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Identification of compounds using HPLC-QTOF-MS online antioxidant activity mapping from aerial parts of *Ligularia stenocephala*

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Abstract

Inflammation, diabetes, and even malignancies are pharmacological effects connected by antioxidant capacity and free radicals. Many antioxidants scavenge free radicals originating from dietary sources such as fruits, vegetables, and teas. To identify the bioactive components of *Ligularia stenocephala*, an effective method combining HPLC-QTOF-MS and bioactivity evaluation was investigated for the first time. Antioxidant agents were isolated from *L. stenocephala*, a folk medicine used for edema and scrofula in Korea, Japan, and China. The phytochemical investigation of the aerial parts of *L. stenocephala* resulted in the separation and determination of six compounds (**1**–**6**). In particular, the chemical structures were identified as hyperoside (**1**), 3,5-dicaffeoylquinic acid (**2**), 3,5-dicaffeoylquinic acid methyl ester (**3**), trifolin (**4**), rutin (**5**), and 3,4-dicaffeoylquinic acid (**6**). Their structures were identified using 1D and 2D NMR spectroscopy and high-resolution electrospray ionization mass spectrometry (HR-ESI-MS) data analysis. The results showed that phenolic components were responsible for the antioxidant inhibitory activity of *L. stenocephala*. Additionally, to understand the mechanisms of the antioxidant inhibitory activity of *L. stenocephala*, a docking simulation study was performed to support the in vitro results. Taken together, this new method is rapid, inexpensive, and can be applied to identify the active components of medicinal herbs without separation.

Keywords *Ligularia stenoceohala*, Asteraceae, Bioactive compound, Active component, Folk medicine

Introduction

Natural bioactive compounds with antioxidant capacity have a positive impact on health [1]. Previous reports have revealed that antioxidants reduce the risk of chronic diseases, including cardiovascular, gut, inflammation, heart diseases, and even cancer [2, 3]. Several bioactive compounds with antioxidant capacity have been discovered, including triterpenoids, saponins, anthocyanins, xanthones, and flavonoids [4]. These substances function as reactive oxygen species (ROS) detoxifiers, hydrogen donors, electron donors, and metal-chelating agents that can reduce ROS-induced damage [5]. The capture of free radicals is often the cause of antioxidant action [6]. Oxygen radicals are extremely hazardous and have been linked to several illnesses, including aging and cancer [7]. DNA damage and lipid peroxidation can cause several degenerative alterations [8]. Thus, continuing studies on bioactive compounds with antioxidant capacity from medicinal plants are necessary for the key benefit of health as a low-cost and safe alternative.

The discovery of bioactive chemicals from complex natural products using conventional techniques such as

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bioassay-guided isolation and structure elucidation is time-consuming and labor-intensive, and it is exceedingly challenging to locate trace components [9]. As a result, practical substitutes have been created, with LC–MS being one of the most notable. Compared to conventional HPLC, new LC technologies, such as HPLC and two-dimensional liquid chromatography, significantly improve the separation power and reduce the analysis time. Advanced MS techniques such as Q-TOF provide accurate mass measurements, enabling reliable identification, even in the absence of reference standards [10]. The application of these methods demonstrated the superiority of rapid compound identification with good accuracy.

With over 27 species utilized as folk medicines, the genus *Ligularia* has been taxonomically assigned to Compositae (*tribe Senecioneae*) [11]. Numerous secondary metabolites from this genus with notable pharmacological properties have been identified as a consequence of comprehensive and thorough phytochemical investigations of *Ligularia* species [11]. Some *Ligularia* species are used in traditional medicine. *Ligularia stenocephala* (Maxim.) Matsum. et Koidz. (Compositae) are widely distributed in Korea, China, and Japan. Whole plants have been used to treat edema and scrofula following Chinese folk medicine. Previous chemical studies have revealed that phenolics, triterpenoids, and benzofuran derivatives are the major constituents [12–14]. The extract and secondary metabolites of this plant have diverse pharmacological properties, such as antiplatelet aggregation, anticoagulation, cytotoxicity, anti-ulcerogenic, and antioxidant effects [15, 16]. Indeed, the water extracts of *L. stenocephala* suppress the formation of nitric oxide by down-regulating the inducible nitric oxide synthase and pro-inflammatory cytokines (eg TNF-α, interleukin (IL)-6, IL-10 and IL-1β expression) through the suppression of NF-κβ activation and mitogen-activated protein kinases phosphorylation in lipopolysaccharide-stimulated macrophage cells [17]. Additionally, benzofuran derivatives from the roots of *L. stenocephala* was found to exhibit potent cytotoxicity against HL-60 (human leukemia cells), Bel-7402 (human hepatoma cells) and HO-8910 (human ovarian neoplasm cells) [18]. As part of our ongoing examination of the pharmacological properties of Korean medicinal herbs [19, 20], we report the chemical profile of *L. stenocephala*. LC-QTOF MS/MS combined with bioassay-guided analysis was also used to identify the active components of *L. stenocephala*. To the best of our knowledge, this is the first study to successfully identify the active components responsible for the antioxidant activities of *L. stenocephala*.

Material and methods

General experimental procedures

1D and 2D NMR experiments were performed using a Bruker 600 MHz spectrometer (Bruker, Billerica, MA, USA). Open column chromatography (CC) was performed using Merck silica gel, 63–200 μM) and YMC RP-18 resins (30–50 μm, Fuji Silysia Chemical Ltd., Kasugai, Aichi, Japan). Thin layer chromatography (TLC) using YMC RP-18 resins was carried out using pre-coated silica gel 60 F_{254} and RP-18 F_{254} (0.30 mm, Merck, Darmstadt, Germany).

LC-QTOF-MS conditions

LC-QTOF-MS combined with a bioassay-guided method was performed as previously reported, with slight modifications [10, 21]. Briefly, using LC-QTOF-MS analysis, the first party provided the chemical composition of the sample. The eluent was collected in the second stage, using a 96-well plate, for 30 s. HPLC analysis was performed using an Agilent 126 series equipment on a C18 column (150 × 4.6 mm, Shiseido CapCell PAK, 5 μm). The mobile phase contained 0.1% formic acid (v/v) (A) in deionized water (solvent A) and acetonitrile (solvent B) with a linear gradient elution: 5% B (0–5 min) and 5–95% B (5–30 min). A UV chromatogram was obtained at 254 nm, with a flow rate of 0.6 mL/min. An Agilent 6530 Q-TOF mass spectrometer (Agilent, Santa Clara, CA, USA) was linked to the HPLC system in negative mode. Fragment ions in the range m/z 50–1700 were detected.

Plant material

The aerial parts of *L. stenocephala* were purchased from Seondahyang Corporation in Gyeongju, Gyeongsangbuk-do, Korea in 2017. The sample was authenticated and identified by Prof. Ki Yong Lee of Korea University. The voucher specimen (KUP-HD106) was stored in the Herbarium of Natural Product Laboratory, College of Pharmacy, Korea University.

Extraction and isolation

The dried aerial parts of *L. stenocephala* (1.1 kg) were extracted three times with 80% aqueous methanol (MeOH) (5.0 L) by sonication for 8 h. The methanol extract was concentrated under reduced pressure to yield the residue (307.01 g). The MeOH extract was suspended in water and successively partitioned with *n*-hexane, EtOAc, and BuOH to obtain *n*-hexane (28.24 g), EtOAc (16.44 g), BuOH (38.1 g), and water (W), respectively.

The EtOAc fraction was separated by column chromatography (CC) using a gradient concentration of

n-hexane–EtOAc (100:1, 50:1, 20:1, 10:1, 5:1, v/v) to obtain nine fractions (fractions – 1 to – 9). Fraction E–7 (2 g) was isolated by YMC RP-C_{18} CC using MeOH-H_2O (1:5, v/v) as the eluent, further purified by Sephadex® LH-20 CC, and eluted with MeOH-H_2O (3:1, v/v) to afford (compound **2**, 9.6 mg), and (compound **3**, 3.6 mg). Finally, fraction E–8 (1.3 g) was separated over silica gel CC and eluted with chloroform-MeOH–water (25:4:1, 10:5:1, v/v/v) to obtain (compound **6**, 4.8 mg), (compound **4**, 3.4 mg), (compound **5,** 60.8 mg), and (compound **1**, 200.5 mg).

Physical and spectroscopic data of active compounds

*Compound **1*** Yellow powder. $C_{21}H_{20}O_{12}$. HR-ESI-MS *m/z* 463.0882 $[M-H]^-$ (calcd. 463.0882); ^{1}H-NMR (600 MHz, CD_3OD) δ_H: 7.85 (1H, d, *J*=2.2 Hz, H-2′), 7.60 (1H, dd, *J*=8.5, 2.2 Hz, H-6′), 6.80 (1H, d, *J*=8.5 Hz, H-5′), 6.41 (1H, d, *J*=1.8 Hz, H-8), 6.21 (1H, d, *J*=2.1 Hz, H-6), 5.18 (1H, d, *J*=7.8 Hz, H-1″); ^{13}C-NMR (150 MHz, CD_3OD) δ_C:179.7 (C-4), 166.2 (C-7), 163.1 (C-5), 158.6 (C-2), 150.1 (C-4′), 145.8 (C-3′), 135.9 (C-3), 123.0 (C-6′), 123.0 (C-1′), 117.9 (C-2′), 116.2 (C-5′), 105.5 (C-1″), 123.0 (C-6), 94.8 (C-8), 77.3 (C-5″), 75.2 (C-3″), 73.3 (C-2″), 70.1 (C-4″), 62.0 (C-6″).

*Compound **2*** White amorphous powder. $C_{25}H_{24}O_{12}$. HR-ESI–MS *m/z* 515.1196 $[M-H]^-$ (calcd. 515.1195). ^{1}H-NMR (600 MHz, CD_3OD) δ_H: 2.02–2.35 (4H, m, 2H-2 and 2H-6), 4.00 (1H, dd, *J*=7.2, 3.0 Hz, H-4), 5.46 (1H, m, H-3), 5.42 (1H, dd, *J*=12.0, 7.2 Hz, H-5), 6.40 (1H, d, *J*=16.2 Hz, H-8″), 6.30 (1H, d, *J*=15.6 Hz, H-8′), 6.81 (2H, d, *J*=7.8 Hz, H-5′ and H-5″), 7.00 (2H, dd, *J*=7.8, 1.8 Hz, H-6′ and H-6″), 7.19 (2H, d, *J*=2.4 Hz) H-2′ and H-2″), 7.65 (1H, d, *J*=15.6 Hz, H-7′), 7.61 (1H, d, *J*=16.2 Hz, H-7″); ^{13}C-NMR (150 MHz, CD_3OD) δ_C: 75.7 (C-1), 36.9 (C-2), 73.5 (C-3), 71.6 (C-4), 73.0 (C-5), 38.6 (C-6), 128.7 (C-1″), 128.9 (C-1′), 116.2 (C-2″), 116.5 (C-2′), 147.7 (C-3″), 147.7 (C-3′), 150.4 (C-4″), 150.5 (C-4′), 117.4 (C-5″), 117.4 (C-5′), 124.0 (C-6′), 123.9 (C-6″), 148.0 (C-7″), 148.2 (C-7′), 116.1 (C-8′), 116.1 (C-8″), 169.3 (C-9″), 169.8 (C-9′), 178.4 (COOH).

*Compound **3*** White amorphous powder. $C_{26}H_{26}O_{12}$. HR-ESI–MS *m/z* 529.1354 $[M-H]^-$ (calculated. 529.1351). ^{1}H-NMR (600 MHz, DMSO) δ_H: 3.60 (3H, s, OCH_3), 3.86 (1H, dd, *J*=6.0 Hz, H-4), 5.16 (1H, m, H-5), 5.19 (1H, m, H-3), 6.15 (1H, d, *J*=18.0 Hz, H-8′), 6.27(1H, d, *J*=12.0, H-8″), 6.79 (2H, dd, *J*=6.0, 12 Hz, H-6′, and H-6″), 7.05 (2H, dd, *J*=6.0 Hz, H-5′ and H-5″), 7.44 (1H, d, *J*=18.0 Hz, H-7′), 7.51 (1H, d, *J*=18.0 Hz, H-7″) ^{13}C-NMR (150 MHz, DMSO) δ_C: 52.4 (OCH_3) 71(C-3), 71.4 (C-1), 70.4 (C-5), 126.1 (C-1″), 125.8 (C-1′), 115.2 (C-2″), 115.1 (C-2), 149.1 (C-4″), 148.2(C-4′), 116.4 (C-5″), 116.3 (C-5′), 121.8 (C-6″), 121.7 (C-6′), 146.1 (C-7″), 146.1 (C-7′), 166.5 (C-9″), 166.0 (C-9′), 174.7 (COO).

*Compound **4*** Yellow powder. $C_{21}H_{20}O_{11}$. HR-ESI–MS *m/z* 447.0992 $[M-H]^-$ (calculated. 447.0992). ^{1}H-NMR (600 MHz, CD_3OD) δ_H: 8.12 (2H, d, *J*=8.8 Hz, H-2′ and H-6'), 6.91 (2H, d, *J*=8.8 Hz, H-3′ and H-5′), 6.44 (1H, d, *J*=2.0 Hz, H-8), 6.24 (1H, d, *J*=2.0 Hz,H-6), 5.17 (1H, d, *J*=7.7 Hz, H-1″); ^{13}C-NMR (150 MHz, CD_3OD) δ:178.3 (C-4), 164.6 (C-7), 161.7 (C-5), 160.2 (C-4′), 157.6 (C-9), 157.1 (C-2), 130.9 (C-6′), 130.9 (C-2′), 114.7 (C-3′), 114.7 (C-5′), 103.5 (C-1″), 98.4 (C-6), 93.3 (C-8), 75.7 (C-3″), 73.6 (C-5″), 71.6 (C-2″), 68.6 (C-4″), 60.8 (C-6″).

*Compound **5*** Yellow powder. $C_{27}H_{30}O_{16}$. HR-ESI–MS *m/z* 609.1463 $[M-H]^-$ (calcd. 609.1461). ^{1}H-NMR (600 MHz, CD_3OD) δ_H: 6.20 (1H, d, *J*=2.0 Hz, H-6), 6.40 (1H, d, *J*=2.0 Hz, H-8), 7.48 (1H, d, *J*=2.0 Hz, H-2′), 6.77 (1H, d, *J*=8.0 Hz, H-5′), 7.56 (1H, dd, *J*=8.0, 2.0 Hz, H-6′), 5.10 (1H, d, *J*=7.5 Hz, H-1″), 4.00 (1H, d, *J*=1.0 Hz, H-1‴), 1.11 (3H, d, *J*=6 Hz, H-6‴). ^{13}C-NMR (150 MHz, CD_3OD) δ_C: 158.7 (C-2), 135.6 (C-3), 179.3 (C-4), 163.0 (C-5), 100.5 (C-6), 167.9 (C-7), 95.3 (C-8), 159.2 (C-9), 105.1 (C-10), 123.1 (C-1′), 116.1 (C-2′), 150.0 (C-3′), 146.0 (C-4′), 117.7 (C-5′), 123.6 (C-6′), 102.5 (C-1″), 75.7 (C-2″), 78.2 (C-3″), 71.3 (C-4″), 77.2 (C-5″), 68.5 (C-6″), 105.1 (C-1‴), 72.1 (C-2‴), 72.1 (C-3‴), 73.9 (C-4‴), 69.7 (C-5‴), 17.9 (C-6‴).

*Compound **6*** Yellow gum; $C_{25}H_{23}O_{12}$. HR-ESI–MS *m/z* 515.1224 $[M-H]^-$ (calculated. 515.1195). ^{1}H-NMR (600 MHz, CD_3OD) δ_H: 8.12 (2H, d, *J*=8.8 Hz, H-2′ and H-6′), 6.91 (2H, d, *J*=8.8 Hz, H-3′ and H-5′), 6.44 (1H, d, *J*=2.0 Hz, H-8), 6.24 (1H, d, *J*=2.0 Hz, H-6), 5.17 (1H, d, *J*=7.7 Hz, H-1″); ^{13}C-NMR (150 MHz, CD_3OD) δ_C: 75.1 (C-1), 40.5 (C-2), 68.7 (C-5), 73.8 (C-4′), 71.0 (C-9), 39.5 (C-2), 169.3 (C-6′), 169.4 (C-2′), 115.7 (C-3′), 148.2 (C-5′), 128.4 (C-1″), 115.9 (C-6), 147.5 (C-8), 150.3 (C-3″), 117.4 (C-5″), 123.8 (C-2″), 176.6 (COOH).

Antioxidant activity assay

Antioxidant activity assays were performed using the diphenylpicrylhydrazine (DPPH) assay, as previously reported with minor modifications [21]. In brief, DPPH exhibits a strong absorption band at 525 nm when it possesses a radical. However, when it undergoes a reaction with an electron donor that supplies hydrogen or electrons, the donor generates either electrons or hydrogen radicals. During this process, the donated electrons irreversibly combine, leading to a gradual fading of the deep purple color and a decrease in absorbance. The measurement of antioxidant activity involves assessing the radical scavenging ability by monitoring the decrease in absorbance as the color of the reaction solution transitions from purple to yellow. The DPPH solution was prepared by dissolving 300 µL of the sample obtained by LC–MS in ethanol. A total of 190 µL of 15 µL DPPH solution in ethanol was added to 10 µL of dissolved material.

A spectrophotometer was used to detect absorbance at 517 nm after 30 min of incubation. The absorbance reading provides information about the extent of DPPH radical scavenging by the sample. The inhibition rate (%), which represents inhibitory activity, was determined using the following formula:

$$\text{Inhibition activity (\%)} = 100 - \left[(S - S_0)/(C - C_0)\right] \times 100,$$

where S_0 and C_0 are the absorbances of the control and inhibitor in ethanol without DPPH solution, and C and S are the absorbances of the control and inhibitor after 30 min.

Molecular docking simulation

To examine the binding affinity and interaction of the active substance with a typical antioxidant protein, molecular docking simulations were performed using AutoDock Vina 1.1.2, in accordance with previously published guidelines [22]. On the RCSB Protein Data Bank website, the crystal structure of Drosophila melanogaster carboxypeptidase D isoform 1 B short was downloaded at a resolution of 2.70 Å (PDB ID:3MN8) [23]. The protein was prepared by deleting water molecules, removing initial ligands, repairing any missing residues, and adding polar hydrogen atoms. The 3D structures of active compounds **2** and **6** were constructed using Chem 3D Pro 20.1 after energy minimization (PerkinElmer Infomatics, 2021). The most stable conformer was selected as a ligand for the docking study.

The docked complex with the lowest binding energy was selected to represent the most favorable interaction between the ligand and the protein. The 2D and 3D molecular docking graphics were designed using LiPlot+ 2.2.5 and PyMol 2.5.4 software, respectively.

Results and discussion

The MeOH extract components were further divided into *n*-hexane, ethyl acetate (EtOAc), *n*-butanol (BuOH), and water fractions owing to the significant antioxidant properties of the extract. The EtOAc fraction exhibited the highest antioxidant activity in a dose-dependent manner. Indeed, the EtOAc fraction inhibited DPPH by over 80% (Table 1) at a concentration of 30 µg/mL. L-Ascorbic acid was used as a positive control. Thus, the EtOAc fraction was selected for further studies to identify the antioxidant components of *L. stenocephala*.

For LC-QTOF-MS coupled with bioassay-guidance, the first phase was to obtain the chemical profile of the MeOH extract of *L. stenocephala* (Fig. 1 and Table 2), and the second phase was to collect the eluent through the column for 30 s per well in a 96-well plate [21]. The collected sample was used for LC–MS coupled with a DPPH-determined free radical-scavenging activity assay (Fig. 1). The results of the DPPH free radical-scavenging activity assay showed that the components showed scavenging activity at 16–18 min on the MS chromatogram. Thus, peaks **d**, **e**, and **f** were predicted to be responsible for the antioxidant activity of the aerial parts of *L. stenocephala*.

Table 1 DPPH radical scavenging activity of extract and fractions from *L. stenocephala*

Sample	Concentrations	DPPH radical scavenging activity (%)
L-Ascorbic acid	50 µM	$61.9 \pm 1.3^{**}$
80% MeOH extract	1 µg/mL	1.0 ± 3.2
	5 µg/mL	7.6 ± 31.4
	10 µg/mL	6.1 ± 1.2
	30 µg/mL	$27.6 \pm 1.1^{*}$
n-Hexane fraction	1 µg/mL	3.3 ± 1.8
	5 µg/mL	5.2 ± 0.2
	10 µg/mL	6.4 ± 1.7
	30 µg/mL	13.3 ± 0.6
EtOAc fraction	1 µg/mL	
	5 µg/mL	$7.4 \pm 0.2^{*}$
	10 µg/mL	$22.8 \pm 1.1^{**}$
	30 µg/mL	$35.9 \pm 1.7^{*}$
n-BuOH fraction	1 µg/mL	4.8 ± 0.3
	5 µg/mL	11.4 ± 1.2
	10 µg/mL	$20.7 \pm 0.4^{**}$
	30 µg/mL	$50.6 \pm 0.9^{***}$
Water fraction	1 µg/mL	3.1 ± 0.1
	5 µg/mL	5.2 ± 0.7
	10 µg/mL	4.2 ± 0.6
	30 µg/mL	$19.7 \pm 1.0^{**}$

The experiment was repeated three times

$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to control. L-Ascorbic acid was used as the positive control

The target compounds were isolated from the *L. stenocephala* MeOH extract to confirm the LC–MS paired with a bioassay-guided method. Using liquid–liquid separation, the crude MeOH extract was divided into layers of *n*-hexane, EtOAc, n-BuOH, and water. Six compounds (**1**–**6**) were extracted from the EtOAc layer by column chromatography over silica gel and C18-reversed phase silica gel. The structures of the single compounds were established by comparison of their experimental and reported 1D and 2D NMR and HR-ESI–MS spectroscopic analyses. Their structures were identified as hyperoside (**1**), 3,5-dicaffeoylquinic acid (**2**), 3,5-dicaffeoylquinic acid methyl ester (**3**), trifolin (**4**), rutin (**5**), and 3,4-dicaffeoylquinic acid (**6**) (Fig. 2). The physical and spectroscopic data of the isolated

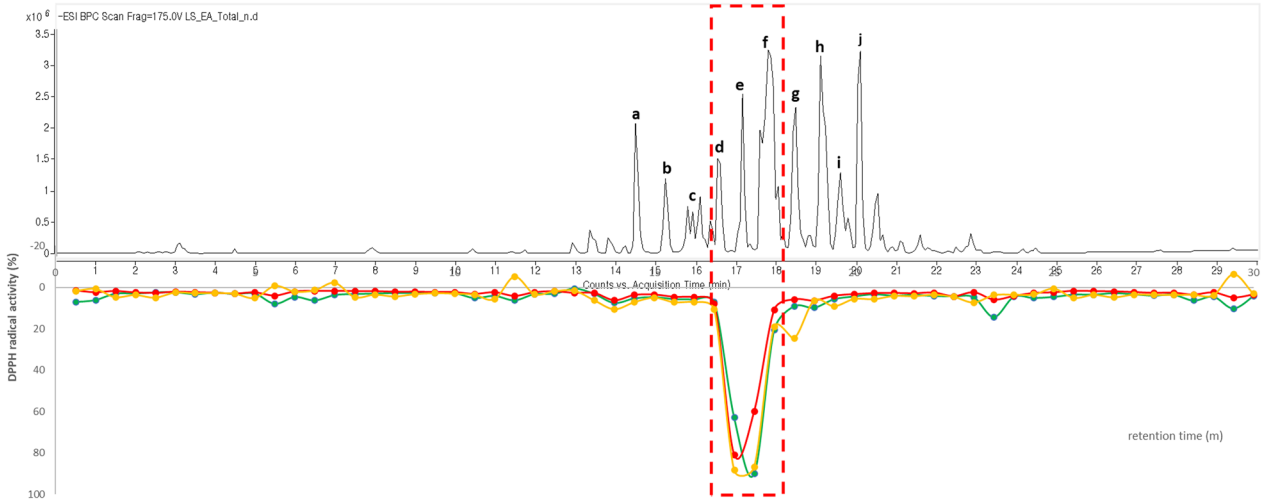

Fig. 1 LC-QTOF-MS coupled with DPPH assay of EtOAc fraction from *L. stenocephala*. MS chromatogram (negative ionization mode); DPPH free radical-scavenging activity of each 30 s and applied directly to DPPH assay. Active components exhibited at the peak around 16–18 min

Table 2 Chemical profile from the aerial parts of *L. stenocephala*

Peaks	Expected compounds	t_R (mins)	Observed *m/z*	Calculated *m/z*	Error *m/z*	Molecular formular $[M-H]^-$	MS/MS fragments (*m/z*)	UV (λ_{max}, nm)	Compd. No.
a	Chlorogenic acid (3-caffeoylquinic acid)	14.471	353.0866	353.0878	0.0012	$C_{16}H_{17}O_9$	191[M-$C_9H_6O_8$-H]$^-$	330	
b	Unidentified	15.220	137.0232	137.0244	0.0012	$C_7H_5O_3$	108[M-29-H]$^-$		
c	Rutin	16.032	609.1452	609.1461	0.0009	$C_{27}H_{29}O_{16}$	300[M-$C_{12}H_{20}O_9$-H]$^-$	265	**5**
d	Quercetin-3-*O*-*β*-D-galactopyranoside	16.532	463.0882	463.0882	0	$C_{21}H_{19}O_{12}$	300[M-$C_6H_{11}O_5$-H]$^-$	265	**1**
e	3,4-Dicaffeoylquinic acid	17.157	515.1190	515.1195	0.0005	$C_{25}H_{23}O_{12}$	353[M-$C_6H_{11}O_5$-H]$^-$	330	**6**
f	3,5-Dicaffeoylquinic acid	17.781	515.1196	515.1195	0.0001	$C_{25}H_{23}O_{12}$	353[M-$C_9H_6O_3$-H]$^-$	245, 330	**2**
g	Unidentified	18.469	203.0918	203.0925	0.0007	$C_9H_{15}O_5$	143[M-60-H]$^-$		
h	3,5-Dicaffeoylquinic acid methyl ester	19.093	529.1354	529.1351	0.0003	$C_{26}H_{25}O_{12}$	367[M-$C_6H_{11}O_5$-H]$^-$	330	**3**
i	Unidentified	19.593	193.0498	193.0506	0.0008	$C_{10}H_9O_4$	133[M-60-H]$^-$		
j	Unidentified	20.093	217.1077	217.1081	0.0004	$C_{10}H_{17}O_5$	157[M-60-H]$^-$		

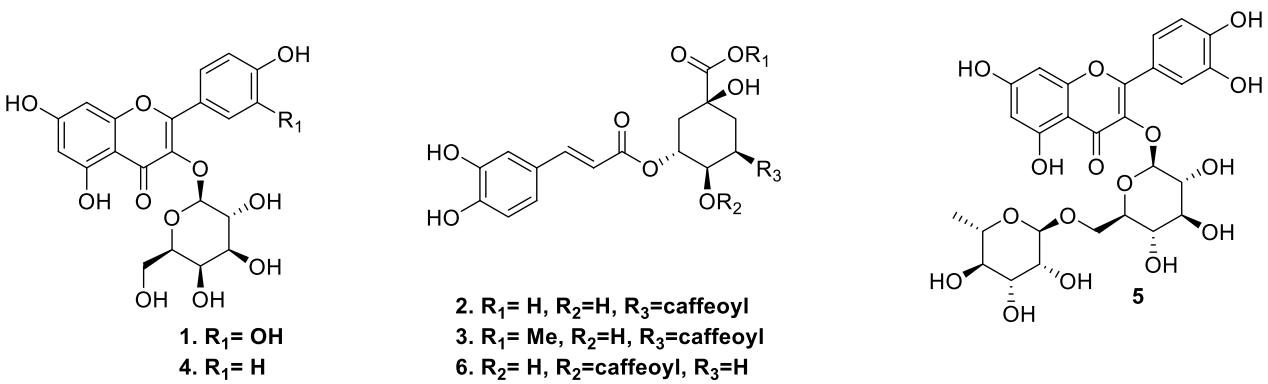

Fig. 2 Structures of isolated metabolites **1**–**6** purified from the MeOH extract of *L. stenocephala*

Table 3 DPPH radical scavenging activity of active components (**1**–**6**) from *L. stenocephala*

Compounds	DPPH radical scavenging activity (%)		
	10 μM	30 μM	50 μM
1	22.6 ± 1.0***	50.7 ± 0.5***	75.0 ± 1.2***
2	23.3 ± 1.0***	63.3 ± 0.7***	89.7 ± 0.2***
3	7.1 ± 4.7	23.7 ± 0.8***	37.0 ± 1.8***
4	8.4 ± 0.2**	24.5 ± 0.1**	40.0 ± 2.8***
5	6.6 ± 2.2	16.3 ± 2.2**	25.4 ± 4.3**
6	38.2 ± 0.7***	67.5 ± 0.9***	90.5 ± 0.5***
L-Ascorbic acid	17.5 ± 0.7**	50.3 ± 0.5***	89.8 ± 2.7**

L-Ascorbic acid was used as the positive control. The experiment was repeated thrice

** $p < 0.01$, *** $p < 0.001$

compounds are reported in the Extraction and Isolation section.

To validate the methods, a DPPH assay was carried out to evaluate target compounds **1**–**6**. As the results (Table 3) exhibited strong activity with IC_{50} values of 29.1 ± 0.1, 21.17 ± 0.2 and 19.5 ± 0.1 μM, respectively, for compounds **1**, **2** and **6**. L-Ascorbic acid (IC_{50} = 30.5 ± 0.1 μM) was used as a positive control. Interestingly, compounds **2** and **6** exhibited the highest activity in the DPPH assay, corresponding to peaks e and f. This peak is observed in Table 1 in line with the in vitro antioxidant activity experiments. Taken together, these results confirmed that LC–MS combined with a bioassay-guided method was appropriate for in vitro experiments. Consequently, this new tool has been used to identify active components in medicinal herbs without separation.

Researchers can characterize small-molecule activity at target protein-binding sites and disclose basic biochemical processes using molecular docking models to depict the atomic-level interaction between a small molecule and a protein [24]. The two main processes in the docking procedure are predicting the ligand shape, location, and orientation at these sites, and determining the binding affinity. The benefits of virtual screening include a small search space, low cost, and high flexibility [22]. These factors can aid in the rapid discovery of a possible target protein inhibitor. Promising possibilities for the discovery and development of novel medications include herbal or phytomedicines originating from conventional herbal medicine systems. To understand the mechanisms of the antioxidant inhibitory activity of *L. stenocephala*, a docking simulation study was performed to support the in vitro results. To examine the binding affinity and interaction of the active substance with a typical antioxidant protein, molecular docking simulations were performed using AutoDock Vina 1.1.2, in accordance with previously published guidelines [22]. On the RCSB Protein Data Bank website, the crystal structure of Drosophila melanogaster carboxypeptidase D isoform 1 B short was downloaded at a resolution of 2.70 Å (PDB ID:3MN8) [23]. From the docking calculations, compounds **2** and **6** had binding energies of − 5.4, and − 6.1 kcal/mol, respectively. Compound **2** exhibited hydrogen bond interactions in the active site with residues ASN 59, ASN 88, GLU 60, and GLU 123 ranging between 2.50 and 3.50 Å, while compound **6** showed hydrogen bonding with ARG 82, GLN 60, GLU 123, and SER 126 ranging from 2.38 to 3.35 Å (Fig. 3). Based on the binding energy, key amino acids, and hydrogen bonds, the results suggest that compounds **2** and **6** isolated from *L. stenocephala* are promising agents as new bioactive compounds with antioxidant capacity.

Natural ingredients are crucial for the development of new drugs. In the past 30 years, the US Food and Drug Administration has authorized approximately 60% of novel small-molecule medications that have been derived from or connected to natural sources [25]. Interestingly, most drugs are oriented toward treating incurable cancers. By reducing the antioxidant capacity of cancer cells, several natural compounds can make cancer cells more susceptible to the oxidative stress caused by chemotherapy and radiation treatment [2]. When the antioxidant defenses of tumors are suppressed, they are less able to counteract oxidative stress, which leads to cell death. Thus, the continued development of bioactive compounds with antioxidant capacity from natural products is an initial step in finding new drugs.

As part of our recent studies on the chemical components and pharmacological effects of Korean medicinal herbs, we described many bioactive compounds such as alkaloids, phenolics, terpenoids derivatives, and saponins [26–33]. These compounds showed diverse bioactivity properties such as antioxidant, cytotoxicity, and anti-inflammatory effects [34–37].

However, when compared to traditional phytochemical methods that involve time-consuming processes such as extraction, isolation, purification, and identification, this technique offers multiple notable benefits. Our study outlines the creation of an efficient HPLC-QTOF-MS method that can quickly and efficiently detect the active compounds in *L. stenocephala*. Through the findings of this study, we have gained a more comprehensive understanding of the fundamental mechanisms responsible for the antioxidant activity of *L. stenocephala*. Additionally, the phytochemical investigation of *L. stenocephala* resulted in the isolation and identification of six compounds **1**–**6**, respectively. Their structures were identified based on 1D and 2D NMR spectroscopy and HR-ESI–MS

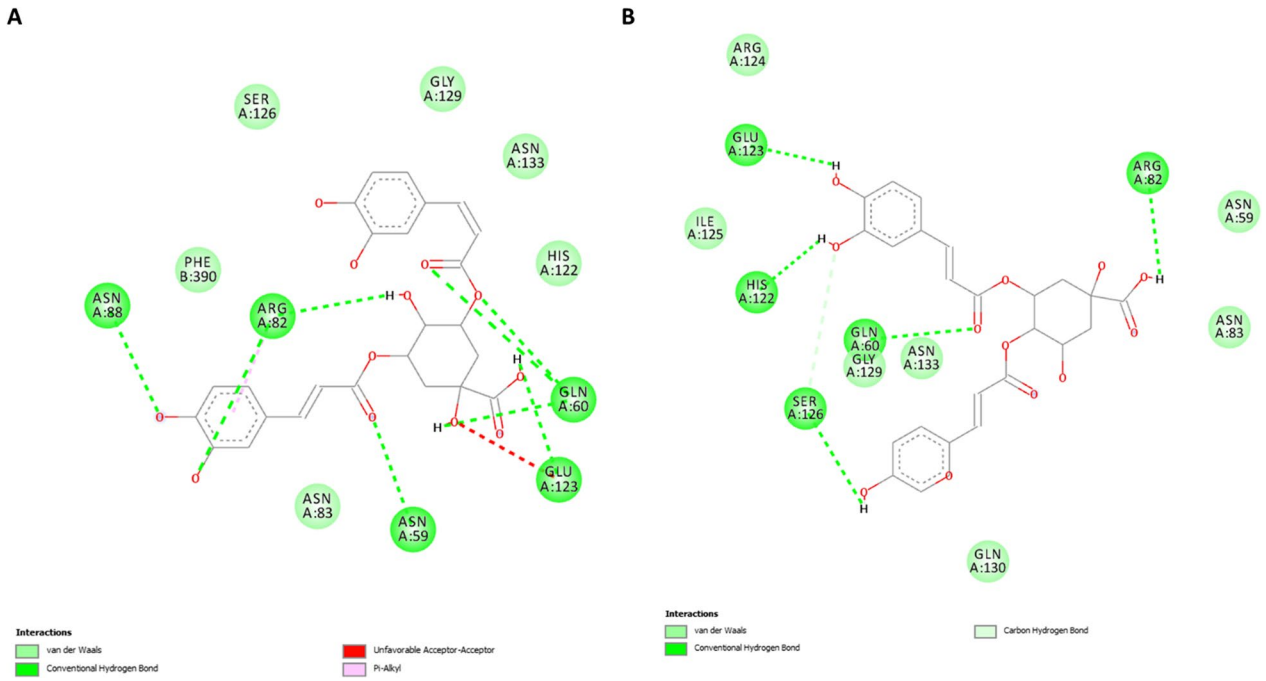

Fig. 3 Molecular docking results of compounds **2** and **6** with the antioxidant protein (PDB: 3MN8): **A** Two-dimensional (2D) docking image of compound **2**. **B** two-dimensional (2D) docking image of compound **6**

data analyses. To validate the methods, a DPPH assay was conducted to compare the antioxidant activity of target compounds **1–6** with the respective peaks obtained from the HPLC-QTOF-MS method combined with a bioassay-guided prediction. As the results (Table 3) exhibited strong activity with IC_{50} values of 21.17 ± 0.2 and 19.5 ± 0.1 μM, respectively, for compounds **2** and **6**. Interestingly, compounds **2** and **6** exhibited the highest activity in the DPPH assay, corresponding to peaks e and f. However, there are concerns that the limitations of the method are complexity, bioassay variability, and the ionization efficiency of the compounds. However, in this study, the results indicate that the HPLC-QTOF-MS method combined with a bioassay-guided successful tool can be used to identify antioxidant components from *L. stenocephala*.

In conclusion, this work reports the first investigation of the antioxidant inhibitory effect of extracts of *L. stenocephala* by combining the HPLC-QTOF-MS method with bioactivity evaluation. As we proposed, compounds **2** and **6** indicated the highest activity in the DPPH activity assay, following LC–MS coupled with a bioassay-guided method. This study contributes to the understanding of the chemical components produced by the aerial parts of *L. stenocephala* as well as their antioxidant properties and could provide a scientific basis for their use as supplementary herbal products for the treatment of antioxidants and other related diseases.

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Author contributions
All authors have their consent to participate. All authors have their consent to publish their work.

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Availability of data and materials
Not applicable.

Declarations

Competing interests
The authors declare no competing financial interest.

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