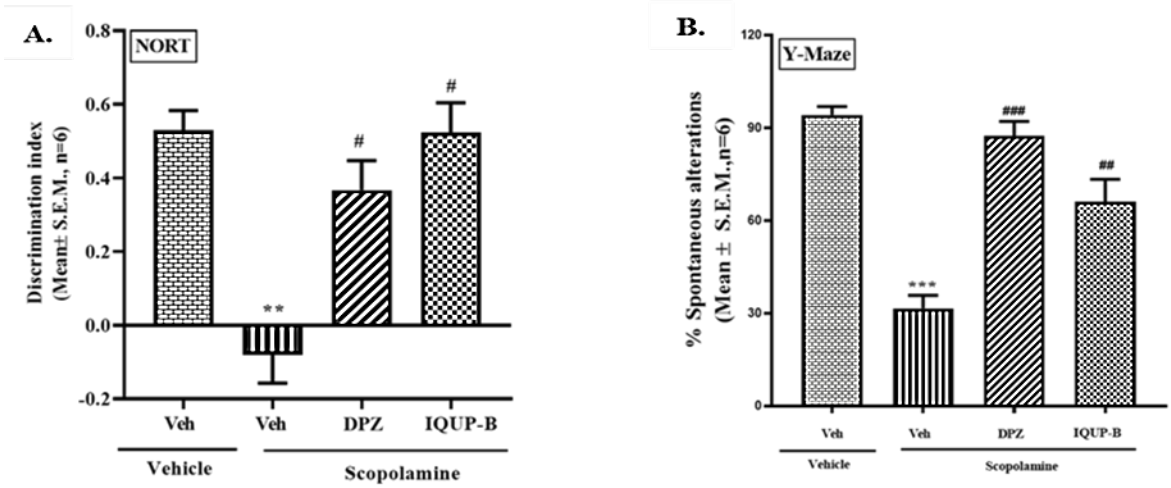

Figure 1: Effect of IQUP-B on discrimination index and short-term memory by using NORT (A) and Y maze (B).

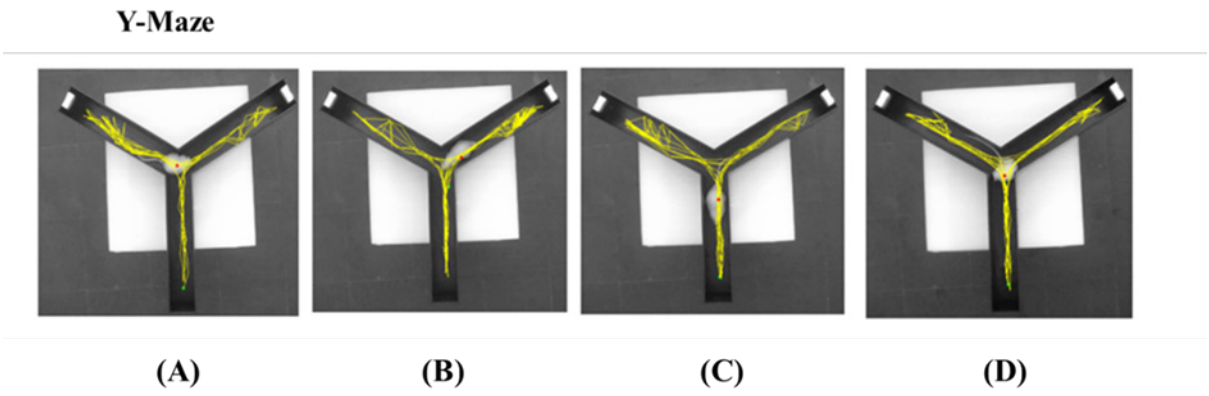

Figure 1C: Tracking's of the animal in Y-Maze. Control Group (A), Diseased group (B), Donepezil group (C), Treatment group (D).

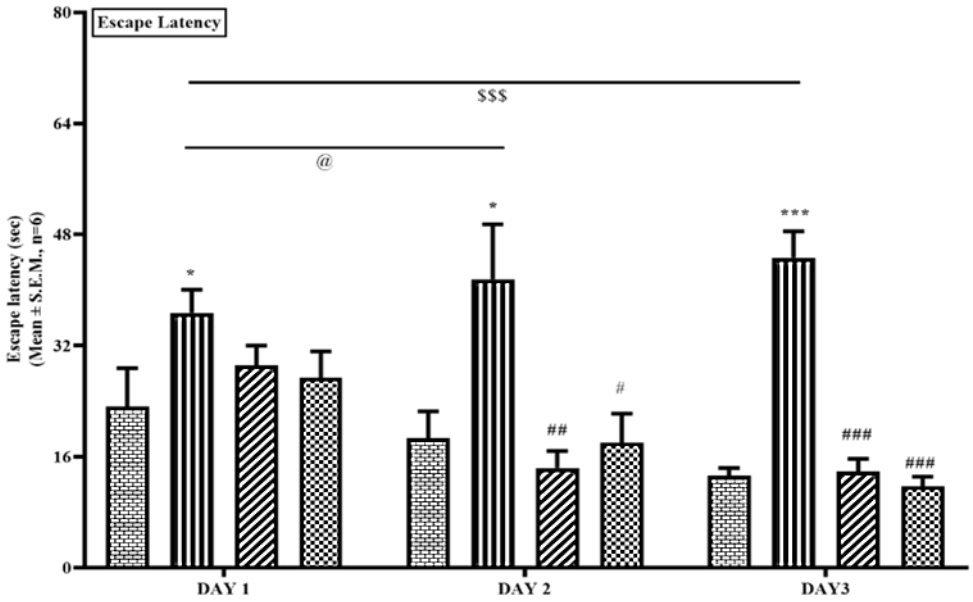

Figure 2A: Effect of IQUP-B on spatial learning in the Morris water maze test. *p<0.05 Vs control group, ## p<0.01 Vs scopolamine alone treated group. $^{@@@}$ p<0.05 Vs-day-2, $^{\$\$\$}$ p<0.001 vs day-3.

target quadrant. But IQUP-B significantly protected the memory decline when compared with group B (Figure 2B). Tracking for the probe the test was shown (Figure 2C).

Effect of IQUP-B on AchE activity

In the hippocampus and plasma, AChE activity in group B rats was considerably higher than group A. One-way ANOVA revealed a significant increase acetylcholinesterase activity in Hippocampus (** p<0.01). In contrast, IQUP-B significantly decreased the AChE activity (p<0.01, #p<0.05) in both hippocampus (Figure 3A) and plasma (Figure 3B) when compared to group B.

Effect of IQUP-B on GSH levels

In the rat hippocampus homogenates, the total content of GSH was measured, and ANOVA revealed significant differences between groups. The levels of GSH were significantly decreased in group B (* p<0.05) when compared to group A. However, Pre-treatment with IQUP-B reduces GSH decline and protects against oxidative stress (p<0.05) when compared to group B (Figure 4A).

Effect of IQUP-B on MDA levels

To elucidate the level of lipid peroxidation, MDA levels were quantified. Statistical analysis revealed a significant increase in MDA levels in group B when compared to group A (##p<0.01). IQUP-B pre-treatment showed a decrease in lipid peroxidation by identifying low MDA (p<0.05) when compared to group B (Figure 4B).

Effect of IQUP-B on SOD levels

The activity of SOD in the hippocampus was measured between the groups. When rats given with scopolamine showed a significant decrease in SOD activity (*p<0.05). The rats pre-treated with IQUP-B had a significant increase in SOD

activity in hippocampus (##p<0.01) when compared with the group B (Figure 4C).

Effect of IQUP-B on nitric oxide

The rats in group B show a substantial surge in nitric oxide (***p<0.001) against group A animals. Pre-treatment with IQUP-B showed significant decrease in level of nitric oxide (###p<0.001) (Figure 4D).

DISCUSSION

The current work adds to our understanding of the possible alternative herbal therapy for Alzheimer's disease using rodent models. In this investigation, we explored the protective outcome of polyherbal formulation against scopolamine prompted cholinergic neuronal damage by scavenging the nitrosative and oxidative free radicals and reducing the activity of cholinergic enzymes that enhance Ach levels which promote cholinergic transmission (Bhuvanendran *et al.*, 2018; Mostafa *et al.*, 2021).

The three well-established memory tasks such as NORT, Y-maze, and Morris water maze test were used. The animals given with scopolamine was showed a significant decline in discrimination index which indicates the memory decline which is consistent to the previous studies (Ponne *et al.*, 2020). In a study conducted by Gupta *et al.*, 2009, the exploring time of the novel item in polyherbal formulation-treated rats was significantly higher than the scopolamine-alone-treated rats (Rajashri *et al.*, 2020) In this study IQUP-B pre-treatment also increases the recognition ability between older and novel object shows that IQUP-B protected the memory decline as per the consideration of previous studies. In Y-maze test spontaneous alternation behaviour has been used to assess spatial working memory. Hritcu *et al.*, (2011) reported that % spontaneous alterations in the y-maze test is decreased by scopolamine administration. In our investigation, scopolamine group showed significant lower spontaneous alterations when compared to control group (Berté *et al.*, 2018). However, oral administration of a IQUP-B improved the spontaneous alternations decline caused by scopolamine when compared with control group and these reports were consistent with previous findings. Escape latency in the MWM task was recorded to investigate the long-term spatial memory of the animal. In

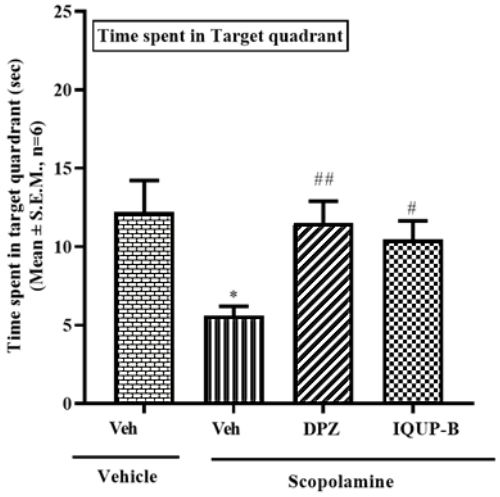

Figure 2B: Effect of IQUP-B on probe trial, the time spent in target quadrant.

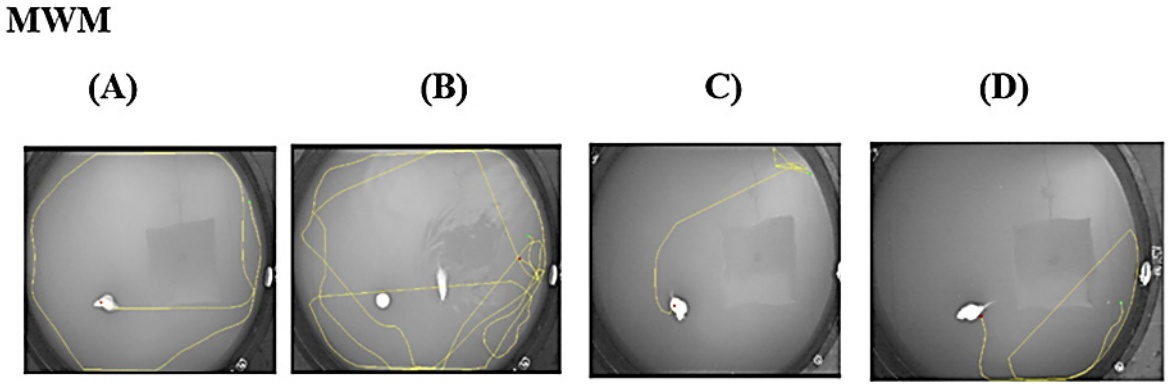

Figure 2C: Trackings of the animals in the MWM test. Control Group (A), Diseased group (B), Donepezil group (C), Treatment group (D).

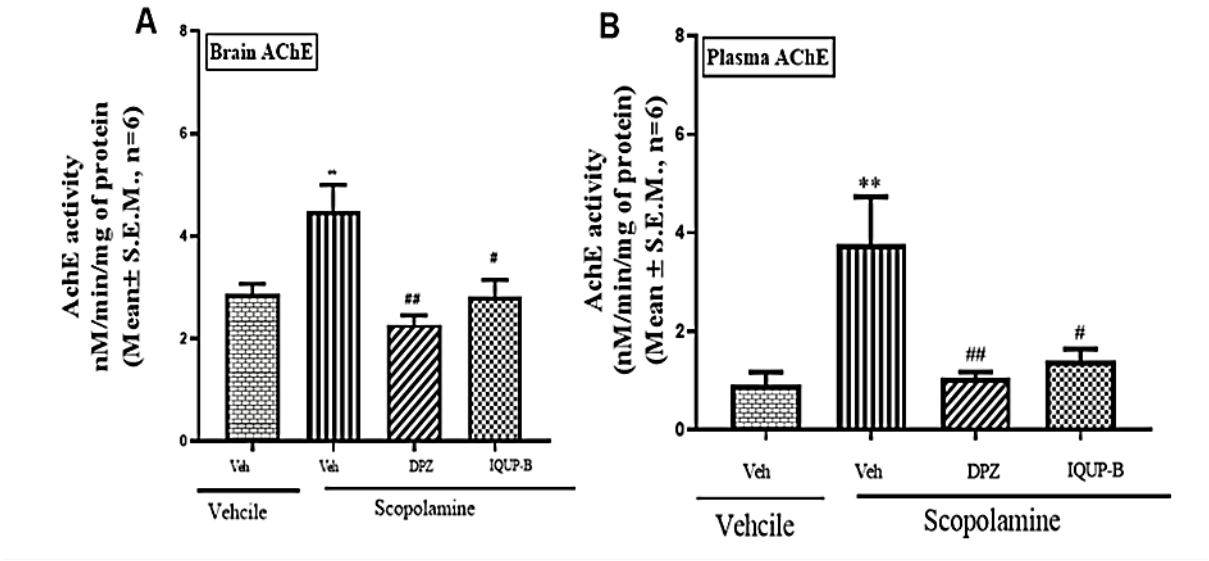

Figure 3: Effect of IQUP-B on AChE activity.

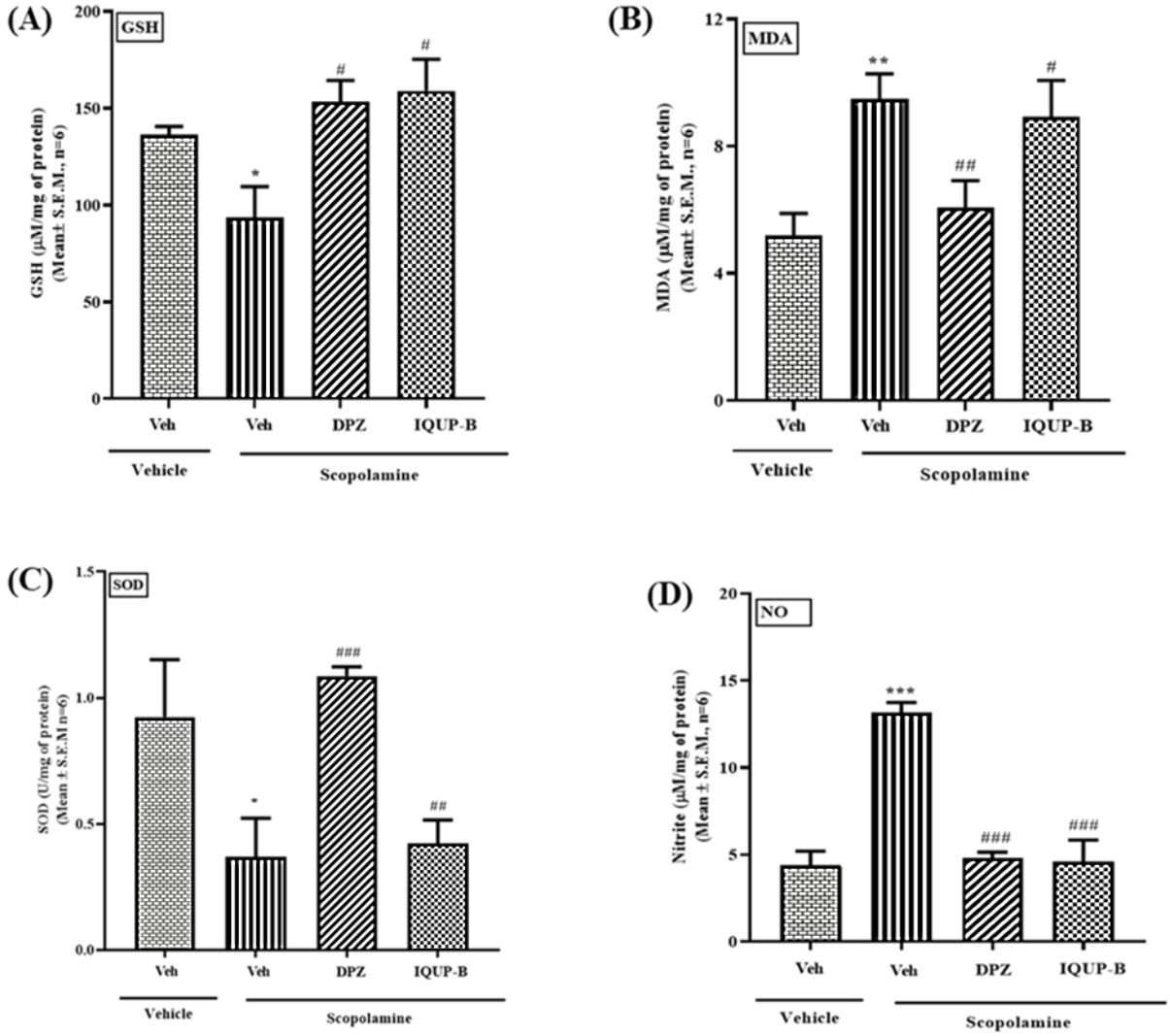

Figure 4: Effect of IQUP-B on GSH, MDA, SOD and NO. GSH (A), MDA (B), SOD (C), Nitrite (D).

literature search, Ishola *et al.*, 2013, opined that scopolamine administration increases the escape latency time when compared to control group (Shabani and Mirshekar, 2018). In the present study scopolamine increased the escape latency whereas, pre-treatment with IQUP-B protected the memory decline caused by scopolamine which is consistent with previous studies indicating improved spatial memory and learning. Furthermore, pre-treatment of rats with IQUP-B increased target quadrant preference than the rats induced with cognitive impairment.

The cholinergic system is essential for learning and memory activities. Cholinergic deficiency is a significant neuropathological characteristic associated with memory loss and is directly related to the severity of cognitive dysfunction in Alzheimer's disease (Blokland 1995). (Fujimori *et al.*, 2022) Inhibiting AChE is the most essential for improving cholinergic function. Scopolamine administration enhanced AChE activity indicating excessive acetylcholine and butyrylcholine hydrolysis. Thus, inhibiting AChE activity could be a promising therapeutic target for the treatment of Alzheimer's disease. In this study IQUP-B pre-treatment significantly attenuates the scopolamine induced enzyme activity in hippocampus which is consistent with the findings of Ijomone and Obi (2013). (Shen *et al.*, 2021) The brain is closely linked to oxidative metabolism and the synthesis of antioxidants enzymes. These enzymes play a crucial role in scavenging free radicals. Both preclinical and clinical investigations have revealed that the imbalance between oxidative free radicals and anti-oxidants during the disease latent period results in the abrupt onset of AD symptoms, including cognitive loss (Sultana *et al.*, 2013) (Alibabaei *et al.*, 2014; Cho *et al.*, 2022). Many researchers stated that increase in oxidative stress is evidenced by increase in lipid peroxidation (MDA), NO and decrease in the activity of SOD and GSH. When our samples were analysed for anti-oxidant capacity and it was found that scopolamine significantly decreases the SOD activity when compared to the control group. Whereas IQUP-B is a polyherbal formulation which showing anti-oxidant as well as anti-inflammatory property and enhanced the levels of GSH and SOD activity significantly (Bae *et al.*, 2020; Foyet *et al.*, 2016; Ishola *et al.*, 2017). On the other hand, it was found that scopolamine enhanced the levels of MDA and NO which increases the oxidative degeneration of cholinergic neurons. IQUP-B pre-treatment neutralises the oxidative free radicals and reduces the extent of lipid peroxidation and NO levels compared to group B.

CONCLUSION

In conclusion, Scopolamine administration caused Alzheimer's-type of dementia in rats by changing the cognitive behaviour of the animals and increasing the AChE level, oxidative stress, and neuro inflammation. This current study demonstrated that pre-treatment with IQUP-B could be able to ameliorate the scopolamine-induced behavioural and biochemical changes in rats. Based on these study results, we can suggest that IQUP-B may become an adjuvant treatment approach which will change the disease progression and improve the quality of life in AD patients.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FUNDING

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ABBREVIATIONS

AD: Alzheimer's disease; **Aβ:** Amyloid beta; **APP:** Amyloid precursor protein; **AChE:** Acetylcholinesterase; **ROS:** Reactive oxygen species; **NF-κB:** Nuclear Factor kappa-beta; **IL-1:** Interleukin-1; **IL-6:** Interleukin-6; **TNF-α:** Tumour Necrosis Factor-α; **BDNF:** Brain Derived neurotrophic factor; **ACTCI:** Acetylthiocholine iodide; **DPPH:** 2,2-Diphenyl-2-picrylhydazyl; **DTNB:** 5,5'-dithiobis-2-nitrobenzoic acid; **OFT:** Open Field Test; **MWM:** Morris Water Maze; **MDA:** Malondialdehyde; **GSH:** Reduced glutathione; **NO:** Nitric Oxide; **SOD:** Super Oxide Dismutase; **IAEC:** Institutional Animal Ethics Committee; **CCSEA:** Committee for control and supervision of Experimental on Animals.

ETHICAL APPROVAL

The animal experimentation protocol of this study was approved by the IAEC-Approved number: 04/IAEC/SVCP/2022.

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