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Original Article

Neuroprotective Effect of *Diplocyclos palmatus* on Aβ (25-35) Induced Alzheimer's Disease in Mice

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ABSTRACT

Objectives: Alzheimer's disease is primarily caused by neurotoxic effects of amyloid beta $A\beta_{(25-35)}$ peptide accumulation and increased levels of Acetylcholinesterase Enzyme (AChE). Acetylcholinesterase inhibitors report for the effective management of cognitive and motor disorders. In the current study the impact of *Diplocyclos palmatus* Methanolic (DPM) seed extract and its Chloroform (DPC) fractions were investigated in mice with Amyloid beta (Aβ)-induced experimental Alzheimer's disease. **Materials and Methods:** Acute toxicity study was performed based on the guidelines of OECD 423 and doses were selected. Mice were administered with standard Donepezil (5 mg/kg/oral) and two doses each of DPM and DPC daily for 21 days (200 mg/kg and 400 mg/kg/oral). Aβ was given by Intra Cerebro Ventricular (ICV) injection in a single dose 3 mg/kg. Cognitive abilities were assessed using the conditioned avoidance test, the rectangular maze and Y-maze. On 22nd day mice were sacrificed, then isolated brain homogenate used for estimation of biochemical parameters such as reduced Glutathione peroxidase (reduced GSH), Malondialdehyde (MDA), nitrite level and AChE levels. **Results:** Administration of DPM and DPC extracts effectively reduces behavioral and biochemical abnormalities in dose dependent way. **Conclusion:** *Diplocyclos palmatus* seeds showed neuroprotective effect on Aβ-induced AD in mice due to their antioxidant and AChE activity.

Keywords: Alzheimer's disease, *Diplocyclos palmatus*, Acetylcholinesterase.

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INTRODUCTION

Alzheimer's disease is a progressive neurological disorder characterized by neuronal loss and extracellular senile plaques results in cognitive difficulties.[1] According to research AD pathogenesis is coupled with an invariant pathogenic cascade triggered by the amyloid beta plaques accumulate in the brain. These plaques formed by the aggregation of Aβ peptides derived from amyloid precursor protein are believed to initiate a cascade of events leading to neuroinflammatory process that may induce neuronal malfunction, cell death and subsequent neurodegeneration.[2] The build-up of aberrant Aβ deposits degeneration and functional impairment within basal forebrain and the brain's weak antioxidant defense make it more susceptible to oxidative injury.[3] It is well known that the intracellular build-up of Aβ in neurons causes a depletion of intracellular GSH, a key endogenous defense enzyme against oxidative stress in the body.[4] Cholinergic neurons are another indication for Alzheimer's disease. Acetylcholine (Ach) being hydrolyzed by activation of Acetylcholinesterase (AChE) around amyloid plaques there is insufficient acetylcholine in the hippocampus in AD pathogenesis.[5] Decreasing the amount of Aβ and enhancing the amount of acetylcholine in the brain so AchE inhibitors with antioxidant capability are used as therapeutic medicines in AD.[6] Moreover, the brain of individuals with AD often exhibits escalated levels of oxidative stress. The generation of pro-inflammatory chemicals such as tumor necrosis factor and Interleukins (ILs) complicate AD pathogenesis. Tumor Necrosis Factor (TNF) is a potent inflammatory cytokine that results in chronic inflammation in the brain, while various interleukins such as IL-1 and IL-6 contributes neuroinflammation and neuronal damage.[7]

In the neurological system, acetylcholine is an important neurotransmitter connected to memory and learning processes. In previous research studies, Cholinesterase enzyme inhibition has been shown to have more effective direct receptor agonist therapy for restoring ACh deficiency through activating either muscarinic or nicotinic receptors.[8] Cholinesterase inhibitors such as donepezil used to treat AD symptoms. This decreases the breakdown of ACh and enhances the concentration of neurotransmitters in the synaptic cleft, by increasing the duration of action and eventually raising cholinergic activity in order to enhance cognitive function. Donepezil appears to improve cognitive function and performance.[9]

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Natural drugs and bio-active compounds from plant extracts are regarded harmless and widely accepted. In past decade the manufactured natural drugs that inhibit Cholinesterase enzyme were shown to be effective.[10] The goal of the current study is to evaluate plant extracts for its novel antioxidant and cholinesterase inhibition activity to use in the treatment of AD. One of the myths about the plant *Diplocyclos palmatus* (DP) states that it can be used as a rejuvenator to treat neurodegeneration and to improve memory. DP seed extracts have been shown to have antioxidant, anticancer, anticonvulsant, antimicrobial, anti-diabetic activity, anti asthmatic and anti-inflammatory properties.[11,12] However, there wasn't enough information on how seed extract affects neurodegenerative disorders. Therefore, an effort is made to study how DP seed extract functions in Aβ induced amnesia in mice.

MATERIALS AND METHODS

Chemicals: β-amyloid peptide, reduced glutathione were purchased from sigma Aldrich, Hyderabad, Donepezil, DPPH and ascorbic acid from Himedia laboratories, Hyderabad, India. The remaining substances were analytical grade.

Plant material and collection

The *Diplocyclos palmatus* plant's seeds were collected in Warangal, Telangana, India. A taxonomist at Kakatiya University of Botany department authenticated and certified the plant seeds.

Extraction of *Diplocyclos palmatus*

The seeds were dried in the shade, crushed into powder, extracted with methanol using Soxhlet apparatus and then fractionated using chloroform.

Animals

With approval number 07/IAEC/UCPSc/KU/2022: CPCSEA 2018-23, the study protocol was sanctioned under IAEC. Male Swiss albino mice (20-22 g) procured from Vyas Lab, Hyderabad. Animals were placed in a lab environment at 12 hr of darkness and 12 hr of light.

Acute oral toxicity study

In accordance with the recommendations of OECD 423, an investigation of acute toxicity for the amount of *Diplocyclos palmatus* Extract (DPE) was made using male Swiss albino mice. Animals were observed for toxicity and mortality signs.

Experiment and treatment plan

Neurotoxicity: It is induced by ICV injection of 10 µg/mL Aβ (25-35) peptide by using stereo toxic equipment to locate the bregma site in the skull.[13]

Grouping and induction of neurotoxicity

Neurotoxicity was induced by Intra Cerebro Ventricular injection of $A\beta_{(25-35)}$ peptide (3 mg/kg). The animals were divided into seven groups, Group 01 (control) oral administration of PBS from day 1 to 21day), Group 02 (Disease control) received 15th day of the experiment a single injection of $A\beta_{(25-35)}$ (3 mg/kg) i.c.v, Group 03 (Standard) received $A\beta_{(25-35)}$ on 15th day a single dose, Donepezil (5 mg/kg) orally from day 15-day 21th day, Group 04 (Low dose) received $A\beta_{(25-35)}$ on the 15th day, a single dose of 3 mg/kg body weight and DPM 200mg/kg from day 1 to day 21, Group 05 (high dose) received $A\beta_{(25-35)}$ on the 15th day, a single dose of 3 mg/kg and DPM 400 mg/kg from day 1-day 21, Group 06 (low dose) received $A\beta_{(25-35)}$ on the 15th day, a single dose of 3 mg/kg and DPC 200 mg/kg. From day 1-day 21, Group 07 (high dose) received $A\beta_{(25-35)}$ on the 15th day, a single dose of 3 mg/kg and DPC 400 mg/kg. From day 1 to day 21, Behavioral data were collected on day 22, after that all animals were killed and their brain tissues were extracted for biochemical analysis and histological evaluations. On the 22nd day, behavioral parameters were observed, all animals were sacrificed and brain tissues collected for biochemical and histopathological investigations.[14]

Assay for DPPH free radical scavenging activity

According to Peterson *et al.* (2001), DPPH activity was evaluated for DPM and DPC extract.[15]

Behavioral parameters

Exteroceptive screening models were used to assess behavioral activity such as Latency period via conditioned avoidance test, escape latency time required to navigate the maze rectangular device and Y-maze used for spontaneous alterations in mice.[16-18]

Biochemical analysis

Determination of Acetylcholineasterase levels

According to the Ellaman method of 1961, the complete brain for AChE activity was assessed in brain homogenate. The spectrophotometer was set at 412 nm.[19]

Test for glutathione peroxidase

The oxidation of glutathione was used to assess Glutathione peroxidase (GSHx) activity. The spectrophotometer rapidly determined based on intensity of the yellow colorant 412 nm. According to Lawrence and Burk (1976), GPx activity was represented as micro moles/min/mg protein.[20]

Measuring lipid peroxidation in brain tissue

Lipid peroxidation measurement in brain tissue was carried out with the following procedure of Ohkawa *et al.* (1979).[21]

Nitrite levels

The formation of Nitric Oxide (NO) was accordant with the buildup of nitrite in the supernatant. The Greiss reagent used for accumulation assessment. The quantity of nitrite in the supernatant was expressed in µmol/mg protein.[22]

Histopathological analysis

On 22nd day buffered formalin preserved brain tissue were sent to histopathological studies.

RESULTS

Study of acute toxicity

In compliance with OECD guideline 423, experiments on these DPM and DPC extracts were undertaken at various graded doses of 100, 500, 1000 and 2000 mg/kg body weight. The DPM and DPC had shown no harmful effects in male Swiss albino mice up to 2000 mg/kg dose.

Free radical scavenging activity by DPPH

DPPH scavenging assay was used to test the antioxidant properties of various fractions. The fractions showed dose-dependent activity. Ascorbic acid's IC_{50} value was determined to be 3.72 µg/mL, while the methanol extract of *Diplocyclos palmatus* (DPM) and its Chloroform fraction (DPC) showed the IC50 values as 28.42 and 37.77 µg/mL, respectively.

Jumping box

The jumping box test results revealed that the latency period in mice had a significantly (p<0.001) higher in the negative control group (29.16±1.60) than in the normal group (12.66±1.24) Whereas mice treated with standard control (14.83±1.07), DPM-low (22.66±1.10), DPM-high dose (16.166±1.34), DPC-low (24.5±1.70) and DPC-high dose (19.33±1.49), significantly decreased latency period compared to the negative control group (Figure 1A).

Rectangular maze

According to the findings in rectangular maze test, the negative control group (144.33±1.63) was significantly (p<0.001) longer maze traverse period when compared to that of normal control group (44.83±1.95). It was found that in mice treated with standard group (64.33±1.75), DPM-low (86.66±1.49), DPM-high dose (72.16±1.57), DPC-low (95.66±1.49) and DPC-high dose (80.66±1.79) decreased maze traverse period compared to the negative control group (Figure 1B).

Y-Maze

The Y-maze results demonstrated that spontaneous changes in mice were considerably (p<0.001) lower in the negative control group (26.166±1.34) compared to that of normal control group (46.66±1.24). It was observed that mice treated with standard control (42.66±1.24), DPM-low (33.166±1.34), DPM-high dose (40.166±1.34), DPC-low (30.33±1.59) and DPC-high dose (37.166±1.57) showed increase in the spontaneous alterations compared to that of the Aβ induced negative control group (Figure 1C).

Biochemical studies

Determination of AChE

The AChE level in the brain tissue was analyzed and is displayed in (Figure 2A). ICV Injection of Aβ significantly (p<0.001) increased AChE levels in negative control group (21.16±0.89) compare to that of Normal control group (6.83±0.68).It was observed on 21st days after treatment that standard drug (11.16±0.68), DPM-low (18.166±0.68), DPM-high dose (15.66±0.94), DPC-low (19.5±0.76) and DPC-high dose (17.16±0.68) comparatively decreases the AChE level when compared with that of the Aβ induced negative control group.

Glutathione peroxidase assay

It was noted in the present work that ICV injection of Aβ induces the oxidative stress in mice thereby causing considerably (p<0.001) reduction in antioxidant enzyme GSH in negative control group (17.33±0.74) than compare to that of Normal control group (32.66±0.94). Mice's treated with donepezil standard group (28.33±0.47), DPM-low dose (22.833±0.37), DPM-high dose (26.16±0.67), DPC-low dose (21.16±0.68) and DPC-high dose (24.66±0.94) it was observed that the antioxidant enzyme levels GSH were significantly restored to normal levels When compared to the negative control group (Figure 2B).

MDA estimation

MDA levels are higher in negative control group (p<0.001, 53.833±0.68) than in the normal control group (22.66±0.74), In the present work it was noted that Mice's treated with the standard drug donepezil (26.16±0.89), DPM-low dose (35.66±0.74), DPM-high dose (29.16±0.68), DPC-low dose (38.33±0.74) and DPC-high dose (32.66±0.74) it showed significantly lowered the levels of MDA when compared to the negative control group (Figure 2C).

Nitrite levels

In the current research work it was observed that Nitrites levels are higher in the Aβ induced negative control group (p<0.001, 267.5±3.40) than in the normal group (77.33±1.10). Significantly decrease in the levels of MDA were noted in animals treated with standard group animals treated with donepezil (89.16±2.26), DPM-low dose (137.16±3.67), DPM-high dose (101.33±2.35), DPC-low dose (163.16±2.11) and DPC-high dose (119.66±2.80) when compared to that of Aβ induced negative control group (Figure 2D).

Histopathology Studies

Histological examination revealed that the cerebral cortex and hippocampus of the normal control mice exhibited typical morphology. In the hippocampus region of Aβ-induced animals, there were numerous small necrosis and apoptosis foci along with apoptotic neurons were seen in the cerebral cortex. The frontal gliosis was present in the standard group animals, but the cerebral cortex's morphology was found to be normal animals treated with DPM-low dose exhibited considerable hippocampus neuron hyperplasia. Animals treated with DPM-high dose exhibited normal frontal cortex but prefrontal cortex gliosis which can be

Behavioral studies

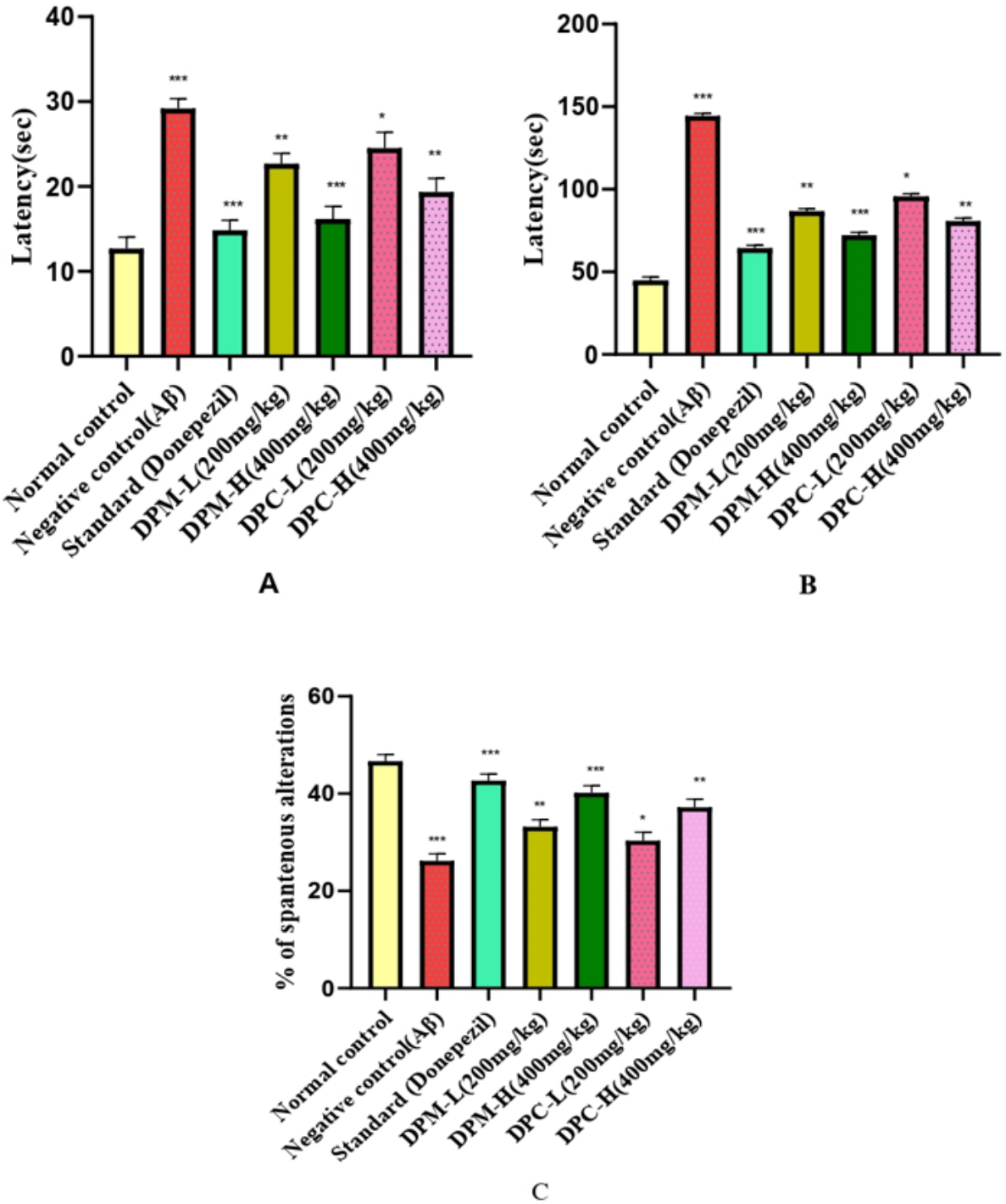

Figure 1: Effect of methanolic extract and its chloroform fraction of *Diplocyclos palmatus* on behavioural parameters in Amyloid beta induced Alzheimer's disease mice: A) Jumping box B) Rectangular maze C) Y-maze activity in brain tissue. Statistical analysis was performed by using one way ANOVA followed by Dunnett's t-test by comparing with negative control group *p<0.05,**p<0.01,***p<0.001. Significant values are expressed as (Mean ± SD, n=6).

compared with that of the standard. In DPC treated mice it was noted that occasional small necrosis and few apoptotic neurons were noted (Figure 3). From this study it can be confirmed that DPM exhibited considerable activity when compared to DPC.

DISCUSSION

Alzheimer's disease is a serious illness in both developed and developing countries, marked as gradual loss of memory and cognitive ability characterized by dementia or impairment of memory loss. Formation of Amyloid beta peptides into fibrillar

Biochemical studies

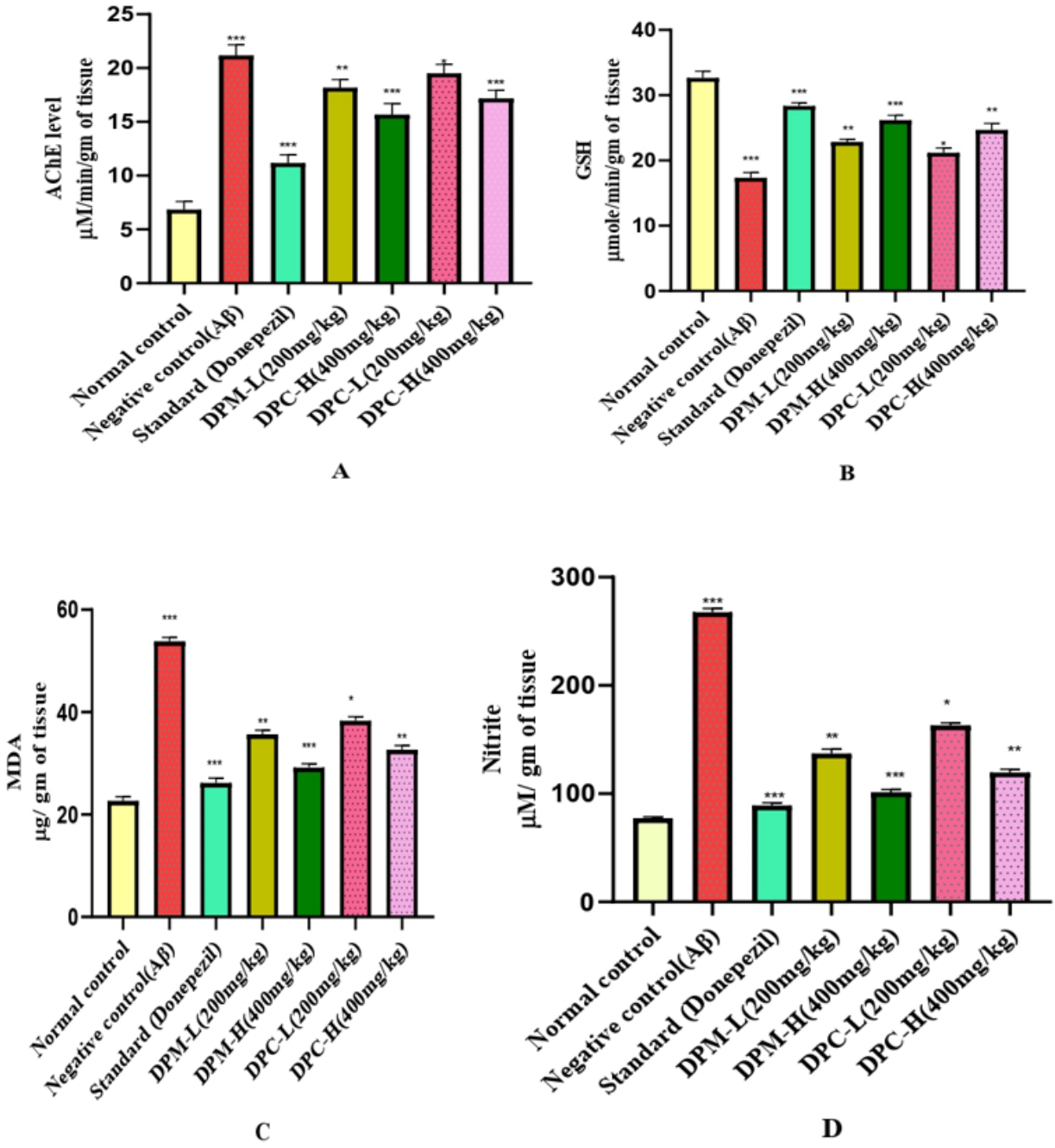

Figure 2: Effect of methanolic extract and chloroform fraction of *Diplocyclos palmatus* on biochemical parameters in Amyloid beta induced Alzheimer's disease mice: A) Acetylcholinesterase activity (AChE) B) GSH level C) MDA level D) Nitrite level in brain tissue. Statistical analysis was performed by using one way ANOVA followed by Dunnett's t-test by comparing with negative control group *p<0.05,**p<0.01,***p<0.001. Significant values are expressed as (Mean ± SD, n=6).

plaques causes nerve damage in striatal and hippocampus region.[23] In modern days of life, Brain tissues are highly susceptible to oxidative stress and often cause neurodegenerative disorders due to increase ROS. In the current study, the methanolic extract of *Diplocyclos palmatus* and its chloroform fraction protects mice from developing AD caused by the compound $A\beta_{(25-35)}$ and donepezil was employed as a reference medication and its activity was compared to that of plant extracts.

DP extract showed antioxidant property and it was proved by DPPH method. Memory impairment was evaluated along with behavioral and biochemical studies.

Histopathology

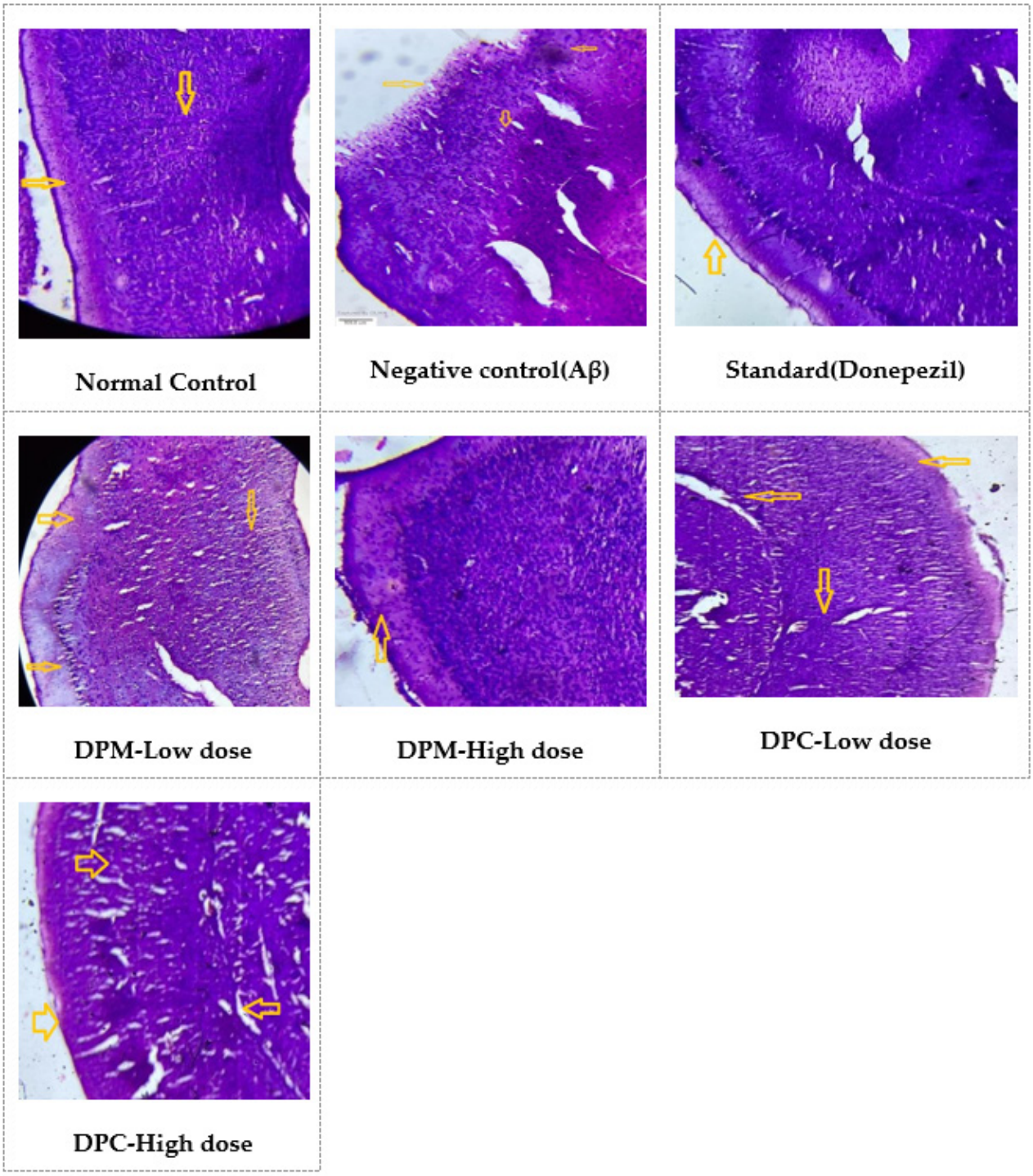

Figure 3: Histopathological examination using light microscope, the pictures are magnified at 40x.

The conditioned avoidance test, rectangular maze and Y-maze are widely used behavioural test for learning and memory in mice. Escape latency taken as parameter for the assessment of cognition in conditioned avoidance test and rectangular maze.[24] In this study the DPM and DPC extracts diminish effect of Aβ and enhance memory suggesting that DPM and DPC possess the memory enhancing activity. A precise and accurate measure of spatial memory of rodents has been carried out using Y-maze and employed in this study was beneficial to evaluate memory since it includes no detrimental stimuli. More significant increase in the percentage of spontaneous alternation on acquisition of memory of the Aβ treated mice within Y-maze task were observed with DPM and DPC extracts. According to the results, the DPM extracts revealed potency-dependent action in every amnesiac mouse statistically significant greater activity than the DPC fractions.

In biochemical analysis, the acetylcholinesterase enzyme was inhibited in the brain indicating an improvement in learning and memory process. The most identifiable alteration that takes place in AD-type dementia is an increase in AChE. AChE was responsible for mediating the transformation of Aβ peptide into fibrillar tangles. Based on significant data, it was believed that AChE's involves free radical generation occurs during the neurodegeneration caused by the Aβ peptide accumulation[25]. According to research, Aβ25-35 peptides enhanced AChE activity in the cerebral cortex and hippocampus, which is likely to cause a persistent ACh deficit and memory impairment. However by inhibiting AChE activity and raising ACh levels in neuronal synaptic clefts, restore memory occurred by pre-treatment with *Diplocyclos palmatus* extract (DPM) and its fraction (DPC). As per the results, DPM extracts showed dose dependent activity in amyloid beta induced Alzheimers disease mice and statistically significant higher activity than the DPC fractions.

The pathology of AD involves oxidative and nitrosative stress. Hippocampus and cortex of AD patients have elevated levels of lipids; nucleic acid and oxidation products. Based on past reports, Aβ-treated mice showed a decline in GSH levels due to oxidative stress. Glutathione deficiency results in failure of hippocampus synaptic plasticity mechanisms which was associated with deficit in spatial memory. The cerebral cortex is a major target for lipid peroxidation that serves as a hallmark of AD. MDA levels are enhanced as final product of lipid peroxidation. Nitric oxide a key nutrient in neurological disorder like Alzheimer's disease was produced by Aβ, a protein that operates on the NF-kB pathway that over expresses the inducible Nitric Oxide Synthase (i-NOS) that promotes ROS generation results in increase nitric oxide levels.[26] In the current study, treatment of DPM and DPC in mice considerably increases GSH levels, decreases MDA levels through reduced TBARS activity in the cortex thereby confirming lipid peroxidation inhibitory effect and reduced NO levels in the brain. This study suggests that administration of DPM and DPC significantly increases the brain antioxidant enzymes and decreases the lipid peroxidation. The findings showed that compared to the DPC fractions, the DPM extracts had statistically significantly higher activity with dose dependent action in amyloid beta induced AD.

Histopathology studies reveals, the hippocampus area and cerebral cortex of Aβ-induced AD mice were shown to contain numerous tiny foci of necrosis and apoptotic neurons. The DP extract therapy prevented Aβ-induced neurodegeneration and the cerebral cortex and hippocampus' histological architecture have been restored.

CONCLUSION

According to the results of the current investigation, the treatment of AD-induced mice with methanolic seed extract of *Diplocyclos palmatus* (DPM) and its Chloroform fraction (DPC) significantly reduces the oxidative stress status and ameliorates the neurodegeneration characteristic of AD in mice. Moreover behavioral, biochemical and histological studies revealed that the high dose of methanolic extract of DP showed a greater interest in improving AD. The effect of seed methanolic extract of DP was achieved through the decreased Acetylcholinesterase enzyme activity and elevates antioxidant capacity of the plant. Therefore, it proved useful to investigate the therapeutic activity of DPM in the management of AD.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

AD: Alzheimer's disease; **DPM:** *Diplocyclos palmatus* methanolic; **DPC:** *Diplocyclos palmatus* chloroform; **ICV:** Intra Cerebro Ventricular; **Aβ:** Amyloid beta; **MDA:** Malondialdehyde; **GSH:** Reduced Glutathione; **ACh:** Acetylcholine; **AChE:** Acetylcholinesterase; **IP:** Intraperitoneal; **NO:** Nitric oxide.

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