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Ethanolic Extract of *Hedysarum Alpinum L* Is Rich in Flavonoids and Shows Free Radical Scavenging and Psychological Modulation Activities

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Abstract: *Hedysarum alpinum L* (HA) is reported to be able to support the immune and nervous systems. However, there is only a few reports about the pharmacological activity of this plant. In current study, we investigated the phytochemical and pharmacological properties of an extract of HA using in vitro and iv vivo models. An extract of the specimens preserved in 70% ethanol was filtered and vacuum dried. Established conventional methods were used for quantitative determination of total phenolic and flavonoids content. Phytochemical characterization showed gallic acid, rutin, and quercetin are rich in the ethanolic extract of HA. In addition, the extract showed free radical scavenging activity on hydroxyl and lipid radicals with IC_{50} 6.72 and 7.73 mg/ml, respectively. Furthermore, in vivo studies were performed on rats with orally applying the HA extract and then assessed their psychological behaviors in elevated plus maze (EPM) and open-field tasks. The HA extract exhibited psychological modulation activity. Our study suggests the ethanolic extract of HA could be potential to be used in the products of mood soother.
Keywords: *Hedysarum alpinum L*; Free radical scavenging; Psychological modulation.

1. Introduction

Hedysarum alpinum L (HA) is one genus of the *Fabaceae* family. Many species of the genus *Hedysarum*, such as HA, *Hedysarum polybotrys*, and *Hedysarum vicioider turch* have been employed in folk medicine in many countries to strengthen the immune system and to treat neuronal diseases [1,2]. HA contains several phytochemicals, including xanthones, flavonoids, saponins, and polysaccharides. These phytoconstituents possess variety of different therapeutic activities that include anti-diabetic, immune-modulation, anti-cancer, and cardioprotective properties [1,2].

Therapeutic effects of the phytochemicals from HA are primarily mediated by their antioxidant and anti-inflammatory activities [3]. Since antioxidative and anti-inflammatory effects of medicinal plants may also beneficial to neuronal disorders, we therefore evaluate the potential pharmacological activity of HA to explore a therapeutic option in the treatment of central nervous system diseases.

The current study examined the phytochemical content of ethanolic extract of HA, and its antioxidative and pharmacological activities in vitro and in vivo, respectively. Our data indicate the HA ethanolic extract could be potential in psychological modulation.

2. Materials and methods

2.1 Animals

Adult male Wister rats used in this study weighing 200 to 250 g, were purchased from the Experimental Animal Center, Ulaanbaatar, Mongolia. All rats were kept under controlled laboratory conditions with a light-dark cycle 12:12 h (lights on at 6:00 a.m.), temperature 20 ± 2ºC, and air humidity between 55% and 60% with free access to food and water. After arrival, the animals were allowed a 2-week period for habituation. Experiments were conducted according to the guidelines of Mongolian and European Animal Ethics Committee.

2.2 Chemicals

All of the chemicals which were of analytical grade, including the reference compounds: rutin, quercetin, gallic acid, kaempferol, apigenin and ketamine were purchased from Sigma Chemical Co. (Sigma Aldrich, St. Louis, MO, USA).

2.3 Plant material

The aerial part of HA (Figure 1A) was collected at Tulu Mountain, Tsaagan soum, Bulgan province, Mongolia in early August 2021. The plant was identified and authenticated by Prof. E. Ganbold, Institute of Botany, Mongolian Academy of Sciences.

2.4 Extraction of phenol and flavonoids of HA with ethanol

Powdered raw material of the dried HA aerial plant (0.5 kg) was preserved in 2 L of 70% ethanol. After 7 days, extract was filtered, the ethanol was evaporated under vacuum below 50ºC and the remaining aqueous solution was lyophilized to get solid extracts. A solid extract (12.5% yield of original material) was stored at 4ºC until use. One hundred mg of the solid extract was dissolved in 10 mL methanol and used for quantitative analysis. For use in vitro and in vivo experiments the extract was dissolved in ethanol and diluted with distil H_2O to the working concentration.

2.5 Preparation of standard solutions

The stock solutions of rutin, quercetin and gallic acid were dissolved in methanol at concentration of 1 mg/mL and stored at 4ºC. Calibration curves were constructed using concentrations of standard solution in the range from 0.01 to 0.5 mg/ml.

2.6 Quantitative analysis of the contents of the ethanolic extract of HA

The total phenolic and flavonoid-contents of the ethanolic extract of HA were determined by UV-visible spectrophotometric methods using the standard calibration curve procedure [1]. The total phenolic content was determined by the Folin-Ciocalteu assay [4,5] and expressed as gallic acid at 760 nm. The total flavonoids were measured following the methods as described [6,7] with slight modification. The absorbance of phenolic solutions was read using formation of acid stable complex with 2% $AlCl_3$ at 430 nm (quercetin) and 500 nm (rutin), respectively. All analyses were performed in triplicate.

2.7 Thin-layer chromatography (TLC) analysis

Ten gram of HA was extracted 3 times with 50 ml of 70% ethanol using the Soxhlet apparatus, and the combined extract was concentrated under reduced pressure. The residue was suspended in H_2O and then successively partitioned with hexane, chloroform, ethyl acetate, and n-butanol. Ethyl acetate, n-butanol and water residues were evaporated and the dry residues was dissolved in methanol and applied to TLC analysis. The samples were spotted of width 5 mm with a Camag microlitre syringe on silica gel (60 GF254, Merck, Germany). The development of the plate has been performed with a mobile phase of hexane-ethyl acetate-formic acid (10:50:1 v/v). After drying at room temperature, the plate was visualized under both 254 and 366 nm UV lights.

2.8 Study of free radical scavenging by electron spin resonance (ESR)

Hydroxyl and lipid radicals were detected using spin trapping agents with an ESR spectroscopy [8]. Briefly, hydroxyl radical was detected in a 50 µL reaction mixture containing 100 mM 5,5-dimethyl-1-pyrroline N-oxide (DMPO), 100 mM hydrogen peroxide (H_2O_2), 0.3 mM Fe (II), 0.15 mM diethylene triamine pentaacetic acid (DTPA), and 30 µL of sample. The reaction was initiated by 0.3 mM Fe(II)/0.15 mM DTPA, and then ESR spectra was recorded for 5 min. Lipid radical was detected in a 50 µL reaction mixture containing 100 mM α-(4-pyridyl-1-oxide)-N-tert-butylnitrone (POBN), 20 mM sodium dodecyl sulfate (SDS), 40 mM linoleic acid (LA), 0.11 mM Cu (II), 0.1 M H_2O_2, 20 mM phosphate-buffered saline (PBS) and 20 µL of sample. The reaction was initiated by Cu (II), and ESR spectra were recorded for 5 min after Cu (II) addition.

2.9 Animal behavior experimental protocol

For assessing anxiolytic activity, rats were randomly divided into 3 groups (n=7) with the oral gavage of vehicle (control group), HA extract 50 mg/kg, and HA extract 100 mg/kg once a day. Forty-five min after vehicle or HA extracts administration the behavior of the animals was assessed in the EPM apparatus.

The animal behavior on the EPM was conduct as described [9]. The apparatus is composed of two open (50 x 10 cm) and two closed (50 x 10 x 40 cm) arms that radiate from a central platform (10 x 10 cm) to form plus sign. The maze was elevated to a height of 50 cm above the floor level by a single central support. During the experiment each animal was placed in the central compartment facing one of the open arms. Number of entries and the time spent in the open arms were recorded for 5 min. An entry was counted when all four paws of rats entered an open or closed arm. An increase in open arms entries and increase in time spent in open arms were interpreted as an index of potential anxiolytic activity. All test sessions were recorded by using a video camera.

We also used ketamine to induce hyperlocomotion behavior on the animals. Four groups of animals were treated with (1) vehicle (orally) + distilled water (i.p., control group); (2) HA extract (orally) + distilled water (i.p.); (3)

vehicle (orally) + ketamine, (25 mg/kg, i.p.); and (4) HA extract (orally) + ketamine (25 mg/kg, i.p.). Two different schedules (acute and chronic) were conducted: (i) animals received once with vehicle or HA extract (200 mg/kg, orally) 45 min before the test, while ketamine (25 mg/kg, i.p) injected 30 min before the open-field test; (ii) animals received vehicle or HA extract (100 mg/kg, orally) and ketamine (25 mg/kg, i.p) for 6 days and open-field test was conducted 30 minute after the ketamine injection on day 6. Five rats (n=5) were used in each group for these two conditions.

Locomotor activity of animal was monitored using the open-field apparatus as previously described [10,11]. The apparatus consisted of a box (transparent walls and black floor, 60 x 60 x 60 cm) illuminated at 300 lux. The floor was marked into 16 squares of 15 x 15 cm. At the beginning of the test, an animal was placed in the rear left of the floor facing away from the observer. The whole experiment was recorded for 5 min by a camera above the test apparatus and the recorded images were used for scoring. The number of squares crossed with all paws was calculated in order to evaluate the hyperlocomotion. Both the EPM and the open-field apparatus were cleaned with 10% alcohol solution before each individual animal session.

2.10 Statistical analysis

Comparisons between experimental groups were performed by two-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test. The values are expressed as mean ± S.E.M. $p < 0.05$ was considered significant.

3. Results

3.1 The quantitative analysis of ethanolic extract of HA

The total phenolic content was determined and expressed as gallic acid with equation of y=0.0819x+0.01, r^2=0.998. The total phenolic content was 8.9 ± 0.96 mg/g as gallic acid equivalents. Total flavonoids content was expressed as quercetin and rutin equivalent, calculated using the equation y=9,0739x+0.0015, r^2=0.997 and y=0.041x+0.0071, r^2=0.999, respectively. The extract of HA contains flavonoids 6.1± 0.55 mg/g as quercetin equivalents, and 9.3 ± 0.87 mg/g as rutin equivalents. TLC results revealed the presence of other flavonoids, including kaempferol, apigenin and other non-identified compounds (Figure1).

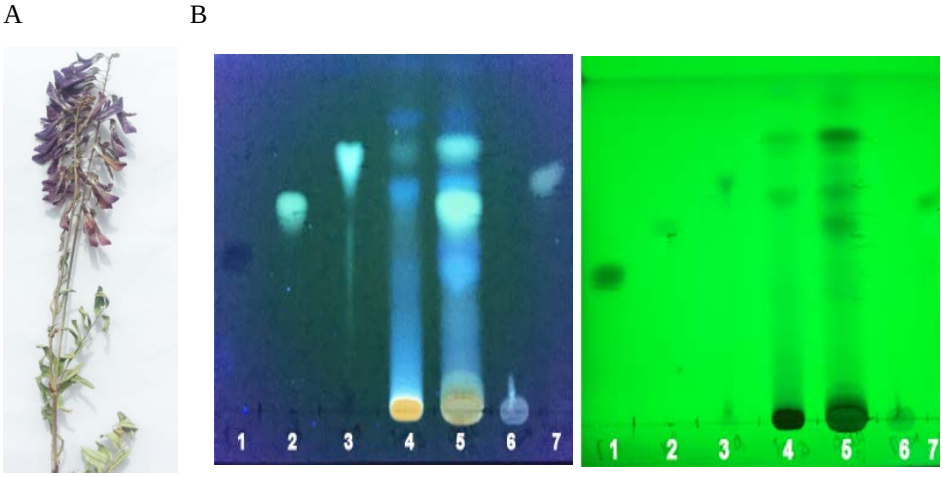

Figure 1. The HA plant and TLC analysis result under different UV wavelength. (A) The aerial part of HA. (B) The TLC plate was visualized under 254 and 366 nm UV lights. The lanes are (1) standard of rutin; (2) standard of quercetin; (3) standard of kaempferol; (4) n-butanol fraction HA; (5) ethyl acetate fraction HA; (6) water residues fraction of HA; and (7) standard of apigenin.

3.2 Free radical scavenging effect of ethanolic extract of HA

Free radical scavenge ability of HA was evaluated using ESR spectroscopy. It's known the height of spectrum signal is directly proportional to the amount of free radical including hydroxyl radical (DMPO/•OH) and lipid radical (POBN/•L). The intensity of DMPO/•OH spin adduct signal was reduced in a dose-dependent manner with the concentration of HA extract, indicating the scavenging of hydroxyl radical (Figure 2A,B). The 50% free radical scavenging concentration (half-maximal inhibitory concentration, IC_{50}) is 6.72 mg/ml. A similar dose-dependent pattern was also observed for the scavenging effect of HA extract on lipid hydroxyl radical. The decreasing intensity of POBN/•L spin adduct IC_{50} is 7.73 mg/ml (Figure 2 C,D).

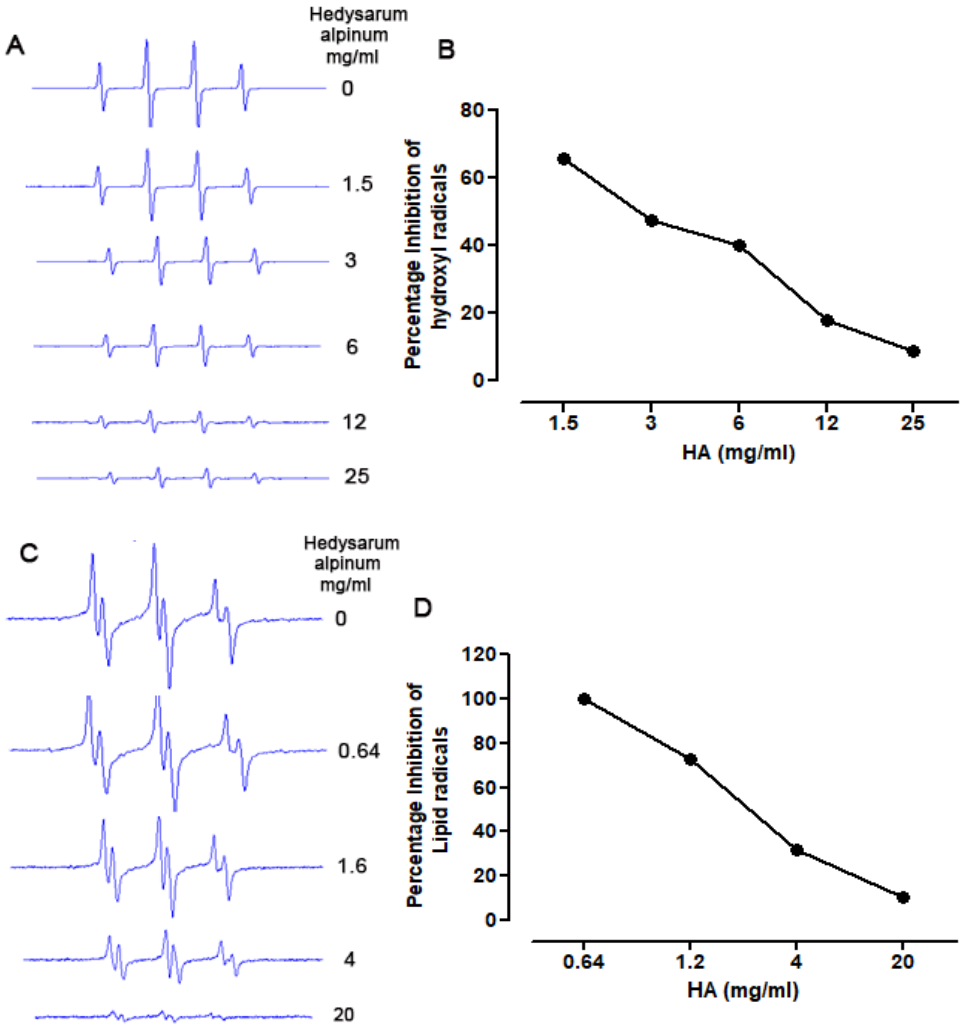

Figure 2. Effects of the HA ethanolic extract on hydroxyl and Lipid radicals detected by ESR. (A) ESR spectra of hydroxyl radicals. (B) Quantitative result of (A). (C) ESR spectra of lipid radicals. (D) Quantitative result of (C).

3.3 The effect of HA extract on the rat behaviors

In the EPM task, the HA extract at a dose of 50 mg/kg significantly increased the time of rats spent in the open arms and the number of entries into open arms compared to the control group ($p < 0.05$), however, a dose of 100 mg/ml did not show significant effect (Figure 3).

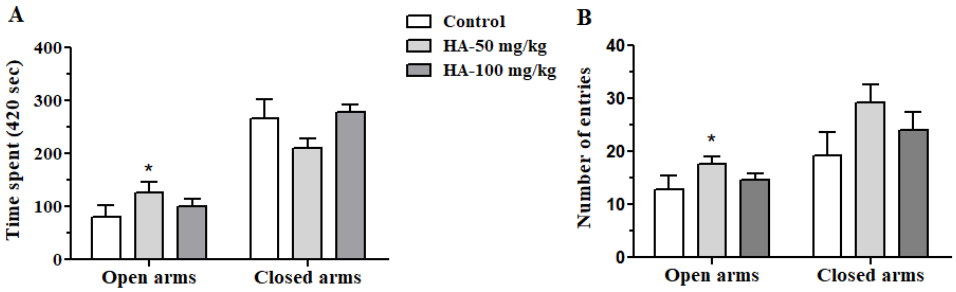

Figure 3. Effects the HA ethanolic extract on EPM test of rats. Data presented as mean ± S.E.M. *, $p < 0.05$ as compared to control group. (n = 7/group)

Both acute and chronic hyperlocomotion behaviors induced by ketamine were also assessed in the open-field test to evaluate the protective effect of HA extract. A dose of 25 mg/kg ketamine induced locomotor activity significantly increased the number of crossings (153.4%) compared to control animals ($p < 0.01$) in the open-field test. An acute pretreatment of HA extract (200 mg/kg) decreased 50% of the number of crossings compared to

ketamine-treated group (Figure 4A). Furthermore, we assessed the chronic administration of HA extract on hyperlocomotion induced by continued ketamine injection. After injection ketamine for 6 days, the numbers of crossing significantly increased (197%) compared to control animals ($p < 0.05$), whereas administration of HA extract (100 mg/kg) significantly decreased 44.5% of crossing numbers compared to ketamine injected animals (Figure 4B).

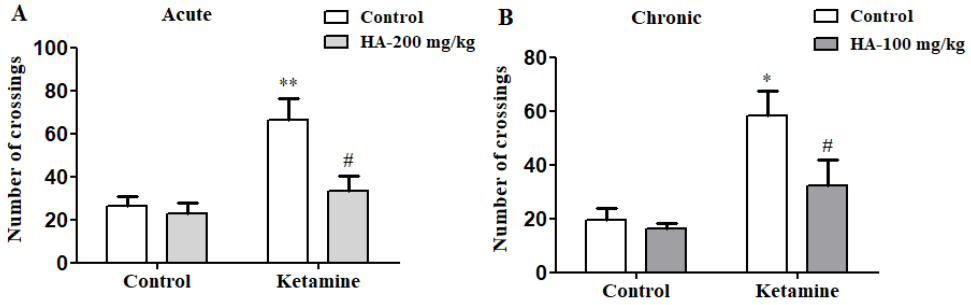

Figure 4. Effects the HA ethanolic extract (200 mg/kg, orally) on ketamine-induced hyperactivity in open-field test in rats. (A) Animals received HA extract (200 mg/kg, orally) and ketamine (25 mg/kg, i.p.) once, 30 min after injection of ketamine, locomotion of rats was detected in the open-field test. (B) Animals received HA extract (100 mg/mg) and ketamine (25 mg/kg) for continuous 6 days. Data presented as mean ± S.E.M. * $p < 0.05$; ** $p < 0.01$ compared to the control group; $^{\#}$ $p < 0.05$ compared to the ketamine-treated group. (n = 5/group)

4. Discussion

The phytochemical analyses indicated that the ethanolic extract of aerial part of HA contains high levels of polyphenols and flavonoids. Compared to the standards, the concentration of gallic acid, rutin and quercetin are 8.9, 9.3, and 6.1 mg/g, respectively. Our finding is consistent with phytochemical compositions of HA reported in previous studies [12].

In this study, we used the ESR to evaluate free radical scavenging capacity of ethanolic extract of HA on both hydroxyl and lipid radicals. The extract showed significant free radical scavenging activity in a concentration dependent manner suppressing ESR amplitude signals for DMPO/•OH and POBN/•L radical spin adduct.

We also assessed the anxiolytic activity of the ethanolic extract of HA using EPM test. The EPM is a well-established system to evaluate the anxiety of animal models [9]. Anxiety is one of the most common psychiatric disorders in the world and become a very important research topic in neuropharmacology. Benzodiazepines are known to be the major class of compounds used to treat anxiety [13]. However, the risk of dependence, withdrawal symptoms upon discontinuation, and cognitive side effects [14], much interest in herbal remedies is growing for anxiety and depression treatment to replace the traditional description in clinics, including benzodiazepines. Various studies demonstrated that polyphenols and flavonoids possess anxiolytic activity [7,15]. To our knowledge, there is a significant lack of studies on this *Hedysarum* species, including its neurological bioactivity. Previous report indicated that the ethanolic extract of HA exerts antidepressant and antipsychotic-like action in behavioral tests [16,17]. In this study, the extract of HA in a dose of 50 mg/kg significantly increased the amount of time spent in the open arms and the number of entries into the open arms compared to the control group ($p < 0.05$) indicating the presence of anxiolytic activity of extract. No significant effect was observed at an increased dose (100 mg/kg). It suggests the sedative action of HA extract should be applied in proper doses. Thus, we attempt to evaluate the effect of HA extract at a higher dose on hyperlocomotion induced by ketamine in the open-field test. As expected, pretreatment HA extract significantly decreased the number of crossings induced by ketamine. In addition, HA extract at dose 100 mg/kg taken simultaneously with ketamine for 6 days maintained the protection from hyperactivity induced by ketamine in open-field test. Ketamine is a dissociative anesthetic drug that acts as an antagonist of NMDA [18,19]. Ketamine induces symptoms which closely mimics the symptoms of schizophrenia, including positive, negative and cognitive impairment [20,21].

Previous studies demonstrated that flavonoids, alkaloids and terpenoids of medicinal plants exert anxiolytic [7,22] and antipsychotic activities [23]. Therefore, the neuroprotective activity of the HA extract could be contributed from the flavonoids which were found abundant in ethanolic extract of HA.

In summary, the ethanolic extract of HA is rich in phytochemicals flavonoids. We believe that this is a pioneer evidence to show that HA ethanolic extract shows free radical scavenging and antipsychotic-like activities. Further investigation will be performed to clarify the mechanism action and identify the neurological active constituents of HA. Our finding suggests the ethanolic extract of HA is highly potential to be developed for neurodegenerative disorders.

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