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Chloroform and ethyl acetate extracts of *Hedysarum alpinum* L. mitigate the ketamine-induced behavioral dysfunction and oxidative stress in schizophrenia-like animals

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The effects of chloroform and ethyl acetate extracts of *Hedysarum alpinum* L. were investigated through a ketamine-induced schizophrenia-like rat model. The quantitation of the total triterpene saponin content of extracts was determined by using oleanolic acid as standard. The ethyl acetate extract had a total triterpene saponin of 10.02 ± 0.02 mg otoacoustic emission (OAE)/g, while the chloroform extract had 8.50 ± 0.05 mg OAE/g, respectively. The neuroprotective effect was evaluated using a schizophrenia-like model induced by ketamine with different behavior tasks, including open-field (for hyperlocomotion evaluation), elevated plus maze (for anxiety examination), and novel object recognition (for memory dysfunction characterization) tests. The rat hippocampus was isolated for biochemical analysis. Ketamine significantly caused hyperlocomotion, anxiety-like behavior, and recognition memory deficit in animals. Oxidative stress induced by ketamine was alleviated by *H. alpinum* chloroform and ethyl acetate extracts at doses of 40 and 80 mg/kg. The level of malondialdehyde (MDA) and superoxide dismutase (SOD) in rat hippocampus were reduced by pretreatments of both *H. alpinum* extracts and droperidol. Chloroform and ethyl acetate extracts of HA exhibit antipsychotic and neuroprotective effects through attenuating oxidative stress. The findings suggest that *H. alpinum* extracts could have the potential to ameliorate schizophrenia-like symptoms.

Key words: *Hedysarum alpinum* L., extracts, neuroprotective, antioxidant effects.

INTRODUCTION

Schizophrenia is a highly complex and heterogeneous neuropsychiatric disorder affecting multiple neuronal systems, whose etiology and mechanisms are still not well-studied (Legge et al., 2021). Dopaminergic and glutamatergic dysfunction as well as oxidative stress are distinguished features among the underlying pathophysiological mechanisms of schizophrenia (Kruse and Bustillo, 2022).

Currently, antipsychotic drugs counteract the symptoms of schizophrenia, which cannot effectively medicate this

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complex disease but exert severe side effects, such as sexual dysfunction, weight gain, and depression (Yoshida and Takeuchi, 2021) and induce withdrawal symptoms upon discontinuation (Cosci and Chouinard, 2020). Consequently, new agents are continually sought for safe and useful combinations of anxiolytic, antidepressant, antipsychotic, and neuroprotective activities.

For several decades, bioactive compounds from medicinal plants with psychotic, neuroprotective, and antioxidant properties have been explored for their potential to target multiple pathways associated with schizophrenia (Vasconcelos et al., 2015). Medicinal plants with proven efficacy may serve as supplements to antipsychotic drugs, enhancing their effectiveness while potentially reducing side effects. It has been reported that while herbal medicine alone may not be effective as monotherapy for psychiatric disorders, its use as an adjunct to first- or second-generation antipsychotic drugs can yield beneficial results (Shi et al., 2021). Despite this, phytochemicals remain a valuable source for developing novel agents with multiple mechanisms of action, which are crucial for the treatment of psychiatric conditions (Berman et al., 2020). Recently, numerous studies have reported that medicinal plant extracts possess an antipsychotic effect against ketamine-induced schizophrenia models. For example, flavonoid extracts from *Peperomia pellucida* L. inhibit hyperlocomotion and improve cognition impairment caused by ketamine-dysregulated oxidative stress and glutamatergic neurotransmission (Venkataramaiah et al., 2021; Temitope et al., 2024). The previous study also indicates that the aqueous extract from the root of *Pueraria lobata* (Puerariae Radix) showed neuroprotective effects in Alzheimer's disease mouse model that received a direct infusion of soluble oligomeric Aβ into the bilateral hippocampal CA1 subregion (Huang et al., 2019). The available research suggests that exploring the effects of medicinal plant extracts and phytochemicals using animal models holds great potential in providing preventive and therapeutic strategies for neurological disorders.

The N-methyl-D-aspartate (NMDA) receptor antagonists, such as ketamine, can induce hallucinations and paranoia that resemble the positive symptoms of schizophrenia, as well as social withdrawal and reduced speech, which are akin to its negative symptoms in humans. Ketamine is a widely used animal model of schizophrenia, as it induces hyperlocomotion, cognitive deficits, anxiety, depression, and impaired social interaction in rodents (Becker et al., 2003). Studies have shown that ketamine disrupts neurotransmitter systems, including dopamine, glutamate, and acetylcholine across various brain regions, such as the prefrontal cortex, striatum, and hippocampus (Wu et al., 2021; Strous et al., 2022. Additionally, numerous studies indicate that ketamine causes severe oxidative damage to the brain (Singh et al., 2020; Ang et al., 2021).

Hedysarum alpinum L. belongs to the genus *Hedysarum* within the Fabaceae family. Various species of the genus, including *H. alpinum*, *Hedysarum polybotrys*, and *Hedysarum vicioides* Turcz have been traditionally used in many countries to strengthen the immune system and treat various disorders (Mo et al., 2022; Gao et al., 2023). Extracts of H. alpinum showed antioxidant, antiradical, and energy-protective effects against carbon tetrachloride-induced liver injury (Toropova et al., 2023). *H. alpinum* contains several phytochemicals, including xanthones, flavonoids, saponins, and polysaccharides (Yurkevich et al., 2021; Toropova et al., 2023; Filonova et al., 2024). These phytoconstituents exhibit diverse bioactivities, including immune-modulatory, anti-cancer, and cardioprotective properties (Jang and Lee, 2023; Pisoschi et al., 2024).

Previous studies demonstrate that ethanolic extract of *H. alpinum* is rich in flavonoids and possesses antioxidant and anxiolytic-like activities in rats with ketamine-induced hyperactivity (Jalsrai et al., 2023). These findings suggest that *H. alpinum* may help alleviate symptoms of psychosis by reducing oxidative stress. However, the neuroprotective effects of various extracts of *H. alpinum* on oxidative stress have not been extensively studied. Therefore, the present study was conducted to evaluate the neuroprotective effects of chloroform and ethyl acetate extracts of *H. alpinum* against ketamine-induced hyperactivity and oxidative stress in rats.

MATERIALS AND METHODS

Chemicals

All of the chemicals were of analytical grade. The reference compounds, including mangiferin (99%), hyperoside (99%), linalool (98%), and geraniol (98%), were purchased from Sigma (Sigma Aldrich, St. Louis, MO, USA). Ketamine hydrochloride was obtained from Rotexmedica (Trittau, Germany).

Plant and extract preparation

The aerial part of *H. alpinum* was collected from the slope of Teluu Mountain in Bulgan province, Mongolia in early August 2023. The plant was identified and authenticated by Prof. E. Ganbold, Institute of Botany, Mongolian Academy of Sciences. The dried aerial parts of the plant (500 g) were powdered and sequentially extracted with hexane, chloroform, ethyl acetate, and n-butanol at room temperature for 48 h. The extracts were filtered, and the solvents were evaporated under vacuum at temperatures below 50°C to obtain solid extracts, which were stored at 4ºC until use. Since the amount of hexane extract was insufficient for the animal study and chloroform and ethyl acetate extracts contained higher concentrations of bioactive components (data not shown), chloroform and ethyl acetate extracts were selected for this study. For in vivo experiments, the extracts were suspended in 2.5% Tween-80.

Sample preparation for analysis of biologically active substances

A sample (30 g) was extracted 4 times with 100 mL of 70% EtOH using the Soxhlet apparatus. The combined extract was

concentrated under reduced pressure, and the residue was suspended in water. The suspension was then sequentially partitioned with hexane, chloroform, ethyl acetate, and n-butanol. The chloroform and ethyl acetate fractions were evaporated, and the dry fractions were dissolved in methanol for thin-layer chromatography (TLC) analyses.

Thin layer chromatography (TLC)

Sample solutions (5 µL) were applied to TLC plates (Merck Silica gel 60 GF 254) at a distance of 1.5 cm from the lower edge of the plate. The plates were developed using different mobile phase compositions for the detection of specific compounds, with ethyl acetate: water: formic acid (8.5: 1.5:0.5 v/v) for mangiferin and hyperoside; toluene: ethyl acetate: acetic acid (14:4:0.5 v/v) for geraniol and linalool, spray with anisaldehyde solution: toluene: ethyl acetate: acetic acid (7:2:0.2 v/v) for oleanolic acid, spray with 10% sulfuric acid. After heating at 100-105ºC for 1-2 min, the plates were observed under natural light or ultraviolet light (254 or 365 nm).

Determination of total triterpene saponins by colorimetric method

The total saponin content in chloroform and ethyl acetate extracts of *H. alpinum* was determined using a previously described method (Le et al., 2018; Mora-Ocación et al., 2022). For analysis, 1 mg of chloroform or ethyl acetate extracts was dissolved in 50 mL of 70% ethanol and heated under reflux at 70 to 80ºC for 1 h. The solution was then cooled, filtered, and transferred to a 50 mL flask, with ethanol added to reach the final volume (Solution A).

Next, 0.4 mL of Solution A was transferred to a 10 mL flask, to which 0.4 mL of a 5% vanillin–acetic acid solution and 2.4 mL of perchloric acid were added. The mixture was heated at 70ºC in a water bath for 15 min, then cooled and ethyl acetate was added to make up the final volume of 10 mL. The absorbance of the test solution was measured at 550 nm using ultraviolet-visible spectrophotometry. 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 using the following equation:

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

where X= Total triterpene saponin content (%), C= A concentrate of 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 ethanol. Then, 1, 2, 3, 4, 5, and 6 mL of this solution were transferred to a separate 10 mL flask, and the steps described previously mentioned were followed to construct a calibration curve.

Animals and behavioral experiments

Adult male Wister rats (weighing 200 to 250 g) were purchased 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 to 60%. Food and water were provided ad libitum. The rats were allowed to acclimate for two weeks before the start of the experiments. All procedures were conducted in accordance with the guidelines of the Mongolian and European Animal Ethics Committee (Approval Number No. 24/042).

Behavioral alterations induced by ketamine were assessed using three tasks, the open field (OF), the elevated plus maze (EPM), and the novel object recognition (NOR) tests. The animals were divided into seven groups (n = 6 per group): Normal control (group 1) received distilled water; negative control (group 2) received 2% Tween-80; positive control (group 3) received droperidol (0,1 mg/kg, a typical antipsychotic), groups 4 and 5, received *H. alpinum* ethyl acetate extract (40 and 80 mg/kg) and groups 6 and 7 received *H. alpinum* chloroform extract (40 and 80 mg/kg) orally for 7 days. All groups except group 1 were co-treated with ketamine (25 mg/kg, i.p.) daily for 7 days. On the sixth day, anxiety and locomotory activity were assessed using the EPM and OF tests 30 min after ketamine injection. On the seventh day, memory impairment was assessed with the NOR test 30 min after ketamine administration. The chloroform and ethyl acetate extracts used were emulsified with 2% Tween-80. Ketamine and droperidol were dissolved in distilled water following previously reported doses (Kozela et al., 2020).

The elevated plus maze (EPM) test

The EPM was conducted as described (Bruijnzeel et al., 2022). The apparatus consists of two open arms and two closed arms (50 × 10 cm each) extending from a central platform (10 × 10 cm), forming a plus sign. The maze was elevated 50 cm above the floor. Each rat was placed in the central compartment facing an open arms at the start of the test. The number of entries and the time spent in the open arms were recorded over a 5 min period. An entry was counted when all four paws of the rat entered an arm. Increased time spent in the open arms and a higher number of open-arm entries were considered indicators of potential anxiolytic effects. All test sessions were recorded using a video camera.

Open-field (OF) test

Locomotor activity was assessed using the OF test as described (Bruijnzeel et al., 2022). The apparatus consisted of a transparent-walled box with a black floor (60 × 60 × 60 cm) illuminated at 300 lux. The floor was divided into 16 squares (15 × 15 cm each). At the start of the test, each rat was placed in the rear left corner of the apparatus, facing away from the observer. The exploratory and emotional behaviors of the animals were observed for 5 min. The entire session was recorded using a camera mounted above the apparatus for later scoring. Behavioral measures included locomotion, the number of squares crossed with all four paws (crossings) was manually counted to assess hyperlocomotion; and anxiety-like behavior, the time spent in the center area (30 × 30 cm) and in the corners of the arena was recorded. The apparatus was cleaned with 70% alcohol solution and dried between each test session.

Novel object recognition (NOR) test

The NOR test was used to assess recognition memory, following the protocol described (Kozela et al., 2020). The same OF apparatus (60 × 60 × 60 cm) was used, with equal light intensity distributed throughout the box. On the first day, rats were individually habituated to the box for 10 min. Trial 1 (T1): on the second day, each rat was placed in the arena for 2 min with two identical objects (wooden cubes, 10 × 10 × 10 cm) positioned in two opposite corners. The rat was then allowed to explore for 5 min. Trial 2 (T2): after a 30 min retention interval in the home cage, one of the familiar objects (F) was replaced with a novel object (N) (a plastic bottle). The rat was then placed back in the center of the box

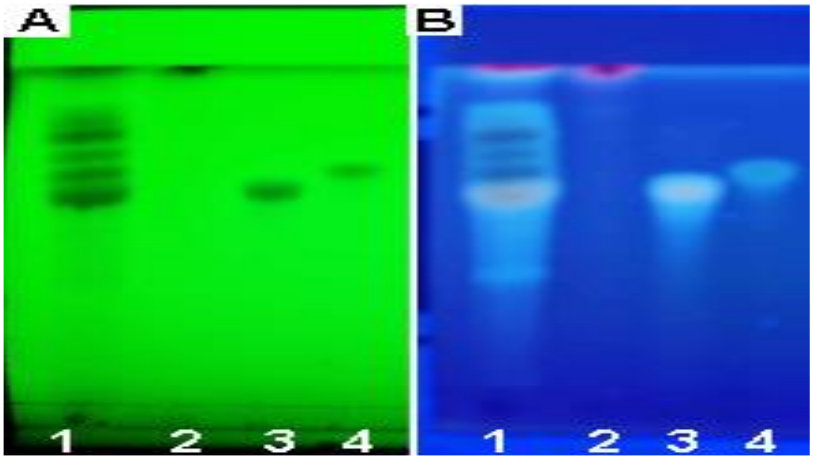

Figure 1. TLC fingerprints of mangiferin and hyperoside in extracts in *H. alpinum*. Lane 1: the ethyl acetate extract; Lane 2: the chloroform extract; Lane 3: the mangiferin standard; and Lane 4: hyperoside standard. A. Observed under UV 254 nm, B. Observed under UV 365 nm.

and allowed to explore for another 5 min. All test sessions were taped using a video camera. The time each rat contacted with the N (TN) and F (TF) was counted, respectively. The Discrimination Index (DI) was calculated as: DI = (TN–TF) / (TN+TF) (Jang and Lee, 2023).

Biochemical analysis

Following the behavioral tests, the rats were decapitated under chloroform anesthesia. The brains were immediately rinsed with ice-cold saline to remove blood, and the hippocampus from both hemispheres was carefully dissected. The hippocampal tissue was homogenized in 20 mM phosphate buffer (pH 7.4) containing 140 mM KCl. The homogenate was then centrifuged at 12,000 g for 10 min at 4ºC, and the resulting supernatant was further used for biochemical analysis. Protein content was quantified using bovine serum albumin as the standard.

Enzyme levels in rat hippocampus related to oxidative stress, including lipid peroxidation (TBARS), superoxide dismutase (SOD), and glutathione peroxidase (GPx), were measured using ELISA kits according to the manufacturer's instructions (Shanghai MLBIO Biotechnology Co. Ltd, China). The enzyme activities were quantified using a microplate ELISA reader (ChroMate-4300, Awareness Technology Co., USA) based on intensity readings.

Statistical analysis

All statistical analyses were conducted using GraphPad Prism, version 5.0. Data were expressed as mean ± standard error mean (SEM) and analyzed using one-way ANOVA following Tukey's multiple comparison test. A P value of 0.05 or less was considered statistically significant.

RESULTS

The phytochemical compositions of *H. alpinum* extract

The chloroform and ethyl acetate extracts from *H. alpinum* were analyzed using TLC to identify the presence of mangiferin, hyperoside, linalool, and geraniol acids using different solvent systems. Two distinct compounds, mangiferin and hyperoside, were confirmed in the extracts based on their Rf values and color, which matched the respective standards: Mangiferin, Rf = 0.6, and Hyperoside, Rf = 0.66 (Figure 1). Spectral comparison of hyperoside, mangiferin, and *H. alpinum* extracts revealed that two bands in the ethyl acetate extract aligned with the positions of mangiferin and hyperoside. However, no spots were detected in the chloroform extract, indicating the absence of these compounds.

After derivatization with anisaldehyde, the bands corresponding to linalool (Rf 0.65) and geranial (Rf 0.57) appeared as brown color spots. Both chloroform and ethyl acetate extracts exhibited two bands with the same Rf value as the standards of linalool and geraniol (Figure 2). However, the intensity of these bands was noticeably weaker in the ethyl acetate extract compared to the chloroform extract (Figure 2). These observations suggest the presence of linalool and geraniol in both extracts.

Additionally, chromatographic bands corresponding to the oleanolic acid standard (Rf = 0.589) were observed in both extracts (Figure 3). The purple-colored spots confirmed the presence of oleanolic acid in both chloroform and ethyl acetate extracts of *H. alpinum*.

TLC analysis revealed that the ethyl acetate extract contained hyperoside and mangiferin, but had a lower fatty acid content compared to the chloroform extract. Conversely, the chloroform extract contained fatty acids but lacked hyperoside and mangiferin. Furthermore, oleanolic acid was detected in both extracts.

The total triterpene saponin content was quantified using oleanolic acid as the standard, with results expressed

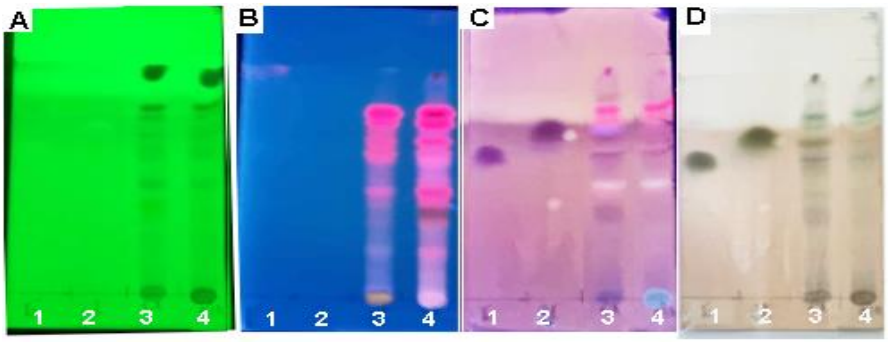

Figure 2. TLC fingerprints of geraniol and linalool in extracts of *H. alpinum*. Lane 1: geraniol standard; Lane 2: linalool standard; Lane 3: chloroform extract; Lane 4: ethyl acetate extract. A. UV 254 nm, B. UV 365 nm, C. Visible light after spraying with 10% sulfuric acid, and D. Sprayed with 10% sulfuric acid, observed under UV 365 nm.

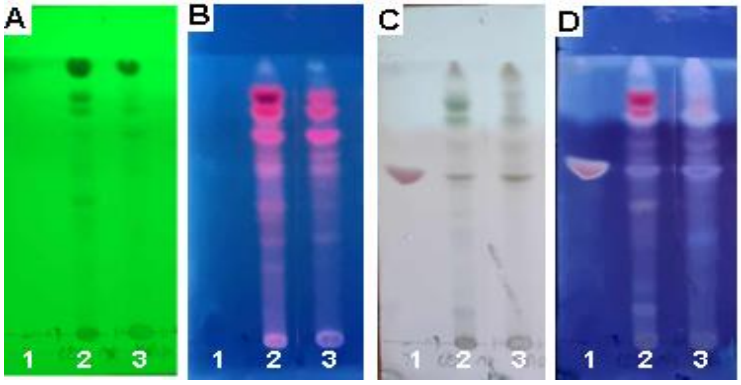

Figure 3. TLC fingerprints of oleanolic acid in extracts of *H. alpinum*. Lane 1: oleanolic acid standard; Lane 2: chloroform extract; Lane 3: ethyl acetate extract. A. UV 254 nm, B. UV 365 nm, C. Visible light after spraying with 10% sulfuric acid, and D. Sprayed with 10% sulfuric acid solution at UV 365 nm.

in mg equivalent to oleanolic acid (mg OAE/g). Oleanolic acid yielded a calibration curve of y = 0.0482x – 0.0606 with a concentration range of 2.0 to 10.0 mg/ml. The ethyl acetate extract had a higher total triterpene saponin content (10.02 ± 0.022 mg OAE/g) compared to the chloroform extract (8.5 ± 0.05 mg OAE/g).

Effect of *H. alpinum* extracts on ketamine-induced behavioral changes in rats

Elevated plus maze (EPM) test

After 6 days of administration, ketamine significantly reduced the percentage of time spent in the open arms of the EPM test. However, the decrease in the open-arm entries was not statistically significant compared to the control group. Similarly, the increased time spent and entries in the closed arms by ketamine-treated rats were not statistically significant, suggesting that ketamine induces partial anxiety-like behavior. Droperidol pretreatment exacerbated ketamine-induced anxiety-like behavior, as droperidol-treated rats spent significantly less time in the open arms and had fewer open-arm entries compared to ketamine-treated rats. This suggests droperidol has an anxiogenic effect (Figure 4).

Meanwhile, pretreatment with *H. alpinum* chloroform extracts at a dose of 40 and 80 mg/kg significantly increased the time spent in the open arms and the number of entries into open arms compared to the ketamine-treated group ($P < 0.05$). No significant difference was observed for the time and number of entries into the closed arms compared with the ketamine-treated group. These results indicate that *H. alpinum* chloroform extract significantly ameliorates ketamine-induced anxiety-like behavior in rats.

Conversely, *H. alpinum* ethyl acetate extract at all doses had no significant effects on the measured EPM

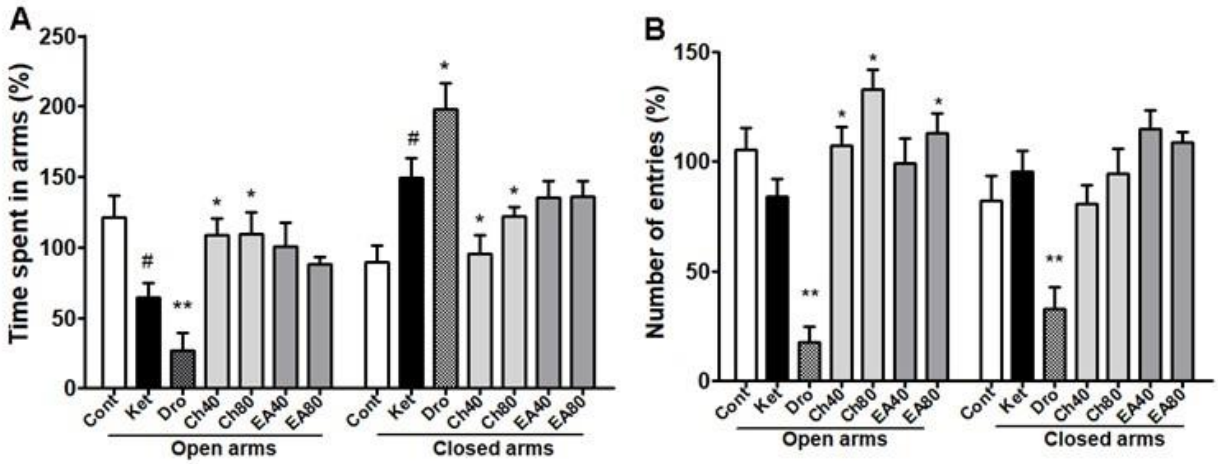

Figure 4. Effects of the *H. alpinum* chloroform and ethyl acetate extracts on ketamine-induced changes in the EPM test in rats. (A) Percentage of time spent in the arms, (B) Percentage of arm entries. Data are presented as mean ± SEM. # $P < 0.05$ compared to control (vehicle) group; * $P < 0.05$ and ** $P < 0.01$ compared to the ketamine-treated group. (n = 5/group).

parameters, though it increased the time spent and number of entries in the open arms compared with the ketamine group. However, it did not significantly decrease the time spent or number of entries in the closed arms (Figure 4). In addition, all extracts significantly increased the time spent and number of entries in the open arms while decreasing the time spent and number of entries in the closed arms compared to the droperidol-treated group (Figure 4).

Open-field (OF) test

Immediately after the EPM test, the rats were placed in the center of the arena of the OF box for 5 min to measure locomotor activity. The number of crossings, as well as the time spent in both the center and the corner of the field, were recorded. Ketamine treatment for 6 days resulted in a 45.86% increase in the number of crossings vs. control, a 50.42% reduction in time spent in the center, and a 15% increase in time spent in the corners. These findings indicate that ketamine-induced anxiety-like behavior.

Droperidol (0.1 mg/kg, administered for 6 days) significantly suppressed ketamine-induced hyperactivity, reducing the number of crossings by 96.35%. However, droperidol also significantly decreased the time spent in the central area by 54.23% and increased the time spent in the corner by 22% relative to the ketamine-treated group. These results indicate that while droperidol effectively suppressed ketamine-induced hyperlocomotion, the anxiety effect persisted.

Treatment with *H. alpinum* ethyl acetate extract (40 and 80 mg/kg, 6 days) significantly suppressed ketamine-induced hyperlocomotion, reducing crossings by 49.65 and 49.83%, respectively. Additionally, both doses of the extract increased the time spent in the central area by 119 and 112%, respectively, suggesting an anxiolytic-like effect. The 40 mg/kg dose significantly reduced the ketamine-induced increase in time spent in the corner, while the 80 mg/kg dose did not (Figure 5).

H. alpinum chloroform extract (40 mg/kg, 6 days) had no significant effect on the OF parameters, while the 80 mg/kg dose reduced the crossings by 39.87% and increased the center time by 61.71%, indicating the higher dose provided significant protection against ketamine-induced anxiety-like behavior (Figure 5).

Novel object recognition (NOR) test

The effect of the *H. alpinum* extracts on ketamine-induced cognitive deficits were evaluated using the NOR test. Cognitive performance was measured through discrimination (DI) and preference (PI) indexes. A negative DI and lower value of PI indicate impaired recognition, with rats spending similar time on familiar and novel objects. Conversely, a positive DI and higher PI suggest recognition of the familiar object, with increased exploration of the novel object. In the normal control group, the DI was positive at 0.36, and the PI was 68.1%, indicating that rats predominantly explored the novel object. In contrast, ketamine-treated rats exhibited a significant ($P < 0.05$) decrease in DI and PI (Figure 6), reflecting cognitive impairment. Specifically, 7 days of ketamine treatment (25 mg/kg) resulted in a negative DI and reduced PI, demonstrating an inability to differentiate between familiar and novel objects (Figure 6).

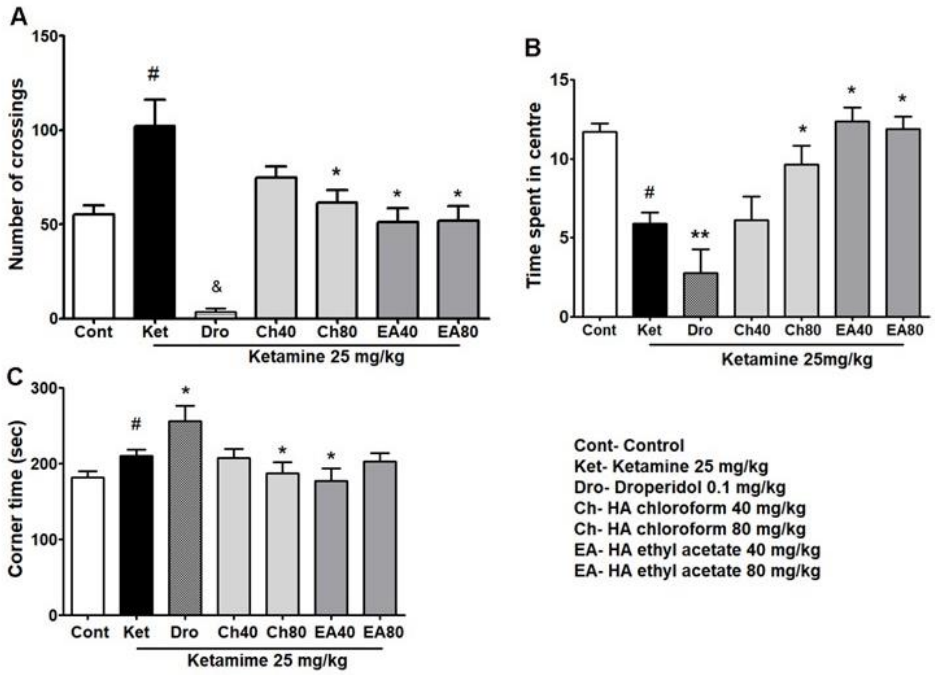

Figure 5. Effects the *H. alpinum* extracts on ketamine-induced hyperactivity in OF test. (A) Relative of number crossing, (B) Time (seconds) of rats spent in the central area, (C) Time (seconds) of rats spent in the corner. Data are presented as mean ± SEM. # P < 0.05 compared to control (vehicle) group; * P < 0.05 and ** P < 0.01 compared to the ketamine-treated group (n = 5/group).

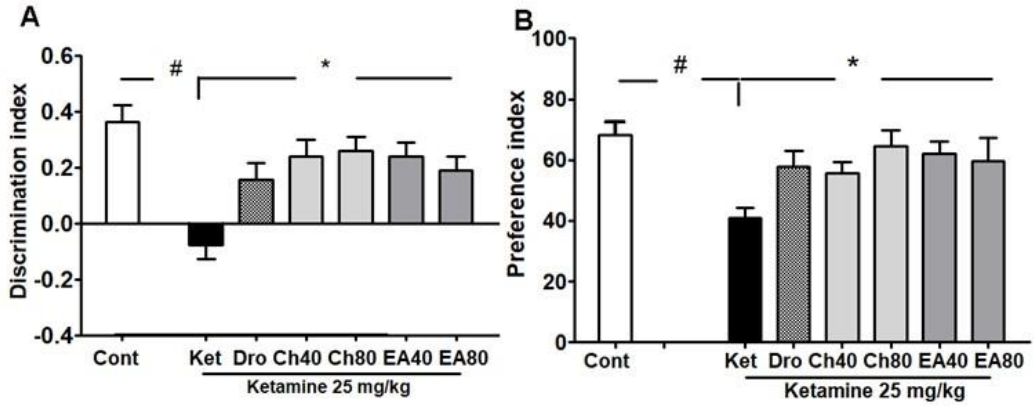

Figure 6. Effects *H. alpinum* extracts on ketamine-induced cognitive dysfunction in rats. (A) The discrimination (DI) indexes, (B) The preference (PI) indexes, Data presented as mean ± SEM. # P < 0.05 compared to the control (vehicle) group; * P < 0.05 compared to the ketamine-treated group (n=5 group) Control, distilled water; Ket, ketamine (25 mg/kg); Dro, droperidol (0.1 mg/kg); Ch40, *H. alpinum* chloroform (40 mg/kg); Ch80, *H. alpinum* chloroform (80 mg/kg); EA40, *H. alpinum* ethyl acetate (40 mg/kg); EA80, *H. alpinum* ethyl acetate (80 mg/kg). n=5 group.

Co-treatment with droperidol (0.1 mg/kg) for 7 days significantly increased DI and PI values compared to the ketamine group, indicating improved object recognition. Similarly, pretreatment with *H. alpinum* chloroform and ethyl acetate extracts (40 and 80 mg/kg) for 7 days significantly improved DI and PI values relative to the ketamine-treated group. Rats in these groups spent more time exploring the novel object, effectively reversing

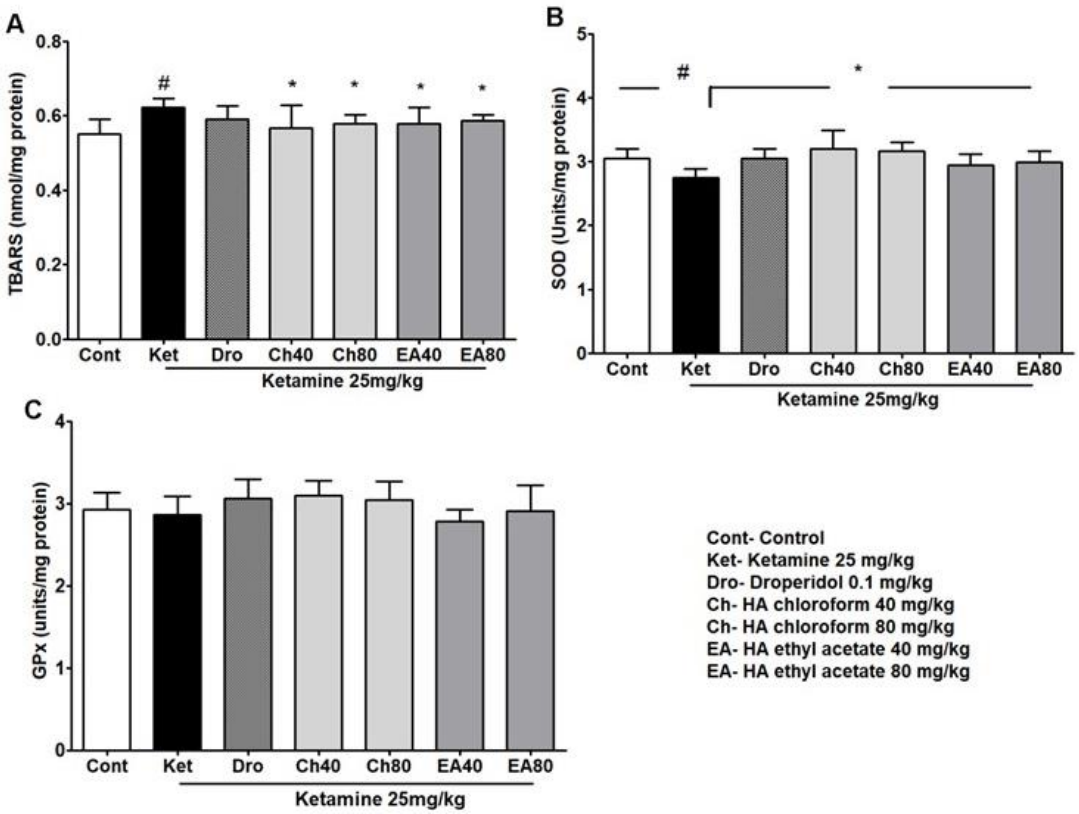

Figure 7. Effects *H. alpinum* extracts on ketamine-induced oxidative stress in rats. (A) The effect on MDA (measured as TBARS), (B) The effect on SOD activity, (C) The effect on GPx activity. # $P < 0.05$ compared to the control (vehicle) group; * $P < 0.05$ compared to the ketamine-treated group. (n=5 group).

memory loss.

Treatment of *H. alpinum* chloroform extract demonstrated a dose-dependent protective effect, with higher doses (80 mg/kg) yielding stronger cognitive behavior improvement. In contrast, the protective effect of *H. alpinum* ethyl acetate extract slightly diminished with increasing doses, although the performance at 80 mg/kg remained comparable to that of droperidol. In summary, pretreatment with droperidol (0.1 mg/kg), *H. alpinum* chloroform (40, 80 mg/kg), and *H. alpinum* ethyl acetate (40, 80 mg/kg) extracts effectively reversed ketamine-induced memory impairment, restoring cognitive function in the NOR test.

Effect of *H. alpinum* extracts on oxidative stress-associated biomarkers

Following the NOR test, the effects of *H. alpinum* extracts on oxidative stress markers, including MDA (measured as TBARS), SOD, and GPx were assessed. Ketamine treatment significantly increased TBARS levels in the hippocampus, indicating elevated lipid peroxidation and oxidative stress. While pretreatment of droperidol (0.1 mg/kg) and *H. alpinum* chloroform and ethyl acetate extracts at the doses of 40 and 80 mg/kg significantly prevented the ketamine-induced increase in TBARS levels ($P < 0.05$). Ketamine also reduced SOD activity, an important antioxidant defense mechanism. Pretreatment with droperidol and *H. alpinum* extracts at both 40 and 80 mg/kg doses effectively prevented the decrease, maintaining SOD activity at normal levels. In contrast, no significant alterations were observed in the activity of GPx enzymes across all groups evaluated in this study, suggesting that GPx levels remained unaffected by ketamine treatment or *H. alpinum* extract interventions (Figure 7).

DISCUSSION

Schizophrenia is one of the most prevalent psychiatric disorders worldwide and remains a significant focus of neuropharmacological research. Despite the availability of drugs to treat psychosis, there are currently no effective remedies for prevention and comprehensive management of schizophrenia (Kantrowitz et al., 2023). A growing body of evidence suggests that natural

polyphenolic compounds possess antipsychotic and neuroprotective effects, primarily attributed to their antioxidant properties (Sarris, 2018). Polyphenols are well-known for their ability to scavenge free radicals, inhibit their production, and regulate oxidative stress (Ribaudo et al., 2020; Khan et al., 2024).

Recent studies demonstrated that *H. alpinum* ethanolic extract significantly suppressed ketamine-induced hyperactivity in the OF test and showed anxiolytic-like activity in the EPM test (Jalsrai et al., 2023). Further characterization revealed that the *H. alpinum* ethanolic extract contains notable levels of polyphenolic compounds, including quercetin (0.6%) and gallic acid (0.9%) (Jalsrai et al., 2023). Based on these findings, it was hypothesized that *H. alpinum* extract may possess an antipsychotic effect linked to its polyphenolic content. To further understand the effects of *H. alpinum* on ketamine-induced schizophrenia-like model, a combination of behavioral paradigms, including EPM, OF, and NOR tasks were conducted on chloroform and ethyl acetate extracts of *H. alpinum*. In the current study, it was found that the H. alpinum ethyl acetate extract contains mangiferin and hyperosides, while the H. alpinum chloroform extract primarily consists of fatty acids, including linalool and geraniol. It is also crucial to note that both extracts contain a high quantity of triterpenoid saponins, specifically identified as oleanolic acids.

The ketamine model of schizophrenia is a well-established tool for studying the cognitive deficits, and dopaminergic and GABAergic dysfunction associated with schizophrenia (Białoń and Wąsik, 2022). Anxiety disorders are frequently observed in patients with schizophrenia (Schirmbeck et al., 2022), making the EPM test relevant in this context (Forro et al., 2022). Studies on the anxiety-related effects of ketamine in animals, however, have been inconsistent, with reports of anxiolytic, anxiogenic, and null effects (Silote et al., 2020). Previous studies suggest that the anxiogenic or anxiolytic effects of ketamine depend on the dosage. Lower doses (10-20 mg/kg) induce anxiogenic effects, while higher doses, such as 50 mg/kg, are associated with anxiolytic effects in the EPM test (Akillioglu and Karadepe, 2021).

In this study, ketamine (25 mg/kg for 6 days) significantly decreased the percentage of time spent in the open arms of EPM, indicating an anxiogenic effect. However, this dose did not reduce the percentage of entries into the open arms, suggesting a partial anxiogenic response. Pretreatment with *H. alpinum* ethyl acetate extract (40 and 80 mg/kg) attenuated anxiety-like behavior induced by ketamine, restoring a more normal behavioral profile in the EPM test. On the other hand, the *H. alpinum* chloroform extract at a lower dose (40 mg/kg) did not significantly affect the anxiety-like behavior, but at a higher dose (80 mg/kg), it significantly reduced anxiety-like behavior, demonstrating an anxiolytic-like effect.

Antipsychotic drugs such as haloperidol and risperidone are well-documented to prevent hyperactivity induced by amphetamine or ketamine, improve memory function, and reduce anxiety levels in rodent models. In the present study, droperidol, a dopamine D2 receptor antagonist, was used as a typical antipsychotic drug for comparison. However, no prior studies have specifically investigated the effect of droperidol on ketamine-induced behavioral and biochemical alterations in rodents. Interestingly, droperidol pretreatment in this study increased anxiety-like behavior induced by ketamine.

In the OF test, the parameters measured included the number of crossings and the time spent in both the center and corner of the field. After administration for 6 days, ketamine-induced hyperlocomotion was characterized by an increased number of crossings. It also promoted anxiety-like behavior, evidenced by reduced time spent in the center and increased time in the corners. It was reported that the hyperlocomotion induced by ketamine is primarily attributed to dopaminergic hyperactivation in the striatal regions of the brain (Sun et al., 2020; Le et al., 2022). Behavioral changes induced by ketamine and other NMDA antagonists, such as phencyclidine, mimic schizophrenia-like symptoms by blocking neurotransmission at NMDA-type glutamate receptors (McCutcheon et al., 2020). This blockade disrupts both dopamine regulation and GABAergic neurotransmissions, contributing to the pathophysiology of schizophrenia (McCutcheon et al., 2020; Szota and Scheel-Krüger, 2020).

Pretreatment with *H. alpinum* ethyl acetate extract (40 and 80 mg/kg) effectively attenuated both hyperlocomotion and anxiety-like behavior in the OF test induced by ketamine. Conversely, the *H. alpinum* chloroform extract (40 mg/kg) did not produce significant differences compared to the ketamine-treated group. A higher dose (80 mg/kg) significantly reduced hyperactivity and anxiety-like behavior, demonstrating both neuroleptic and anxiolytic-like effects. These findings suggest that the reversal of hyperlocomotion and anxiety-like behavior by *H. alpinum* extracts in ketamine-treated animals may underly glutamatergic and GABAergic pathways. These results align with the previous reports, supporting the antipsychotic potential of *H. alpinum* ethanolic extract (Nasir et al., 2020; Szota and Scheel-Krüger, 2020; Jalsrai et al., 2023). Furthermore, droperidol pretreatment exhibited similar effects to other antipsychotic drugs resulting in diminished ketamine-induced hyperactivity. However, it also increased anxiety-like behavior in the OF test. These results underscore droperidol's role in modulating hyperactivity while highlighting its anxiogenic side effects in ketamine-induced models.

The NOR test evaluates recognition memory, which is impaired in schizophrenia patients (Gebreegziabhere et al., 2022) and disrupted by ketamine in young healthy volunteers (Santiago et al., 2023). As expected, ketamine (25 mg/kg, i.p. for 7 days) induced cognitive impairment in the NOR test, reducing DI to a negative

value and decreasing the PI compared to the normal control group. Numerous studies associate ketamine-induced memory impairment with oxidative stress, glutamatergic neurotransmission antagonism, and acetylcholine blockade in cortical and hippocampal areas (Li et al., 2020). Droperidol pretreatment successfully prevented memory dysfunction induced by ketamine in the NOR test. Similarly, H. alpinum ethyl acetate and chloroform extracts (40 and 80 mg/kg, 7 days) significantly reversed memory dysfunction, demonstrating their potential to mitigate memory deficits.

Oxidative stress plays a critical role in the pathophysiology of schizophrenia, defined as an imbalance between reactive oxygen species (ROS) production and antioxidant defense system (Ermakov et al., 2021). Levels of MDA are elevated, and SOD and GPx are decreased in schizophrenia patients (Buosi et al., 2021). Ketamine has been reported to increase oxidative stress and decrease antioxidant enzyme activity in the brain, including the frontal cortex, hippocampus, and striatum (Reus et al., 2018; Moghaddam et al., 2021). In the current study, ketamine increased TBARS levels, and decrease SOD activity in rat hippocampus compared to the control group, while GPx activity remained unchanged. These findings align with previous studies (Reus et al., 2018; Moghaddam et al., 2021). Pretreatment with droperidol decreased TBARS levels and increased SOD activity, ameliorating ketamine-induced oxidative damage in the hippocampus. Interestingly, these data contradict the reports that haloperidol, a typical antipsychotic, exacerbates ketamine-induced oxidative damage in rodents (Valvassori et al., 2021). However, atypical antipsychotic drugs such as risperidone and clozapine have shown antioxidant effects against ketamine-induced oxidative stress (Fujikawa et al., 2024). Both *H. alpinum* chloroform and ethyl acetate extracts (40 and 80 mg/kg) significantly prevented ketamine-induced oxidative stress. These results align with previous reports showing that antioxidant therapy decreased oxidative stress and improved antioxidant enzyme activity in schizophrenia patients (Madireddy and Madireddy, 2020; Ermakov et al., 2021).

Chemical analysis revealed that *H. alpinum* ethyl acetate extract is rich in flavonoids and xanthones, whereas *H. alpinum* chloroform extract is dominated by fatty acids such as linalool, gingerol, and oleic acid. Both extracts also contain high levels of triterpenoid saponins, identified as oleanolic acids. The significant protective effects of both extracts against ketamine-induced behavioral and oxidative alterations suggest a key role for oleanolic acid. Notably, oleanolic acid has been reported to prevent MK-801-induced schizophrenia-like behaviors in mice, including hyperlocomotion and memory impairment in the NOR test (Park et al., 2014; Chen et al., 2023). The observations indicate that the reversal of hyperlocomotion and anxiety-like behavior and memory dysfunction by *H. alpinum* extracts in ketamine-treated animals might be attributed to the oleanolic acids present in *H. alpinum* extracts.

Interestingly, the anxiolytic effects observed with *H. alpinum* extracts contrast with the anxiogenic effects of droperidol. This suggests that *H. alpinum* extracts may act through additional mechanisms beyond oxidative stress regulation. For instance, glutamatergic neurotransmission plays a well-established role in anxiety-like behavior (Nasir et al., 2020), which could be modulated by components of H. alpinum extracts. Additionally, flavonoids and fatty acids found in H. alpinum extracts may contribute to their anxiolytic effects, as multiple studies have reported that essential oils and flavonoids can reduce anxiety by modulating GABAergic or serotonergic systems (de Sousa et al., 2023; Khan et al., 2023).

Conclusion

The study demonstrated the antipsychotic potential of *H. alpinum* extracts in attenuating hyperactivity, and anxiety-like behavior, and reversing cognitive deficits induced by ketamine in rodent models. These therapeutic effects are likely mediated through their bioactive components, particularly oleanolic acids, flavonoids, and fatty acids. Given their ability to target both oxidative stress and anxiety-related behaviors, *H. alpinum* extracts may offer advantages over traditional antipsychotic drugs in managing schizophrenia-like symptoms.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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