

Research Article**Ameliorative effect of Betulinic acid on ageing and Scopolamine-induced learning and memory impairment in rats****Poonam Ruhel, Dinesh Dhingra****Pharmacology Division, Department of Pharmaceutical Sciences, Guru Jambheshwar University of Science and Technology, Hisar -125001, Haryana (India).*

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Abstract

Background: Ageing is a natural process which includes a progressive decline in cognitive functions as a result of maladaptation of cholinergic neuronal activity. The reason behind ageing-induced cholinergic neuronal loss is largely unknown, however, oxidative stress is speculated to be majorly involved in its aetiology. **Objectives:** In the present study, the effect of betulinic acid was evaluated on learning and memory in aged rats as well as scopolamine-induced amnesic rats. **Material and methods:** Betulinic acid (25 and 50 mg/kg; p.o.) was administered to separate groups of rats for 7 consecutive days. Donepezil (1 mg/kg; i.p.), an acetylcholinesterase inhibitor was used as a standard drug. Behavioral models such as Morris water maze and elevated plus maze were used to evaluate the effect of drugs on learning and memory of rats. After behavioral studies, animals were sacrificed and their brain was isolated and further processed for estimation of various biochemical parameters such as acetylcholinesterase activity, oxidative and nitrosative stress markers and histological examinations. **Results:** Betulinic acid significantly improved learning and memory of aged as well as scopolamine-induced amnesic rats. Further, betulinic acid significantly reduced oxidative-nitrosative stress, as indicated by decreased lipid peroxidation and nitrite level and increased the levels of reduced glutathione and superoxide dismutase, in both aged as well as scopolamine-induced amnesic rats. Further, the AChEs activity was found to be significantly reduced after administration of high dose (50 mg/kg) of betulinic acid in aged rats as well as scopolamine-induced amnesic rats. In addition, histopathological evaluation showed that betulinic acid-treated aged rats have less number of pyknotic neurons in hippocampal CA1 region as compared to aged control rats. **Conclusion:** The present study provides the pharmacological evidence for neuroprotective and memory enhancing effect of betulinic acid in aged as well as scopolamine-induced amnesic rats, possibly through its anti-acetylcholinesterase activity and anti-oxidant activity. Further, the current findings support the usefulness of betulinic acid in the management of age-related cognitive dysfunction.

Keywords: Acetylcholinesterase; ageing; betulinic acid; dementia; learning; memory

Introduction

Ageing is a natural process that accounts for significant economic and societal costs due to age-related non-communicable diseases. Though more than 300 theories have been put forward to explain the ageing process (Medvedev, 1990), the "free radical theory" is considered to be the most

acceptable theory of ageing (Kirkwood and Kowald, 2012; Sohal and Orr, 2012). In addition, a brain is considered to be the most vulnerable organ to this ageing process as neurons lack a robust regenerative capacity and have low anti-oxidant defense mechanism (Friedman, 2011). Though the exact cause of ageing brain cells is not clearly known, the oxidative stress is thought to be the critical factor in the initiation of ageing-induced cognitive decline (Di Domenico et al., 2016; Fukui et al., 2001, Harman, 1956; Levin et al., 2005). Ample body of evidence showed that oxidative stress during ageing cause degeneration of glial cells (Streit et al., 2008; Zalfa et al., 2016) and disturb mitochondrial electron transport chain (Bonomini et al., 2015; Tarantini et al., 2018), which can be correlated with altered synaptic and non-synaptic

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communication between neurons and glia and may further lead to memory loss (Popa-Wagner et al., 2013). Ageing of brain cells results in a decline in cognitive performance in domains of reasoning, problem-solving skills, attention, processing speed, working memory and episodic memory (Simen et al., 2011). Ancient Indian Ayurveda also describes that core cognitive function of the brain starts declining from the fourth decade of life onwards, and after the eighth decade of life, the loss of decision-making capacity becomes prominent, leading to senile dementia (Singh, 2013). In addition, a pace of world population ageing is accelerating due to advancement in healthcare facilities and remarkable increases in life expectancy. By 2050, older persons are projected to account for one in every five people globally, with most of the increase in developing countries (World Population Prospects, 2015). Therefore, interventions that possess anti-ageing activity and improve cognitive function in older people are needed to reduce this burden on society.

An allopathic system of medicine is yet to provide a satisfactory remedy for ageing and related disorders. Therefore, researchers all over the world are looking for new directions and alternative strategies for managing ageing-induced cognitive decline. More importantly, around 80% of the world's population use herbal medicinal products as a primary source of healthcare (Ekor, 2014). Plants like *Bacopa monniera* (Pandareesh et al., 2016), *Withania somnifera* (Ahmed et al., 2013), *Centella asiatica* (Xu et al., 2012), *Zingiber officinale* (Joshi and Parle, 2006), *Glycyrrhiza glabra* (Dhingra et al., 2004), *Inula britannica* (Chen et al., 2016) as well as *Evolvulus alsinoides* (Sethiya et al., 2018) have been investigated for their anti-ageing and cognitive enhancing properties.

It is known that acetylcholine is needed in adequate amount for successive neuronal transmission. However, Alzheimer's disease patients and other age-related disorders show deficits in the cholinergic level that is believed to be responsible for cognitive impairments (Kumar and Calache, 1991). Further, it is quite difficult to administer acetylcholine (ACh) directly to the patients due to its unstable nature. Thus, the best approach to increase ACh level at the synapse is to inhibit the hydrolysis of ACh by means of cholinesterase inhibitors. Plant derived cholinesterase inhibitors such as physostigmine (*Physostigma venenosum*) and galantamine (*Galanthus caucasicus*) are known to have symptomatic relief in Alzheimer's disease. However, the central and peripheral side effects (anorexia, bradycardia, insomnia, weight loss, nausea, diarrhoea) associated with anti-cholinesterases such as rivastigmine, galantamine, tacrine etc. limit their clinical utility (Schneider, 2000; Yaari et al., 2008). Previous studies have documented the anti-ageing or memory-enhancing effect of some bioactive compounds such as berberine (Kumar et al.,

2016), bacoside-A and B (Chatterji et al., 1965; Basu et al., 1967) and huperzine-A (Yang et al., 2013). However, in phase II clinical trials huperzine-A failed to show the cognitive enhancing effect in patients with mild to moderate Alzheimer's disease (Rafii et al., 2011).

Betulinic acid, a pentacyclic triterpenoid, is widely found in the medicinal plants (Gauthier et al., 2011), including *Bacopa monniera* (Viji et al., 2010), *Diospyros bipindensis* (Brusotti et al., 2017), *Centella asiatica* (James and Dubery, 2009), *Zizyphi spinosi* (Qian et al., 2012) etc. Betulinic acid was reported to have a wide spectrum of pharmacological activities such as, antioxidant (Yamashita et al., 2002), anti-inflammatory (Viji et al., 2010), antimalarial (de Sá et al., 2009), antidiabetic (Alqahtani et al., 2013), antidepressant (Machado et al., 2013), anti-HIV (Fujioka et al., 1994), anti-hepatitis C (Lin et al., 2015), antineoplastic (Fulda, 2009) and immunomodulatory activities (Pang et al., 2018). In addition, betulin or its bioactive derivative betulinic acid showed neuroprotective effects in cerebral ischemia-reperfusion injury (Lu et al., 2011), Parkinson's disease (Tsai et al., 2017), epilepsy (Muceniece et al., 2008), amyloid beta-induced neurotoxicity (Planchard et al., 2012) and multiple sclerosis (Blazevski et al., 2013). Anti-oxidant and anti-inflammatory mechanisms are suggested to be involved in the neuroprotective effect of betulinic acid. In addition, *in vitro* enzyme inhibition study revealed the inhibitory activity of betulinic acid against acetylcholinesterase (AChE) with the IC₅₀ values of 13.5–28.5 μM as compared to that of the reference standard, physostigmine (Jamila et al., 2014). Further, it is found to have good high safety profile upto a dose level of 500 mg/kg (Pisha et al., 1995) and longer elimination half-life (Udeani et al., 1999). In addition, betulinic acid also has high permeability through blood brain barrier as evident from *in silico* data (Khan et al., 2018), thus making it a suitable candidate to study for treatment of brain disorders.

Recently, betulinic acid has been reported to show protective effect against intracerebroventricular streptozotocin-induced cognitive deficits and neuronal damage in rats (Kaundal et al., 2018). But the effects of betulinic acid on ageing-induced and scopolamine-induced cognitive deficits have not been reported so far. Therefore, the present study was aimed to elucidate the potential of betulinic acid in reversing ageing-induced learning and memory loss. Scopolamine administered to young human volunteers induced memory deficits, comparable to those in the aged population (Drachman and Leavitt, 1974; Crook et al., 1986), so we also observed the effect of betulinic acid on scopolamine-induced amnesic rats. Further, the histopathological evaluation of betulinic acid on neurons in

hippocampal CA1 region of rat brain was also carried out.

Materials and methods

Animals

Wistar young male rats (3 months old) and aged female rats (18 months old) were procured from Disease Free Small Animal House, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar (Haryana, India). The animals were maintained under standard laboratory conditions with alternating light and dark cycles of 12 h each and had free access to food and water. The animals were acclimatized for at least 5 days before behavioral experiments. Experiments were carried out between 09:00 and 17:00 h. The experimental protocol was approved by the Institutional Animals Ethics Committee (IAEC/2016/10-17; 5-09-2016) and care of laboratory animals was taken as per guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Environment, Forests, and Climate Change, Govt. of India, New Delhi.

Drugs

Betulnic acid (purity >98.0%) was purchased from Aktin Chemicals, Inc (Chengdu, China). Donepezil was obtained as a gift sample from Ranbaxy Laboratories Pvt. Ltd., Gurgaon (Haryana), India. Scopolamine was purchased from Sigma-Aldrich (St. Louis, MO, USA). Betulnic acid was suspended in 0.25 % w/v sodium carboxy methyl cellulose and was given

through oral gavage as per body weight (not exceeding 5 ml/kg b.w.). Donepezil and scopolamine were dissolved in normal saline and administered intraperitoneally.

Experimental protocol

In this study, there were 22 groups of rats and each group comprised of a minimum of eight animals. The time schedule for entire experimental protocol has been depicted in Figure 1. The details of experimental groups were as follows:

Ageing rat model

Groups for elevated plus maze

Group I (Control group for young female rats): Vehicle (Normal saline) was administered i.p. to young female rats for 7 consecutive days. Transfer latency (TL) was measured 30 min after the injection of normal saline on the 6th day and 7th day.

Group II: (Control group for aged female rats): Vehicle (Normal Saline) was administered i.p. to aged female rats for 7 consecutive days. TL was measured 30 min after the injection of normal saline on the 6th day and 7th day.

Group III: Donepezil (1 mg/kg; i.p.) was administered to aged female rats for 7 consecutive days. TL was measured 30 min after the injection of donepezil on the 6th day and 7th day.

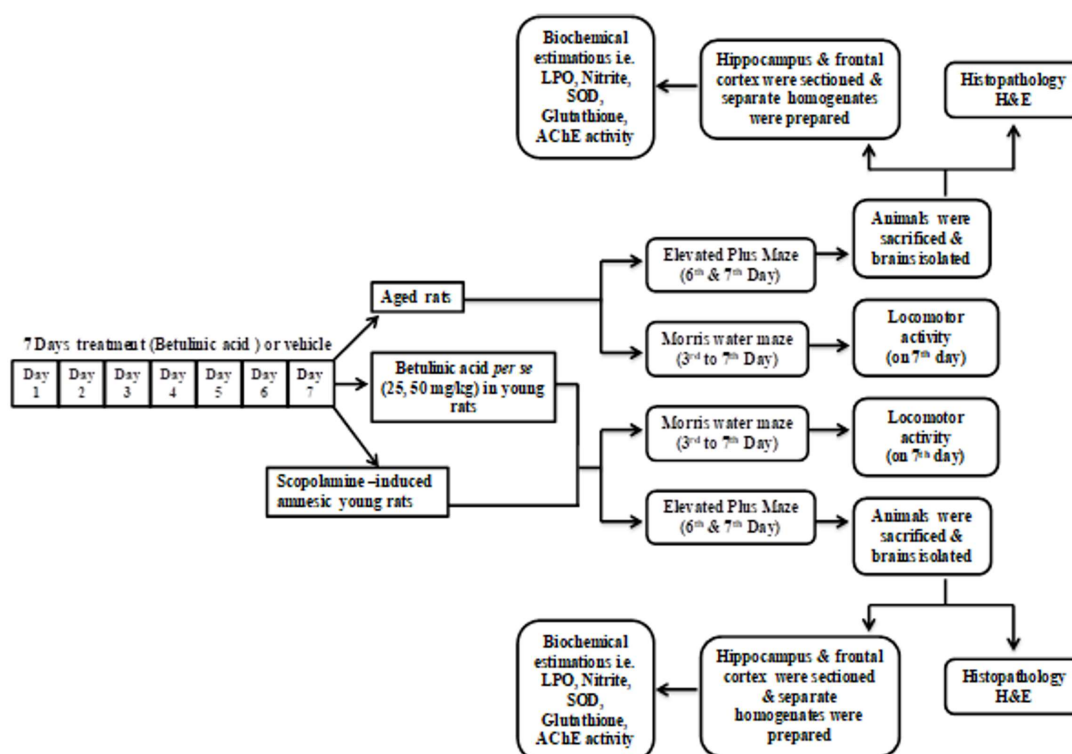


Figure 1. Illustration of experimental procedures for ageing-induced amnesia and scopolamine-induced amnesia rat models. Drug treatment was carried out for 7 consecutive days. The behavioral observations were started from day 3 onwards, and the animals were sacrificed after the last behavioral test on day 7. The hippocampus and frontal cortex of rat brain were isolated to carry out biochemical and histopathological examinations. AChE -acetylcholinesterase, H&E -hematoxylin and eosin, LPO -lipid peroxidation; SOD -superoxide dismutase

Group IV and V: Betulinic acid (25 and 50 mg/kg; p.o., respectively) was administered to aged female rats for 7 consecutive days. TL was measured 30 min after the injection of betulinic acid on the 6th day and 7th day.

After behavioral testing on elevated plus maze, animals were tested for locomotor activity using actophotometer, which was followed by sacrificing of animals by cervical dislocation and their brain was isolated for biochemical (TBARS, nitrite, GSH, SOD and AChE activity) and histopathological studies. Doses and route of administration of betulinic acid and donepezil were selected on the basis of previous literature (Silva et al., 2016; Sonkusare et al., 2005).

Groups for Morris water maze

Group VI (Control group for young female rats): Normal saline was administered i.p. to young rats for 7 consecutive days. EL was measured on 3rd to 6th day and TSTQ was measured on the 7th day.

Group VII (Control group for aged female rats): Normal saline was administered i.p. to aged rats for 7 consecutive days. EL was measured on 3rd to 6th day and TSTQ was measured on the 7th day.

Group VIII: Donepezil (1 mg/kg; i.p.) was administered to aged female rats for 7 consecutive days. EL was measured on 3rd to 6th day and TSTQ was measured on the 7th day.

Group IX and X: Betulinic acid (25 and 50 mg/kg; p.o., respectively) was administered to aged female rats for 7 consecutive days. EL was measured on 3rd to 6th day and TSTQ was measured on the 7th day.

Scopolamine-induced amnesic rat model

Groups for elevated plus maze

Group XI: Vehicle (Normal saline) was administered i.p. to young male rats for 7 consecutive days. TL was measured 30 min after the injection of normal saline on the 6th day and 7th day.

Group XII: Vehicle (Normal saline) was administered i.p. to young male rats for 6 consecutive days. TL was measured on the 6th day. Scopolamine (1 mg/kg, i.p.) was administered on the 7th day and TL was measured 30 min after scopolamine injection.

Group XIII and XIV: Betulinic acid (25 and 50 mg/kg; p.o. respectively) was administered to young male rats for 7 consecutive days. TL was measured 30 min after the injection of betulinic acid on the 6th day and 7th day.

Group XV and XVI: Betulinic acid (25 and 50 mg/kg; p.o., respectively) was administered to young male rats for 6 consecutive days. On the 7th day, scopolamine was administered 30 min before the injection of betulinic acid. TL was measured 30 min after the injection of betulinic acid on 7th day.

After behavioral testing on elevated plus maze, animals were sacrificed by cervical dislocation and their brain was isolated for

biochemical estimations (TBARS, nitrite, GSH, SOD and AChE activity) and histopathological studies.

Groups for Morris water maze

Group XVII: Vehicle (Normal saline) was administered i.p. to young male rats for 7 consecutive days. EL was measured on 3rd to 6th days and TSTQ was measured on the 7th day.

Group XVIII: Vehicle (Normal saline) was administered i.p. to young male rats for 6 consecutive days. EL was measured on 3rd to 6th days. Scopolamine (1 mg/kg, i.p.) was administered to young male rats on the 7th day and TSTQ was measured 30 min after scopolamine injection.

Group XIX and XX: Betulinic acid (25 and 50 mg/kg; p.o. respectively) was administered to young male rats for 7 consecutive days. EL was measured on 3rd to 6th days and TSTQ was measured on the 7th day.

Group XXI and XXII: Betulinic acid (25 and 50 mg/kg; p.o., respectively) was administered to young male rats for 6 consecutive days. EL was measured on 3rd to 6th days. On the 7th day, scopolamine was administered 30 min before the injection of betulinic acid. TSTQ was measured on 30 min after injection of betulinic acid on 7th day.

After measuring TSTQ in Morris water maze on the 7th day, animals were tested for locomotor activity using actophotometer.

Behavioral assessments

Elevated plus maze and Morris water maze were used as behavioral models to evaluate the effect of drugs on learning and memory of rats. The details of these models are as follows:

Elevated plus-maze

Elevated plus-maze consisted of two opposite open arms (50×10 cm), crossed with two closed arms of the same dimensions with 40 cm high walls (Sharma and Kulkarni, 1992). The arms were connected with central square (10×10 cm) and the entire maze was elevated to a height of 50 cm from the floor. Each rat was placed at the end of one of the open arms, facing outwards. The time taken by the animal with all its four paws in the closed arm on the 6th day (acquisition trial) was noted and was called as initial transfer latency (ITL). Cut-off time was fixed at 90 s, and in case a rat could not find the closed arm within this period, it was gently pushed into one of the closed arms and allowed to explore the maze for another 30 s. The second trial (retention trial) was performed 24 h after the acquisition trial (day 7), and retention transfer latency (RTL) was noted. connected with central square (10×10 cm) and the entire maze was elevated to a height of 50 cm from the floor. Each

rat was placed at the end of one of the open arms, facing outwards. The time taken by the animal with all its four paws in the closed arm on the 6th day (acquisition trial) was noted and was called as initial transfer latency (ITL). Cut-off time was fixed at 90 s, and in case a rat could not find the closed arm within this period, it was gently pushed into one of the closed arms and allowed to explore the maze for another 30 s. The second trial (retention trial) was performed 24 h after the acquisition trial (day 7), and retention transfer latency (RTL) was noted.

Morris water maze performance task

The acquisition and retention of a spatial navigation task were examined using a Morris water maze (Morris, 1984; Ruhel and Dhingra, 2018). Water maze consists of a cylindrical pool (180 cm in diameter and 50 cm in height) filled with water maintained at approximately 28±1 °C and measuring 30 cm deep. Water was made opaque by using non-toxic and non-irritant dye (titanium dioxide). The tank was divided into four equal quadrants (Q1–4), and a submerged platform (10×10 cm²) was placed 2 cm below the surface of the water in the middle of the target quadrant (north Q1). The position of the platform was kept unaltered throughout the training session. The water maze was also kept in the same position throughout the study. During testing, the investigator wearing a white lab coat stood at the west edge of the pool.

Acquisition test (Learning)

All the rats underwent training over four consecutive days, starting from day 3 of drug treatment, and consisting of 4 swimming trials per day, each at an interval of 30 min approximately. Each animal was subjected to training trials for 4 consecutive days, the starting position was changed with each exposure as mentioned below and target quadrant (Q1) remained constant throughout the training period:

Day 1 Q1 Q2 Q3 Q4

Day 2 Q2 Q3 Q4 Q1

Day 3 Q3 Q4 Q1 Q2

Day 4 Q4 Q1 Q2 Q3

For each trial, the rat was placed at the edge of the pool in the center of the appropriate quadrant, facing the wall of water maze; and latency to find the platform was recorded. Cut off time for finding the platform was kept 90 s. If the rat could not find the submerged platform in 90 s, then the animal was gently placed on it and allowed to stay there for the next 20s. Escape latency (EL) time to locate the hidden platform in water maze was noted as an index of acquisition or learning.

Retention test (Memory)

Following training for 4 days, a retention test was performed on

day 5 (day 7 of drug treatment). The platform was removed from water maze. Each rat was placed in the quadrant (Q3) opposite to the target quadrant (Q1) and allowed to explore the target quadrant for 90 s. Time spent in the target quadrant (TSTQ) in search of the missing platform was noted which indicated index of retrieval or retention.

Assessment of locomotor activity

Immediately after recording TSTQ on the 7th day, the locomotor activity (horizontal activity) of animals was assessed using actophotometer (INCO, Ambala, India). This instrument operates on photoelectric cells which are connected in circuit with a counter. When the beam of light falling on the photocell is cut off by the animal, a count is recorded. Each animal was placed in the actophotometer for a period of 5 min and locomotor counts were recorded (Kulkarni and Dhir, 2008). The apparatus was placed in a sound-attenuated and ventilated room.

Biochemical assessments

Following behavioral assessments, the animals were sacrificed by cervical dislocation and their brain was isolated. Frontal cortex and hippocampus were separated and then weighed. Tissue homogenates 10% (w/v) of both frontal cortex and hippocampus were prepared in 0.1 M phosphate buffer (pH 7.4). The homogenates were centrifuged at 10,000×g at 4°C for 15 min. Aliquots of supernatants were separated and used for biochemical estimations. UV–vis spectrophotometer (SPECTROstar® Nano, Ortenberg, Germany) was used as an instrument for various biochemical estimations.

Estimation of acetylcholinesterase activity

AChE activity was assessed in the hippocampal and cortical regions by the method of (Ellman et al., 1961). The assay mixture contained 50 µl of tissue homogenate, 3 ml of sodium phosphate buffer (pH 8.0), 100 µl of acetylthiocholine iodide, and 100 µl of 0.01 M 5,5' dithio-bis-2-nitro benzoic acid (DTNB, Ellman reagent). The change in absorbance was measured for 2 min at a 30s interval at 412 nm using a UV–vis spectrophotometer. Results were expressed as µM of acetylthiocholine iodide hydrolyzed per min per mg of protein.

Measurement of lipid peroxidation

The quantitative measurement of TBARS, an indicative of lipid peroxidation (LPO) was carried out according to the method as described by Wills (1966) with slight modifications. In brief, 0.5 ml of supernatant from tissue homogenate was incubated with 0.5 ml of Tris HCl for 2 hrs at 37°C. After the incubation, it was treated with 1 ml of ice-cold trichloroacetic acid (10% w/v) reagent followed by addition

of 1 ml thiobarbituric acid (0.67% w/v) and placed in boiling water bath for 15 min, cooled, centrifuged and then the clear supernatant was removed. The absorbance of the supernatant was measured at 535 nm against blank using UV-vis spectrophotometer. The values were calculated using molar extinction coefficient of chromophore ($1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$).

Estimation of reduced glutathione

Reduced glutathione (GSH) was estimated according to the method described by Ellman (1959). A 1.0 ml of homogenate was precipitated with 1.0 ml of 4% w/v sulfosalicylic acid by keeping the mixture at 4 °C for 1 h, and the samples were immediately centrifuged at 1,200×g for 15 min at 4°C. The assay mixture contained 1.0 ml of supernatant, 2.0 ml of phosphate buffer (0.1 M, pH 8.0), and 0.2 ml of 0.01 M DTNB. The yellow color developed was read immediately at 412 nm using a UV-vis spectrophotometer. Results were calculated using the molar extinction coefficient of chromophore ($1.36 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) and expressed as nM of GSH per mg of protein.

Superoxide dismutase activity

Superoxide dismutase (SOD) activity was assayed according to the method of Kono (1978), wherein the reduction of nitro blue tetrazolium was inhibited by SOD and measured at 560 nm using a UV-vis spectrophotometer. In brief, the reaction was initiated by the addition of the 500 µl of hydroxylamine hydrochloride to the assay mixture containing 2 ml nitroblue tetrazolium and 100 µl tissue homogenate sample. The results were expressed as units/mg protein where one unit of enzyme is defined as the amount of enzyme-inhibiting the rate of reaction by 100 percent.

Estimation of nitrite

Accumulation of nitrite, an indicator of the production of nitric oxide (NO), was determined with a colorimetric assay with Greiss reagent as described by Green et al. (1982). In brief, 500µl of supernatant and 500 µl of Greiss reagent (250 µl of 1.0% w/v sulfanilamide and 250 µl of 0.1% w/v N-naphthylethylenediamine) were mixed, and the mixture was incubated in the dark for 10 min at room temperature. Absorbance was recorded at 546 nm with a UV-vis spectrophotometer. The concentration of nitrite in the supernatant was determined from a sodium nitrite standard curve.

Protein estimation

Protein estimation was done by biuret method using bovine serum albumin as standard (Gornall et al., 1949).

Assessment of histological changes

After behavioral testing on elevated plus maze, animals were sacrificed by cervical dislocation and their brain was isolated for histopathological studies. The brains were rapidly removed and fixed by immersion in formalin (10 %v/v). The brain tissues were

cut into 3mm thickness, and its blocks were embedded in paraffin. The brain sections (4 µm thick) were prepared and stained with haematoxylin and eosin stain. Furthermore, hippocampal CA1 region of the brain was examined under bright field illumination using AHB-T-51 microscope (Olympus Vanox Research Microscope, Japan) and photographed (Kim et al., 2014).

Statistical analysis

Graph Pad Prism (Graph Pad Software, San Diego, CA, USA) was used for all statistical analysis. The results are expressed as mean ± SEM. The behavioral data of learning in Morris water maze was analyzed by repeated measures two-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test for multiple comparisons. The data of biochemical parameters, elevated plus maze, locomotor activity and retention of memory in Morris water maze were analyzed using one-way ANOVA followed by Tukey's test for multiple comparisons. In all tests, $p < 0.05$ was considered statistically significant.

Results

Effect of betulinic acid on transfer latencies of aged rats using elevated plus maze

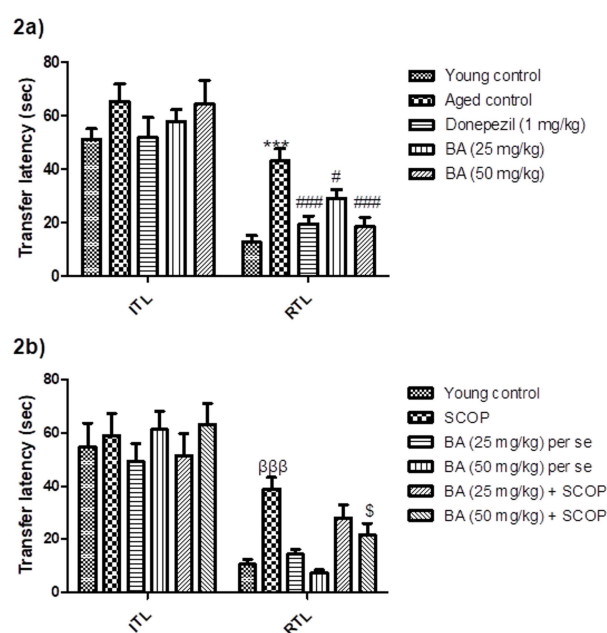


Figure 2. Effect of betulinic acid on transfer latency of aged rats (2a) and scopolamine-induced amnesic rats (2b) in elevated plus maze. Data are in mean ± SEM (n=7 per group). Data were analyzed by one-way ANOVA followed by Tukey's test for multiple comparisons. BA-betulinic acid; SCOP-scopolamine. ITL –Initial transfer latency; RTL – Retention transfer latency

In figure 2a, *** $p < 0.001$ versus young control, # $p < 0.05$ versus aged control, ### $p < 0.001$ versus aged control. For ITL (6th day): $F(4,30)=1.055$; $p=0.3956$. For RTL (7th day): $F(4,30)=12.06$; $p < 0.0001$.

In figure 2b, ### $p < 0.001$ versus young control, \$ $p < 0.05$ versus SCOP. For ITL (6th day): $F(5,36)=0.5097$; $p=0.7670$. For RTL (7th day): $F(5,36)=11.94$; $p < 0.0001$.

ITL on day 6 was found to be fairly unaltered in all the groups irrespective of the treatment given. On the 7th day (that is, 24 h after first exposure to elevated plus maze), significantly high TL was observed in aged control rats as compared to young control rats. However, betulinic acid significantly decreased TL on day 7 (i.e. RTL) in aged rats as compared to aged control animals, indicating an improvement of memory (Figure 2a). Memory enhancing effect of betulinic acid (50 mg/kg) was found to be comparable to the standard drug i.e. donepezil (1 mg/kg).

Effect of betulinic acid on scopolamine-induced amnesia in young rats using elevated plus maze

There was no significant effect of betulinic acid per se on TL of young rats on 6th and 7th days, indicating a non-significant effect on learning and memory. Scopolamine significantly increased TL on the 7th day in young male rats, indicating impairment of memory. Higher dose (50 mg/kg) betulinic acid significantly reversed scopolamine-induced amnesia (Figure 2b).

Effect of betulinic acid on escape latency and time spent in target quadrant by aged rats in Morris water maze

There was a significant increase in escape latency of aged female rats on 5th and 6th days as compared to young female rats,

indicating impairment of learning. There was a decrease in time spent in target quadrant in aged rats as compared to young rats, indicating impairment of memory. Betulinic acid (50 mg/kg) and donepezil (1 mg/kg) significantly increased learning and memory of aged rats as evident by decrease in EL on 5th and 6th days; and an increase in time spent in target quadrant in probe trial on 7th day (Figure 3a and 3b).

Effect of betulinic acid on scopolamine-induced amnesia in young male rats using Morris water maze

Scopolamine administered 30 min before recording TSTQ on 7th day significantly decreased TSTQ, indicating impairment of memory in young rats. Betulinic acid (50 mg/kg) significantly reduced scopolamine-induced spatial memory impairment, as indicated by reversal of scopolamine-induced decrease in TSTQ. However, betulinic acid per se treatment in young rats did not alter mean EL (Figure 3c) and TSTQ (Figure 3d) as compared to control rats, indicating non-significant effect on learning and memory.

Effect of betulinic acid on locomotor activity of young and aged rats

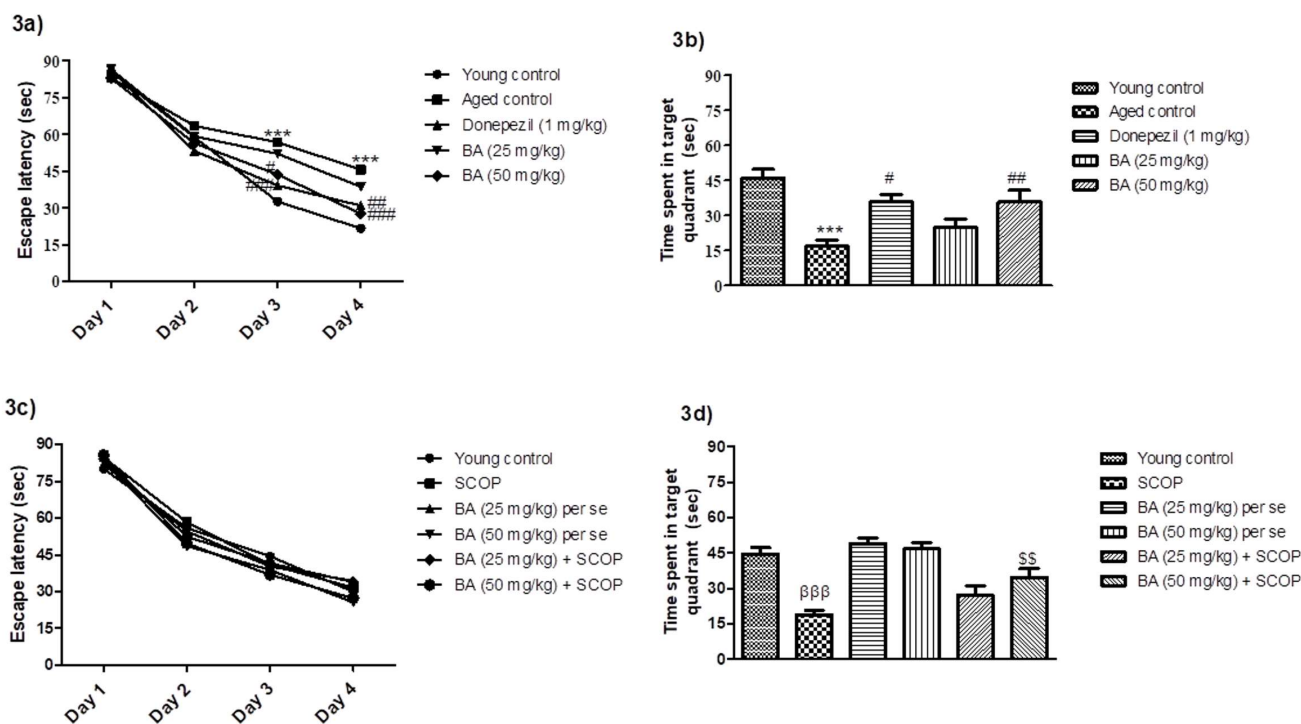


Figure 3. Effect of betulinic acid on escape latency and time spent in target quadrant in Morris water maze test in aged rats (3a and 3b respectively) and scopolamine-induced amnesic rats (3c and 3d respectively). Data are in mean \pm SEM (n=7 per group). Acquisition data (escape latency in 3a & 3c), as plotted in line diagram, were analyzed by two-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. Retention data (time spent in target quadrant in 3b & 3d) in probe trial was analyzed by one-way ANOVA followed by Tukey's test for multiple comparisons. BA -betulinic acid; SCOP - scopolamine.

For Figure 3a & 3b, $^{***}p < 0.001$ versus young control, $^{\#}p < 0.05$; $^{##}p < 0.01$ and $^{###}p < 0.001$ versus aged control. Interaction (time \times treatment) for Escape Latency. Time; $F(3,75) = 223.8$; $p < 0.0001$. Treatment; $F(4,75) = 21.48$; $p < 0.0001$. $F(4,25) = 9.335$; $p < 0.0001$ for TSTQ

For Figure 3c & 3d, $^{###}p < 0.001$ versus young control, $^{ss}p < 0.01$ versus SCOP. Interaction (time \times treatment) for Escape Latency. Time; $F(3,90) = 359.9$; $p < 0.0001$. Treatment; $F(5,90) = 2.157$; $p = 0.0856$. $F(5,30) = 16.29$; $p < 0.0001$ for TSTQ

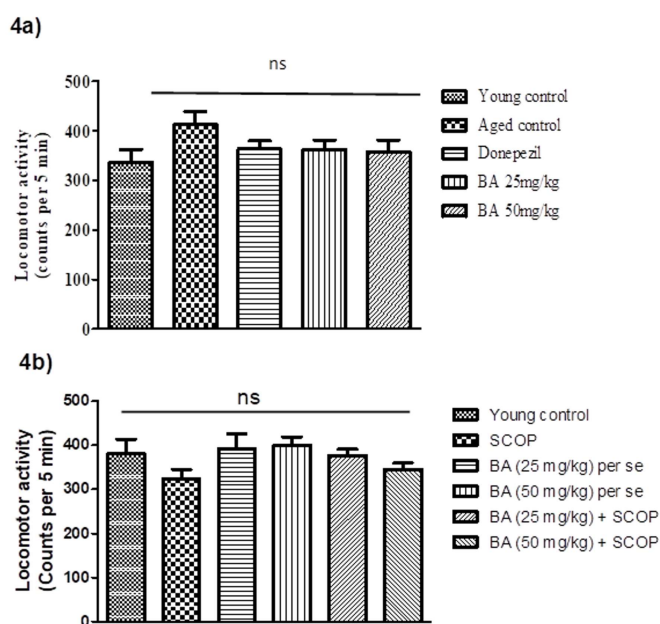


Figure 4. Effect of betulinic acid on locomotor activity of aged rats (4a) and scopolamine-induced amnesic rats (4b). Data are in mean \pm SEM ($n=7$ per group). Data were analyzed by one-way ANOVA followed by Tukey's test for multiple comparisons. BA- betulinic acid; ns- non significant; SCOP- scopolamine.

For Figure 4a, $F(4,30)=1.451$; $p=0.2416$.

For Figure 4b, $F(5,36)=1.452$; $p=0.2297$.

Spontaneous locomotor activity scores did not differ significantly among all the treatment groups as assessed on the last day of treatment (i.e. day 7) in both aged (Figure 4a) as well as young rats (Figure 4b).

Effect of betulinic acid on brain acetylcholinesterase activity in aged rats and scopolamine-induced amnesic young rats

Cholinergic function was determined in hippocampus and frontal cortex in terms of AChE activity. In the present study, AChE activity in hippocampus and frontal cortex of aged control animals was found to be reduced significantly ($p<0.01$ and $p<0.05$, respectively) as compared to young control animals. Betulinic acid (50 mg/kg) reduced the AChEs activity in aged rats as compared to vehicle-treated aged control rats, suggesting its AChEs inhibitory activity (Figure 5a). Scopolamine significantly increased AChE activity in hippocampus and frontal cortex of young rats. Betulinic acid (50 mg/kg) significantly reversed the scopolamine-induced increase in AChE activity in both hippocampus and frontal cortex of young rats (Figure 5b).

Effect of betulinic acid on brain TBARS level, GSH, SOD, and nitrite level in aged rats

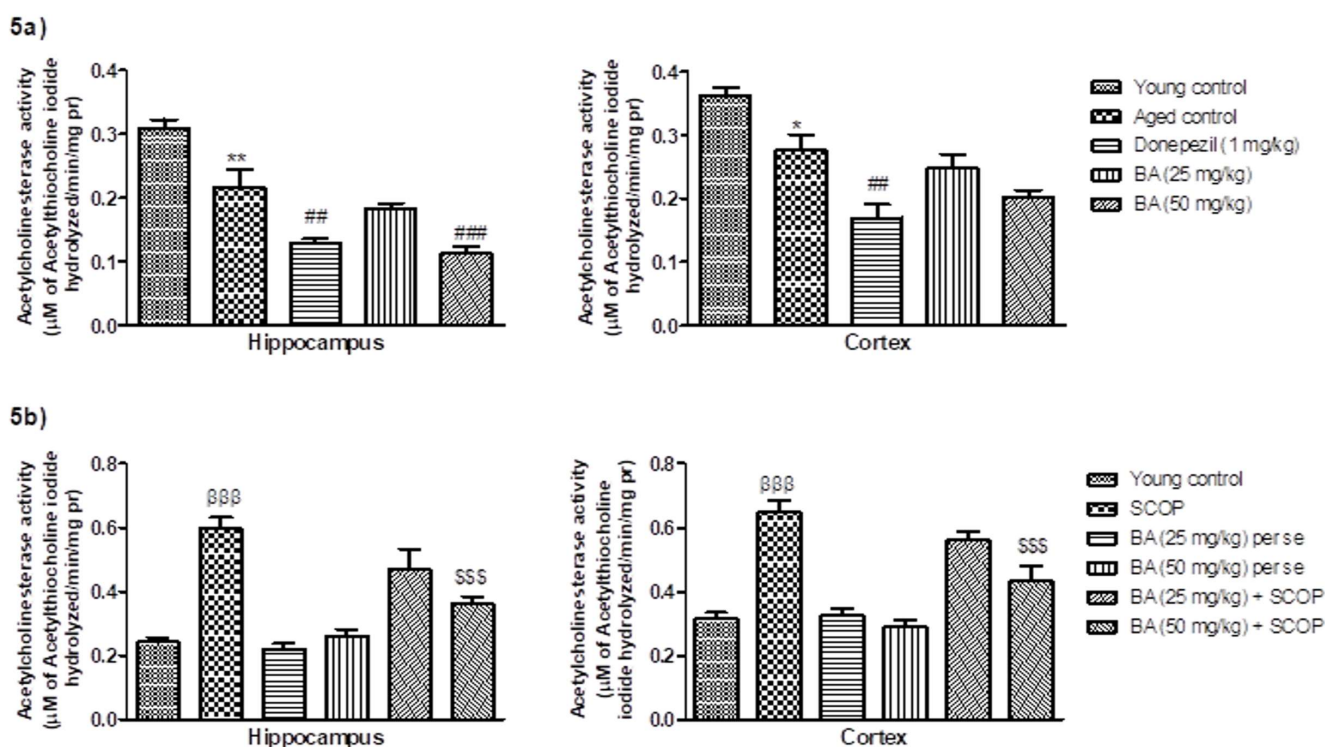


Figure 5. Effect of betulinic acid on brain acetylcholinesterase activity in aged rats (5a) and scopolamine-induced amnesic rats (5b). Data are in mean \pm SEM ($n=7$ per group). Data were analyzed by one-way ANOVA followed by Tukey's test for multiple comparisons. BA- betulinic acid; SCOP- scopolamine; pr- protein.

For Figure 5a, $^*p<0.05$ and $^{**}p<0.01$ versus young control, $^{##}p<0.01$ and $^{###}p<0.001$ versus aged control. $F(4,30)=26.78$; $p<0.0001$ (Hippocampus). $F(4,30)=14.67$; $p<0.0001$ (Cortex)

For Figure 5b, $^{###}p<0.001$ versus young control, $^{SSS}p<0.001$ versus SCOP; $F(5,36)=19.58$; $p<0.0001$ (Hippocampus). $F(5,36)=24.79$; $p<0.0001$ (Cortex)

Oxido-nitrosative stress was found to be markedly high in aged control rats as compared to young control rats. TBARS levels were increased significantly in both hippocampus and frontal cortex of vehicle-treated aged rats ($p<0.001$ and $p<0.001$, respectively) as compared to vehicle-treated young rats (Figure 6a). Further, treatment with donepezil significantly reduced

elevated TBARS level in both hippocampus ($p<0.05$) and frontal cortex ($p<0.01$) of aged rats as compared to vehicle-treated aged rats. Though, high dose of betulinic acid (50 mg/kg) significantly reduced TBARS levels in both hippocampus ($p<0.05$) and frontal cortex ($p<0.001$) as compared to aged control rats, but the low dose (25 mg/kg)

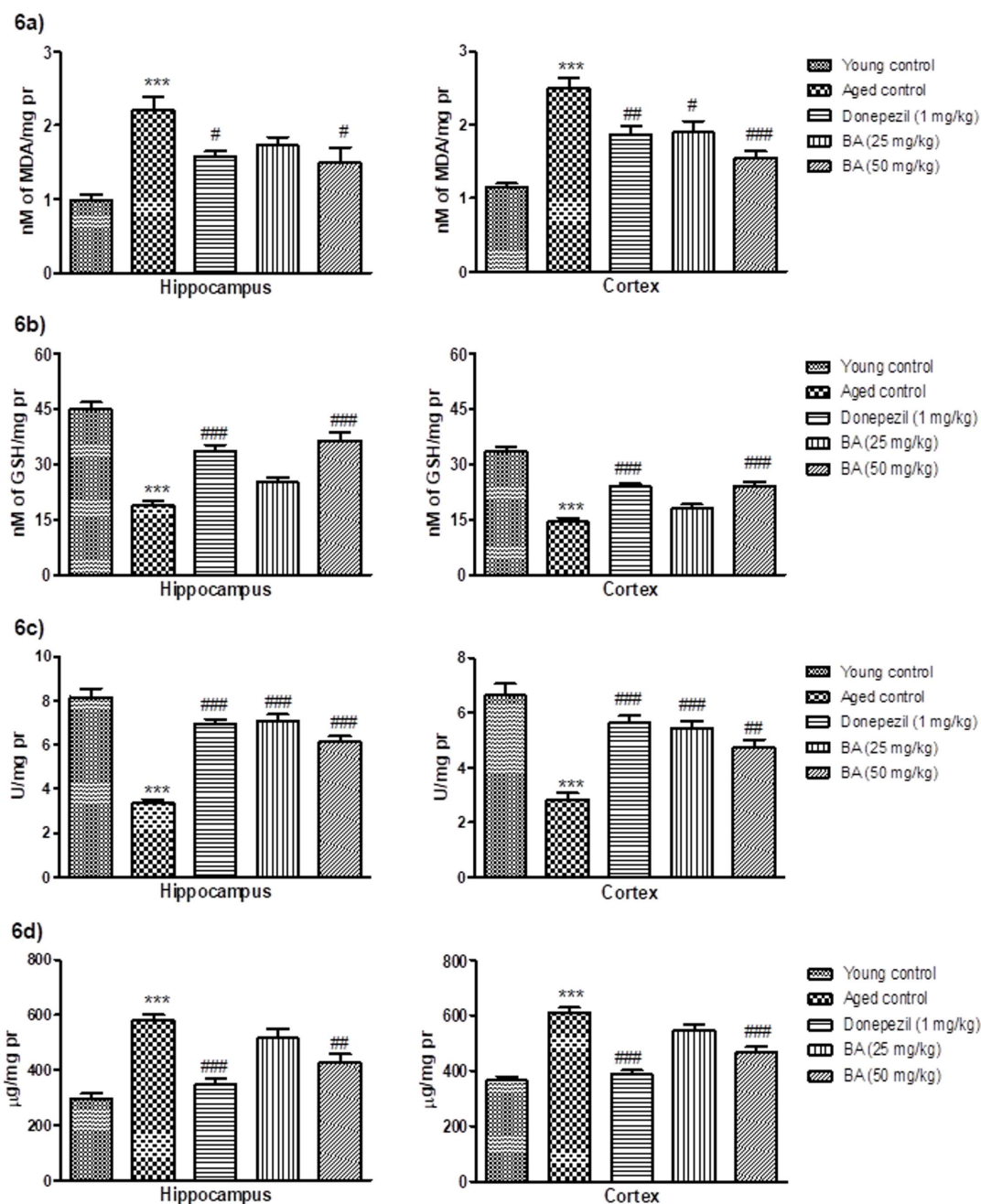


Figure 6. Effect of betulinic acid on brain lipid peroxidation (6a), reduced glutathione (6b), superoxide dismutase (6c), and nitrite levels (6d) in aged rats. Data are in mean \pm SEM ($n=7$ per group). Data were analyzed by one-way ANOVA followed by Tukey's test for multiple comparisons. BA- betulinic acid; pr –protein.

*** $p<0.001$ versus young control, # $p<0.05$, ## $p<0.01$ and ### $p<0.001$ versus aged control.

For Figure 6a, $F(4,28)=9.457$; $p<0.0001$ (Hippocampus). $F(4,30)=17.20$; $p<0.0001$ (Cortex)

For Figure 6b, $F(4,30)=36.01$; $p<0.0001$ (Hippocampus). $F(4,30)=47.00$; $p<0.0001$ (Cortex)

For Figure 6c, $F(4,30)=49.86$; $p<0.0001$ (Hippocampus). $F(4,30)=21.13$; $p<0.0001$ (Cortex)

For Fig 6d, $F(4,30)=18.78$; $p<0.0001$ (Hippocampus). $F(4,30)=32.19$; $p<0.0001$ (Cortex)

could able to reduce TBARS level only in frontal cortex (Figure 6a).

GSH levels and SOD activity was found to be decreased significantly in both hippocampus and frontal cortex of aged rats as compared to vehicle-treated young rats. Donepezil significantly ($p<0.001$) increased GSH levels and SOD activity as compared to vehicle-treated aged rats. Betulinic acid (50 mg/kg) significantly ($p<0.001$) reversed the ageing-induced decrease in GSH levels in both hippocampus and frontal cortex (Figure 6b). In addition, betulinic acid (25 and 50 mg/kg)

significantly restored the decreased SOD activity in both hippocampus ($p<0.001$) and frontal cortex ($p<0.001$ and $p<0.01$, respectively) of aged rats as compared to aged control rats (Figure 6c).

Nitrite levels were also found to be increased significantly ($p<0.001$) in both hippocampus and frontal cortex of aged rats as compared to vehicle-treated young rats (Figure 6d). Donepezil significantly ($p<0.001$) decreased nitrite levels in both hippocampus and frontal cortex as compared to aged control rats. Betulinic acid (50 mg/kg) significantly

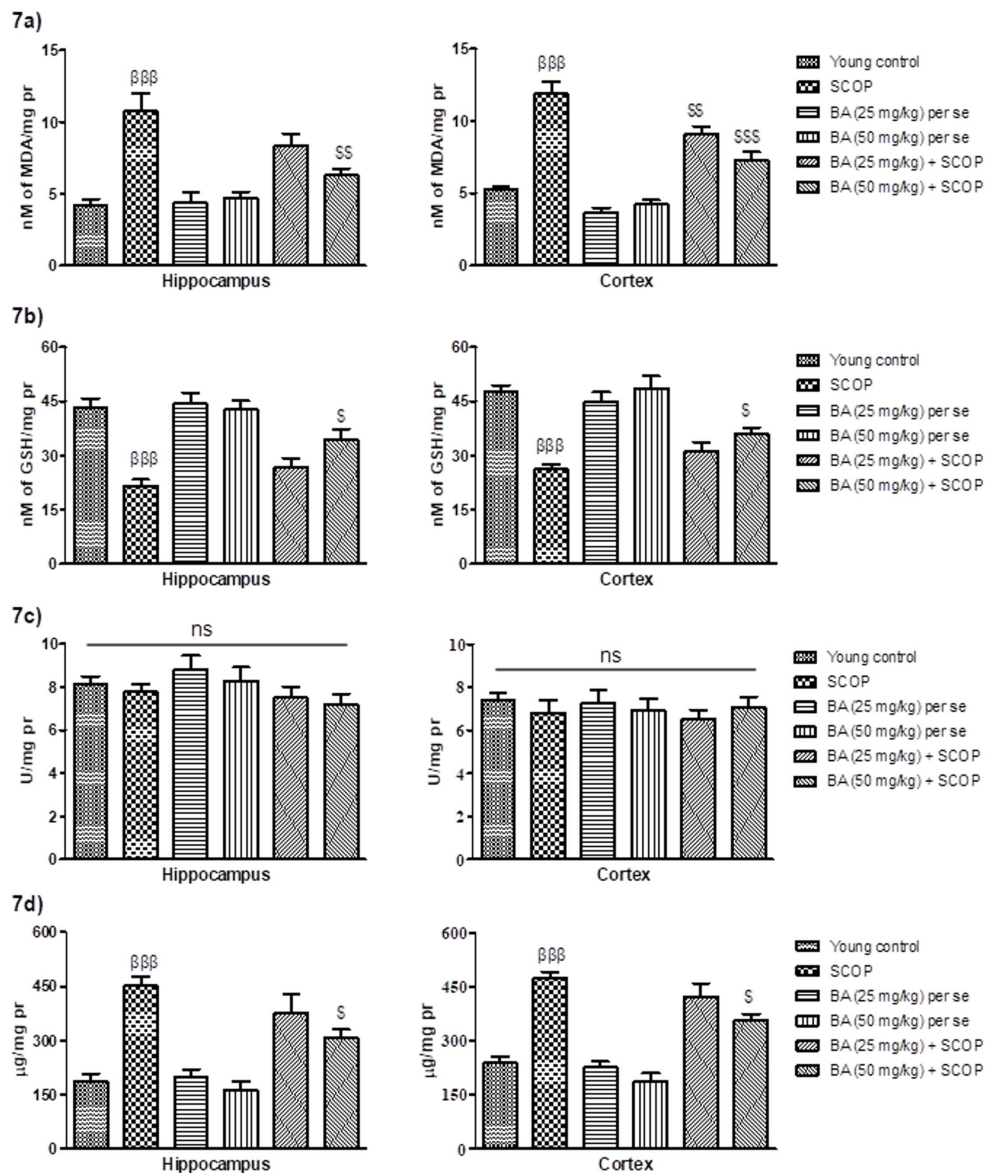


Figure 7. Effect of betulinic acid on brain lipid peroxidation (7a), reduced glutathione (7b), superoxide dismutase (7c), and nitrite levels (7d) in scopolamine-induced amnesic rats. Data are in mean \pm SEM (n=7 per group). Data were analyzed by one-way ANOVA followed by Tukey's test for multiple comparisons. BA-betulinic acid; ns – non-significant; SCOP-scopolamine; pr - Protein

$\beta\beta\beta$ $p<0.001$ versus young control, S $p<0.05$, SS $p<0.01$ and SSS $p<0.001$ versus SCOP.

For Figure 7a, $F(5,36) = 12.11$; $p<0.0001$ (Hippocampus). $F(5,36) = 40.75$ $p<0.0001$ (Cortex)

For Figure 7b, $F(5,36) = 14.16$; $p<0.0001$ (Hippocampus). $F(5,36) = 16.00$; $p<0.0001$ (Cortex)

For Figure 7c, $F(5,36) = 1.267$; $p=0.2995$ (Hippocampus). $F(5,36) = 0.3994$; $p=0.8460$ (Cortex)

For Figure 7d, $F(5,34) = 13.45$; $P<0.0001$ (Hippocampus). $F(5,34) = 27.8$; $p<0.0001$ (Cortex)

ameliorated ageing-induced increase in nitrite level in both hippocampus ($p < 0.01$) and frontal cortex ($p < 0.001$). However, low dose of betulinic acid (25 mg/kg) failed to show any significant effect on nitrite levels in aged rats (Figure 6d).

Effect of betulinic acid on brain TBARS level, GSH, SOD, and nitrite level in scopolamine-induced amnesic young male rats

Scopolamine administration significantly raised the oxidative stress as evidenced by an increase in TBARS levels (Figure 7a) and decrease in GSH level (Figure 7b) in both hippocampus ($p < 0.001$) and frontal cortex ($p < 0.001$) as compared to young control rats. However, SOD level was found to be unaltered in scopolamine treated rats as compared to young control rats (Figure 7c). Interestingly, betulinic acid (50 mg/kg) ameliorated the increased TBARS level in both hippocampus ($p < 0.01$) and frontal cortex ($p < 0.001$) of scopolamine treated rats. Further, betulinic acid could able to raise the decreased GSH level in both hippocampus and frontal cortex of scopolamine treated rats. On the contrary, treatment with betulinic acid *per se* (25 and 50 mg/kg) did not alter brain TBARS level, GSH level and SOD activity as compared to young control group. Scopolamine significantly ($p < 0.001$) increased nitrite level in hippocampus of young rats. Betulinic acid (50 mg/kg) significantly ($p < 0.05$) ameliorated scopolamine-induced increase in nitrite levels in both hippocampus and frontal cortex (Figure 7d).

Effect of betulinic acid on neurons in hippocampal CA1 region in aged rats

Microscopic histopathological analysis showed that hippocampal CA1 neurons in young control (Figure 8: Panel 1a & 1b; at 10x and 40x magnifications, respectively) were healthy with robust and oval shape and arranged linearly. On the other hand, photomicrographs from hippocampus CA1 region of aged control rat brain showed a significant loss of neurons as compared to young control group (Figure 8: Panel 2a & 2b; at 10x and 40x magnifications, respectively). Further, microscopic examination revealed that neurons in the aged control were large and sparsely arranged. Few of the degenerated cells were sickle-shaped or with an altered morphology. Donepezil (1 mg/kg; Figure 8: Panel 3a & 3b; at 10x and 40x magnifications, respectively) significantly decreased the neuronal loss in the hippocampus of aged rats. Betulinic acid (25 mg/kg, Figure 8: 4a, 4b and 50 mg/kg, Figure 5a, 5b; at 10x and 40x magnifications, respectively) showed more number of healthy neurons with an oval shape and clear cytoplasm as compared to aged control rats indicating their protective action. Further, betulinic acid with higher dose showed more protection in aged rats as compared to low dose.

Discussion

In the present study, we investigated the effect of betulinic acid on learning and memory of aged and young rats by means of behavioral, biochemical and histological examinations. The aged rats performed poorly in memory evaluation task as compared to young control rats, which can be assumed due to increased oxidative stress and nitrite level as well as decreased AChE activity. Further, aged rats showed more number of damaged neurons with shrunk or sickle-shaped and irregular morphology in the hippocampal CA1 region as compared to young control rats. However, these abnormalities were significantly attenuated by treatment with betulinic acid. Further, the observed effects were reproduced in scopolamine-induced amnesic young rats. To rule out the neuroprotective and nootropic effects of estrogen, young male rats were used in place of young female rats for scopolamine-induced amnesic rat model.

Betulinic acid significantly improved learning and memory of aged rats as compared to vehicle-treated aged rats when assessed using elevated plus maze and Morris water maze paradigms. Further, betulinic acid also improved the memory of scopolamine-induced amnesic rats. In addition, we did not observe any significant differences in locomotor activity of rats in any of the group, indicating that memory-improving effect of betulinic acid is independent of the effect on locomotor activity. Betulinic acid significantly ameliorated increased AChE activity in scopolamine-induced amnesic rats. On the contrary, the AChE activity was found to be decreased in aged rats as compared to young control rats. These results are in line with the previous findings where AChEs activity was found to be decreased in ageing and related disorders including Alzheimer's disease (Das et al., 2001). Such decrease in AChEs activity can be speculated due to significant loss of cholinergic innervations during ageing (Perry et al., 1992). In normal physiology, AChE hydrolysis ACh to acetate and choline that results in the termination of the effect of ACh at the cholinergic post-synapse (Soreq and Seidman, 2001). Therefore, a decrease in the release of ACh neurotransmitter and its increased breakdown by AChE enzyme at the synapse is thought to be responsible for memory loss and cognitive dysfunction in ageing and related disorders. Betulinic acid significantly increased memory and decreased AChE activity in aged rats. The memory improving effect of betulinic acid was comparable to that of standard drug i.e. donepezil. Our result is also supported by *in silico* study where betulinic acid was found to have a higher binding energy against AChE esterase receptors (Manigandan, 2014) and has also been reported to inhibit AChE activity (Jamila et al., 2014).

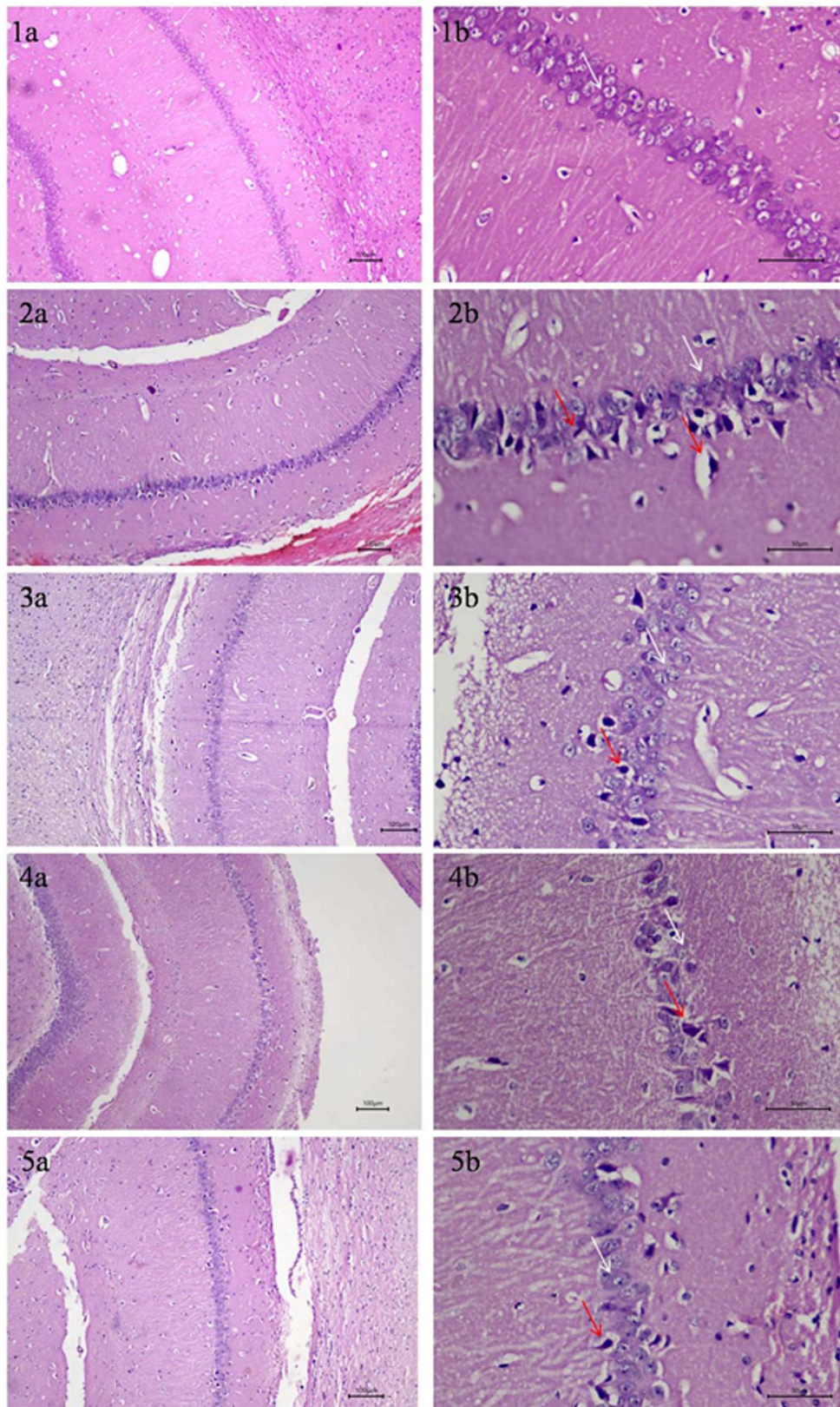


Figure 8. Effect of betulinic acid on histopathological changes in the CA-1 region of hippocampus of aged rats. Photomicrographs (10x & 40x) of H&E stained brain hippocampus CA1 sections. Panel 1a & 1b (at 10x and 40x magnifications respectively): Vehicle-treated young control group showing healthy neurons; Panel 2a & 2b (at 10x and 40x magnifications respectively): Aged control group showing dark stained degenerated neurons; Panel 3a & 3b (at 10x and 40x magnifications respectively): Donepezil (1 mg/kg) significantly decreased the neuronal loss in the hippocampus of aged rats; Panel 4a & 4b (at 10x and 40x magnifications respectively): BA (25 mg/kg) showed neuroprotection in aged rats as indicated by more number of healthy neurons with oval shape and clear cytoplasm as compared to aged control rats; Panel 5a & 5b (at 10x and 40x magnifications respectively): BA (50 mg/kg) showed more neuroprotection as compared to lower dose (25 mg/kg) of BA in aged rats; *BA*: betulinic acid.

Oxidative stress, which occurs due to the imbalance between reactive oxygen species and endogenous antioxidant defense system, can be measured by checking MDA level, GSH, and SOD activity. In the present study, betulinic acid significantly reduced the increased level of MDA and also increased the antioxidant GSH levels in both hippocampus as well as frontal cortex of aged rats as compared to aged control rats. Further, the treatment of betulinic acid could able to restore endogenous SOD activity which is found to be decreased in aged control rats. Meanwhile, brain nitrite level was also found to be significantly high in aged control rats as compared to young control rats. Although, NO derived from endothelial nitric oxide synthase (NOS) plays a role in preserving and maintaining the brain's microcirculation (Fu et al., 2011; Steinkamp-Fenske et al., 2007), NO derived from inducible NOS or neuronal NOS may have detrimental effects to the brain cells (Kanao et al., 2012; Toda et al., 2009), as NO reacts with superoxide ions and generates highly reactive peroxynitrite (ONOO⁻), which further trigger a cascade of harmful events (Ljubuncic et al., 2010; Maruyama et al., 2001; Pryor and Squadrito, 1995). NO is a gaseous free radical with a very short half-life in vivo. It is converted into more stable NO metabolites, nitrite (NO²⁻) and nitrate (NO³⁻), within few seconds of its release. Thus, measurements of the stable end products of NO in tissue homogenate provide an indirect measure of NOS activity and NO production (Lundberg et al., 2008). Herein, we found that betulinic acid administration significantly reduced the nitrosative stress as assessed by decreased nitrite level in both hippocampus as well as frontal cortex of aged rats as compared to aged control rats. This antioxidant effect of betulinic acid is consistent with previous findings where betulinic acid decreased LPO and simultaneously inhibited NO generation, thereby reduced oxidative or nitrosative stress (Blazevski et al., 2013; Lu et al., 2011). In addition, these antioxidant effects of betulinic acid were also observed in scopolamine-induced amnesic rats that showed an increased level of oxidative stress markers, which is in line with the previous findings (Budzynska et al., 2015; Fan et al., 2005).

To further confirm the protective effect of betulinic acid on brain cells, we performed the histopathological examination. The hippocampus CA1 region of aged control rat brain showed a significant loss of healthy neurons and increase in pyknotic neurons as compared to young control group. The morphological characterization of pyknotic neurons were observed as large and sparsely arranged, sickle-shaped neurons or with an altered morphology. Betulinic acid treated groups showed less number of pyknotic neurons and more number of healthy neurons with an oval shape and clear cytoplasm in the hippocampal CA1 region of aged rats, indicating its neuroprotective effect. Further, the protective effect was more

significant at high dose (50 mg/kg) than low dose (25 mg/kg) of betulinic acid.

Conclusion

In conclusion, the present findings depict the effectiveness of betulinic acid in improving age-related learning and memory impairment. The antioxidant and AChEs inhibitory activity of betulinic acid can be speculated to be involved in memory enhancing effect of betulinic acid. In addition, betulinic acid treatment also increased number of healthy neurons in the hippocampal CA1 region of aged rats, further supporting neuroprotective action. Thus, our findings revealed the importance of betulinic acid in preventing age-related learning and memory impairment. However, additional studies are warranted to further explore the downstream signalling pathways involved in its neuroprotective effect.

Conflict of interest

There is no conflict of interest among authors.

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