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ORIGINAL ARTICLE

Steroidal Saponin Dioscin Attenuated Aluminum Chloride–Induced Alzheimer's-Like Pathology in Rat Model via Modulation of Oxidative and Inflammatory Markers

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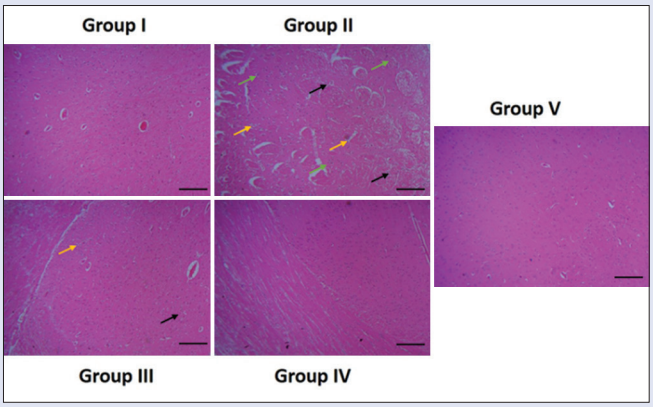
ABSTRACT

Background: In this study, we elucidated the neuroprotective effect of dioscin, a plant-derived steroidal saponin utilized in Chinese medicine to treat various diseases. To achieve this, we established the aluminum chloride–induced Alzheimer's disease rat model. **Materials and Methods:** Young, healthy, male Wistar rats were grouped into five: control, Alzheimer's disease–induced, Alzheimer's disease–induced and treated with 10 mg/kg dioscin, Alzheimer's disease–induced and treated with 20 mg/kg dioscin, and Alzheimer's disease–induced and treated with standard neuroprotective drug donepezil (1.5 mg/kg/day) by oral route. After completion of the treatment period, the animals were subjected to behavioral, biochemical, molecular, and histological analyses. Morris water maze and open-field tests were conducted to analyze the behavior of the animals. Acetylcholinesterase, glutamate, neurotransmitters, and oxidative stress markers were quantified to assess the biochemical changes in Alzheimer's-induced and dioscin-treated rats. Furthermore, quantitative polymerase chain reaction (qPCR) analysis was done to assess the mRNA expression of proinflammatory genes, and the neuroprotective effect of dioscin was confirmed with histopathological analysis of hippocampal region. **Results:** Aluminum chloride–induced neurodegeneration in rats was evident with behavioral, biochemical, molecular, and histological analyses. The results of behavioral analysis revealed that dioscin treatment increased the memory in Alzheimer's-induced rats. It also decreased the oxidative stress markers, and increased the level of norepinephrine and dopamine neurotransmitters involved in cognition. The mRNA expression of proinflammatory cytokines was significantly decreased in Alzheimer's-induced dioscin-treated rats. Histopathological analysis revealed that dioscin protects the hippocampus from aluminum chloride–induced neurodegeneration. **Discussion:** Overall our results confirmed that dioscin is a potent neuroprotectant and can be utilized to treat Alzheimer's disease in the future.

Key words: Alzheimer's disease, dioscin, neurodegenerative diseases, neuroprotectant, phytochemical, rat model

SUMMARY

- Dioscin increased the levels of cholinergic and biogenic neurotransmitters and decreased the levels of glutamate, thereby preventing $AlCl_3$-induced learning and memory impairment.
- Dioscin reduced oxidative stress, inhibited inflammation, and prevented the accumulation of Aβ42 in the hippocampus, and thereby prevented $AlCl_3$-induced Alzheimer's-like disease in rats.



Abbreviations used: ROS: reactive oxygen species; TBARS: Thiobarbituric acid reactive substance; SOD: superoxide dismutase; $AlCl_3$: Aluminum chloride.

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INTRODUCTION

Globally, the count of older population has drastically increased in recent decades. The World Health Organization (WHO) has reported that the population of older people above 60 years will increase from 12% to 22% by 2050.[1] The geriatric care given to these people will be a greater task for both the families and also the country. Even though cancer and cardiovascular disease are considered to be the leading causes of mortality in old people, neurodegenerative diseases tend to be a major concern since the treatment for these diseases are costly and the available drugs are not much potent. More than 50 million individuals were affected with dementia and the incidence rate is increasing 20%, annually. By 2030, approximately 66 million individuals will be affected with Alzheimer's disease and it is predicted to be doubled by 2050.[2-4] Alzheimer's disease is a multifactorial, progressive neurodegenerative

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disease which impairs the brain functions. Memory loss and decline in cognition are the major pathology of Alzheimer's disease; apart from this, patients suffer with behavioral disturbances, attention deficiency, and language, which impair their daily activities.[5,6]

Treatment costs for Alzheimer's was estimated to be 305 billion dollars in 2020 and it is expected to increase by 1 trillion dollars by 2050.[7] At present, there is no potent drug which completely cures Alzheimer's disease whereas few drugs are available to delay the progress of disease and treat symptoms such as memory and behavior impairment. Most of the drugs discovered failed in clinical trials and also rendered serious side effects.[8,9] Hence it is the need of the hour to discover a potent cost-effective drug to treat Alzheimer's disease. Since oxidative stress plays a crucial role in neurodegenerative diseases, phytomedicine which are potent antioxidants are boom to treat these diseases. Dioscin is one such phytochemical derived from plants such as *Dioscorea nipponica* and *Dioscorea zingiberensis*.[10] Dioscin is a steroidal saponin which exhibits antioxidant, anti-inflammatory, antifungal, antiviral, antitumor properties.[11–13] It is also a potent immunostimulant and lipid lowering drug.[14,15]

Since Alzheimer's disease is a multifactorial disease, the exact causative agent has not yet been discovered. Heavy metal pollution of the environment had been proven to impair brain development and induce neurodegeneration.[16] Aluminum is a heavy metal which can induce Alzheimer's disease. Increased exposure to aluminum leads to neurofibrillary tangles, neuroinflammation, and amyloid aggregation which are the pathologies observed in patients with Alzheimer's disease.[17,18] The rat model of Alzheimer's disease induced by aluminum chloride ($AlCl_3$) is a widely accepted model which mimics the human Alzheimer's disease.[17,19,20] Therefore, in this study, we aimed to evaluate the neuroprotective effect of steroidal saponin dioscin against Alzheimer's disease using $AlCl_3$-induced Alzheimer's disease rat model.

MATERIALS AND METHODS

Chemicals

$AlCl_3$ crystals (purity: 99%), dioscin (purity: ≥95%), and donepezil (purity: ≥95%) were procured from Sigma Aldrich, USA. Analytical grade and high-purity chemicals were used in this study.

Animals

In this study, we used three-month-old male Wistar rats weighing around 210–260 g. The animals were procured from the institutional animal house after obtaining permission from the institutional ethical committee. The rats were acclimatized for a week to the laboratory conditions with a temperature of 25 ± 2°C, relative humidity of 55 ± 5%, and 12-hour light–dark cycles. The rats were caged in a polypropylene cage bedded with rice husk. The cages were cleaned thrice a day, and the bedding was changed daily. Animals were fed standard laboratory rat diet pellets and water *ad libitum*. Only the protocols approved by the institutional ethical committee were conducted on animals, and the animals were treated with utmost care and concern.

Experimental design

40 healthy male Wistar rats were divided into five groups, each consisting of eight rats. Group I animals were treated with vehicle alone (control group). Group II animals were induced with Alzheimer's disease by treating them with $AlCl_3$ orally once a day for 15 consecutive days. $AlCl_3$ solution was freshly prepared in distilled water and administered orally with 0.5 mL/100 g b.w.[21] Group III animals were treated orally with 10 mg/kg/day of dioscin for 21 days concomitant with $AlCl_3$ for 15 days in the same manner. Group IV rats were treated orally with 20 mg/kg/day of dioscin for 21 days concomitant with $AlCl_3$ for 15 days. Group V rats were treated orally with 1.5 mg/kg/day of donepezil (positive control) for 21 days concomitant with $AlCl_3$ for 15 days.

Behavioral analysis

Morris water Maze

The animals were subjected to Morris water maze test.[22] The Morris water maze test is a circular pool with a diameter of 160 cm filled with water till a depth of 30 cm. The experiment was conducted in two parts: acquisition and PROBE trials. While performing the acquisition trial, a transparent platform was placed in the middle of the maze at a height of 1 inch from below the water level. The animals were placed on the platform for 20 seconds and then placed gently into the water at the south corner of the maze. Then, the animals were allowed to fetch the platform for 60 seconds, but if they failed, the animals would then be guided to the platform. The animals were trained for three days with three consecutive trials per day. The temperature of the water was maintained at 26°C, and the training was conducted daily between 8 and 12 pm. Then, the platform was removed, and the animals were allowed to swim freely for 60 seconds. The number of times the animals crossed the platform, the location, and the time spent in that area were recorded and tracked.

Open Field test

The open field apparatus is a rectangular cage made of high-density non-porous plastic with a dimension of 100 cm × 100 cm × 40 cm. The cage floor was divided into 25 equal squares and the open field was well illuminated with light. The animals were placed in the middle of the open field and allowed to explore for five minutes. The activities of animals were video recorded and assessed with SMART video-tracking software. The rearing behavior of rats and the number of squares crossed were counted to analyze the locomotory behavior of rats. The open field cage was wiped with 90% ethanol and allowed to dry completely before the initiation of the experiment with the other animals.

Biochemical Analysis

Preparation of tissue homogenate

The hippocampal tissues were isolated from the control and experimental animals after the ether anesthesia and sacrifice. The tissue homogenate was prepared by homogenizing 300 mg of hippocampal tissue with 500 µL of phosphate-buffered saline (PBS). The homogenized mixture was subjected to ultrasonication to break the cell membrane completely. Then, the samples were centrifuged at 7,500 rpm for 10 minutes at 4°C; supernatant was collected and store at -20°C for further analysis.

Quantification of acetylcholinesterase activity

Acetylcholinesterase activity was quantified using assay kit as per the manufacturer's protocol (Cusabio, Houston, USA). The reagents and working standards were prepared according to the manufacturer's protocol. Briefly, 100 µL of brain tissue homogenate was added to each well and incubated for two hours at 37°C. After incubation, the samples were discarded, and 100 µL of biotin antibody was added and incubated for one hour. The biotin antibody was aspirated and the wells were rinsed thrice with 200 µL of wash buffer. 100 µL of horse radish peroxidase (HRP)-avidin was added and incubated for an hour. After one hour, the HRP-avidin complex was removed and the plates were rinsed for five times with wash buffer. Then, 90 µL of TMB (3,3',5,5'-Tetramethylbenzidine) substrate was added and incubated in dark for 15 minutes. Then, the reaction was arrested by adding 50 µL of

stop solution. The absorbance of the sample was quantified at 450 nm using a microplate reader.

Quantification of glutamate levels

Glutamate levels in the brain tissue samples were measured based on the competitive enzyme immunoassay kit (MyBiosoure, USA). The assay was conducted based on the instructions provided by the manufacturer. The final absorbance of the sample solution was measured at 450 nm using the microplate reader.

Quantification of neurotransmitters

Noradrenaline and dopamine levels in the brain tissues were measured using the commercially available assay kits procured from LifeSpan BioSciences, Inc. The optical densities of the samples were measured at 450 nm using microplate reader.

Quantification of oxidative stress markers

Oxidative stress markers in the brain tissues were measured using commercially available kits (Elabscience, USA). Briefly, dichlorofluorescin is oxidized to dichlorofluorescin in the presence of reactive oxygen species (ROS), and the levels of dichlorofluorescin can be measured with an excitation wavelength of 502 nm and the emission wavelength of 525 nm to quantify the level of ROS is samples. Lipid peroxidation was measured by quantifying the levels of thiobarbituric acid reactive substances (TBARS), the byproduct of lipid peroxidation. It was measured at an excitation wavelength of 520 nm and an emission wavelength of 550 nm using microplate reader. Superoxide dismutase (SOD) inhibits the formation of superoxide anion, thereby inhibiting the oxidization of hydroxylamine to form nitrite. The absorbance was measured at 550 nm. Glutathione levels were measured using competitive enzyme-linked immunosorbent assay (ELISA), and the absorbance of the sample was measured at 450 nm. To form a reddish azo compound that can be measured at 550 nm. The absorbance values are directly proportional to the levels of nitric oxide. Next, catalase activity was measured calorimetrically by measuring the absorbance at 405 nm using microplate reader.

qPCR analysis

The mRNA expression of proinflammatory cytokines interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α and major histocompatibility complex (MHC) class II marker for microglial activation was measured via real-time PCR analysis. Total RNA was isolated with MagMAX mirVana Total RNA Isolation Kit (Thermo Fischer, USA). The isolated RNA was converted to cDNA using Maxima H Minus Double-Stranded cDNA Synthesis Kit (Thermo Scientific, USA). Then, qPCR analysis was done using PowerTrack SYBR green master mix in CFX Opus 96 Real Time PCR system (BioRad, USA).

Quantification of amyloid-beta 42 levels

The level of amyloid-beta 42 (Aβ42) in the brain tissue samples was measured by using a commercially available assay kit (MyBiosource). The assay was conducted based on the manufacturer's instructions provided with the kit. The absorbance of the samples was measured at 450 nm using the microplate reader. Concentration of the samples was estimated based on the standard curve plotted with standards.

Histological analysis

Hippocampal tissues of control and experimental animals were fixed with Bouin's fixative solution (0.2 g of picric acid and 2 g of paraformaldehyde in distilled water) for 24 hours. The fixed tissue was processed according to the protocol of Bancroft and Gamble (2002).[23] The sliced tissues (4 μ thick) were stained with hematoxylin and eosin. The stained sections were viewed and photographed under light microscope.

Statistical analysis

The data were statistically analyzed with one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls *post hoc* test by using SPSS software version 16.0 (SPSS Inc., IBM, USA). $P < 0.05$ was considered as statistically significant.

RESULTS

Effect of dioscin on the behavioral analysis of $AlCl_3$-exposed rats

Morris water maze

Figure 1 shows the results of the Morris water maze test. According to the results, the mean latency time taken by the animals to reach the platform, mean time spends on target quadrant, and the number of times the animals crossed the original platform were calculated. On day 1, there was no difference in the mean latency time between the experimental groups; however, there was a significant difference on days 2 and 3. On day 3, the mean latency time of $AlCl_3$-treated group increased to 70 ± 3 seconds, whereas the rats concomitantly treated with low- and high-dose dioscin showed latency of 45 ± 5 seconds and 40 ± 3 seconds, respectively. Control- and donepezil- (standard drug) treated groups showed less latency of 20 ± 2 seconds and 25 ± 4 seconds, respectively. On day 5, the mean latency of 20 mg/kg dioscin + $AlCl_3$ treated group significantly ($P < 0.05$) decreased to 28 ± 4 seconds, which is comparatively equal to the mean latency time of control- and donepezil-treated rats. Figure 1b shows the mean time spends on the target quadrant. $AlCl_3$-treated animals showed less time spent of 20 ± 4 seconds, whereas rats concomitantly treated with low- and high-dose dioscin showed mean time spent of 55 ± 7 seconds and 45 ± 6 seconds, respectively. Figure 1c represents the number of times the rats crossed the original platform. Control, $AlCl_3$ + low-dose dioscin treated, $AlCl_3$ + high-dose dioscin treated, and donepezil-treated animals showed no significant difference. $AlCl_3$ alone treated animals crossed the original platform less than seven times only.

Open field test

The memory capacity of the rats was evaluated using the open field test. Figures 1c and 1d show the number of squares crossed by the rats and the number of rearing performed. Compared to the control animals, $AlCl_3$-treated animals significantly crossed ($P < 0.05$) less number of squares. There was no significant difference between $AlCl_3$ + high-dose dioscin-treated and donepezil-treated animals [Figure 1c]. Compared to $AlCl_3$-treated animals $AlCl_3$ + low-dose and $AlCl_3$ + high-dose dioscin-treated animals showed increased rearing performance. There was no significant difference between $AlCl_3$ + low-dose and $AlCl_3$ + high-dose dioscin-treated and donepezil-treated animals [Figure 1d].

Effect of dioscin on biochemical parameter in $AlCl_3$-exposed rats

Effect of dioscin on acetylcholinesterase activity

Figure 2a illustrates the activity of acetylcholinesterase in control and experimental animals. $AlCl_3$ drastically increased the activity of acetylcholinesterase to 6.5 ± 0.8 U/mg protein compared to control (4.8 ± 1.1 U/mg protein). Both low- and high-dose dioscin significantly ($P < 0.05$) reduced the activity of acetylcholinesterase to 5.8 ± 0.7 and 4.1 ± 1.2 U/mg protein, respectively. There was no significant difference between high-dose dioscin-treated rats and control animals. Donepezil-treated animals showed significant reduction in the activity of acetylcholinesterase compared to the control animals.

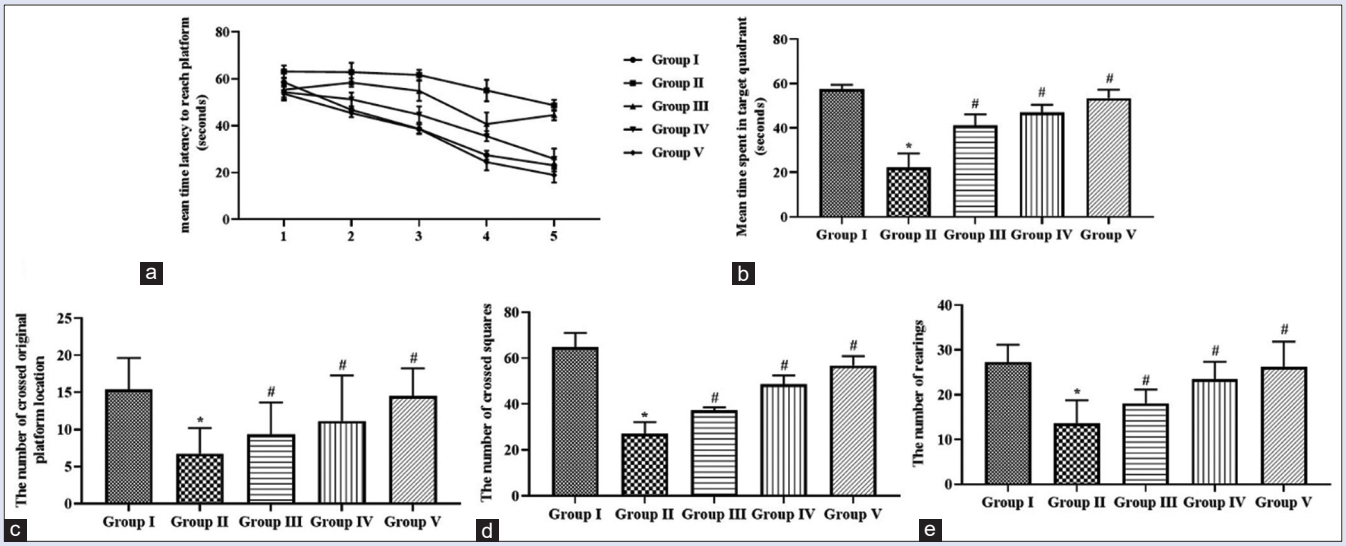

Figure 1: Effect of steroidal saponin dioscin on behavioral analysis in $AlCl_3$-exposed rats. Morris water maze test: (a) Mean latency time taken by the rats to reach the platform; (b) Mean time spend on target quadrant; (c) Number of times the rats crossed the original platform. Open field test: (d) Number of squares crossed by the rats; (e) Number of rearings performed. Data were statistically analyzed with one-way ANOVA followed by Student–Newman–Keuls *post hoc* test. $P < 0.05$ was considered as statistically significant, * control vs others, # $AlCl_3$ vs others

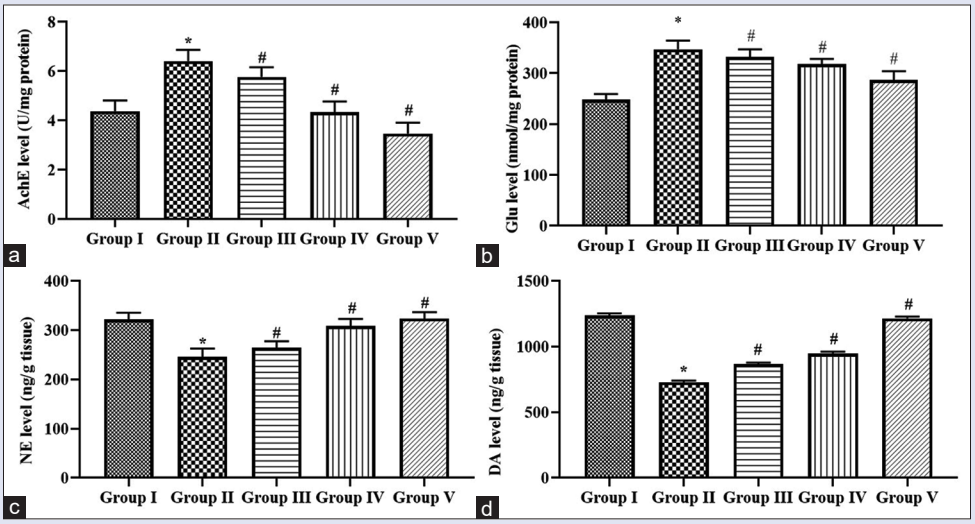

Figure 2: Effect of steroidal saponin dioscin on biochemical parameter in $AlCl_3$-exposed rats. (a) Acetylcholinesterase activity; (b) Glutamate; (c) Nor-epinephrine; (d) Dopamine. The estimation of biochemical parameters was performed with commercially available kits. Data were statistically analyzed with one-way ANOVA followed by Student–Newman–Keuls *post hoc* test. $P < 0.05$ was considered as statistically significant, * control vs others, # $AlCl_3$ vs others

Effect of dioscin on glutamate

Figure 2b depicts the results of glutamate in control and experimental animals. According to the results, there was a significant increase in the level of glutamate in $AlCl_3$-treated animals (355 ± 2.1 nmol/mg protein) compared to the control animals (263 ± 3.5 nmol/mg protein). Compared to $AlCl_3$-treated animals, $AlCl_3$ + high-dose dioscin-treated animals showed significant ($P < 0.05$) decrease in the levels of glutamate (310 ± 1.8 nmol/mg protein). There was no significant difference between $AlCl_3$ + high-dose dioscin-treated rats and donepezil-treated rats.

Effect of dioscin on neurotransmitters

Figure 2c represents the levels of norepinephrine in the control and experimental animals. Norepinephrine was significantly reduced in $AlCl_3$-treated animals (235 ± 4.2 ng/g tissue) compared to the control animals (318 ± 3.6 ng/g tissue), $AlCl_3$ + low-dose dioscin-treated animals (252 ± 4.5 ng/g tissue), and $AlCl_3$ + high-dose dioscin-treated animals (295 ± 3.5 ng/g tissue). There was no significant difference between $AlCl_3$ + high-dose dioscin-treated animals and donepezil-treated animals (310 ± 3.8 ng/g tissue). Figure 2d shows the levels of dopamine in control and experimental animals. $AlCl_3$ + low-dose dioscin-treated animals (920 ± 15 ng/g tissue) and $AlCl_3$ + high-dose dioscin-treated animals (955 ± 8 ng/g tissue) showed significantly ($P < 0.05$) increased levels of dopamine compared to the $AlCl_3$-treated animals (780 ± 11 ng/g tissue). There was no significant difference between the control (1320 ± 15 ng/g tissue) and donepezil-treated animals (1250 ± 10 ng/g tissue).

Effect of dioscin on oxidative stress markers in $AlCl_3$-treated rats

In this study, we measured the level of ROS, lipid peroxidation, superoxide dismutase, and glutathione in control and experimental animals; their results are illustrated in Figure 3. Figure 3a shows the level of total ROS. Compared to control animals (2250 ± 20 fluorescence/mg protein), $AlCl_3$-treated rats showed significant increase in the levels of ROS (5750 ± 30 fluorescence/mg protein). Dioscin significantly ($P < 0.05$) decreased the levels of ROS. Compared to low-dose dioscin (5250 ± 25 fluorescence/mg protein), high-dose dioscin significantly ($P < 0.05$) reduced the levels of ROS (4950 ± 20 fluorescence/mg protein). Figure 3b shows the results of TBARS levels in control and experimental animals. High-dose dioscin significantly decreased the levels of TBARS (2.7 ± 0.3 nmol/MDA equivalents/mg protein) compared to the $AlCl_3$-treated animals (3.8 ± 0.1 nmol/MDA equivalents/mg protein). There was no significant difference between low-dose dioscin-treated (3.6 ± 0.08 nmol/MDA equivalents/mg protein) and the $AlCl_3$-treated animals. $AlCl_3$ significantly decreased the activity of SOD (270 ± 2.9 U/mg protein) compared to the control animals (430 ± 2.2 U/mg protein). Both low-dose (355 ± 4.7 U/mg protein) and high-dose dioscin (370 ± 3.8 U/mg protein) significantly ($P < 0.05$) increased the activity of SOD. There was no significant difference between high- and low-dose dioscin and donepezil-treated animals [Figure 3c]. GSH levels were also significantly decreased in $AlCl_3$-treated animals (9.6 ± 0.8 nmol/mg protein) compared to the control animals (14.8 ± 0.5 nmol/mg protein). Compared to $AlCl_3$-treated animals, both the low- (11.5 ± 0.8 nmol/mg protein) and high-dose (13.8 ± 0.6 nmol/mg protein) dioscin-treated animals showed significant ($P < 0.05$) increase in the GSH levels [Figure 3d].

Figure 4 represents the levels of nitric oxide and activity of catalase in the experimental animals. Nitric oxide levels were significantly increased in $AlCl_3$-treated animals (13.6 ± 0.8 µmol/mg protein) compared to the control group animals (3.9 ± 0.4 µmol/mg protein). Dioscin significantly ($P < 0.05$) reduced the levels of nitric oxide (9.7 ± 0.7, 7.2 ± 0.5 µmol/mg protein low- and high-dose dioscin, respectively). Catalase activity was significantly ($P < 0.05$) increased in both low- (5.8 ± 0.2 H_2O_2 decomposed/min/mg protein) and high-dose dioscin (8.2 ± 0.5 H_2O_2 decomposed/min/mg protein) group compared to the $AlCl_3$-treated group (4.7 ± 0.4 H_2O_2 decomposed/min/mg protein) [Figure 4b].

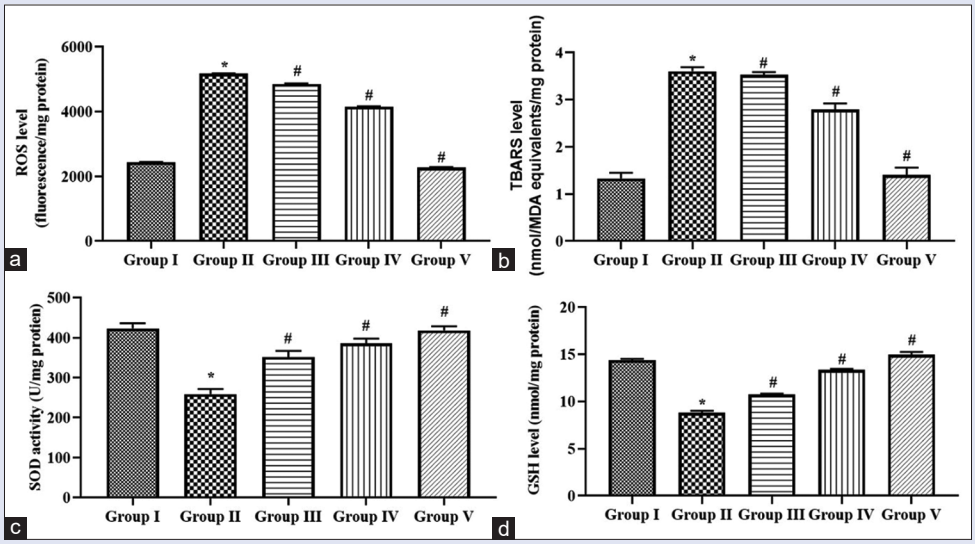

Figure 3: Effect of steroidal saponin dioscin on oxidative stress markers in $AlCl_3$-exposed rats. (a) Total reactive oxygen species, (b) Lipid peroxidation, (c) Superoxide dismutase, and (d) Glutathione were quantified in control and experimental, using commercially available kits. Data were statistically analyzed with one-way ANOVA followed by Student–Newman–Keuls *post hoc* test. $P < 0.05$ was considered as statistically significant, * control vs others, # $AlCl_3$ vs others

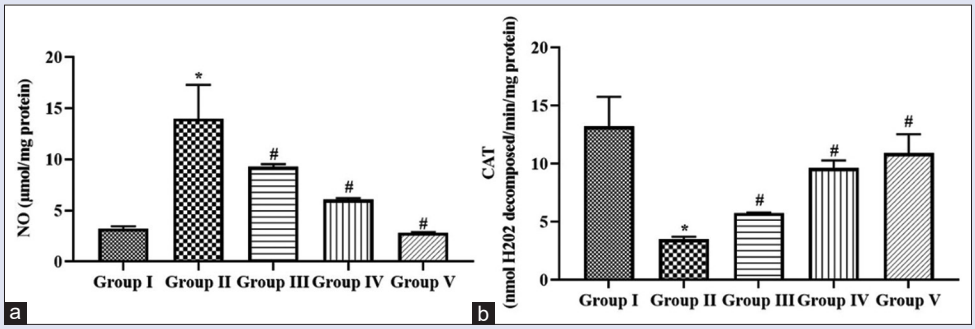

Figure 4: Effect of steroidal saponin dioscin on oxidative stress markers in $AlCl_3$-exposed rats. (a) Nitric Oxide and (b) Catalase levels in the control and experimental rats. Data were statistically analyzed with one-way ANOVA followed by Student–Newman–Keuls *post hoc* test. $P < 0.05$ was considered as statistically significant, * control vs others, # $AlCl_3$ vs others

Effect of dioscin on proinflammatory cytokines in $AlCl_3$-exposed rats

The mRNA expression of proinflammatory cytokines such as IL-1β, IL-6, TNF-α, and major histocompatibility complex class II marker for microglial activation was measured using real-time PCR analysis and the results were depicted in Figure 5. The expression of proinflammatory cytokines were significantly increased in $AlCl_3$-treated rats compared to the control rats. $AlCl_3$ + dioscin treatment significantly ($P < 0.05$) decreased the proinflammatory cytokines mRNA expression compared to the $AlCl_3$ alone treated rats. No significant difference was seen between the standard drug donepezil-treated rats and dioscin-treated rats.

Effect of dioscin on Aβ42 in $AlCl_3$-exposed rats

Figure 6 shows the results of Aβ42. According to the results, $AlCl_3$ significantly increased the levels of Aβ42 (57 ± 2.7 pg/mL), whereas simultaneous treatment with dioscin decrease the Aβ42 (38 ± 1.5, 27 ± 1.8 pg/mL, low- and high-dose dioscin, respectively). No significant difference was observed between the donepezil (25 ± 1.3 pg/mL) and high-dose dioscin (27 ± 1.8 pg/mL).

Effect of dioscin on the histomorphology of the hippocampus in $AlCl_3$-exposed rats

Figure 7 illustrates the light microscopic images of hematoxylin and eosin (H&E)–stained hippocampal tissue of control and experimental animals. According to the results, the control and $AlCl_3$ + donepezil-treated animals showed normal histoarchitecture of hippocampal region with normal neurons. There were increased number of pyknotic and vacuolated cells in $AlCl_3$-exposed animals (Group II). Cellular shrinkage, chromatolysis, and necrotic cells were viewed in $AlCl_3$-exposed rats. Compared to $AlCl_3$-treated animals, the $AlCl_3$ + dioscin-treated animals showed decreased number of necrotic, vacuolated, and pyknotic cells. The hippocampal damage was reduced in high-dose dioscin-treated rats (Group IV) compared to the low-dose dioscin-treated rats (Group III).

DISCUSSION

So far, there is no potent drug available to cure Alzheimer's disease. Drugs such as cholinesterase inhibitors and glutamate regulators have been used to treat symptoms of Alzheimer's, but these drugs show side effects such as swelling of the brain, microhemorrhages, and fall. We analyzed the potency of phytochemical dioscin to protect brain from Alzheimer disease. Aluminum, a heavy metal, increases the formation of ROS, which interrupt various signaling pathways in the central nervous system.[24] Increased exposure to aluminum leads to neuroinflammation, amyloid aggregation, and formation of neurofibrillary tangles which are characteristic pathology of Alzheimer's disease.[25,26] Therefore, we induced Alzheimer's disease in rats with $AlCl_3$ which is a widely accepted model to induce Alzheimer's in a rat model. The hippocampus region is involved in learning and memory function of the brain and is the most vulnerable part of the brain which is affected in Alzheimer's disease.[27] Therefore, in this study, we selected hippocampus region of interest to assess the potency of dioscin against neuronal damage.

The Morris water maze test is a behavioral study performed to analyze spatial learning and memory, which is used to assess the potency of discovered drugs on cognition.[28–30] In our study, $AlCl_3$ exposure significantly increased the mean latency time of the rats to reach the platform. $AlCl_3$-treated animals were unable to find the original platform, and the time spent on platform was less compared to the control and dioscin-treated animals. However, dioscin-treated rats showed reduced mean latency time and were able to find the original platform region during PROBE trial, which this proves that dioscin protected the hippocampal region from $AlCl_3$-induced damage. The exploratory behavior of rats was assessed with open field test.[31] $AlCl_3$-treated rats were in stressed condition and roamed along the sides of the wall of the open field, whereas $AlCl_3$ + dioscin-treated animals showed exploratory behavior, which may be because dioscin protected the neurons from $AlCl_3$-induced behavioral changes.

Learning and memory are regulated by the cholinergic neurotransmitter called acetylcholine. Acetylcholine regulates hippocampus function to optimize different phases of memory. It is metabolized by an enzyme acetylcholinesterase which is found to be elevated in patients with Alzheimer's disease.[32,33] Therefore, inhibiting the activity of acetylcholinesterase might be able to protect the hippocampus from neurodegeneration and improve memory in patients with Alzheimer's disease. In this study, dioscin significantly decreased the levels of acetylcholinesterase activity, which demonstrates its neuroprotective effect. Glutamate, an excitatory neurotransmitter, plays a key role in synaptic plasticity involved in learning and memory.[34] Increased levels of glutamate leads to excitotoxicity, thereby causing neuroinflammation and eventually neuronal loss, which are hallmarks of Alzheimer's disease.[35] Norepinephrine regulates the spatial memory; it retrieves contextual and spatial memory in Alzheimer's rat model and also in patients with Alzheimer's disease.[36,37] Alterations in the levels of

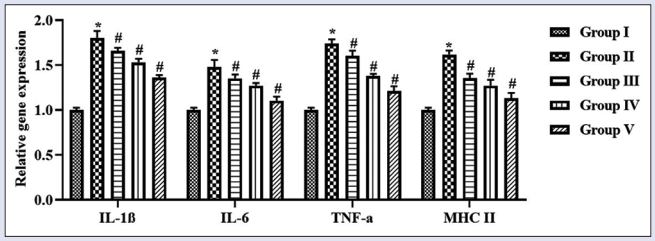

Figure 5: Effect of steroidal saponin dioscin on proinflammatory cytokines in $AlCl_3$-exposed rats m-RNA expression of proinflammatory cytokines interleukin-1β, interleukin-6, tumor necrosis factor-α and major histocompatibility complex class II marker for microglial activation were measured with real-time PCR analysis. Fold change of each gene expression is depicted in the figure. Data were statistically analyzed with one-way ANOVA followed by Student–Newman–Keuls *post hoc* test. $P < 0.05$ was considered as statistically significant, * control vs others, # $AlCl_3$ vs others

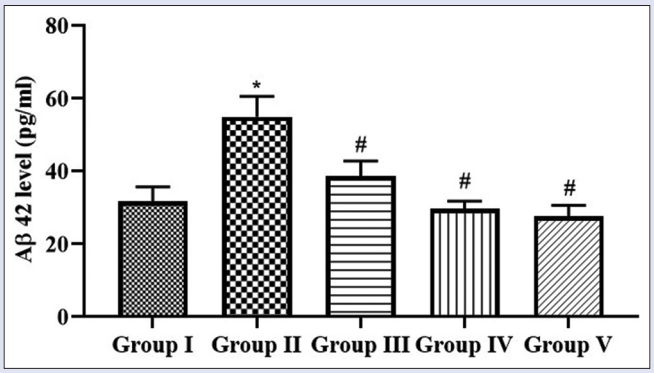

Figure 6: Effect of steroidal saponin dioscin on amyloid beta 42 levels in $AlCl_3$-exposed rats. Amyloid beta 42 levels were estimated with commercially available ELISA kit. Data were statistically analyzed with one-way ANOVA followed by Student–Newman–Keuls *post hoc* test. $P < 0.05$ was considered as statistically significant, * control vs others, # $AlCl_3$ vs others

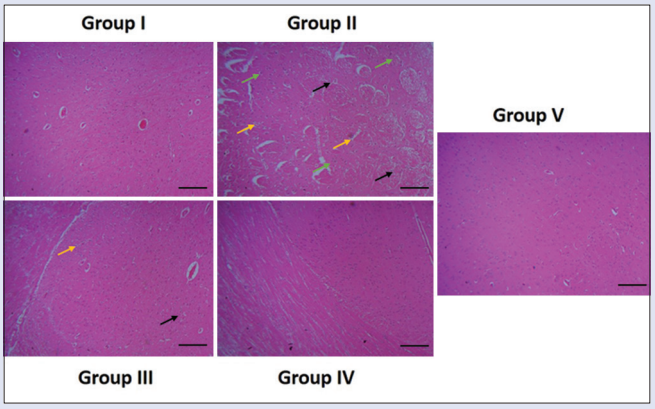

Figure 7: Effect of steroidal saponin dioscin on hippocampus histomorphology in $AlCl_3$-exposed rats. Group I: Control animals demonstrates typical histological arrangements. Group II: $AlCl_3$-exposed rats show increased number of pyknotic and vacuolated cells (green arrows), cellular shrinkage (yellow arrows), and necrotic cells (black arrows). Group III and IV: $AlCl_3$ + dioscin at low and high dose treated rats show decreased number of necrotic, vacuolated, and pyknotic cells. Group V: Standard drug donepezil-treated animals exhibit reduced hippocampal damages. Hippocampal tissue of control and experimental rats were processed and stained with hematoxylin and eosin stain. The representative light microscopic images were taken at 40 × magnification and scale bar 50 μm

norepinephrine lead to impaired cognition and mood disorders.[38] Dopamine is the key modulator of hippocampal synaptic plasticity which encodes memory. Reduced levels of dopamine have been observed in patients with Alzheimer's disease.[39] Dioscin significantly decreased acetylcholinesterase activity and glutamate levels and increased the levels of norepinephrine and dopamine, thereby preventing hippocampal region from $AlCl_3$-induced neuronal damage.

Brain is the most vulnerable organ for oxidative stress as it utilizes high oxygen supply. The neurons contain increased amounts polyunsaturated fatty acids, which readily interacts with ROS, causing lipid peroxidation.[40,41] Increased oxidative stress markers such as malondialdehyde, nitric oxide, and 4-hydroxynonenal and decreased levels of antioxidant enzymes have been detected in patients with Alzheimer's disease.[42,43] Dioscin reduced the levels of ROS and nitric oxide, and reduced the amount of lipid peroxidation in the hippocampus of $AlCl_3$-treated rats. It also increased the activity of SOD and catalase, which agree with the results of a previous study where dioscin inhibited hepatic ischemic injury via suppressing oxidative-nitrative stress.[44]

Inflammation is the key pathologic factor of Alzheimer's disease which increases the amyloid beta plaques, neurofibrillary tangles, and co-localization with microglial, thereby causing neurodegeneration.[45] Activated microglial cells produce proinflammatory cytokines which causes neuronal apoptosis, which in turn leads to learning and memory deficits.[46,47] $AlCl_3$ increased the expression of IL-1β, IL-6, TNF-α, and MHC class II marker for microglial, whereas dioscin significantly reduced the levels of proinflammatory cytokines. Dioscin also reduced the levels of Aβ42, which are hydrophobic and fibrillogenic beta amyloid deposited in patients with Alzheimer's disease. The histological analysis of hippocampal tissue further confirmed the anti-inflammatory and neuroprotective effect of dioscin. Dioscin reduced oxidative stress, inhibited inflammation, prevented the accumulation of Aβ42 in hippocampus, thereby preventing the formation of $AlCl_3$-induced Alzheimer's-like disease in rats.

CONCLUSION

To conclude, natural steroidal saponin dioscin decreased the $AlCl_3$-induced oxidative stress, inflammation, and Aβ42 peptides deposition in the hippocampus. It increased the levels of cholinergic and biogenic neurotransmitters and decreased the levels of excitatory neurotransmitter glutamate levels, thereby preventing the animals from $AlCl_3$-induced learning and memory impairment. The results of the behavioral analysis further confirmed the neuroprotective effect of dioscin. Overall, our results demonstrate that dioscin is a potent neuroprotectant agent. However, further research is still warranted for the better understanding of mode of action of dioscin against the neurological deficits in order to develop it as a potent alternative medicine to treat patients with Alzheimer's disease.

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Authors contributions

Research theme and methodology designing: Huibo Guan, Xinmin Yao, and Miao Yu; Supervising, preparation of manuscript, results interpretation: Yanyan Zhou, Xinmin Yao, and Miao Yu; Experiments, data validation, and proof reading: Huibo Guan and Yanyan Zhou.

Conflicts of interest

There are no conflicts of interest.

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