Protection Against Oxidative Stress-Induced Retinal Cell Death by Compounds Isolated From *Ehretia asperula*

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Abstract

*Ehretia asperula* (*E asperula*) is a well-known traditional medicinal plant in Vietnam with potent activity against a wide range of diseases, including hepatitis B and various cancers. Although *E asperula* has been used in traditional medicine, the efficacy of *E asperula* and its bioactive components on retinal degenerative diseases has not been fully evaluated. In present this study, we found that ethanolic extracts of *E asperula* increased cell viability in retinal precursor cells exposed to glutamate/BSO-induced excitotoxicity/oxidative stress. The major responsible bioactive compounds were rosmarinic acid and methylrosmarinic acid. First, 10 known compounds were isolated from *E asperula* leaves. Their chemical structures were determined using 1D and 2D nuclear magnetic resonance, and compared with published data. Using high-performance liquid chromatography, we determined the content of 4 compounds in *E asperula* extract: rosmarinic acid, lithospermic acid B, astragalin, and kaempferol 3-rutinoside. The most abundant of these compounds was lithospermic acid B. The protective effects of the pure compounds and ethanolic extracts against excitotoxicity and oxidative stress-induced retinal cell death were tested in R28 cells. Both 70% and 95% ethanolic extracts of *E asperula* increased cell viability in these conditions. Rosmarinic acid and methyl rosmarinic acid were more effective at protecting against retinal cell death and elevated reactive oxygen species in cells subjected to glutamate/BSO-induced excitotoxicity/oxidative stress. These findings suggested that *E asperula* could potentially be used to treat retinal degeneration.

Keywords

*Ehretia asperula*, boraginaceae, reactive oxygen species, chromatographic profile, phenolics, bioactivity

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Introduction

As the population ages, an increasing number of people will suffer from aging-related visual impairments, such as decreased visual acuity and visual field loss, and struggle to perform routine activities¹. Optic neuropathy is typically caused by age-related loss of sensory activity in the central nervous system, and degeneration of the optic nerve eventually leads to glaucoma, which causes visual field loss and irreversible blindness²,³. This condition affects an estimated 60 million people worldwide, with approximately 13% of the affected suffering from bilateral blindness⁴. In fact, because glaucoma can be asymptomatic until a relatively late stage, a much greater number of patients than estimated could suffer optic neuropathy-related visual impairment⁵. Despite the availability of glaucoma medications, these medications are commonly used to treat IOP, and there are no available modalities to treat optic nerve degeneration.

The death of retinal ganglion cells (RGCs), a population of neurons in the inner layer of the retina, is the most important factor in glaucoma-related blindness. RGCs, like many other neurons, are post-mitotic and do not recover or regenerate after being damaged. Glaucoma is classified as a neurodegenerative disorder, similar to Alzheimer’s and Parkinson’s disease⁶,⁷. Oxidative stress is a common pathological mechanism of neurodegenerative diseases⁸, and is caused by imbalanced generation and removal of reactive oxygen species (ROS). An overabundance of ROS results in oxidative...
damage to cellular macromolecules such as DNA, proteins, and lipids, ultimately leading to neuronal cell death. Since the 1980s, when the link between glaucoma and oxidative stress was first proposed, numerous studies in experimental animal models of glaucoma and clinical samples have supported this link.

Therefore, antioxidant substances that inhibit oxidative stress in RGCs may be promising candidates for protection against optic nerve degeneration, and preventing RGC death by correcting the overabundance of ROS characteristic of glaucoma is considered to be a viable potential therapeutic approach.

_Ebrelia asperula_ plant is endemic to Northern Vietnam. _E. asperula_ leaves have traditionally been used as a folk medicine to treat a variety of ailments such as hepatitis, liver cirrhosis, and cancer.

Furthermore, _E. asperula_ prevents or alleviates diabetes, hypertension, and acne. Some researchers have recently revealed the potential pharmacological effects of _E. asperula_ in cancer treatment, but there are no trial results using _E. asperula_ in cancer therapy. One study identified that the leaves of _E. asperula_ contained several constituents with anti-cancer properties, but the underlying mechanisms responsible for these effects are unknown. Only a few reports to date have characterized the chemical components of this plant and their biological effects, particularly on eye disease. Therefore, the aim of the present study was to investigate the chemical and chromatographic profiles of _E. asperula_ extracts, and their antioxidative activities against oxidative/excitotoxic stress-induced retinal cell death.

**Results and Discussion**

Ethanolic extract of _E. asperula_ (EEEA) was subjected to open column chromatography, isolating 10 known compounds (1-10). Comparison of the compounds’ physical and spectroscopic data (please see Supplemental file) with those reported in the literature identified the components as lithospermic acid B (1)\(^1\)\(^5\), ethyl lithospermate (2)\(^1\)\(^6\), clinopodic acid B (3)\(^1\)\(^7\), rosmarinic acid (4)\(^1\)\(^8\), methyl rosmarinic acid (5)\(^1\)\(^8\), kaempferol 3-rutinoside (6)\(^1\)\(^9\), astragalin (7)\(^1\)\(^9\), caffeic acid (8)\(^2\)\(^1\)\(^9\), β-aminyrin (9)\(^2\)\(^2\)\(^2\)\(^2\)\(^2\)\(^2\)\(^2\), and α-amyrin (10)\(^2\)\(^2\)\(^2\)\(^2\)\(^2\)\(^2\)\(^2\) (Figure 1).

High-performance liquid chromatography with diode-array detection (HPLC-DAD) analyses identified 4 major compounds, 7, 8, 5, and 2, at retention times of 21.3, 23.7, 28.0, and 36.2 min, respectively, at 260 nm (Figure 2), based on the comparison of the retention times and absorption spectra of the unknown peaks in the 70% ethanolic extract with the data for the isolated compounds.

The calibration curves for 4 compounds were calculated using the linear relationships between concentration (x) and peak area (y) of each compound and are shown in Table 1 as regression equations (y = ax + b). All 4 calibration curves demonstrated acceptable linearity (r\(^2\) ≥ 0.9996). The limits of detection (LOD) ranged from 0.196367 to 2.447649 μg/mL, and limits of quantification (LOQ) ranged from 0.595053 to 7.417119 μg/mL for 4 standard compounds.

Quantitative analysis of rosmarinic acid, lithospermic acid B, astragalin, and kaempferol 3-rutinoside in _E. asperula_ extract was performed using the analytical method. These compounds were found in concentrations ranging from 0.303 to 13.439 mg/g (Table 1). The most abundant of these 4 compounds was lithospermic acid B (13.439 ± 0.375 mg/g).

To determine the _in vitro_ cytoprotective effect of _E. asperula_ on retinal cells, 10%, 30%, 50%, 70%, and 95% ethanol extracts were tested. These extracts did not significantly affect the cell viability of untreated R28 rat retinal precursor cells at concentrations of 10 and 50 μg/mL (Figure 3A). To determine if the EEEAs were cytoprotective, R28 cells treated with glutamate and l-buthionine-[S,R]-sulfoximine (BSO) were examined in the presence of different types of EEEA. Glutamate is a major retinal excitatory transmitter that induces retinal cell death when present in high concentration.

The addition of glutamate to R28 cell culture causes severe damage in vitro, including decreased viability and cell death, mimicking excitotoxicity in the retina. Furthermore, BSO is a potent inhibitor of glutathione synthesis and is used to enhance the cytotoxic effects of various drugs. As expected, treatment with glutamate and BSO resulted in a decrease in cell viability, but the antioxidant, N-acetylcysteine, reversed the glutamate/BSO-induced cytotoxicity in R28 cells (Figure 3B). Interestingly, the 70% and 95% EEEA effected the cell viability under identical experimental conditions (Figure 3B).

Next, the bioactive compounds obtained from the 70% ethanol extract were investigated. Compounds 1, 2, 4, 5, 6, 7, 9, and 10 did not affect the viability of R28 cells in the MTT assay after 24 h at concentrations ranging from 10 to 100 μM (Figure 4A). Among these compounds, the addition of 4 and 5 (33 and 100 μM) significantly increased the survival rate under glutamate/BSO-induced cytotoxicity conditions (Figure 4B). However, these increases in survival rates were not observed with other compounds.

Glutamate-induced cytotoxicity is generally accompanied by ROS production, so the antioxidant effects of compounds 4 and 5 were evaluated. Intracellular ROS levels were increased by H\(_2\)O\(_2\), O\(_2^-\), and OH\(^-\) treatment of R28 cells. However, pretreatment with 33 and 100 μg/mL EEEA significantly decreased intracellular ROS levels despite the presence of H\(_2\)O\(_2\), O\(_2^-\), and OH\(^-\) (Figure 5A to C). In addition, pretreatment with 33 and 100 μM of 4 and 5 also decreased intracellular ROS levels under identical experimental conditions. These results suggested that compounds 4 and 5 had antioxidant properties.

Rosmarinic acid (4) is found in a variety of plants, and has liver protective, anti-inflammatory, and neuroprotective properties. Some preclinical studies of neurodegenerative diseases suggest that compound 4 has strong neuroprotective effects in the treatment of Parkinson’s and Alzheimer’s diseases. The efficacy of compounds 4 and 5 confirmed the cytoprotective effects of EEEA against glutamate/BSO-induced oxidative stress in R28 cells.

In conclusion, 10 known compounds (1-10) were isolated from _E. asperula_ leaves with an ethanolic extraction. The chromatographic profile of the 70% ethanolic leaf extract was obtained by comparison of the retention times. Furthermore, the ethanolic extract and the isolated compounds, 4 and 5, demonstrated significant protection against excitotoxicity/oxidative stress-induced retinal cell death.
stress-induced retinal cell death, implying that *E. asperula* could be used to treat ocular diseases after further research.

## Materials and Methods

### Chemicals and Apparatus

The 1D and 2D nuclear magnetic resonance spectra were obtained using a 500 MHz Bruker Avance DRX spectrometer, and chemical shifts were recorded in parts per million. Silica gel (Merck, 63-200 μm particle size) and RP-C18 (Merck, 75 μm particle size) particles were used for column chromatography. Thin-layer chromatography (TLC) was performed using Merck 60 F254 and RP-C18 F254 silica gel plates. Isolated compounds were visualized after spraying with aqueous 20% H₂SO₄ and heating for approximately 5 min. Analytical-grade acetonitrile and distilled HPLC-grade water were purchased from Fisher Scientific. Open column chromatography was performed using silica gel (Merck) and Sephadex LH-20 (Pharmacia).

### Plant Material and Extraction

Leaves of *E. asperula* (5 kg) were collected in Northern Vietnam in 2018. Botanical identification was performed by Msc. Trong.
Duc Nghiem. A herbarium specimen was deposited at the Department of Botany, Hanoi University of Pharmacy. Air-dried leaves were ground and extracted with 70% ethanol at room temperature 3 times (3 h × 20 L). The ethanol extract was obtained by solvent evaporation.

**Isolation and Identification**

The ethanol extract (180 g) was resuspended in distilled water and successively partitioned using hexane (Hx) (12 g), methylene chloride (MC) (28 g), ethyl acetate (EtOAc) (15 g), and n-butanol (BuOH) (20 g). The MC-soluble fraction was subjected to silica gel column chromatography using a stepwise gradient of Hx–EtOAc (10:5 to 0:1) to yield an isomeric mixture of 9 and 10. The EtOAc fraction was loaded onto a silica gel column using an EtOAc-MeOH-Water (100:4:3) solvent system to obtain 5 subfractions (EA 1-5). Subfraction EA-2 was then subjected to RP-C18 and Sephadex LH-20 open column elution using MeOH–H2O to yield compounds 2 (2.2 mg), 3 (2.3 mg), 4 (26.2 mg), 5 (33.1 mg), and 8 (3.4 mg). Similarly, compound 7 (11.3 mg) was obtained from the subfraction EA-3. Compounds 1 (4.3 mg) and 6 (3.4 mg) were then obtained from the BuOH fraction after subjecting it to an isolation protocol using repeated Sephadex LH-20 open column elution in MeOH–H2O (2:10-10:1).

**HPLC Analysis**

The analyses were conducted using an Agilent series 1200 liquid chromatography system and a YMC pack pro C18 column packed with 5 µm particles (4.6 × 250 mm) maintained at 25 °C in a column oven. The HPLC method is presented in Table 2. Formic acid (0.1%) was added to both acetonitrile and water mobile phases to obtain a better peak shape. The injection volume was 10 µL. The flow rate was 0.7 mL/min, and UV detection was performed at 260 nm.

**Sample Preparation for HPLC Analysis**

Ethanol extract of *E. asperula* leaves (10 mg) was dissolved in methanol (1 mL) and filtered through a syringe filter (0.45 µm). Four standard isolated compounds with purities ≥98% as evaluated using HPLC analysis, were dissolved in methanol to make stock solutions (1 mg/mL), and then further diluted with methanol to obtain various concentrations for quantitative analysis.

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### Table 1. Regression Equation, Linear Range, LOD, LOQ, and Content of 4 Compounds.

| Compound                        | Linear range (µg/mL) | Slope (a) | Intercept (b) | \( R^2 \) | LODb (µg/mL) | LOQc (µg/mL) | Content (mg/g) |
|---------------------------------|----------------------|-----------|---------------|----------|--------------|--------------|---------------|
| Rosmarinic acid                 | 7.8125 to 250        | 15.121    | 27.579        | 0.9996   | 2.447649     | 7.417119     | 7.782 ± 0.221 |
| Lithospermic acid B             | 7.8125 to 250        | 12.466    | -3.2366       | 0.9998   | 1.128552     | 3.419855     | 13.439 ± 0.375 |
| Astragalin                      | 1.953125 to 62.5     | 14.874    | 5.2139        | 0.9999   | 0.196367     | 0.595053     | 0.303 ± 0.008 |
| Kaempferol 3-rutinoside         | 1.953125 to 62.5     | 12.865    | 5.0396        | 0.9998   | 0.286691     | 0.868761     | 2.109 ± 0.063 |

LOD, limit of detection; LOQ, limit of quantification.

\(^a\) means peak area; \(x\); means concentration.

\(^b\) LOD = 3.3 × (SD of intercept/slope of the calibration curve).

\(^c\) LOQ = 10 × (SD of intercept/slope of the calibration curve).

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**Figure 3.** Protective effect of ethanol extract of *E. asperula* (EEEa) against glutamate/BSO-induced cell death in R28 cells. (A) R28 cells were treated with DMSO (vehicle), 10 and 50 µg/mL of 10%, 30%, 50%, 70%, and 95% EEEa or N-acetyl-L-cysteine (NAC) for 24 h. Cell viability of R28 cells was measured by MTT assay. (B) R28 cells were pretreated with DMSO (vehicle), 10 and 50 µg/mL of 10%, 30%, 50%, 70%, and 95% EEEa or NAC (1 mM) as a positive control for 1 h, and then incubated with 10 mM glutamate and 0.5 mM BSO for 24 h. Cell viability of R28 cells was measured by MTT assay (Con: untreated control; Veh: vehicle). Data are expressed as mean ± SEM. ***P<.001.
Calibration Curve, LOD, and LOQ

The calibration curves for 4 standard compounds were determined from the peak areas of the standard solutions at various concentrations: rosmarinic acid (7.8125-250 μg/mL), lithospermic acid B (7.8125-250 μg/mL), astragalin (1.953125-62.5 μg/mL), and kaemferol 3-rutinoside (1.953125-62.5 μg/mL). All measurements were performed in triplicate to produce calibration curves. The LOD and LOQ for the 4 standard compounds were determined using the standard deviation (SD) of the intercept and slope of the calibration curve, as derived by the following equations:

\[
LOD = 3.3 \times \left( \frac{SD \text{ of the intercept}}{Slope \text{ of the calibration curve}} \right)
\]

\[
LOQ = 10 \times \left( \frac{SD \text{ of the intercept}}{Slope \text{ of the calibration curve}} \right)
\]

Cell Culture

Immortalized rat retinal precursor cells (R28 cells) were purchased from Kerafast (USA). R28 cells were cultured in 75 cm² culture flasks in Dulbecco’s modified Eagle’s medium (DMEM; HyClone) supplemented with 10% fetal bovine serum (FBS; HyClone) and 100 U/mL penicillin/streptomycin (HyClone). The cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO₂.

Cell Viability

R28 cells were seeded in 96-well plates and incubated for 24 h. After 1 h of pretreatment with various concentrations of EEEA, glutamate (10 μM) and 1-buthionine-(S,R)-sulfoximine (BSO; 0.5 mM) mixture (glutamate/BSO) was added to the culture and kept for 24 h. To measure cell viability, MTT (3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide)
solution was added to the cells (final concentration: 0.5 mg/mL) at 37 °C and maintained for 1 h. The optical density of the solubilized formazan product was measured using a spectrophotometer (BioTek Instruments, VT, USA) at a test wavelength of 570 nm and reference wavelength of 690 nm.

Assessment of ROS Production

Intracellular ROS levels were determined using dichlorodihydrofluorescein diacetate (DCFH-DA) and ROS levels were assayed as previously described. Briefly, R28 cells were pretreated with the indicated concentrations of the compounds or extracts for 1 h, and the cells were then loaded with the DCFH-DA radical probe (10 µM) and incubated for 20 min at 37 °C. After removing the excess probe, 1 mM H2O2 (H2O2 radical), 1 mM H2O2 plus 100 µM iron (II) perchlorate hexahydrate (•OH), or KO2 at 1 mM (O2−) were added to generate the radical species. Fluorescence was measured after the ROS-generating compounds were present for various time periods, using excitation/emission wavelengths of 485/535 nm (luminescence spectrometer LS50B, PerkinElmer, UK).

Statistical Analysis

Data are expressed as mean percentage of the control value ± standard error of the mean (SEM). Statistical comparisons were performed using one-way analysis of variance followed by Dunnett’s test. Statistical analyses were conducted using GraphPad Prism, version 7.0 (GraphPad). Differences were considered statistically significant at *P < .05.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplemental material

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