Isolation and Evaluation of the Antioxidant Capacity of Compounds from *Ehretia asperula* Zoll. & Moritzi

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Abstract: Ehretia asperula *Zoll. & Moritzi was a common medicinal herb found in several Asian nations. This herb was found in many provinces in northern Vietnam, especially Hoa Binh province. Leaves of* E. asperula *cultivated in Tay Ninh Province were used to isolate compounds and evaluate their potential as antioxidants by five methods, including TAC, RP, FRAP, DPPH, and ABTS$^{\bullet+}$. Seven compounds were isolated and elucidated from the ethyl acetate fraction, including kaempferol (**1**), astragalin (**2**), nicotiflorin (**3**), rutin (**4**), caffeic acid (**5**), (-)-loliolide (**6**), and daucosterol (**7**), where compounds **1**, **4**, **6**, and **7** were found from this species for the first time. All isolated compounds from* E. asperula *leaves exhibited antioxidant activity, consisting of 5.70 ± 0.01 to 299.13 ± 10.19 μg/mL for EC_{50} values. Especially compound **5** had a very strong antioxidant effect based on five methods TAC (EC_{50} = 8.43 ± 0.15 μg/mL), RP (EC_{50} = 6.79 ± 003 μg/mL), FRAP (EC_{50} = 12.72 ± 006 μg/mL), DPPH (EC_{50} = 5.57 ± 0.02 μg/mL), and ABTS$^{\bullet+}$ (EC_{50} = 5.70 ± 0.01 μg/mL). From our results, Tay Ninh Province's* E. asperula *leaves are abundant in naturally occurring antioxidants, indicating their potential use as therapeutic materials.*

Keywords: *antioxidant;* Ehretia asperula*; kaempferol; (-)-loliolide; rutin*

■ INTRODUCTION

In this age, most individuals believe that free radicals are essential to our lives because they participate in most living processes of living organisms. Reactive oxygen and reactive nitrogen species, also known as ROS and RNS, are needed for defence mechanisms such as fighting diseases, participating in signaling systems, inducing mitotic reactions, and catalyzing metabolic processes. When they are excessively produced by exogenous chemicals or due to the body's metabolism for the oxidation of nucleic acids, lipids, proteins, gene mutations, damage, and cell death [1], excessive production of ROS and RNS damages the organism, leading to immunodeficiency and favorable conditions for opportunistic bacteria to invade imports. Natural compounds are crucial in the development of novel active pharmaceuticals because they have antioxidant activity and few side effects [2-3].

Ehretia asperula Zoll. & Moritzi was a species of the Boraginacae family that was often discovered in nations including China, Thailand, Myanmar, and Vietnam. In Vietnam, *E. asperula* grows in many provinces, including Ha Nam, Ninh Binh, Thien-Hue, Gia Lai, and many others. The leaves of this herb have been used for tea as a good drink for people's health by ethnic minorities in the north of Vietnam. Many studies have found that it can be employed to treat a lot of diseases, such as liver detoxification, ulcers, tumors, antioxidation, inflammation, and improving the body's resistance. Additionally, Kuo and Kuo's examination of the ethanol

extraction from *E. asperula* stems revealed that the extraction may be resistant to colon cancer, liver cancer, and HIV replication activity in H9 lymphocyte cells [4-6].

Eight compounds including phenolic and flavonoids, were isolated from the leaves of *E. asperula* in Hoa Binh Province, Vietnam, by Kim et al. [7]. The compounds were then tested for their cytotoxic action on cell lines, including those from the human lung (Hep-G2, LU-1), cervical (HeLa), breast (MCF-7), and rhabdomyosarcoma (RD) cancer. The results showed that some compounds showed good cytotoxic activity. Le et al. [8] found that the ethanolic extract of *E. asperula* increased cell viability when retinal precursor cells were exposed to glutamate and 1-buthionine-(*S,R*)-sulfoximine-induced excitotoxicity or redox imbalance. Besides, ten compounds belong to classes such as phenolic, triterpenes, and flavonoids were isolated from this species by the above researchers. So far, little study has been done on the chemical composition and biological activities of *E. asperula* species, particularly about its antioxidant capacity. As a result, this study advances knowledge about this species chemical composition and antioxidant capacity.

■ EXPERIMENTAL SECTION

Materials

In Tay Ninh province's Thai Binh commune and Chau Thanh district, *E. asperula* was planted and the leave texture can be seen in Fig. 1. This herb was confirmed by Dr. Luu Hong Truong (Institution of Ecology in Ho Chi Minh City, Vietnam) and kept in the Biochemistry laboratory with code number (Ea.2021-TN001), Can Tho University, Vietnam. In this investigation, the following solvents from Chemsol-Vietnam were utilized to macerate, extract, fractionate, isolate, recrystallize, and purify compounds as acetone (Ac), dichloromethane (DC), chloroform ($CHCl_3$), ethanol (EtOH), ethyl acetate (EtOAc), *n*-hexane, and methanol (MeOH). Besides, a number of other chemicals were employed to assess the compounds' antioxidant activity, including 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma-Aldrich, Germany), 2,2'-azino-bis(3-ethylbenzothiazoline-6 sulfonic acid) (ABTS, Sigma-Aldrich, Germany), ferric chloride (Sigma-Aldrich, Germany), potassium hexacyanoferrate(III) (Merck, Germany), trichloroacetic acid (Merck, Germany), trolox (Merck, Germany), acetic acid (Xilong, China), dimethyl sulfoxide (Xilong, China), sulfuric acid (Xilong, China), sodium phosphate (Xilong, China), and vanillin (Xilong, China).

Instrumentation

With the help of the Buchi R-300 rotavapor system (Buchi, Switzerland), the crude extract was composed. The compounds' melting point was measured on the X-4 Series melting-point apparatus with microscope (Zhejiang, China). FTIR/4600Type A (Jasco, Japan) infrared spectrometer was used to record the infrared spectra. The Bruker Avance 600 MHz spectrometer (Rheinstetten, Germany) recorded the NMR spectra.

Fig 1. Leave texture of *Ehretia asperula* Zoll. & Moritzi

Column chromatography (CC) used silica gel with particle sizes of 0.040–0.063 mm (230–400 mesh) (Merck, Germany). Pre-coated aluminium silica gel 60 F_{254} plates (Merck, Germany) with a thickness of 0.2 mm was utilized in thin-layer chromatography. Envision was accomplished by using UV light (254 and 365 nm), spraying the vanillin solution, or heating.

Procedure

Extraction and isolation

After the sample (31 kg of fresh *E. asperula* leaves) was brought to the laboratory, they were washed, dried at a temperature of 50 °C, ground into dry powder (15 kg) with 3.66% moisture, and kept at −20 °C until use. The powder was extracted by soaking it in ethanol 96 °C (3 × 40 L) for 24 h. Using an evaporator, the ethanol extract (1.5 kg) was collected and stored at 4 °C for use in subsequent experiments. The *n*-hexane (750 g) and EtOAc (250 g) extracts were made by liquid-liquid extraction of the ethanol extract using solvents with increasing polarity.

The EtOAc extracts of *E. asperula* that was used to isolate compounds in this study. To get 10 fractions, the EtOAc extract was treated with silica gel CC gradient elutions of *n*-hexane-EtOAc-MeOH (100:0:0–0:70:30) in vacuum liquid chromatography (E1–E10) based on TLC profiles. Fraction E2 (1.3 g) was eluted by DC-MeOH (100:0–70:30), yielding 9 subfractions (E2.1–E2.9); subfraction E2.6 (0.35 g) was eluted by $CHCl_3$-MeOH (100:0–80:20), yielding 5 subfractions (E2.6.1–E2.6.5); compound **1** (25 mg) was separated using silica gel CC with $CHCl_3$-MeOH (90:10) from subfraction E2.6.4. Fraction E7 (24.1 g) had yellow crystals (0.1 g), that are denoted as E7.CT (crystals), compound **2** (30 mg) was obtained by eluting subfraction E7. CT (0.1 g) with $CHCl_3$-MeOH (90:10) in silica gel CC. Similar, fraction E8 (47.53 g) also had yellow crystals (70 mg), that are denoted as E8.CT, using silica gel CC with $CHCl_3$-MeOH (80:20), compound **3** (32 mg) was obtained. Likewise, fraction E9 (20.6 g) had yellow crystals (90 mg), that are denoted as E9.CT, subfraction E9. CT (90 mg) was subjected to silica gel CC by $CHCl_3$-MeOH (70:30) to provide compound **4** (25 mg). Fraction E4 (1.3 g) was fractionated by silica gel CC and eluted with *n*-hexane-EtOAc-MeOH (100:0:0–0:80:20), yielding eight subfractions (E4.1–E4.8); subfraction E4.4 (0.45 g) was eluted by DC-MeOH (100:0–75:25), yielding five subfractions (E4.4.1–E4.4.5), using silica gel CC with DC-MeOH (90:10), compound **5** (15 mg) was separated from subfraction E4.4.3. Fraction E3 (2.9 g) was eluted by DC-MeOH (100:0–80:20) to afford eleven subfractions (E3.1–E3.11); by using a recrystallization and crystal purification method, compound **6** (20 mg) was obtained from subfraction E3.2. Fraction E5 (19.2 g) had white crystals (0.12 g), that are denoted as E5. CT, by recrystallization that amount of crystals (E5.CT) many times with the solvent system DC-MeOH (1:1), compound **7** (15 mg) was obtained.

Antioxidant assays

Total antioxidant capacity (TAC) Assay. Make the necessary adjustments and apply them in accordance with the methods stated by Nasir et al. [9]. The phosphomolybdenum method was used to assess the total antioxidant capacity of the samples spectrophotometrically. Each sample (300 µL) was mixed with 900 µL of the test solution (0.6 M H_2SO_4, 28 mM sodium phosphate, and 4 mM ammonium molybdate). For 90 min, the reaction solution was incubated at 95 °C. The reaction mixture was then allowed to settle to ambient temperature before being measured at 695 nm.

Measurement of the reducing power (RP) assay. Make the necessary adjustments and apply them in accordance with the methods stated by Fatima et al. [10], 500 µL of each sample, 500 µL of phosphate buffer (0.2 M, pH = 6.6), and 500 µL of 1% $K_3Fe(CN)_6$ made up the reaction mixture. Following a 20 min incubation period at 50 °C, 500 µL of 10% Cl_3CCOOH was added, and the mixture was centrifuged for 10 min at 3,000 rpm. Following centrifugation, 500 µL was taken out and well shaken before being combined with 500 µL of water and 100 µL of 0.1% $FeCl_3$. At 700 nm, the reaction mixture's spectral absorbance was measured.

Ferric reducing antioxidant power (FRAP) assay. Make the necessary adjustments and apply them in accordance with the methods stated by Song et al. [11]. This method's reduction of the ferric-tripyridyltriazine

complex serves as its foundation. For 30 min in the dark, FRAP solution (990 µL) was added to each sample (10 µL). The 593 nm wavelength was used to calculate the optical density.

DPPH assay. Make the necessary adjustments and apply them in accordance with the methods stated by Baliyan et al. [12]. First, 40 µL of DPPH (1,000 µg/mL) and 960 µL of each sample made up the reaction mixture. For 30 min, the reaction mixture was incubated at 30 °C in the dark. Next, at 517 nm, the absorbance of DPPH was determined.

ABTS assay. Make the necessary adjustments and apply them in accordance with the methods stated by Hussen and Endalew [13]. Initially, 2.45 mM potassium persulfate and 7 mM ABTS were reacted to create $ABTS^{\bullet+}$. Before being used, the mixture was allowed to sit at room temperature in the dark for 12 to 16 h. Subsequently, the blend was thinned out to get an optical density of 0.70 ± 0.05 at 734 nm. To conduct the survey, react 10 µL of each sample for 6 min at room temperature with 990 µL of $ABTS^{\bullet+}$. The spectral absorbance of the reaction mixture was then measured at 734 nm.

■ RESULTS AND DISCUSSION

Isolation of Compounds

From the EtOAc extracts of the leaves of *E. asperula*, seven compounds were isolated and identified. The structures of the separated compounds were ascertained by spectroscopic methods such as FTIR, ESI-MS, 1D, and 2D-NMR. Seven compounds were isolated including kaempferol (**1**) [14], astragalin (**2**) [15], nicotiflorin (**3**) [16], rutin (**4**) [17], caffeic acid (**5**) [18], (-)-loliolide (**6**) [19], and daucosterol (**7**) [20] (Fig. 2). As far as we are aware, four compounds including **1**, **4**, **6**, **7** were the first time to be isolated in the species.

Kaempferol (1): yellow amorphous powder, m.p. 262 °C; FTIR (KBr) ν_{max}: 3310 cm^{-1} (OH); 1656 cm^{-1} (C=O); 1601, 1504, and 1453 cm^{-1} (aromatic ring, C=C); ^{1}H-NMR (600 MHz, DMSO-d_6), δ_H (ppm): 6.19 (1H, *d*, *J* = 2.0 Hz, H-6), 6.44 (1H, *d*, *J* = 2.0 Hz, H-8), 6.93 (2H, *d*, *J* = 8.0 Hz, H-3', H-5'), 8.04 (2H, *d*, *J* = 8.0 Hz, H-2', H-6'), 12.50 (1H, *s*, 5-OH); ^{13}C-NMR (150 MHz, DMSO-d_6), δ_C (ppm): 146.8 (C-2), 136.0 (C-3), 176.0 (C-4), 160.7 (C-5), 98.2 (C-6), 164.0 (C-7), 93.5 (C-8), 156.2 (C-9), 103.0 (C-10), 121.7(C-1'), 129.5 (C-2'), 115.4 (C-3'), 159.2 (C-4'), 115.4 (C-5'), 129.5 (C-6').

Kaempferol-3-*O*-β-D-glucopyranoside (astragalin) (2): yellow amorphous powder, m.p. 181 °C; FTIR (KBr) ν_{max}: 3256 cm^{-1} (OH); 1650 cm^{-1} (C=O); 1602, 1509, and 1433 cm^{-1} (aromatic ring, C=C); ^{1}H-NMR (600 MHz, DMSO-d_6), δ_H (ppm): 3.09 (2H, *m*, H-3'', H-4''), 3.18 (1H, *m*, H-2''), 3.22 (1H, *m*, H-5''), 3.32 (1H, *s*, H-6''a), 3.57 (1H, *d*, *J* = 12.0 Hz, H-6''b), 5.46 (1H, *d*, *J* = 7.2 Hz, H-1''), 6.20 (1H, *d*, *J* = 2.0 Hz, H-6), 6.42 (1H, *d*, *J* = 2.0 Hz, H-8), 6.88 (2H, *d*, *J* = 9.0 Hz, H-3', H-5'), 8.04 (2H, *d*, *J* = 9.0 Hz, H-2', H-6'), 12.60 (1H, *s*, 5-OH); ^{13}C-NMR (150 MHz, DMSO-d_6), δ_C (ppm): 156.2 (C-2), 133.2 (C-3), 177.4 (C-4), 161.2 (C-5), 98.7 (C-6), 164.4 (C-7), 93.6 (C-8), 156.4 (C-9), 103.9 (C-10), 120.9 (C-1'), 130.8 (C-2'), 115.1 (C-3'), 159.9(C-4'), 115.1 (C-5'), 130.8 (C-6'), 100.9 (C-1''), 74.2 (C-2''), 77.4 (C-3''), 69.9 (C-4''), 76.4 (C-5''), 60.8 (C-6''); HR-ESI-MS *m/z* 449.1072 $[M+H]^+$, calculated $C_{21}H_{20}O_{11}$, *m/z* 448.1006.

Kaempferol-3-*O*-β-D-rutinoside (nicotiflorin) (3): yellow amorphous powder, m.p. 198 °C; FTIR (KBr) ν_{max}: 3410 cm^{-1} (OH); 1656 cm^{-1} (C=O); 1601, 1505, and 1446 cm^{-1} (aromatic ring, C=C); ^{1}H-NMR (600 MHz, DMSO-d_6), δ_H (ppm): 0.98 (3H, *d*, *J* = 6.0 Hz, H-6'''), 3.04 (1H, *m*, H-4''), 3.09 (1H, *d*, *J* = 9.6 Hz, H-4'''), 3.16 (1H, *m*, H-2''), 3.22 (1H, *m*, H-3''), 3.27 (4H, *m*, H-5'', H-6''a, H-3''', H-5'''), 3.35 (1H, *m*, H-2'''), 3.69 (1H, *d*, *J* = 10.2 Hz, H-6''b), 4.38 (1H, *d*, *J* = 1.2 Hz, H-1'''), 5.30 (1H, *d*, *J* = 7.8 Hz, H-1''), 6.20 (1H, *d*, *J* = 2.0 Hz, H-6), 6.41 (1H, *d*, *J* = 2.0 Hz, H-8), 6.88 (2H, *dt*, *J* = 9.0 Hz, 4.8 Hz, H-3', H-5'), 8.00 (2H, *dt*, *J* = 9.0 Hz, 4.8 Hz, H-2', H-6'), 12.55 (1H, *s*, 5-OH); ^{13}C-NMR (150 MHz, DMSO-d_6), δ_C (ppm): 156.5 (C-2), 133.2 (C-3), 177.4 (C-4), 161.2 (C-5), 98.7 (C-6), 164.2 (C-7), 93.7 (C-8), 156.8 (C-9), 104.0 (C-10), 120.9 (C-1'), 130.8 (C-2'), 115.1 (C-3'), 159.9 (C-4'), 115.1 (C-5'), 130.8 (C-6'), 101.3 (C-1''), 74.2 (C-2''), 76.4 (C-3''), 69.9 (C-4''), 75.7 (C-5''), 66.9 (C-6''), 100.7 (C-1'''), 70.3 (C-2'''), 70.6 (C-3'''), 71.8 (C-4'''), 68.2 (C-5'''), 17.7 (C-6'''); HR-ESI-MS *m/z* 595.1667 $[M+H]^+$, calculated $C_{27}H_{30}O_{15}$, *m/z* 594.1585.

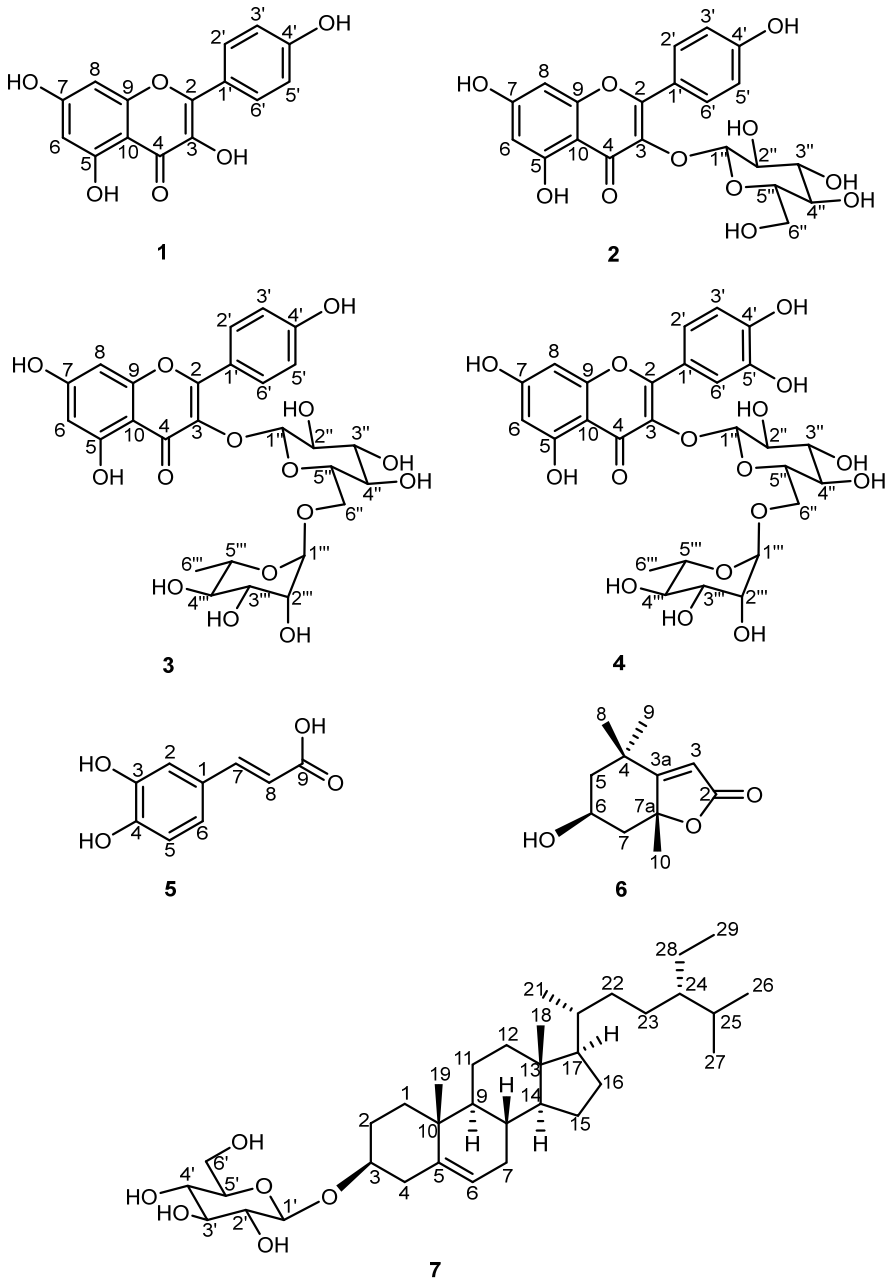

Fig 2. Chemical structures of compounds **1**-**7**

Quercetin-3-*O*-β-D-rutinoside (rutin) (4): yellow amorphous powder, m.p. 195 °C; FTIR (KBr) ν_{max}: 3332 cm^{-1} (OH); 1650 cm^{-1} (C=O); 1596, 1501, and 1454 cm^{-1} (aromatic ring, C=C); ^{1}H-NMR (600 MHz, DMSO-d_6), δ_H (ppm): 0.99 (3H, *d*, *J* = 6.0 Hz, H-6'''), 3.71, (1H, *d*, *J* = 10.8 Hz, H-6''b), 4.38 (1H, *s*, H-1'''), 5.34 (1H, *d*, *J* = 7.2 Hz, H-1''), 6.19 (1H, *d*, *J* = 2.4 Hz, H-6), 6.38 (1H, *d*, *J* = 1.8 Hz, H-8), 6.84 (2H, *d*, *J* = 8.4 Hz, H-5'), 7.53 (1H, *d*, *J* = 2.4 Hz, H-2'), 7.55 (1H, *dd*, *J* = 8.4, 2.4 Hz, H-6'), 12.60 (1H, *s*, 5-OH); ^{13}C-NMR (150 MHz, DMSO-d_6), δ_C (ppm): 156.4 (C-2), 133.3 (C-3), 177.3 (C-4), 161.2 (C-5), 98.6 (C-6), 164.1 (C-7), 93.5 (C-8), 156.5 (C-9), 103.9 (C-10), 121.1 (C-1'), 115.2 (C-2'), 144.7 (C-3'), 148.4 (C-4'), 116.2 (C-5'), 121.5 (C-6'), 101.2 (C-1''), 74.0 (C-2''), 76.4 (C-3''), 70.0 (C-4''), 75.9 (C-5''), 66.9 (C-6''), 100.7 (C-1'''), 70.5 (C-2'''), 70.3 (C-3'''), 71.8 (C-4'''), 68.2 (C-5'''), 17.7 (C-6''').

3,4-Dihydroxycinnamic acid (caffeic acid) (5): yellowish-brown crystal, m.p. 213 °C; FTIR (KBr) ν_{max}:

3305 cm^{-1} (OH); 1690 cm^{-1} (C=O); 1642, 1611, and 1510 cm^{-1} (aromatic ring, C=C); ^{1}H-NMR (600 MHz, MeOD), δ_H (ppm): 6.24 (1H, *d*, *J* = 15.6 Hz, H-8), 6.80 (1H, *d*, *J* = 8.4 Hz, H-5), 6.95 (1H, *dd*, *J* = 8.4, 2.4 Hz, H-6), 7.06 (1H, *d*, *J* = 2.4 Hz, H-2), 7.55 (1H, *d*, *J* = 15.6 Hz, H-7); ^{13}C-NMR (150 MHz, MeOD), δ_C (ppm): 127.8 (C-1), 115.1 (C-2), 147.0 (C-3), 149.4 (C-4), 116.5 (C-5), 122.8 (C-6), 146.8 (C-7), 115.6 (C-8), 171.0 (C-9); HR-ESI-MS *m/z* 181.0494 $[M+H]^+$, 163.0391 $[M+H-H_2O]^+$, calculated $C_9H_8O_4$, *m/z* 180.0423.

(6S,7aR)-6-hydroxy-4,4,7a-trimethyl-5,6,7,7a-tetrahydro-1-benzofuran-2(4*H*)-one((-)loliolide) (6): white crystal, m.p. 142 °C; FTIR (KBr) ν_{max}: 3427 cm^{-1} (OH); 1713 cm^{-1} (C=O); 1619 cm^{-1} (C=C); ^{1}H-NMR (600 MHz, $CDCl_3$), δ_H (ppm): 1.27 (3H, *s*, CH_3 at C-9), 1.47 (3H, *s*, CH_3 at C-8), 1.53 (1H, *dd*, *J* = 14.4, 3.6 Hz, H-5α), 1.77 (1H, *d*, *J* = 3.6 Hz, H-7α), 1.79 (3H, *s*, CH_3 at C-10), 1.99 (1H, *dt*, *J* = 14.4, 2.4 Hz, H-5β), 2.47 (1H, *dt*, *J* = 14.4, 2.4 Hz, H-7β), 4.33 (1H, *s*, H-6), 5.70 (1H, *s*, H-3); ^{13}C-NMR (150 MHz, $CDCl_3$), δ_C (ppm): 172.0 (C-2), 112.8 (C-3), 183.0 (C-3a), 35.9 (C-4), 47.3 (C-5), 66.7 (C-6), 45.6 (C-7), 86.8 (C-7a), 26.5 (C-8), 30.7 (C-9), 27.0 (C-10); HR-ESI-MS *m/z* 197.1181 $[M+H]^+$, 179.1076 $[M+H-H_2O]^+$, calculated $C_{11}H_{16}O_3$, *m/z* 196.1099.

β-Sitosterol-3-O-β-D-glucopyranoside (daucosterol) (7): white powder, m.p. 275 °C; FTIR (KBr) ν_{max}: 3403 cm^{-1} (OH); 1460 cm^{-1} (C=C-H); ^{1}H-NMR (600 MHz, DMSO-d_6), δ_H (ppm): 0.65 (3H, *s*, CH_3 at C-18), 1.00 (3H, *s*, CH_3 at C-19); 2.90–3.64 (6H, *m*, H-2' – H-6'), 4.22 (1H, *d*, *J* = 7.8 Hz, H-1'), 5.32 (1H, *t*, H-6); ^{13}C-NMR (150 MHz, DMSO-d_6), δ_C (ppm): 36.8 (C-1). 29.2 (C-2), 76.8 (C-3), 39.3 (C-4), 140.4 (C-5), 121.2 (C-6), 31.4 (C-7), 31.3 (C-8), 49.6 (C-9), 36.2 (C-10), 20.6 (C-11), 38.3 (C-12), 41.8 (C-13), 56.1 (C-14), 23.8 (C-15), 27.8 (C-16), 55.4 (C-17), 11.6 (C-18), 19.1 (C-19), 35.4 (C-20), 18.9 (C-21), 33.3 (C-22), 25.4 (C-23), 45.1 (C-24), 28.7 (C-25), 18.6 (C-26), 19.7 (C-27), 22.6 (C-28), 11.8 (C-29), 100.8 (C-1'), 73.4 (C-2'), 76.9 (C-3'), 70.1 (C-4'), 76.7 (C-5'), 61.1 (C-6').

Antioxidative Activities of Seven Compounds

Evaluate the antioxidant activity of seven compounds isolated from *E. asperula* leaves by using five methods such as TAC, RP, FRAP, DPPH, and $ABTS^{\bullet+}$ (Table 1). According to Wahyuningsih et al. [21], the degree of antioxidant power was separated into four levels very strong (EC_{50} < 50 μg/mL), strong (EC_{50} = 50–100 μg/mL), moderate (EC_{50} = 101–150 μg/mL), and weak (EC_{50} > 15 0μg/mL).

Based on the reduction of Mo(VI) to Mo(V) and the creation of a clear blue phosphate/Mo(V) complex in the phosphomolybdenum technique, the total antioxidant activity (TAC) of seven compounds was calculated. In the assay, **5** showed the strongest total antioxidant activity (EC_{50} = 8.43 ± 0.15 μg/mL), 5.89 times higher than the trolox (EC_{50} = 49.65 ± 0.17 μg/mL).

Antioxidants can inhibit the activity of common oxidative stress due to their capacity to convert Fe^{3+} into Fe^{2+}. The ability to reduce a compound was one of the most important characteristics for evaluating the

Table 1. The results of $Abs_{0.5}$ or EC_{50} concentration (μg/mL) of seven compounds

Compounds and Trolox	The $Abs_{0,5}$ or EC_{50} values of 07 compounds				
	TAC	RP	FRAP	DPPH	$ABTS^{\bullet+}$
1	$48.35^{e} \pm 0.88$	$23.43^{e} \pm 0.09$	$41.10^{f} \pm 0.33$	$6.91^{e} \pm 0.07$	$64.85^{d} \pm 0.72$
2	$70.34^{d} \pm 0.41$	$173.33^{a} \pm 0.23$	$52.06^{d} \pm 0.33$	$17.93^{b} \pm 0.43$	$96.63^{b} \pm 1.24$
3	$73.49^{d} \pm 0.45$	$142.40^{b} \pm 0.27$	$155.85^{a} \pm 0.71$	$18.64^{a} \pm 0.06$	$9.33^{e} \pm 0.09$
4	$84.80^{c} \pm 0.10$	$20.54^{f} \pm 0.08$	$47.18^{e} \pm 0.19$	$6.01^{f} \pm 0.04$	$8.95^{e} \pm 0.06$
5	$8.43^{f} \pm 0.15$	$6.79^{g} \pm 0.03$	$12.72^{g} \pm 0.06$	$5.57^{f} \pm 0.02$	$5.70^{f} \pm 0.01$
6	$299.13^{a} \pm 10.19$	$130.24^{c} \pm 1.86$	$119.80^{c} \pm 0.36$	$17.12^{c} \pm 0.15$	$136.78^{a} \pm 0.51$
7	$187.50^{b} \pm 0.74$	$54.52^{d} \pm 0.21$	$142.82^{b} \pm 2.40$	$13.82^{d} \pm 0.15$	$92.65^{c} \pm 0.90$
Trolox	$49.65^{e} \pm 0.17$	$5.19^{g} \pm 0.05$	$2.45^{h} \pm 0.02$	$0.64^{g} \pm 0.00$	$2.29^{g} \pm 0.01$

*Letter-separated values indicate statistically significant differences at the 5% level. The lowercase letters denote column comparisons: a, b, c, d, e, f, g, h

antioxidant capacity of that compound. RP and FRAP are used to determine reduced activity in this study. Assay results showed that compounds **1**-**7** were relatively able to reduce Fe^{3+} to Fe^{2+}. Particularly, **5** had a very powerful capacity to convert Fe^{3+} to Fe^{2+} (RP, EC_{50} = 6.79 ± 0.03 μg/mL; FRAP, EC_{50} = 12.72 ± 0.06 μg/mL), but was still weaker than trolox.

The compounds **1**-**7** showed that the free radical neutralizing activity significantly depended on these compounds' concentration. Compound **5** exhibits stronger DPPH free radical neutralization properties than the remaining compounds (EC_{50} = 5.57 ± 0.02 μg/mL). The ABTS compound was oxidized by oxidizing agents to form an $ABTS^{\bullet+}$ cation with a dark blue color. In this method, the ability to decolorize antioxidants in *E. asperula* leaves as a result of the interaction between $ABTS^{\bullet+}$ radicals and compounds isolated from them was used to assess the antioxidant capacity. Similarly, like DPPH, compounds isolated from *E. asperula* leaves also show demonstrate free radical neutralizing activity, in which **5** (EC_{50} = 5.70 ± 0.01 μg/mL) was very strong antioxidant sample but still 2.49 times weaker than trolox (EC_{50} = 2.29 ± 0.01 μg/mL).

By employing five methods, our study's findings are in line with other investigations on the antioxidant potential of **5**, including TAC, RP, FRAP, DPPH, and $ABTS^{\bullet+}$ [22-25]. Specifically, Sharma and Chandra [22] isolated **5** from the roots of *Bryophyllum binnatum* (Lam.) Kurz and investigated its antioxidant capacity. With an EC_{50} value of 66.58 ± 0.49 μg/mL, it was shown to have a higher antioxidant activity than root extract, which had an EC_{50} value of 139.52 ± 2.62 μg/mL, according to the DPPH free radical neutralization method. In the same year, Chiou et al. [23] also isolated **5** from the flowers of *Echinacea purpurea* and reported that it might block the α-amylase enzyme. The inhibition capacities of acarbose, **5**, and chlorogenic acid at 2.5 mg/mL were 85.23, 95.10, and 76.86%, respectively. Besides, the study of Girsang et al. [24] reported on the antioxidant properties of **5** from the methods like DPPH (IC_{50} = 8.72 μg/mL), scavenging H_2O_2 (IC_{50} = 15.23 μg/mL), $ABTS^{\bullet+}$ (IC_{50} = 6.23 μg/mL). Finally, Bovilla et al. [25] announced that **5** had strong antioxidant activity through the FRAP and DPPH methods. According to many studies, most basic phenols with several hydroxyl groups at the aromatic ring's *ortho* location were exceedingly efficient as oxidation inhibitors. This may be explained by the bond strengths or bond dissociation energies (enthalpy change) of hydroxyl groups, which are frequently used to evaluate how effectively an antioxidant reduces the impact of free radicals. Therefore, the lower the bond dissociation energy of these groups, the faster the reaction of the antioxidant with free radicals. Otherwise, the less the bond strengths that hydroxyl groups in phenols had, the easier it was to disperse the free radical and convert a hydrogen-radical. Likewise, the hydroxyl groups of hydroxycinnamic acids were responsible for their antioxidant characteristics. An effective way to assess an antioxidant's level of antioxidant activity is to look at its bond dissociation energy for the hydroxyl groups. Therefore, the weaker the bond between oxygen atoms and hydrogen atoms of the hydroxyl groups, the better the compounds exhibit antioxidant resistance through their ability to donate hydrogen atoms to neutralize free radicals. Furthermore, as with **5**, phenolic compounds with two OH groups oriented in *ortho* location demonstrated strong antioxidant activity [26-28].

Countless studies have demonstrated flavonoids' various biological abilities, counting anti-cancer, anti-inflammatory, and so on. Nonetheless, the most remarkable property of flavonoids is their antioxidant capacity. Much of their antioxidant activity is related to their chemical structures. The existence and location of hydroxyl groups linked to aromatic rings A and B or the C-2, C-3 double bond in amalgamation with a 4-carbonyl group of the flavonoids show the strongest antioxidant activity. However, the antioxidant capacity will be reduced when a sugar unit is linked to the flavonoid skeleton at C-3 to form a flavonoid glycoside compound. As antioxidants, flavonoids may work through different mechanisms, but the transfer of hydrogen atoms from the hydroxyl groups to neutralize detrimental free radicals seems to be the most significant mechanism [29-31]. In this study, four flavonoids compounds including **1**, **2**, **3**, **4**, all showed antioxidant

capacity with the EC_{50} values in five methods from 6.01 ± 0.04 to 173.33 ± 0.23 μg/mL.

Rutin (**4**) was the strongest antioxidant compound among the four flavonoids listed above. Two methods, DPPH (EC_{50} = 6.01 ± 0.04 μg/mL) and $ABTS^{\bullet+}$ (EC_{50} = 8.95 ± 0.06 μg/mL) showed that **4** was a very strong antioxidant. In addition, three methods such as TAC, RP, and FRAP also showed that **4** was a strong antioxidant compound with the following EC_{50} values of 84.0.10 ± 0.23, 20.54 ± 0.08, and 47.18 ± 0.19 μg/mL, respectively. Compound **4** has also been evaluated for its antioxidant activity by using many methods such as TAC, RP, FRAP, DPPH, $ABTS^{\bullet+}$, PCL, superoxide anion, and hydrogen peroxide scavenging [32-34]. Our result is similar to the research conclusions on this compound, which is a good antioxidant. Especially, this is the first time **4** has been isolated from *E. asperula*. The study by *Rusmana et al.* [32] announced rutin's *in vitro* antioxidant capacity. The results showed that **4** had a strong ability to neutralize DPPH and $ABTS^{\bullet+}$ free radicals with the EC_{50} values such as 5.56 ± 0.05 μg/mL and 17.16 ± 0.23 μg/mL. Additionally, in 2019, Tian et al. [33] showed that **4** might have a significant role as an adjuvant in the management of oxidative stress and inflammatory illnesses. Using three different methods, they assessed the compound's antioxidant capability and came to the following conclusions, which are DPPH (IC_{50} = 5.18 μg/mL), $ABTS^{\bullet+}$ (IC_{50} = 0.85 μg/mL) and FRAP (0.028 mmol Fe^{2+} μg/mL).

Many other studies on three flavonoids compounds **1**, **2**, and **3** have also shown their antioxidant properties. In our study, **1** was a strong to very strong antioxidant in all five methods. This result is analogous to those of other studies about this compound. Following the study of Han et al. [35], results utilizing the DPPH and FRAP essays, **1** had a substantial antioxidant activity. In 2017, the anti-free radical properties of **1**, which were studied by Dar et al. [36]. They used methods like RP, DPPH, TBARS to demonstrate the compound's strong antioxidant capacity. Pursuant to the research of Wang et al. [37], **1** was a flavonoid that has anticancer and antioxidant qualities. By free radical capture methods (DPPH, $ABTS^{\bullet+}$), the antioxidant capacity of **1** was investigated with EC_{50} values achieved of 47.93 ± 0.01 μM, 0.337 ± 0.02 mM, respectively. Besides, **1** also had the ability to cause cytotoxicity to cell lines such as the human hepatoma cell line Hep-G2 (EC_{50} = 30.92 ± 0.05 μM), mouse colon cancer cell line CT26 (EC_{50} = 88.02 ± 0.01 μM), mouse melanoma cell line B16F1 (EC_{50} = 70.67 ± 0.05 μM). Additionally, this is the first time that **1** has been isolated from *E. asperula* and evaluated for antioxidant activity utilizing the TAC method.

Astragalin (**2**) was a strong to very strong antioxidant in four methods, including TAC, FRAP, DPPH, and $ABTS^{\bullet+}$ with EC_{50} values from 17.93 ± 0.43 to 96.63 ± 1.24 μg/mL. But in the RP method, **2** was a weak antioxidant (EC_{50} = 173.33 ± 0.23 μg/mL). This consequence is similar to many studies that reported that **2** exhibited good antioxidant properties in three methods, such as DPPH [38], FRAP [39], $ABTS^{\bullet+}$ [40] and it showed weak antioxidant capacity in the RP method through the research of Dong et al. [41]. However, almost no research papers have evaluated **2**'s antioxidant activity using the TAC method, as our study did.

A ubiquitous flavonoid in plants, nicotiflorin (**3**) had a wide range of biological actions, including anti-inflammatory, anti-nociceptive, and antioxidant properties that guard against acute oxidative liver damage caused by CCl_4 [42-43]. In this research, its antioxidant ability of **3** was very strong in the DPPH, $ABTS^{\bullet+}$ methods, with EC_{50} values achieved of 18.64 ± 0.06 μg/mL, 9.33 ± 0.09 μg/mL, respectively, and strong in the TAC method (EC_{50} = 73.49 ± 0.45 μg/mL). Other studies have used some of the same methods as ours, including RP, FRAP, DPPH, $ABTS^{\bullet+}$ [44-45], nevertheless, very few have used the TAC method.

For daucosterol (**7**), this compound was isolated from *E. asperula* for the first time, and the DPPH method showed that it has a very strong antioxidant potential (EC_{50} = 13.82 ± 0.15 μg/mL), strong antioxidant capacity in two methods (RP, EC_{50} = 54.52 ± 0.21 μg/mL; $ABTS^{\bullet+}$, EC_{50} = 92.65 ± 0.90 μg/mL), moderate and weak antioxidant capacity in the FRAP and TAC methods. According to different reports about daucosterol's free radical scavenging ability using the

DPPH method, it exhibited good antioxidant activity, as demonstrated by its IC_{50} value of 11.42 ± 0.07 μg/mL [46]. Furthermore, several studies have examined this compound's antioxidant properties using a variety of methods, including the oil stability index (OSI), TAC, RP, FRAP, DPPH, and $ABTS^{\bullet+}$ [47-48].

The final compound was (-)-loliolide (**6**), which was isolated for the first time from this species and overall exhibited negligible antioxidant capacity. Our outcome was consistent with the study of Silva et al. [49] that **6** had weak antioxidant activity through three methods including DPPH, FRAP and oxygen radical absorbance capacity (ORAC). However, by using the MTT method, the neuroprotective effects of this compound were assessed. Furthermore, **6** also decreased the production of pro-inflammatory cytokines TNF-α and IL-6 and lowered nitric oxide generation.

Taking everything into consideration, a lot of published research has provided a clear explanation of the aforementioned compounds' antioxidant action. This shows that the presence of the above compounds in *E. asperula* makes the EtOAc extract have strong antioxidant activity as well as other biological activities of this species.

■ CONCLUSION

This research has isolated seven compounds from the EtOAc extract of *E. asperula* leaves. Four compounds are isolated for the first time in the species. In addition, all compounds isolated have been evaluated for their antioxidant activity. It is evident that *E. asperula* Zoll. & Moritzi, which is cultivated in Tay Ninh Province, Vietnam, has the potential to be utilized as a herbal remedy.

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■ CONFLICT OF INTEREST

There are no conflicting interests, according to the authors.

■ AUTHOR CONTRIBUTIONS

Dai Thi Xuan Trang, Tran Chi Linh, and Chong Kim Thien Duc performed experiments, prepared extracts, isolated the compounds, and evaluated the antioxidant activity. Nguyen Quoc Chau Thanh and Pham Quoc Nhien determined the structures of the compounds from experimental spectral data and compared them with other results. Nguyen Trong Tuan, Luu Thai Danh, and Chong Kim Thien Duc wrote, edited, supplemented, and completed the manuscript. The completed version of this work has been authorized by each individual author.

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