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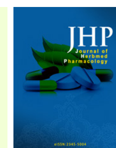

n-Butanol fraction from *Hedysarum alpinum* L. attenuates scopolamine-induced memory impairment in rats

Aldarmaa Jalsrai[1*], Anu Altangerel[1], Myadagbadam Urtnasan[1], Saranchimeg Borchuluun[1], Kristina Battsogt[2], Yue Cong[3], Hsiu Mei Hsieh-Li[4*]

[1]Institute of Traditional Medicine and Technology, Ulaanbaatar, Mongolia
[2]Department of Molecular Biology and Biochemistry, Rutgers University–New Brunswick
[3]Pharmaceutical College, Henan University, Kaifeng City, China
[4]National Natural University of Taiwan, Department of Life Sciences, Taipei, Taiwan

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ABSTRACT

Introduction: *Hedysarum alpinum* L. (HA) contains considerable amounts of polyphenols and saponins, and exhibits immune-enhancing and antiviral properties. Evidence indicates that triterpenoid saponins possess neuroprotective effects against neurodegenerative damage. The present study evaluated the effect of an HA saponin-rich n-butanol fraction (HABF) on cognitive deficits induced by scopolamine in a rat model.

Methods: Cognitive impairment was assessed using passive avoidance (PA) and novel object recognition (NOR) tasks. Total triterpene saponins of HABF were quantified by the colorimetric method using oleanolic acid as a standard compound. Biochemical analyses of hippocampus tissue included measurements of acetylcholinesterase (AChE) activity, choline acetyltransferase (ChAT), catalase (CAT), and tumor necrosis factor-α (TNF-α).

Results: HABF contained 131 ± 0.02 mg oleanolic acid equivalent (OAE)/g of total triterpene saponins. Scopolamine significantly impaired memory, as shown by a decreased discrimination index in the NOR test and reduced retention latency in the PA test. Pretreatment with HABF (40 and 80 mg/kg) significantly alleviated the memory deficits in both tasks. Scopolamine significantly increased AChE activity and TNF-α levels, and decreased CAT activity compared to the vehicle-treated group. These alterations were significantly reversed by HABF treatment, whereas ChAT activity remained unchanged compared with the MPTP-treated group.

Conclusion: HABF, rich in triterpene saponins, ameliorates scopolamine-induced memory impairment, likely through the inhibition of AChE activity and modulation of oxidative stress and inflammation. These findings may suggest its potential neuroprotective effect in cognitive dysfunction.

Implication for health policy/practice/research/medical education:
The n-butanol fraction of *Hedysarum alpinum* extract may exert a neuroprotective effect against cognitive impairment by attenuating oxidative stress and inflammation. These findings provide a scientific rationale for in-depth analysis of triterpenoid saponins from *H. alpinum* as potential therapeutic agents for neurodegenerative diseases.

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Introduction

Alzheimer's disease (AD) is pathologically characterized by the accumulation of insoluble plaques and neurofibrillary tangles, leading to an extensive neuronal loss and brain atrophy (1). It accounts for approximately 50%-60% of all dementia cases in individuals over the age of 65 and is among the most rapidly increasing progressive neurodegenerative diseases worldwide (2). Currently, there is no cure for AD, and existing therapies are mainly symptomatic, aiming to slow down disease progression rather than reverse it (2). The cornerstone pharmacological treatments are the acetylcholinesterase (AChE) inhibitors and N-methyl-D-aspartate receptor antagonists, which act via distinct action mechanisms and have side effects

***Corresponding authors**: Aldarmaa Jalsrai, Email: jaldarmaa@gmail.com; Hsiu Mei Hsieh-Li, Email: hmhsieh@ntnu.edu.tw

(2). Nevertheless, the exact molecular mechanisms underlying AD remain unclear. Given the growing global burden of AD and the lack of effective therapies, there is an urgent need for more promising treatments that can target its root causes and provide sustained improvements in cognitive function and quality of life.

Numerous reports have shown that oxidative stress is a critical factor in the AD progression (3). Notably, free radical scavengers and antioxidants have shown promising potential in mitigating AD-related pathology (4). Consequently, phytochemicals and medicinal herbs have garnered increasing attention as potential sources of neuroprotective compounds that may slow down or prevent the progression of AD (5).

Hedysarum alpinum L. (HA) is a species within the genus *Hedysarum,* which belongs to the Fabaceae family. The chemical components of HA comprise xanthones, flavonoids, saponins, and polysaccharides (6). Our previous research demonstrated that extracts of HA possessed antioxidant, protective, and anti-amnestic effects, likely due to their high content of polyphenols and flavonoids, in a ketamine-induced behavioral dysfunction rat model (7). Despite these findings, the neuroprotective potential of HA in neurodegenerative diseases remains poorly investigated. Increasing evidence indicates that natural triterpenoid saponins exhibit significant neuroprotective effects in various neurodegenerative disease models, including AD (8). Notably, HA is rich in triterpenoid saponins (6), suggesting its potential neuroprotective effects against AD.

Scopolamine (scop) is a non-selective muscarinic acetylcholine receptor antagonist that readily crosses the blood-brain barrier, and is widely used in rodents to induce cognitive and memory impairment, simulating AD-like symptoms (9). Scopolamine raises reactive oxygen species by altering the antioxidant enzymes, which promotes amyloid plaque accumulation and heightens neuroinflammation, leading to progressive neuronal atrophy and degeneration (10). The objective of this study was to determine the neuroprotective potential of the n-butanol fraction of HA in the scop-induced AD-like rat model.

Materials and Methods

Drugs and chemicals

Solvents for extraction were obtained from Merck (Germany). Scopolamine hydrobromide, donepezil, and the reference compound oleanolic acid (98%) were purchased from Sigma-Aldrich (USA). ELISA kits for the estimation of tissue AChE, choline acetyltransferase (ChAT), catalase (CAT), and tumor necrosis factor-α (TNF-α) were sourced from MLBIO Biotechnology (Shanghai, China).

Plant material collection and identification

The aerial parts of HA were collected from the slope of Tuluu Mountain in Bulgan province, Mongolia, in early August 2024. The plant was identified and authenticated by Prof. E. Ganbold, Department of Botany, Botanic Garden and Research Institute, Mongolian Academy of Sciences (Herbarium code: LINN 921.54). The aerial parts were dried in the shade at room temperature for 10 days, then powdered.

Extract preparation

The aerial part powder (500 g) was extracted with 1.5 L of 70% ethanol for 5 days at room temperature. The ethanol extract was filtered and concentrated using a rotary evaporator under vacuum at 50 °C. The extract was sequentially partitioned with hexane, chloroform, and ethyl acetate to remove nonpolar and moderately polar constituents. The remaining aqueous layer was further extracted with n-butanol using a solvent-separation tunnel. The partitioning was repeated 4 times using 100 mL of n-butanol each time. The n-butanol fractions were then combined, the solvent was removed under reduced pressure using a rotary evaporator at temperatures below 50 °C, and the resulting material was dried by freeze-drying. The resulting fraction, which was identified as a triterpene-saponin-rich extract, was stored at 4 °C until further use (9).

Determination of total triterpene saponins by colorimetric method

The total triterpene saponin content in HA n-butanol fraction (HABF) was determined according to a previously described method (2,11). For analysis, 1 mg of HABF was suspended in 50 mL of 70% ethanol and heated under reflux at 70-80 °C for one hour. The solution was then cooled, filtered, and transferred to a 50 mL flask, with ethanol added to reach the final volume of 50 mL (Solution A). Next, 0.4 mL of the solution A was transferred to a 10 mL flask, to which 0.4 mL of 5% vanillin–acetic acid solution and 2.4 mL of perchloric acid were added. The mixed solution was heated at 70 °C in a water bath for 15 minutes. Ethyl acetate was added to the cooled mixture to reach a final volume of 10 mL. A UV-Vis spectrophotometer was used to determine the absorbance of the test solution at 550 nm. A blank solution was prepared using 0.4 mL of 96% ethanol instead of Solution A. The total triterpene saponin content was calculated as oleanolic acid equivalent (OAE) using the following equation:

$$X = \frac{C \times V_1 \times V_3 \times 100}{a \times V_2 \times (100 - W)} \times 100$$

X: Total triterpene saponin content (%)
C: A concentrated test solution from the calibration curve (mg/mL)
a: Sample (mg)
V1, V2, V3: Dilution of test solution (mL: 50, 0.4, 10)
W: Moisture (%)

A standard solution was prepared by dissolving 1 mg of oleanolic acid in 50 mL of ethanol. Subsequently, 1, 2, 3, 4, 5, and 6 mL aliquots of this solution were transferred into individual 10 mL volumetric flasks. The steps described above were followed to construct a calibration curve for the determination of total triterpene saponins in HABF.

Animals and treatments

Wistar rats (weighing 200 to 250 g) were obtained from the Experimental Animal Center in Ulaanbaatar, Mongolia. The animals were housed under controlled laboratory conditions with a 12:12-hour light-dark cycle (lights on at 6:00 a.m.), a temperature of 20 ± 2 °C, and an air humidity of 55-60%. Food and water were provided ad libitum. The rats were allowed to acclimate for two weeks before the start of the experiments. All animal procedures complied with the guidelines of and were approved by the Mongolian and European Animal Ethics Committees (approval number No. 24/042).

Animals were randomized into five groups, each containing six rats (n = 6). Donepezil and the HABF extract were administered orally once a day for 14 days. From days 3 to 14, all groups, with the exception of the normal control (group 1; G-I), were treated with scopolamine (scop, 2 mg/kg, i.p.) once daily for 12 days to induce memory dysfunction (13). Memory performance was assessed using the NOR on day 12 and PA on days 13-14. G-I (normal control): received distilled water. G-II (negative control): received distilled water and scop (2 mg/kg). G-III (positive control): received donepezil (3 mg/kg) and scop (2 mg/kg); G-IV: received HABF (40 mg/kg) and scop (2 mg/kg). G-V: received HABF (80 mg/kg) and scop (2 mg/kg). HABF, donepezil, and scop were all dissolved in distilled water following previously reported doses (14,15).

Novel object recognition (NOR) test

The NOR test was used to assess recognition memory, following the protocol described (16). An open box (62 × 62 × 40 cm, without cover) was used, with uniform light intensity distributed throughout the arena. The objects to be discriminated were of different shapes. Prior to testing, the rats were habituated to the box for 10 minutes. On the following day, the rats were allowed to explore the empty arena for 2 minutes. During the first trial (T1), two identical objects (wooden cubes, 10 cm^3) were placed in opposite corners near the sidewalls. Each rat was placed in the center of the box, and the total time in contact was recorded for a period of 5 min. In trial 2 (T2): after a 60 minutes retention interval in the home cage, one familiar object (F) was substituted with a novel (N), unfamiliar (a plastic bottle) object. The rat was then returned to the center of the box for another 5 minutes. All test sessions were recorded by a video camera. The time each rat spent in contact with the N (TN) and F (TF) was counted, respectively. The Discrimination Index (DI) was determined according to the formula DI = (TN−TF) / (TN+TF) as described.

Passive avoidance (PA) test

The PA equipment (Ugo Basile, Italy) consisted of two equal light and dark compartments (22 × 22 × 30 cm) with an electric grid floor separated by a sliding door (17). In the acquisition trial, rats were initially placed in the light compartment, and the door between the two compartments was opened after 30 seconds. After the rat entered the dark chamber, the door was shut, and a 1 mA foot shock was delivered for 3 seconds through the stainless-steel rods. A retention trial was performed 24 hours after the acquisition trial. The rat was reintroduced into the light compartment and the step-through latency period was recorded without an electric foot shock after the door was opened. If the rat did not enter the dark chamber within 300 seconds, the step through latency was recorded as 300 seconds.

Tissue preparation

Following the behavioral tests, the rats were decapitated under chloroform inhalation anesthesia. To remove blood, brain tissues were immediately rinsed in ice-cold saline, and the hippocampus from both hemispheres was carefully dissected. The hippocampal tissue was homogenized in phosphate buffer (20 mM, pH 7.4) containing 140 mM KCl. The tissue homogenates were centrifuged (1000 g × 15 minutes, 4 °C), and the supernatant was further recovered for biochemical analysis. Protein content was quantified using bovine serum albumin as the standard (17).

Biochemical analysis

The levels of AchE, ChAT, CAT, and TNF-α in hippocampal tissue were measured using ELISA assay kits (Shanghai MLBIO Biotechnology, China), following the manufacturer's protocol. The enzyme activities were quantified using a microplate ELISA reader (ChroMate-4300, Awareness Technology Co., USA) based on absorbance intensity.

Statistical analysis

Statistical evaluation was performed using GraphPad Prism, version 5.0. Data are presented as mean ± SEM. Statistical differences among groups were determined using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test, with statistical significance set at $P < 0.05$.

Results

The total triterpene saponin content in HABF

Total triterpene saponin was quantified using oleanolic acid as the standard, with results expressed in mg equivalent to oleanolic acid (mg OAE/g). A calibration curve (y = 0.0482x − 0.0606) was obtained with an

oleanolic acid concentration ranging from 2.0 to 10.0 mg/mL. The n-butanol extract had higher total triterpene saponin content (131 ± 0.083 mg OAE/g) than the ethanol extract (8.91 ± 0.022 mg OAE/g).

Effect of HABF on scop-induced memory impairment in rats

Cognitive performance was measured through the DI in a NOR test. A negative value of DI indicated impaired recognition, with rats spending similar time on familiar and novel objects. Conversely, a positive value of DI indicated recognition of the familiar object, with increased exploration of the novel object. In the normal control group, the DI was 0.57, indicating that rats predominantly preferred the novel object. In contrast, scop treatment (2 mg/kg for 12 days) resulted in an inability to discriminate the familiar object from a novel one. Scop-treated rats exhibited a significant ($P < 0.01$) decrease in DI (DI = -0.25), reflecting cognitive impairment. A significant difference in the DI was observed between donepezil (0.54) and the scop-treated group ($P < 0.01$). Pretreatment with HABF (40 and 80 mg/kg) significantly increased DI values (0.34 and 0.48, $P < 0.05$) compared to the scop-treated-only group, suggesting improved object recognition. Rats in the HABF-treated group spent a longer time discovering the novel object, effectively reversing memory loss (Figure 1A). Moreover, DI for all HABF-treated groups was increased, comparable to the positive group, which was treated with donepezil (3 mg/kg). These results suggest HABF (40 and 80 mg/kg) effectively reversed scop-induced memory impairment.

In the retention trials of the PA test, the scop-treated group significantly reduced the latency to enter the dark chamber (58.34 ± 9.90 seconds) compared to the control group (222.60 ± 18.35 seconds; $P < 0.05$), indicating impairment of PA memory (Figure 1B). Donepezil significantly increased the scop-induced shortened latency to enter the dark chamber ($P < 0.01$). Pretreatment with HABF (80 mg/kg) increased latency significantly compared to the scop-treated-only group ($P < 0.05$), whereas no significant effect was observed at the lower dose (40 mg/kg). HABF at the higher dose (80 mg/kg) effectively attenuated scop-induced PA memory impairment, demonstrating an effect comparable to that of donepezil (Figure 1B).

To clarify the effects of HABF on cholinergic function, the activities of AChE and ChAT were assessed in the homogenates of hippocampal tissue. ELISA results revealed that AChE levels were significantly elevated in scop-treated rats compared with controls, whereas ChAT levels did not differ significantly between the scop-treated and vehicle control groups. The donepezil-treated group showed a significant decrease in scop-induced AChE activity, while simultaneously increasing ChAT level compared to the scop-treated group. Pretreatment with HABF (40 or 80 mg/kg) attenuated the scop-induced elevation of AChE activity.

We also evaluated the levels of inflammatory and antioxidant markers in the hippocampus. Scop significantly increased TNF-α levels relative to control animals. Pretreatment with HABF at 80 mg/kg significantly reduced scop-induced upregulation of TNF-α, while the 40 mg/kg did not show a significant change. In addition, scop treatment significantly decreased CAT activity compared to the vehicle-treated group. Pretreatment with HABF at 80 mg/kg significantly increased CAT activity compared with the scop-treated group, whereas 40 mg/kg produced no significant effect. The donepezil-treated group reduced scop-induced inflammation and restored CAT levels (Table 1).

Discussion

Hedysarum alpinum is known to be rich in triterpenoid saponins, but there is limited literature specifically addressing its neuroprotective effects, while increasing evidence highlights the neuroprotective effects of triterpenoids in AD. For example, oleanonic acid has been shown to exert protective effects against $A\beta_{25-35}$-induced

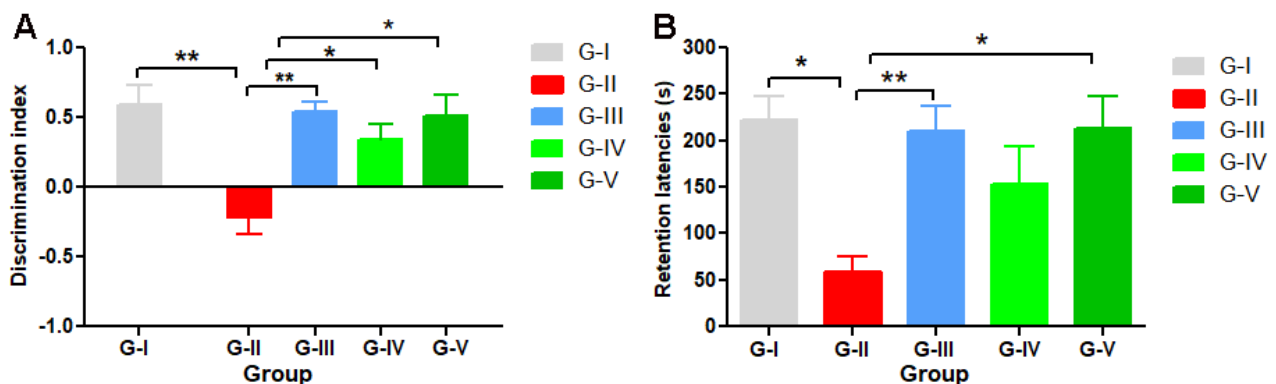

Figure 1. Effects of *Hedysarum alpinum* n-butanol fraction (HABF) on scopolamine (scop)-induced memory impairment in rats. **(A)** Discrimination index in the novel object recognition test. **(B)** The latency (sec) in the passive avoidance test. Values represent mean ± SEM (n=6). G-I: normal control; G-II: negative control (scopolamine, 2 mg/kg); G-III: positive control (donepezil, 3 mg/kg); G-IV and G-V: HABF-pretreated groups. *$P < 0.05$; **$P < 0.01$.

Table 1. Effect of *Hedysarum alpinum* n-butanol fraction (HABF) on biochemical parameters in hippocampus of scopolamine (scop)-induced Alzheimer's disease in rats

Groups	AChE (mU/mg)	ChAT (mU/mg)	TNF-α (ng/ml)	CAT (U/mg)
G-I normal control,	24.86 ± 0.7	51.79 ± 0.55	39.64 ± 1.47	136.9 ± 1.89
G-II AD model group (scop 2 mg/kg)	31.75 ± 1.02#	49.88 ± 1.34	45.88 ± 0.67#	125.1 ± 2.9#
G-III donepezil (3 mg/kg) + scop (2 mg/kg)	25.35 ± 1.08***	53.80 ± 1.21	37.77 ± 1.1**	142 ± 3.26***
G-IV HABF (40 mg/kg) + scop (2 mg/kg)	27.03 ± 1.06*	49.03 ± 0.75	41.13 ± 2.01	127 ± 1.55
G-V HABF (80 mg/kg) + scop (2 mg/kg)	27.26 ± 0.57*	49.19 ± 1.17	39.14 ± 1.54*	133.9 ± 7*

AChE: acetylcholinesterase enzyme; ChAT: choline acetyltransferase enzyme; TNF-α: tumor necrosis factor; CAT: catalase enzyme. Values represent the mean ± SEM. #$P < 0.05$ vs G-I (normal control); *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs G-II (AD model) (n = 6).

memory deficits, indicating a potential role in alleviating cognitive impairment associated with AD (18). Oleanolic acid is implicated in multiple pathological pathways and has been shown to exhibit antioxidative and anti-inflammatory activities (19). In this study, we investigated the potential protective effects of HABF on scop-induced memory impairment in AD rats, using the NOR and PA tests. First, phytochemical analysis showed that HABF contained triterpene saponins at a concentration of 131 mg/g (calculated as OAE). Consistent with previous findings, scop administration induced significant memory deficits in both the NOR and PA behavioral tests (20). After 12 days of scop injection, the DI in the NOR test was markedly reduced compared to the control group, indicating impaired recognition memory. Pretreatment with donepezil at 3 mg/kg and HABF at both doses of 40 and 80 mg/kg significantly improved DI, effectively reversing the scop-induced inability to distinguish between familiar and novel objects. In the PA test, the scop-treated animals presented a significant reduction in the latency in entering the dark compartment, indicating notable memory impairment. Pretreatment with donepezil (80 mg/kg) significantly increased latency compared with the scop-only treated group, demonstrating its protective effect against memory deficits caused by scop. HABF at the 40 mg/kg dose did not show a significant effect, suggesting a probable dose-dependent response. Notably, the 40 mg/kg dose may represent a threshold for initiating HABF's neuroprotective activity, although its effects were not significantly different. In contrast, an 80 mg/kg dose of HABF showed effects comparable to those of donepezil across both behavioral tests, indicating a robust neuroprotective efficacy at higher doses.

The hippocampus plays a critical role in cognitive functions, particularly in learning and memory formation (21). Moreover, scop administration has been shown to reduce neuronal density in the hippocampus (22,23). Neuronal degeneration in both the cortex and hippocampus has been reported as a major contributor to cognitive impairment in AD (24). The hippocampus analysis showed that scop injection significantly increased AChE activity compared with the control group. This observation is consistent with previous studies demonstrating that scop elevates hippocampal AChE activity in rodents (25). Pretreatment with HABF at both 40 and 80 mg/kg effectively reversed the scop-induced increase in AChE activity, thereby offering protection against learning and memory impairments. In contrast, our analysis revealed no significant changes in hippocampal ChAT activity across the experimental groups. Although previous evidence indicates that scop can reduce ChAT levels in the CA1 region of the hippocampus and in the amygdala, a dose of 0.1 mg/kg has been reported to have no effect on hippocampal ChAT levels (26). Therefore, it is possible that, in our experiment, the scopolamine dose was too low, or the injection period was not long enough to produce a significant change in hippocampal ChAT levels. Donepezil (3 mg/kg) significantly suppressed scop-induced AChE activity, increasing ChAT level compared to the scop group.

To further elucidate the mechanisms involved in the protective effects of HABF, we analyzed CAT activity and hippocampal levels of the inflammatory marker TNF-α. Scop injection markedly decreased CAT activity and increased TNF-α level compared to the vehicle group. Pretreatment with donepezil and HABF at 80 mg/kg significantly restored CAT activity and reduced TNF-α levels in the rat hippocampus compared with the scop-treated group, demonstrating a protective effect against scop-induced neuroinflammation and oxidative damage. In contrast, the 40 mg/kg dose of HABF did not produce significant changes in either CAT activity or TNF-α levels, suggesting a dose-dependent protective effect. These results are consistent with the findings from behavioral experiments, in which HABF at a lower dose partially restored scop-induced memory deficit.

This study has some limitations. While we observed significant improvements in behavioral tests and specific biochemical markers, the precise molecular pathways and specific saponin compounds responsible for these effects remain to be identified. Future work should focus on isolating individual triterpene saponins from HABF to evaluate their specific contributions and investigate their impact on other AD-related pathologies, such as amyloid-β aggregation and tau phosphorylation, to provide a more comprehensive understanding of their therapeutic potential.

Conclusion

HABF exhibits neuroprotective effects by attenuating oxidative stress and inflammation, suggesting potential therapeutic use against AD-like symptoms induced by scop injection.

Our results suggest that further analysis of HA is necessary to determine its detailed chemical composition and pharmacological properties, thereby enabling a full assessment of the therapeutic potential of this plant.

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Declaration of AI-assisted tools in the writing procedure

The authors utilized the Gemini AI language model (Google) for assistance in English language editing and manuscript refinement. The final content and conclusions remain the sole responsibility of the authors.

Authors' Contribution

Conceptualization: Hsiu Mei Hsieh-Li.
Data curation: Kristina Battsogt, Anu Altangerel, Myadagbadam Urtnasan, Saranchimeg Borchuluun, Aldarmaa Jalsrai.
Formal analysis: Aldarmaa Jalsrai, Kristina Battsogt.
Investigation: Anu Altangerel, Myadagbadam Urtnasan, Aldarmaa Jalsrai.
Methodology: Aldarmaa Jalsrai.
Supervision: Aldarmaa Jalsrai, Hsiu Mei Hsieh-Li.
Visualization: Aldarmaa Jalsrai, Hsiu Mei Hsieh-Li.
Writing–original draft: Aldarmaa Jalsrai.
Writing–review & editing: Kristina Battsogt, Yue Cong, Anu Altangerel, Myadagbadam Urtnasan, Hsiu Mei Hsieh-Li.

Conflict of interests

The authors claim to have no conflicts of interest.

Ethical considerations

Animal experimental procedures were approved by the Mongolian Health Ministry (approval number No. 24/042).

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