



Characterization of the potential antihypertensive activity of *Aster pseudoglehnii* leaf extracts

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Abstract

Chronic hypertension is closely associated with oxidative stress and endothelial dysfunction, both of which contribute to cardiovascular disease development. This study evaluated the potential antihypertensive and vasoprotective effects of *Aster pseudoglehnii* leaf extract and identified its major bioactive metabolites. Dried leaves were extracted with 70% ethanol and fractionated into two subfractions (F1 and F2) using preparative liquid chromatography. Antioxidant capacity was evaluated by measuring total phenolic and flavonoid contents, DPPH radical-scavenging activity, and oxygen radical absorbance capacity, while angiotensin-converting enzyme (ACE) inhibitory activity was assessed to determine antihypertensive potential. F2 exhibited stronger antioxidant and ACE inhibitory activities than F1. In EA.hy926 endothelial cells, F2 suppressed inflammatory adhesion molecules (ICAM-1 and VCAM-1) and enhanced endothelial nitric oxide synthase (eNOS) activation through phosphorylation. Untargeted metabolomic analysis revealed enrichment of cynarine, 4,5-dicaffeoylquinic acid, kaempferol-3-*O*-rutinoside, and lysophosphatidylglycerol (16:0) in F2. These findings indicate that F2 exerts potential antihypertensive effects through coordinated antioxidant and endothelial-protective mechanisms.

Keywords *A. pseudoglehnii* · Hypertension · Antioxidant activity · Angiotensin-converting enzyme · Endothelial dysfunction

Introduction

Hypertension is a major modifiable risk factor for cardiovascular disease (CVD) and all-cause mortality worldwide (Mills et al., 2020; Wang and He, 2024). Persistent elevation of blood pressure imposes both mechanical and oxidative stress on the vascular wall, leading to endothelial dysfunction and chronic vascular inflammation (Gallo et al., 2021). This pathological condition is characterized by excessive production of reactive oxygen species (ROS), reduced bioavailability of nitric oxide (NO), and enhanced leukocyte–endothelial interactions, which collectively contribute to arterial stiffness and target organ damage (Kim et al., 2025). The vascular endothelium plays a critical role in maintaining vascular homeostasis by regulating vasodilation

and suppressing inflammatory responses through endothelial NO synthase (eNOS)-mediated NO production (Yamagata, 2019). Under hypertensive conditions, angiotensin II (Ang II) promotes oxidative stress and disrupts eNOS activity, while pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α) stimulate the expression of adhesion molecules, including intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (Drozd et al., 2023; Zhao et al., 2015). Therefore, strategies aimed at reducing oxidative stress and suppressing endothelial inflammation are considered promising approaches for the prevention and management of hypertension.

In recent years, polyphenol-rich plant resources have received considerable attention because of their antioxidant, anti-inflammatory, and endothelial-supportive properties (Zhang and Tsao, 2016). Accumulating experimental evidence suggests that polyphenols can enhance NO bioavailability, attenuate ROS generation, and regulate the expression of adhesion molecules, thereby contributing to blood pressure regulation (Fraga et al., 2019). Among these plant resources, species belonging to the *Aster* genus have long been used in traditional medicine for their anti-inflammatory

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and cardioprotective properties (Rolnik and Olas, 2022; Sharma et al., 2022). *A. pseudoglehnii*, a species native to Korea, is known to contain abundant flavonoids and phenolic compounds. However, despite its phytochemical richness, the potential antihypertensive activity of this plant and its underlying molecular mechanisms have not yet been thoroughly investigated.

Therefore, in the present study, leaves of *A. pseudoglehnii* were extracted with 70% ethanol and subsequently fractionated using preparative liquid chromatography. The antioxidant capacity and antihypertensive potential of the resulting fractions were then systematically evaluated. Furthermore, the endothelial-protective effects of the fractions were investigated in Ang II- and TNF- α -stimulated EA.hy926 endothelial cells by measuring intracellular ROS production, eNOS phosphorylation, and the expression levels of ICAM-1 and VCAM-1. In addition, an untargeted UHPLC–Orbitrap MS/MS-based metabolomic analysis was conducted to identify bioactive constituents potentially responsible for the observed vascular-protective effects. By integrating antioxidant, enzymatic, cellular, and metabolomic analyses, this study provides comprehensive mechanistic insights into the antihypertensive potential of *A. pseudoglehnii*.

Materials and methods

Chemicals and reagents

All chemicals and reagents used in this study were of analytical grade unless otherwise specified. General laboratory reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), Bio-Rad (Hercules, CA, USA), and Daejung Chemicals (Siheung, Korea). The stable free radical compound 2,2-diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Haverhill, MA, USA). The EA.hy926 human vascular endothelial cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM; 25 mM glucose) supplemented with stable L-glutamine and sodium pyruvate (Biowest, Nuaille, France), 10% fetal bovine serum (FBS), and 1% penicillin–streptomycin (Gibco, Grand Island, NY, USA). Cells were maintained at 37 °C in a humidified incubator with an atmosphere containing 5% CO₂. For the cell viability assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Samchun Chemical (Seoul, Korea). Primary antibodies against ICAM-1 (#4915), VCAM-1 (#13662), eNOS (#9572), and phosphorylated eNOS (phospho-eNOS; Ser1177, #9570) were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies against β -actin and horseradish peroxidase (HRP)-conjugated secondary

antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

Preparation of *A. pseudoglehnii* extract

All experiments were conducted using *A. pseudoglehnii* cultivated on Ulleungdo Island, Korea. The leaves and stems were freeze-dried at –50 °C for 72 h and then ground into a fine powder using an electric blender (UNB-A9001, Ningbo, China). Two grams of the powdered sample were extracted with 100 mL of 70% ethanol (1:50, w/v) using a Sonics Vibra-Cell™ VCX 750 ultrasonic processor (Sonics & Materials Inc., Newtown, CT, USA). Sonication was performed on ice for three cycles (5 min per cycle at 60% amplitude, with 3 s pulses on and 10 s pulses off). The extracts were centrifuged at 15,000 $\times$ g for 10 min, and the resulting supernatants were filtered through Whatman No. 1 filter paper (GE Healthcare, UK). The filtrates were concentrated using a rotary vacuum evaporator (Eyela, Tokyo, Japan) and subsequently lyophilized to obtain dried extract powder. The dried extracts were stored at –50 °C until further use.

Fractionation of *A. pseudoglehnii* ethanol extracts

The ethanol extract of *A. pseudoglehnii* was fractionated using preparative high-performance liquid chromatography (Prep-HPLC; LC-Forte/R, YMC Co., Kyoto, Japan) equipped with a diode array detector (DAD). UV detection was performed at 240, 280, and 320 nm.

Chromatographic separation was carried out on a YMC Triart C18-S column (250 $\times$ 15 mm, 20 μ m) at a flow rate of 10 mL/min, with an injection volume of 5 mL (15 mg/mL). The mobile phase consisted of distilled water (A) and acetonitrile (B). The gradient program was as follows: 0 min, 0% B; 20 min, 40% B; 25 min, 100% B; and maintained at 100% B until 45 min. Under these conditions, the extract was separated into two major fractions, designated F1 and F2, which were collected based on peak resolution and used for subsequent biological and metabolomic analyses.

Measurement of total phenolic content (TPC)

TPC of the fractions obtained from the ethanol extract of *A. pseudoglehnii* was determined using the Folin–Ciocalteu colorimetric method with slight modifications (Singleton and Rossi, 1965). Briefly, 100 μ L of appropriately diluted sample was mixed with 500 μ L of 0.2 M Folin–Ciocalteu reagent. After incubation at room temperature for 5 min, 400 μ L of 10% (w/v) sodium carbonate (Na₂CO₃) solution was added. The reaction mixture was then incubated at 37 °C for 1 h in a water bath. Absorbance was measured at 750 nm using a NanoPhotometer (IMPLEN, Munich, Germany). TPC was calculated from a gallic acid standard calibration

curve and expressed as milligrams of gallic acid equivalents (GAE) per gram of dry weight (mg GAE/g dry weight).

Measurement of total flavonoid content (TFC)

The TFC of the fractions obtained from the ethanol extract of *A. pseudoglehnii* was determined using a colorimetric assay with quercetin as the calibration standard, with slight modifications (Atanassova et al., 2011). Briefly, 100 μ L of sample solution (1 mg/mL) was mixed with 400 μ L of distilled water, followed by the addition of 30 μ L of 5% (w/v) sodium nitrite (NaNO_2). After incubation at room temperature for 5 min, 30 μ L of 10% (w/v) aluminum chloride (AlCl_3) was added, and the mixture was allowed to react for an additional 6 min. Subsequently, 200 μ L of 1 M sodium hydroxide (NaOH) and 240 μ L of distilled water were added. Absorbance was measured at 415 nm using a NanoPhotometer (IMPLEN, Munich, Germany). TFC was calculated from a quercetin standard calibration curve and expressed as milligrams of quercetin equivalents (QE) per gram of dry weight (mg QE/g dry weight).

DPPH free radical scavenging assay

The free radical scavenging activity of the samples was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay with minor modifications (Mensor et al., 2001). A 0.2 mM DPPH solution and ascorbic acid (used as a positive control) were prepared in methanol. For the assay, 200 μ L of each sample or ascorbic acid solution was mixed with 100 μ L of 0.2 mM DPPH solution in a 96-well microplate. The reaction mixture was incubated in the dark at 37 °C for 30 min. Absorbance was then measured at 517 nm using a microplate reader (Molecular Devices, San Jose, CA, USA). DPPH radical scavenging activity (%) was calculated using the following equation:

DPPH radical scavenging activity(%)

$$= 100 - \left(\frac{\text{Sample Abs}}{\text{Blank Abs}} \times 100 \right)$$

The antioxidant activity was expressed as the half-maximal inhibitory concentration (IC_{50} , mg/mL), defined as the concentration required to scavenge 50% of DPPH radicals.

Oxygen radical absorbance capacity (ORAC) assay

The oxygen radical absorbance capacity (ORAC) assay was performed according to (Huang et al., 2002) with slight modifications. All reagents were prepared in 75 mM potassium phosphate buffer (pH 7.0). Briefly, 50 μ L of sample or Trolox (used as a positive control) was mixed with 50 μ L

of 3 μ M fluorescein sodium salt and pre-incubated at 37 °C in the dark for 15 min. Subsequently, 25 μ L of 221 mM 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH; Thermo Fisher Scientific, Waltham, MA, USA) was added to initiate the reaction. Fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm at 1-min intervals for 90 min using a fluorescence microplate reader. Antioxidant capacity was calculated from the area under the fluorescence decay curve and expressed as micromoles of Trolox equivalents per gram of dry weight ($\mu\text{mol TE/g dry weight of sample}$).

Angiotensin-converting enzyme (ACE) inhibitory activity

The ACE inhibitory activity of fractions derived from the ethanol extract of *A. pseudoglehnii* was determined as described by (Cushman and Cheung, 1971) with slight modifications. ACE (0.05 U/mL; Sigma-Aldrich, St. Louis, MO, USA) and 15 mM hippuryl-L-histidyl-L-leucine (HHL; Sigma-Aldrich) were prepared in 20 mM sodium borate buffer containing 0.3 M NaCl (pH 8.3). Equal volumes (100 μ L each) of sample solution, ACE solution, and HHL substrate were sequentially added to a 96-well microplate. The reaction mixture was incubated at 37 °C for 2 h and subsequently terminated by the addition of 1 N HCl. Absorbance was measured at 390 nm using a microplate reader. ACE inhibitory activity (%) and IC_{50} values were determined as previously described (Suo et al., 2022).

Cell culture

EA.hy926 human umbilical vein-derived endothelial cells were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. Cells were maintained at 37 °C in a humidified incubator containing 5% CO_2 (Edgell et al., 1983). When cell confluence reached approximately 80%–90%, the cells were subcultured at a density of 4.0×10^5 cells per 100-mm culture dish. Only cells with passage numbers below 20 were used for all experiments.

Cell viability assay

Cell viability was assessed using the MTT assay (Mosmann, 1983). EA.hy926 cells were seeded in 96-well plates at a density of 1×10^4 cells per well and incubated for 24 h. The cells were then treated with the fractions at concentrations of 30, 50, 100, or 200 $\mu\text{g/mL}$ for 20 h. After treatment, 20 μ L of MTT solution (1 mg/mL in DPBS) was added to each well, followed by incubation for 4 h. The culture medium was subsequently removed, and 200 μ L of dimethyl sulfoxide

(DMSO) was added to dissolve the resulting formazan crystals. Absorbance was measured at 570 nm using a microplate reader. Cell viability was expressed as a percentage relative to the untreated control.

Measurement of intracellular ROS

Intracellular ROS levels were measured using CM-H2DCFDA (Invitrogen, Carlsbad, CA, USA), a cell-permeable fluorogenic probe. EA.hy926 cells were seeded in 96-well plates at a density of 1×10^4 cells per well and incubated for 24 h. To evaluate the protective effects of the fractions, cells were pretreated with the indicated concentrations of the samples and then stimulated with angiotensin II (Ang II, 1 μ M) for 30 min to induce ROS production (Griendling et al., 1994). After stimulation, the cells were washed with Dulbecco's phosphate-buffered saline (DPBS) and incubated with 20 μ M CM-H2DCFDA for 30 min in the dark. The cells were washed three times with DPBS, and fluorescence intensity was measured using a microplate reader at excitation and emission wavelengths of 492 and 522 nm, respectively. Relative ROS levels were expressed as a percentage of the untreated control.

Western blot analysis

eNOS and phospho-eNOS expression

EA.hy926 cells were seeded in 60-mm culture dishes at a density of 2.5×10^5 cells per dish and grown to approximately 90% confluence. To evaluate eNOS phosphorylation, the cells were incubated under serum-free conditions for 2 h in the presence or absence of the samples.

Inflammatory marker expression (ICAM-1 and VCAM-1)

To establish an endothelial inflammation model, EA.hy926 cells were pretreated with the samples for 1 h and subsequently stimulated with TNF- α (10 ng/mL) for 6 h (Han et al., 2017). The expression levels of ICAM-1 and VCAM-1 were then analyzed to evaluate the anti-inflammatory effects of the samples.

Western blot

After treatment, cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in RIPA buffer containing protease and phosphatase inhibitors. The lysates were centrifuged at $15,000 \times g$ for 15 min at 4 °C, and protein concentrations were determined using the bicinchoninic acid (BCA) assay with bovine serum albumin as the standard. Equal amounts of protein (30 μ g) were separated on 10% SDS-polyacrylamide gels and subsequently transferred to

polyvinylidene difluoride membranes (Invitrogen, Waltham, MA, USA). The membranes were blocked with 5% bovine serum albumin in TBST for 1 h and then incubated overnight at 4 °C with primary antibodies against eNOS (1:1000; Cell Signaling Technology, Danvers, MA, USA), phospho-eNOS (Ser1177, 1:1000), ICAM-1 (1:1000), VCAM-1 (1:1000), and β -actin (1:2000; Santa Cruz Biotechnology, Dallas, TX, USA). After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000) for 2 h at room temperature. Immunoreactive bands were visualized using enhanced chemiluminescence reagents (Bio-Rad, Hercules, CA, USA) and detected using an ImageQuant 800 system (Cytiva, Marlborough, MA, USA). Band intensities were quantified by densitometric analysis using ImageQuant TL software (version 10.2).

LC-MS/MS analysis

The F2 fraction was analyzed using an ultra-high-performance liquid chromatography system coupled with Orbitrap tandem mass spectrometry (UHPLC-Orbitrap MS/MS; Q Exactive Plus, Thermo Fisher Scientific, Waltham, MA, USA). Chromatographic separation was performed on a Hypersil GOLD C18 column (2.1×100 mm, 1.9 μ m) maintained at 36 °C. The mobile phases consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B), delivered at a flow rate of 0.3 mL/min. The gradient program was as follows: 0–5 min, 5% B; 5–18 min, 5–100% B; and 18–21 min, 100% B. Mass spectrometric detection was performed in negative ion mode with a spray voltage of 3.5 kV and a capillary temperature of 320 °C. Full-scan mass spectra were acquired over an m/z range of 100–1,000 at a resolution of 70,000. MS/MS fragmentation was performed using a normalized collision energy of 30%.

Data were processed using Compound Discoverer software (version 3.3, Thermo Fisher Scientific). The data processing parameters included a retention time window of 0–20 min, mass range of 100–1500 Da, mass tolerance of 5 ppm, signal-to-noise ratio > 3, and a minimum peak intensity of 1×10^5 ions.

Metabolite identification

Untargeted metabolite profiling was performed using Compound Discoverer software (version 3.3) integrated with the mzCloud database. Metabolites were annotated based on accurate mass measurements and MS/MS fragmentation patterns through comparison with reference databases, including mzCloud, MassBank of North America (MoNA), FoodB, PubChem, and the Human Metabolome Database (HMDB).

Statistical analysis

All experimental results are expressed as mean $\pm$ standard deviation (SD) from three independent replicates ($n=3$), except for the LC–MS/MS metabolomic analysis, which was conducted with six replicates ($n=6$). Statistical analyses were performed using SPSS version 12.0 (Statistical Package for the Social Sciences; IBM SPSS Statistics, Chicago, IL, USA). Differences among groups were evaluated by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. A value of $p < 0.05$ was considered statistically significant. IC_{50} values were calculated using probit analysis. Graphs and data visualizations were generated using GraphPad Prism version 9.4 (GraphPad Software Inc., San Diego, CA, USA).

Results and discussion

Extraction yield and fractionation of *A. pseudoglehnii*

The leaves of *A. pseudoglehnii* were extracted with 70% ethanol using probe-assisted sonication. The extraction yield was calculated based on the dry weight of the freeze-dried plant powder and expressed as the mean $\pm$ standard deviation (SD). The ethanol extraction yielded $26.93 \pm 0.61\%$. The crude ethanol extract was subsequently separated into two fractions (Fraction 1 and Fraction 2) by preparative liquid chromatography (Prep-LC) according to their UV

chromatographic profiles. The UV intensity patterns of each fraction are presented in Fig. 1. Fraction 1 (retention time: 0–7 min) exhibited a yellow color, whereas Fraction 2 (retention time: 7–15 min) appeared nearly colorless, indicating differences in chemical composition and polarity between the fractions. The yield of F1 was $55.45 \pm 2.99\%$, whereas F2 accounted for $9.74 \pm 1.25\%$ of the crude extract.

Phenolic enrichment and antioxidant capacity

As shown in Table 1, F2 exhibited significantly higher TPC (169.78 ± 2.93 mg GAE/g) and TFC (306.80 ± 79.36 mg QE/g) than F1 (73.71 ± 1.92 mg GAE/g and 24.87 ± 21.02 mg QE/g, respectively). The substantial increases in both TPC and TFC in F2 indicate that preparative liquid chromatography effectively concentrated phenolic and flavonoid constituents in this fraction. Considering that phenolic compounds are major contributors to the antioxidant potential of plant extracts, this enrichment suggests a direct relationship between fractionation and bioactivity.

Consistent with its higher phenolic composition, F2 demonstrated significantly stronger antioxidant capacity. The DPPH radical-scavenging assay revealed a markedly lower IC_{50} value for F2 (0.04 ± 0.01 mg/mL) than for F1 (0.30 ± 0.02 mg/mL), indicating enhanced electron-donating ability. Furthermore, the ORAC assay, which reflects hydrogen atom transfer (HAT)-based antioxidant mechanisms, showed that F2 exhibited a significantly higher value (2046.13 ± 237.59 μ mol TE/g) than F1 (774.05 ± 48.64 μ mol TE/g). The consistency between the DPPH and ORAC

Fig. 1 Preparative chromatographic fractionation of *A. pseudoglehnii* ethanolic extract. The crude extract was separated by preparative liquid chromatography and monitored at 240, 280, and 320 nm using a diode array detector (DAD). The chromatograms represent UV absorbance profiles recorded at each wavelength. Fractions F1 (0–7 min) and F2 (7–15 min) were collected based on peak resolution and absorbance intensity. The distinct retention times and UV spectral patterns indicate compositional differences between the two fractions

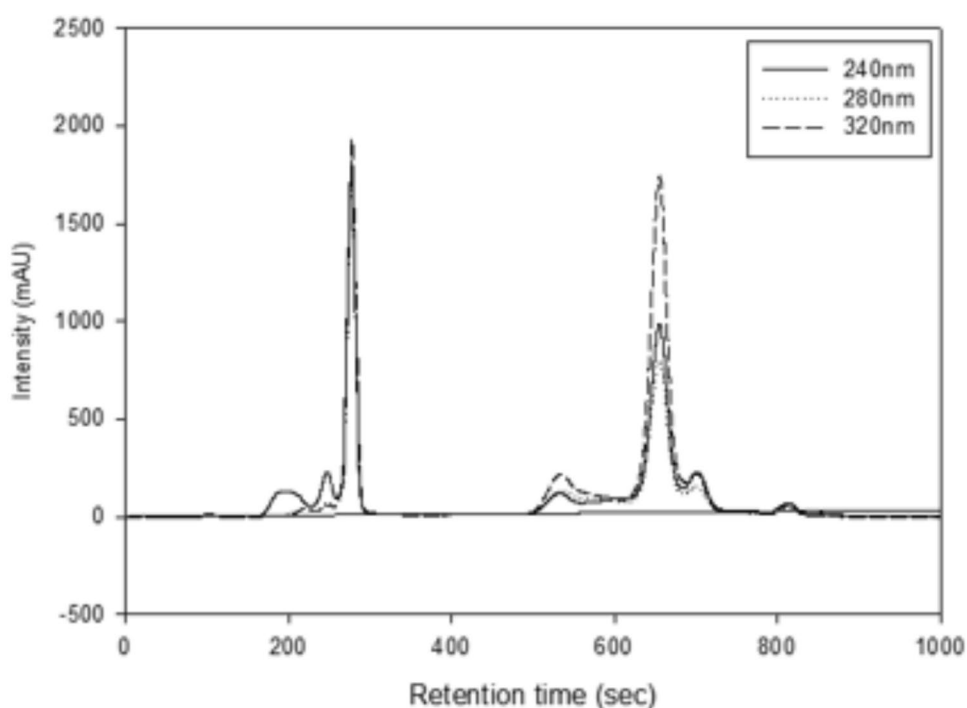


Table 1 Phenolic enrichment and antioxidant capacity of F1 and F2 fractions from *A. pseudoglehnii*

Sample	TPC (mg GAE/g of dry weight of sample)	TFC (mg QE/g of dry weight of sample)	DPPH free radical scavenging activity IC ₅₀ (mg/mL)	ORAC (μmol TE/g dry weight of sample)
F1	73.71 ± 1.92 ^b	24.87 ± 21.02 ^b	0.3 ± 0.02 ^a	774.05 ± 48.64 ^b
F2	169.78 ± 2.93 ^a	306.8 ± 79.36 ^a	0.04 ± 0.01 ^b	2046.13 ± 237.59 ^a

TPC and TFC are expressed as mg GAE and mg QE per g dry weight of sample, respectively. DPPH radical scavenging activity is presented as IC₅₀ (mg/mL), and ORAC values are expressed as μM Trolox equivalents (TE)/g dry weight of sample. Data are presented as mean ± SD (*n* = 3). Different superscript letters within the same column indicate significant differences (*p* < 0.05)

results suggests that F2 possesses broad-spectrum radical-scavenging activity through multiple antioxidant mechanisms.

Metabolomic profiling identified an enrichment of caffeoylquinic acid derivatives and kaempferol glycosides in F2. Caffeoylquinic acids contain catechol moieties capable of donating hydrogen atoms and stabilizing free radicals through resonance structures (Clifford, 2000; Calderon-Montano et al., 2011; Santana-Galvez et al., 2017). Similarly, kaempferol derivatives possess hydroxyl groups that contribute to redox modulation and metal-chelating properties, thereby enhancing antioxidant defense (de Champlain et al., 2004). Importantly, oxidative stress is a key contributor to endothelial dysfunction and hypertension-associated vascular damage. Excessive ROS reduce NO bioavailability and promote inflammatory signaling cascades. Therefore, the strong antioxidant capacity of F2 may not only reflect chemical radical-scavenging activity *in vitro* but also suggest potential physiological relevance in mitigating oxidative stress-related vascular dysfunction. Taken together, the phenolic enrichment observed in F2 directly correlates with its superior antioxidant performance, supporting the functional significance of its polyphenolic composition.

Evaluation of ACE inhibitory activity

F2 demonstrated markedly stronger ACE inhibitory activity (IC₅₀ = 0.30 ± 0.02 mg/mL) than F1 (IC₅₀ = 5.40 ± 0.19 mg/mL), representing an approximately 18-fold difference in potency. This substantial disparity indicates that the bioactive constituents responsible for ACE inhibition were selectively enriched in F2 during the fractionation process. The lower IC₅₀ value of F2 reflects its greater inhibitory efficiency at relatively low concentrations, suggesting that active compounds were functionally concentrated in this fraction. ACE is a key regulatory enzyme in the renin–angiotensin system (RAS), catalyzing the conversion of angiotensin I to angiotensin II. Angiotensin II is a potent vasoconstrictor that increases vascular resistance and blood pressure while also stimulating oxidative stress, endothelial dysfunction, and pro-inflammatory signaling pathways. Persistent

activation of ACE therefore contributes not only to elevated blood pressure but also to vascular remodeling and increased cardiovascular risk.

Polyphenolic compounds may inhibit ACE through interactions with the active site of the enzyme. Such interactions may stabilize the enzyme–inhibitor complex and reduce catalytic efficiency. Given that metabolomic profiling revealed an enrichment of caffeoylquinic acids and kaempferol glycosides in F2, the enhanced ACE inhibitory activity observed in this fraction is consistent with its polyphenolic composition. In addition, several studies have indicated that polyphenols may exert indirect vasoprotective and potential antihypertensive effects by modulating cellular redox balance and enhancing NO bioavailability, which further influences vascular tone. Therefore, the strong ACE inhibition exhibited by F2, together with its pronounced antioxidant capacity, supports a multifunctional role in endothelial protection and vascular homeostasis. Collectively, these results indicate that phenolic enrichment in F2 contributes not only to enhanced radical-scavenging activity but also to potent ACE inhibition, reinforcing its potential as a bioactive fraction with vasoprotective and potential antihypertensive relevance.

Effects of subfractions on cell viability

Cell viability was assessed using the MTT assay following treatment with F1 at concentrations of 30, 50, 100, and 200 μg/mL for 24 h. Treatment with F1 significantly increased cell viability at all tested concentrations (30–200 μg/mL), with values ranging from 153.56 ± 12.77% to 174.51 ± 17.63% relative to the control group. F2 exhibited a concentration-dependent response. At 30 and 50 μg/mL, cell viability remained above 100% (119.10 ± 6.09% and 106.89 ± 12.80%, respectively). However, cell viability significantly decreased at 100 and 200 μg/mL (70.10 ± 8.32% and 65.57 ± 1.10%, respectively), indicating cytotoxic effects at higher concentrations. Although a gradual decrease in viability was observed with increasing concentrations of F2, cell viability remained above 80% at concentrations up to 50 μg/mL. Based on these results, concentrations ≤ 50 μg/

Table 2 Tentative identification of discriminant metabolites in each group

<i>m/z</i>	RT (min)	Tentative identification	MS/MS fragment			VIP value	P (corr)	Error (ppm)
837.4832	17.545	Unknown	80.9641	225.0071	837.4858	3.65	0.9934	–
515.1196	10.142	Cynarine	191.0555	179.0344	135.0443	2.42	0.9760	0.23
499.1251	10.601	Unknown	191.0555	179.0344	135.0443	2.20	0.9960	–
593.1515	9.957	Kaempferol-3-rutinoside	285.0403	284.0328	255.0299	2.12	0.9961	0.52
361.093	10.457	Unknown	119.0493	93.0335	163.0393	2.01	0.9885	–
839.4988	17.913	Unknown	80.9641	225.0071	839.4988	1.98	0.9924	–
515.1197	10.309	4,5-dicaffeoylquinic acid	173.0449	179.0343	191.0555	1.84	0.9740	0.34
483.2731	15.052	LPG 16:0	255.233	152.9951	78.9579	1.59	0.9760	0.53

Metabolites were tentatively identified using UHPLC–Orbitrap MS/MS analysis. RT indicates retention time. MS/MS fragments represent major product ions detected in the fragmentation spectra. VIP values and *p*(corr) correlation coefficients were obtained from OPLS-DA analysis. Mass error is expressed in parts per million (ppm)

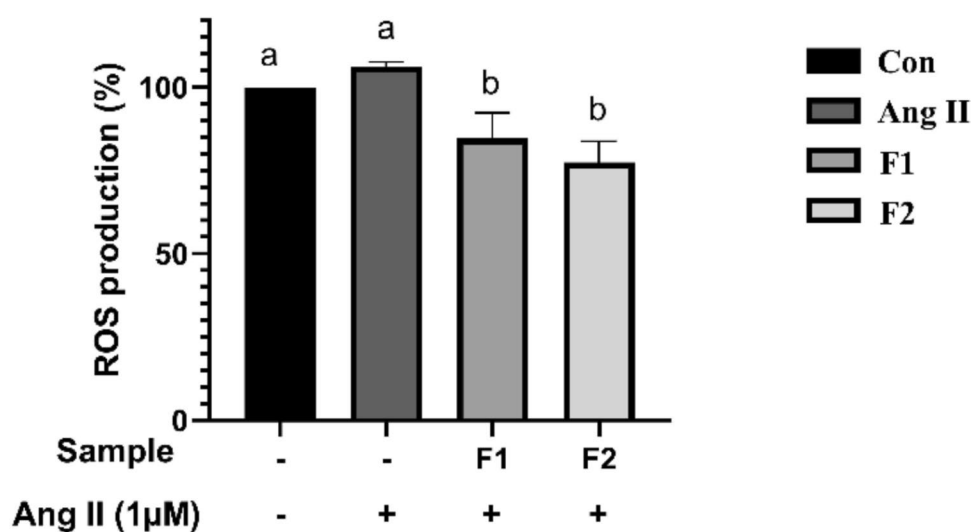


Fig. 2 Effects of *A. pseudoglehnii* subfractions on angiotensin II-induced intracellular ROS production in EA.hy926 endothelial cells. Cells were pretreated with F1 or F2 (50 μg/mL) for 1 h prior to stimulation with angiotensin II (Ang II, 1 μM). Intracellular reactive oxygen species (ROS) levels were measured using the CM-H2DCFDA

fluorescent probe. ROS production is expressed as a percentage of the untreated control group. Data are presented as mean ± SD from three independent experiments (*n* = 3). Different letters indicate statistically significant differences among groups (*p* < 0.05)

mL were considered non-cytotoxic and were therefore selected for subsequent experiments.

Suppression of angiotensin-II induced intracellular ROS production

Intracellular ROS levels were measured in EA.hy926 cells following stimulation with angiotensin II (Ang II, 1 μM). As shown in Fig. 2, Ang II significantly increased ROS production to $106.26 \pm 1.15\%$ compared with the control group (*p* < 0.05), confirming successful induction of oxidative stress. Pretreatment with both fractions (50 μg/mL) attenuated Ang II-induced ROS generation. F1 reduced ROS levels

to $84.65 \pm 6.26\%$, whereas F2 produced a greater reduction to $77.43 \pm 5.26\%$. These findings indicate that F2 more effectively suppressed Ang II-induced oxidative stress than F1. Ang II is known to stimulate NADPH oxidase-dependent ROS generation, which decreases NO bioavailability and contributes to endothelial dysfunction (Griendling et al., 1994). The stronger inhibitory effect observed with F2 suggests more effective modulation of oxidative signaling pathways, likely associated with its higher phenolic content and greater antioxidant capacity.

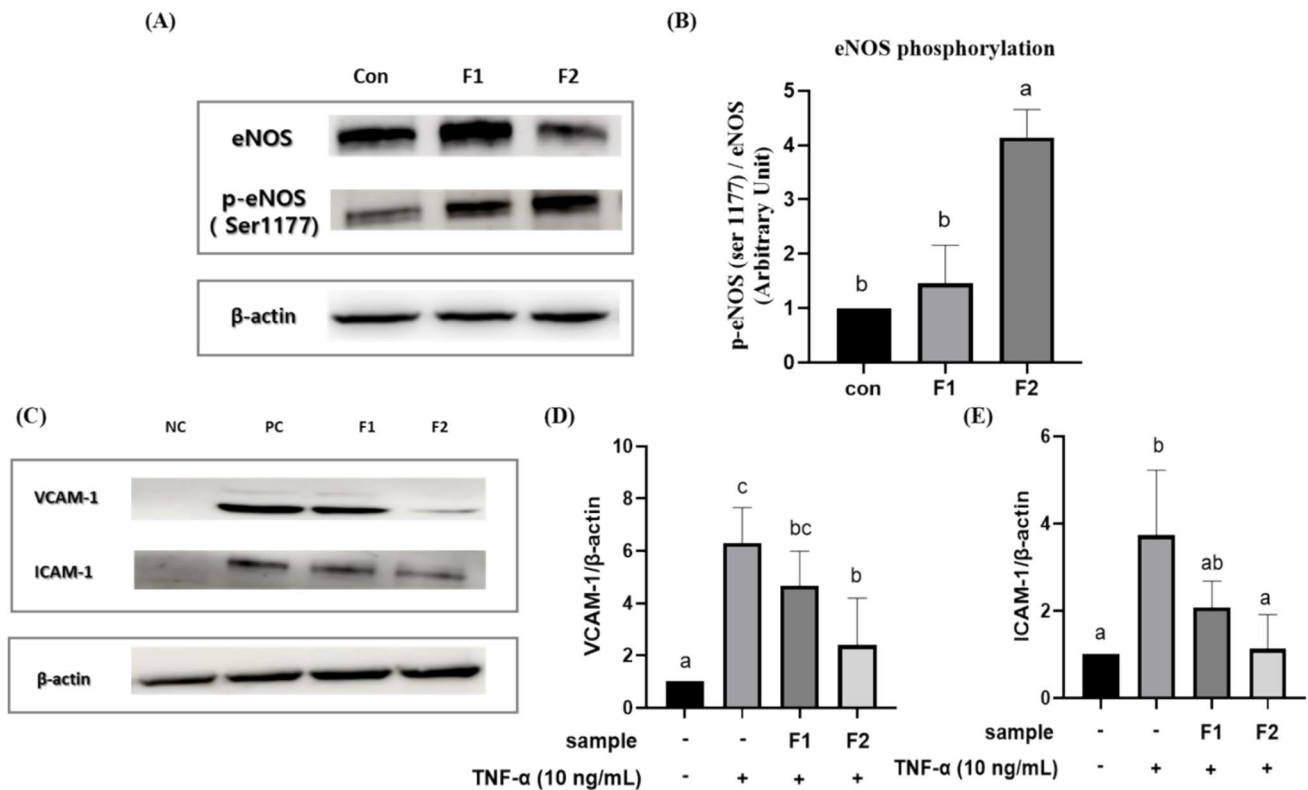


Fig. 3 Effects of *Aster pseudoglehnii* subfractions on endothelial function and inflammatory marker expression in EA.hy926 cells. EA.hy926 cells were serum-starved and treated with F1 or F2 (50 µg/mL) for 2 h. Untreated cells served as the control group. **A** Representative immunoblots of total endothelial nitric oxide synthase (eNOS) and phosphorylated eNOS (Ser1177). **B** Densitometric analysis of the p-eNOS/eNOS ratio. For inflammatory marker analysis, cells were pretreated with F1 or F2 (50 µg/mL) for 1 h and subsequently

stimulated with tumor necrosis factor-α (TNF-α, 10 ng/mL) for 6 h. **C** Representative immunoblots of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). **D** Quantitative analysis of ICAM-1 expression normalized to β-actin. **E** Quantitative analysis of VCAM-1 expression normalized to β-actin. Data are expressed as mean ± SD ($n=3$). Different letters indicate significant differences among groups ($p<0.05$; one-way ANOVA followed by Duncan's multiple range test).

Effects of subfractions on eNOS phosphorylation in EA.hy926 cells

EA.hy926 cells were treated with F1 or F2 (50 µg/mL) for 2 h, and eNOS phosphorylation at Ser1177 was analyzed by western blotting (Fig. 3A, B). Both fractions significantly increased phospho-eNOS levels compared with the control group. Although total eNOS expression was slightly reduced, the phospho-eNOS/eNOS ratio was significantly elevated in both treatment groups, with F2 producing a greater increase than F1. Phosphorylation at Ser1177 enhances eNOS enzymatic activity and NO production, thereby improving endothelial function (Fleming, 2010). The stronger activation observed with F2 may be attributed to its higher content of polyphenolic constituents, such as kaempferol derivatives, which are known to stimulate the PI3K/Akt/eNOS signaling pathway.

Suppression of adhesion molecule expression during vascular endothelial inflammation

Endothelial inflammation is characterized by the upregulation of adhesion molecules, including ICAM-1 and VCAM-1, which is primarily mediated through activation of NF-κB (Libby et al., 2002). To evaluate the anti-inflammatory effects of the subfractions, ICAM-1 and VCAM-1 expression levels were measured in TNF-α-stimulated EA.hy926 cells. Cells were pretreated with F1 or F2 (50 µg/mL) for 1 h prior to stimulation with TNF-α (10 ng/mL) for 6 h. TNF-α markedly increased ICAM-1 and VCAM-1 protein expression compared with the untreated control (Fig. 3C), confirming the successful induction of endothelial inflammation. Pretreatment with both fractions attenuated TNF-α-induced adhesion molecule expression, with F2 producing a significantly greater reduction than F1. ICAM-1 and VCAM-1 play

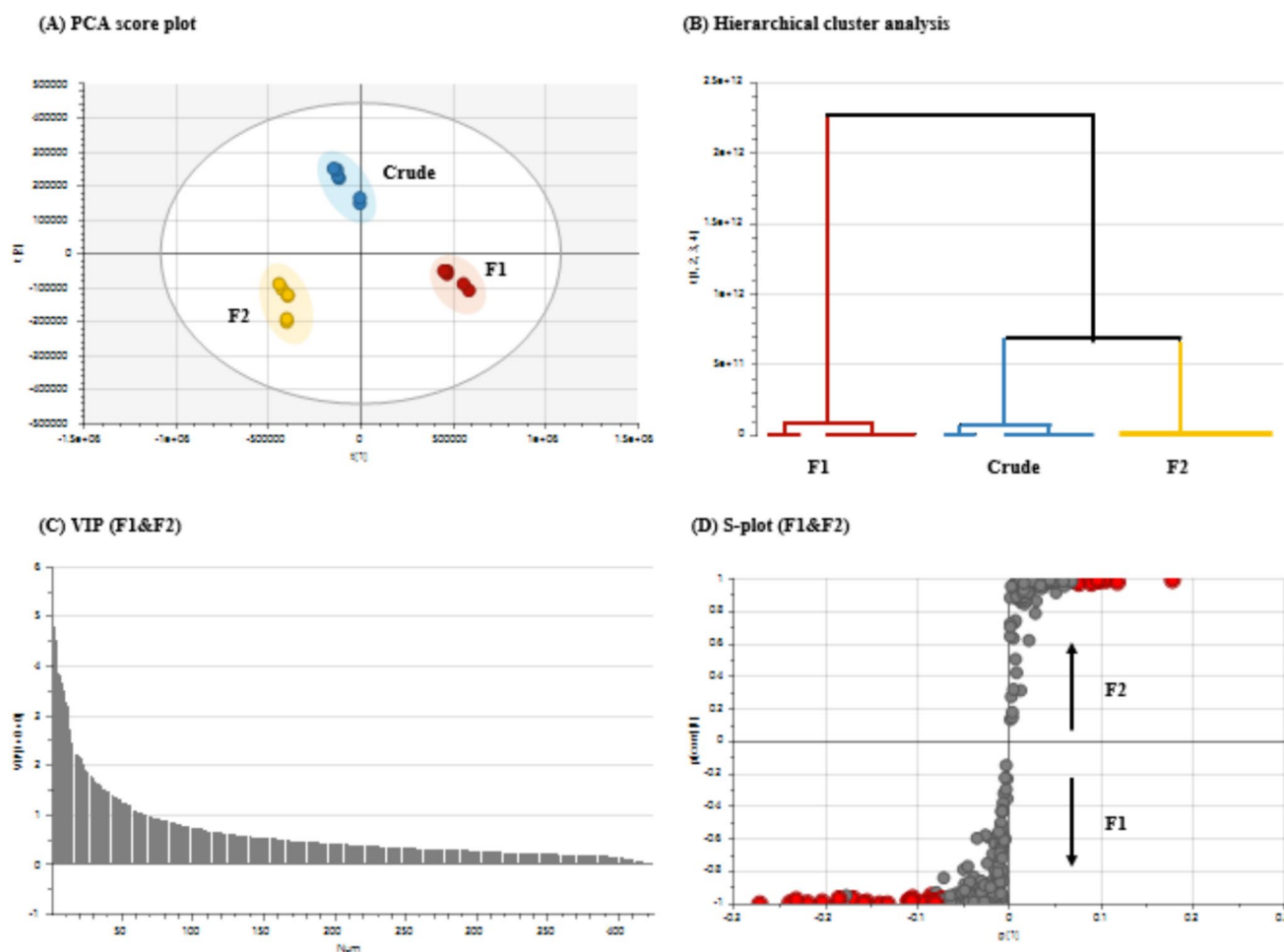


Fig. 4 Multivariate metabolomic analysis of the 70% ethanol extract of *A. pseudoglehnii* and its subfractions. **A** PCA score plot showing clear separation among the crude extract, F1, and F2 ($R^2X[1]=0.804$; $R^2X[2]=0.135$). **B** Hierarchical clustering analysis (HCA) den-

drogram confirming distinct clustering of the three groups. **C** VIP plot from OPLS-DA identifying eight discriminant metabolites ($VIP \geq 1.5$, $p < 0.05$). **D** S-plot indicating metabolites enriched in F2 (upper quadrant) and F1 (lower quadrant)

critical roles in leukocyte adhesion and the progression of vascular inflammation, and their expression is regulated by redox-sensitive transcriptional pathways, including NF- κ B. Chlorogenic acid derivatives and kaempferol glycosides, which were enriched in F2, have been reported to inhibit NF- κ B activation and suppress adhesion molecule expression in endothelial cells (Chang et al., 2010; Chen et al., 2004). Therefore, the stronger inhibitory effect of F2 on ICAM-1 and VCAM-1 expression may be attributed to modulation of redox-sensitive inflammatory signaling pathways.

Metabolomic analysis by UHPLC–Orbitrap MS/MS and multivariate statistical analysis

Untargeted UHPLC–Orbitrap MS/MS analysis performed in negative ion mode detected 424 metabolite features from the 70% ethanol extract of *A. pseudoglehnii* and its subfractions (F1 and F2). Principal component analysis

(PCA) revealed clear separation among the crude extract, F1, and F2 (Fig. 4A), with PC1 and PC2 explaining 80.4% ($R^2X[1]=0.804$) and 13.5% ($R^2X[2]=0.135$) of the total variance, respectively. F2 was distinctly separated from both the crude extract and F1 along the PC1 axis, indicating substantial compositional differences among the fractions. Hierarchical clustering analysis further supported this separation, demonstrating strong similarity within groups (Fig. 4B). To identify metabolites responsible for the discrimination between F1 and F2, orthogonal partial least squares discriminant analysis (OPLS-DA) was performed. Eight discriminant metabolites were selected based on variable importance in projection (VIP) values > 1.5 and $p < 0.05$. In the S-plot (Fig. 4D), these metabolites were positioned at the extremities of the covariance and correlation axes, indicating a strong contribution to group separation. Metabolites located in the upper quadrant were enriched in F2, whereas those in the lower quadrant were relatively enriched in F1. Tentative

metabolite identification based on accurate mass measurements, retention time, MS/MS fragmentation patterns, and database matching (Table 2) revealed that phenolic acids, flavonoid glycosides, and lipid-related compounds were the predominant classes among the selected metabolites. Notably, 4,5-dicaffeoylquinic acid, cynarine, kaempferol-3-O-rutinoside, and lysophosphatidylglycerol (LPG 16:0) were preferentially enriched in F2. Caffeoylquinic acid derivatives contain catechol moieties capable of scavenging and reactive oxygen species, thereby attenuating oxidative stress-associated endothelial dysfunction and preserving nitric oxide (NO) bioavailability (Santana-Galvez et al., 2017). Polyphenols have also been reported to modulate redox-sensitive signaling pathways, including Nrf2/HO-1 and NF- κ B pathways, which are closely associated with endothelial homeostasis and vascular inflammatory responses.

In particular, kaempferol glycosides exhibit antioxidant and endothelial-supportive activities through suppression of NF- κ B-mediated inflammatory signaling and enhancement of antioxidant defense systems (Calderon-Montano et al., 2011). In addition, dietary polyphenols have been shown to regulate endothelial nitric oxide synthase (eNOS) activity and NO production through intracellular signaling pathways associated with vascular tone regulation (Serreli and Deiana, 2023). These biochemical properties provide a plausible mechanistic basis for the enhanced antioxidant capacity and ACE inhibitory activity observed in F2. In addition, polyphenols have been reported to interact with the catalytic site of ACE and modulate NO-related pathways (Fleming, 2010; Guerrero et al., 2012), which is consistent with the functional data obtained in this study. Although LPG 16:0 is not typically classified as a classical antioxidant, lysophospholipids have been reported to influence membrane organization and intracellular signaling cascades, potentially affecting endothelial responses.

In summary, the metabolomic profile of F2 supports its potential antihypertensive and vasoprotective properties through coordinated modulation of oxidative stress, ACE activity, NO production, and inflammatory marker expression. These findings suggest that the phenolic enrichment of F2 contributes not only to enhanced antioxidant capacity but also to ACE inhibition and endothelial protection. Nevertheless, further in vivo and pharmacokinetic studies are necessary to confirm its systemic efficacy and bioavailability.

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Declarations

Conflict of interest The authors declare that they have no competing interests.

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