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Postprint: Chemical Constituents and Anti-inflammatory Activity of Ligularia virgaurea (Maxim.) Mattf., a Tibetan Medicine

Authors: Zhang Xinyu, Luo Ricuo, Wang Hongling, Liang Wenjuan

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Abstract

Ligularia virgaurea is one of the original plants of the Tibetan medicine Rixiao, possessing heat-clearing, detoxifying, and yellow-water-eliminating efficacy. To investigate the anti-inflammatory active constituents of Ligularia virgaurea, this study utilized silica gel column chromatography, gel column chromatography, ODS reversed-phase column chromatography, and other methods for separation and purification, identified the structures of the compounds through various spectroscopic techniques, and employed a lipopolysaccharide (LPS)-induced RAW264.7 cell model to evaluate the inhibitory activity of the compounds on nitric oxide (NO) production. The results demonstrated that: 1) A total of 21 compounds were isolated from the petroleum ether and n-butanol fractions of Ligularia virgaurea, which were identified as spiroeuryolide (1), cacalol acetate (2), oplopenone (3), 8-ethyl palmosalide A (4), 1-hydroxy-3,7-dimethyl-2-(pent-3-enyl)benzofuran (5), syringaresinol O-β-D-glucoside (6), pinoresinol O-β-D-glucopyranoside (7), isoeucommin A (8), eucommin A (9), 6,7-dimethoxycoumarin (10), ferulic acid (11), ethyl caffeate (12), methyl caffeate (13), methyl ferulate (14), ethyl ferulate (15), caffeic acid (16), 2-[(E)-3 ,7 -dimethyl-2 ,6 -octadienyl]-4-methoxy-6-methylphenol (17), 2,8-dimethyl-6-methoxy-2-(4-methylpent-3-enyl)chromene (18), β-sitosterol (19), dodecyl (Z)-9-hexadecenoate (20), and hexacosanal (21). Among these, compounds 1-4, 6, 11-16, 18, 20, and 21 were obtained from Ligularia virgaurea for the first time. 2) In vitro anti-inflammatory assays revealed that compounds 1-3, 6, 11-16, 17, and 19 significantly inhibited NO release at all tested concentrations (1.56–50.00 $\mathrm{mol \cdot L^{-1}}$) ($P < 0.05$ or $P < 0.01$). Compound 5 showed no inhibitory effect on NO release at 50.00 $\mathrm{mol \cdot L^{-1}}$, but demonstrated inhibitory activity at concentrations of 12.50 and 25.00 $\mathrm{mol \cdot L^{-1}}$ ($P < 0.05$). These findings enrich the understanding of the chemical constituents and biological activities of Ligularia virgaurea, and provide a theoretical foundation for the development and utilization of its anti-inflammatory properties.

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Full Text

Preamble

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Title: Study on Chemical Constituents and Anti-inflammatory Activity of Tibetan Medicine Ligularia virgaurea

Authors: Zhang Xinyu[1], Luo Ricuo[1], Wang Hongling[1]*, Liang Wenjuan[2]
Affiliations:
[1]Research Center of Chinese Medicine Resource and National Medicine, Jiangxi University of Traditional Chinese Medicine, Nanchang 330004, China
[2]College of Food Science and Technology, Yunnan Agricultural University, Kunming 650201, China

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Abstract

Ligularia virgaurea is one of the original plants of the Tibetan medicine "Rixiao," traditionally used for clearing heat, detoxification, and removing "yellow water." To investigate its anti-inflammatory active constituents, this study employed silica gel column chromatography, gel column chromatography, and ODS reversed-phase column chromatography for separation and purification. Compound structures were identified using various spectroscopic methods, and their inhibitory effects on nitric oxide (NO) were evaluated using a lipopolysaccharide (LPS)-induced RAW264.7 cell model. The results showed that: (1) Twenty-one compounds were isolated from the petroleum ether and n-butanol fractions of L. virgaurea, identified as spiroeuryolide (1), cacalol acetate (2), oplopenone (3), 8-ethyl-palmosalide A (4), 1-hydroxy-3,7-dimethyl-2-(pent-3-enyl)benzofuran (5), syringaresinol-O-β-D-glucopyranoside (6), pinoresinol-O-β-D-glucopyranoside (7), isoeucommin A (8), eucommin A (9), 6,7-dimethoxycoumarin (10), ferulic acid (11), ethyl caffeate (12), methyl caffeate (13), methyl ferulate (14), ethyl ferulate (15), caffeic acid (16), 2-[(2 E)-3 ,7 -dimethyl-2 ,6 -octadienyl]-4-methoxy-6-methylphenol (17), 2,8-dimethyl-6-methoxy-2-(4 -methylpent-3 -enyl)-chromene (18), β-sitosterol (19), dodecyl(Z)-9-hexadecenoate (20), and hexacosanal (21). Among these, compounds 1-4, 6, 11-16, 18, 20, and 21 were isolated from L. virgaurea for the first time. (2) In vitro anti-inflammatory assays demonstrated that compounds 1-3, 6, 11-16, 17, and 19 significantly inhibited NO release at concentrations ranging from 1.56 to 50.00 $\mathrm{mol \cdot L^{-1}}$ ($P < 0.05$ or $P < 0.01$). Compound 5 showed no inhibitory effect on NO release at 50.00 $\mathrm{mol \cdot L^{-1}}$, but exhibited inhibition at 12.50 and 25.00 $\mathrm{mol \cdot L^{-1}}$ ($P < 0.05$). These findings enrich the understanding of the chemical composition and biological activity of L. virgaurea, providing a theoretical foundation for the development and utilization of its anti-inflammatory properties.

Keywords: Ligularia virgaurea; sesquiterpenes; chemical composition; struc-

tural identification; anti-inflammatory activity

Introduction

Ligularia virgaurea (Asteraceae) is a perennial herb and one of the original plants of the Tibetan medicine "Rixiao," documented in the *Drug Standards of the Ministry of Health of the People' s Republic of China: Tibetan Medicine* (1995 edition) and the *Qinghai Tibetan Medicine Standard* (1992 edition). It is primarily distributed in northeastern Tibet, northwestern Yunnan, Sichuan, Qinghai, and Gansu provinces. The whole plant is used medicinally to clear latent heat, detoxify and heal sores, remove "yellow water" (Qinghai Provincial Health Department, 1992), and dispel wind-dampness (Liu et al., 2006). Previous studies have reported that ethanol extracts of L. virgaurea inhibit scab pathogens (Luo et al., 2015), and its chemical constituents include sesquiterpenes, lignans, steroids, and phenylpropanoids (Wu et al., 2004; Wu et al., 2005a, 2005b; Zhang et al., 2007; Dong et al., 2015; Tori, 2016; Qi et al., 2017; Nakashima et al., 2018; Saito et al., 2019). Sesquiterpenes are the major components, and some sesquiterpenes and phenylpropanoids have demonstrated anti-inflammatory activity (Guo et al., 2018; Liao et al., 2023). Our research group previously isolated 12 compounds from the ethyl acetate fraction of L. virgaurea (Wang et al., 2022). To further investigate its anti-inflammatory active constituents, this study isolated and identified 21 compounds from the petroleum ether and n-butanol fractions, among which compounds 1–4, 6, 11–16, 18, 20, and 21 were obtained from L. virgaurea for the first time. Thirteen potential anti-inflammatory active constituents were discovered, providing a chemical and pharmacological basis for the development and utilization of L. virgaurea.

1. Instruments and Materials

Instruments

The following instruments were used: AX-600 NMR spectrometer (Bruker, Germany); Waters e2695 HPLC system (Waters, USA); Eclipse XD-C18 analytical column (250 mm × 4.6 mm, 5 m, Agilent, USA); Agilent 1260 HPLC system (Agilent, USA); ZORBAX SB-C18 semi-preparative column (250 mm × 9.4 mm, 5 m, Agilent, USA); Triple TOF 5600 HR-QTOF-MS (AB SCIEX, USA); CO_2 incubator (2014-88759, Esco, Singapore); Rotavapor R-210 rotary evaporator (BUCHI, Switzerland); and Multiskan Go microplate spectrophotometer (Thermo Fisher Scientific, USA).

Reagents and Materials

Reagents included Sephadex LH-20 dextran gel (Amersham Pharmacia, Switzerland); GF254 thin-layer chromatography silica gel (Yantai Huayang New Mate-

rials, China); ODS reversed-phase silica gel (Fuji, Japan); Nitric Oxide Detection Kit (Beyotime Biotechnology, China); Cell Counting Kit-8 (Dalian Meilun Biotechnology, China); RAW264.7 mouse mononuclear macrophages (Cell Bank of Chinese Academy of Sciences); chromatographic-grade methanol (TEDIA, USA); deuterated reagents (Cambridge Isotope Laboratories, USA); organic reagents (Xilong Chemical, China); DMEM high-glucose medium and fetal bovine serum (Gibco Life Technologies, USA).

Plant Material

Ligularia virgaurea was collected in Ganzi, Sichuan Province in August 2020 and identified as the dried whole herb of Ligularia virgaurea (Asteraceae) by researcher Zhong Guoyue. A voucher specimen (20200801) is deposited at the Research Center of Chinese Medicine Resource and National Medicine, Jiangxi University of Traditional Chinese Medicine.

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2. Methods

2.1 Extraction and Isolation

Dried L. virgaurea (5.0 kg) was extracted twice with 75% ethanol. The combined extracts were concentrated to obtain a total extract, which was sequentially partitioned with petroleum ether, ethyl acetate, and n-butanol (Wang et al., 2022) to yield petroleum ether (Fr.1, 73.8 g), ethyl acetate (Fr.2), n-butanol (Fr.3, 159.1 g), and aqueous fractions (Fr.4).

The petroleum ether fraction Fr.1 (73.8 g) was subjected to silica gel column chromatography eluted with petroleum ether-ethyl acetate (100:2 to 7:3) to obtain six subfractions (Fr.1-1 to Fr.1-6). Subfraction Fr.1-2 (12.4 g) was further purified by silica gel column chromatography (petroleum ether-dichloromethane 9:1 to 7:3), Sephadex LH-20 gel chromatography (methanol), and ODS reversed-phase column chromatography (methanol-water 6:4 to 9:1) to yield compounds **3** (32.0 mg), **18** (37.2 mg), **20** (21.3 mg), and **21** (24.3 mg). Subfraction Fr.1-3 (9.2 g) was separated by silica gel column chromatography (petroleum ether-dichloromethane 7:3 to 5:5), followed by ODS reversed-phase chromatography (methanol-water 4:6 to 7:3) and Sephadex LH-20 gel chromatography (methanol) to afford compounds **1** (42.8 mg), **2** (21.4 mg), **4** (8.7 mg), **5** (48.6 mg), **10** (12.3 mg), and **17** (10.2 mg).

The n-butanol fraction Fr.3 (159.1 g) was chromatographed on a silica gel column eluted with dichloromethane-methanol (100:5 to 8:2) to give six subfractions (Fr.3-1 to Fr.3-6). Subfraction Fr.3-1 (10.1 g) was separated by silica gel column chromatography (petroleum ether-ethyl acetate 100:1 to 6:4), Sephadex LH-20 gel chromatography (methanol), and ODS reversed-phase column chromatography (methanol-water 4:6 to 8:2) to yield compounds **14** (34.7 mg), **15** (45.1 mg), and **19** (107.1 mg). Subfraction Fr.3-2 (6.0 g) was subjected to

silica gel column chromatography (petroleum ether-ethyl acetate 8:2 to 5:5) and Sephadex LH-20 gel chromatography (methanol) to obtain compound **11** (48.0 mg), which was further purified by semi-preparative HPLC (Agilent) using methanol-water (37:63, 228 nm) to afford compounds **12** (10.8 mg, tR = 32.4 min) and **13** (50.1 mg, tR = 40.6 min). Subfraction Fr.3-4 (5.6 g) was separated by ODS reversed-phase silica gel column chromatography (methanol-water 1:9 to 5:5), followed by silica gel column chromatography and Sephadex LH-20 gel chromatography (methanol) to yield compounds **6** (73.2 mg), **7** (8.6 mg), **8** (5.9 mg), and **9** (3.4 mg). Subfraction Fr.3-5 (6.1 g) was chromatographed on an ODS reversed-phase silica gel column with methanol-water (1:9 to 5:5) to obtain compound **16** (20.0 mg).

2.2 Anti-inflammatory Activity Evaluation

Cytotoxicity Assay The cytotoxicity of compounds **1**–**3**, **5**, **6**, **11**–**16**, **17**, and **19** against RAW264.7 cells was assessed. Logarithmic-phase RAW264.7 cells were seeded in 96-well plates at a density of 3×10^4 cells per well and cultured for 24 h. After removing the culture medium, cells were divided into blank, control, and treatment groups (four replicates per well). Treatment groups received fresh medium containing various drug concentrations (6.25–100.00 $\mathrm{mol \cdot L^{-1}}$). Following treatment, CCK-8 solution was added and incubated for 30 min, and absorbance was measured at 450 nm. Cell viability was calculated according to the literature (Guo et al., 2022) to determine safe concentration ranges.

NO Inhibition Assay Logarithmic-phase RAW264.7 cells were seeded in 96-well plates (3×10^4 cells per well) and divided into blank (CON), model (MOL), methotrexate (MTX), and treatment groups (four replicates per well). After 24 h incubation, the old medium was removed. Except for the blank group, all groups were stimulated with LPS (1.00 $\mathrm{g \cdot mL^{-1}}$) for 1 h. Treatment groups were then administered different drug concentrations (1.56–50.00 $\mathrm{mol \cdot L^{-1}}$) based on cytotoxicity results, the methotrexate group received MTX (0.06 $\mathrm{mol \cdot L^{-1}}$), and model and blank groups received fresh medium. After 24 h incubation, 50 L of supernatant from each well was transferred to a new 96-well plate, and Griess reagents A and B (50 L each) were added sequentially in the dark. Absorbance was measured at 540 nm to calculate NO concentration.

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3. Results

3.1 Structural Identification

Compound 1

Pale yellow oil; molecular formula $C_{15}H_{18}O_2$; ESI-MS m/z: 231.1 $[M+H]^+$. ^{1}H-NMR (600 MHz, Methanol-d_4) δH: 6.52 (1H, d, J = 1.4 Hz, H-6), 5.69 (1H, s, H-9), 2.23 (1H, m, H-4), 2.07 (3H, s, H-14), 1.94–2.06 (5H, m, H-1, 2, 3α), 1.71

(1H, m, H-3β), 1.90 (3H, s, H-13), 0.76 (3H, d, J = 7.1 Hz, H-15). ^{13}C-NMR (150 MHz, Methanol-d$_4$) δC: 38.6 (C-1), 25.4 (C-2), 35.4 (C-3), 48.9 (C-4), 156.2 (C-5), 117.3 (C-6), 144.6 (C-7), 147.8 (C-8), 119.2 (C-9), 57.5 (C-10), 112.8 (C-11), 174.5 (C-12), 7.8 (C-13), 23.5 (C-14), 14.1 (C-15). The data were consistent with literature reports (Huang et al., 2013), identifying compound **1** as spiroeuryolide.

Compound 2

White solid; molecular formula $C_{17}H_{20}O_3$; ESI-MS m/z: 273.1 $[M+H]^+$. ^{1}H-NMR (600 MHz, Chloroform-d) δH: 7.23 (1H, d, J = 1.4 Hz, H-12), 3.22–3.27 (1H, m, H-4), 2.81–2.85 (1H, m, H-1α), 2.57 (3H, s, H-14), 2.39 (3H, s, H-17), 2.37 (3H, d, J = 1.4 Hz, H-15), 1.75–1.91 (4H, m, H-2, 3), 1.19 (3H, d, J = 7.1 Hz, H-13). ^{13}C-NMR (150 MHz, Chloroform-d) δC: 23.6 (C-1), 16.7 (C-2), 30.1 (C-3), 29.1 (C-4), 125.1 (C-5), 135.6 (C-6), 127.2 (C-7), 145.3 (C-8), 131.5 (C-9), 127.0 (C-10), 116.9 (C-11), 141.6 (C-12), 11.4 (C-13), 14.4 (C-14), 20.7 (C-15), 168.9 (C-16), 21.5 (C-17). The data were consistent with literature reports (Arellano et al., 2018), identifying compound **2** as cacalol acetate.

Compound 3

Yellow solid; molecular formula $C_{15}H_{24}O$; ESI-MS m/z: 221.2 $[M+H]^+$. ^{1}H-NMR (600 MHz, Chloroform-d) δH: 4.63 (1H, m, H-10α), 4.53 (1H, m, H-10β), 2.66–2.70 (1H, m, H-3), 2.32–2.35 (1H, m, H-7β), 2.15 (3H, s, H-15), 1.03–1.10 (1H, m, H-6β), 0.87 (3H, d, J = 6.9 Hz, H-12), 0.62 (3H, d, J = 6.9 Hz, H-13). ^{13}C-NMR (150 MHz, Chloroform-d) δC: 27.4 (C-1), 28.6 (C-2), 56.1 (C-3), 52.1 (C-4), 49.3 (C-5), 26.6 (C-6), 35.3 (C-7), 150.9 (C-8), 51.8 (C-9), 103.6 (C-10), 29.6 (C-11), 22.0 (C-12), 15.7 (C-13), 211.7 (C-14), 29.0 (C-15). The data were consistent with literature reports (Joseph-Nathan et al., 1989), identifying compound **3** as oplopenone.

Compound 4

Pale yellow oil; molecular formula $C_{17}H_{26}O_3$; ESI-MS m/z: 279.2 $[M+H]^+$. ^{1}H-NMR (600 MHz, Chloroform-d) δH: 5.57 (1H, m, H-1), 3.42–3.47 (1H, m, H-16α), 3.22–3.27 (1H, m, H-16β), 2.85 (1H, d, J = 14.3 Hz, H-9α), 2.74 (1H, d, J = 13.0 Hz, H-6α), 1.95 (1H, d, J = 13.0 Hz, H-6β), 2.16 (1H, m, H-2α), 2.03 (1H, m, H-2β), 2.40–2.44 (1H, m, H-9β), 1.89 (3H, d, J = 1.5 Hz, H-13), 1.67–1.73 (1H, m, H-4), 1.41–1.48 (2H, m, H-3), 1.16 (3H, t, J = 7.0 Hz, H-17), 1.00 (3H, d, J = 7.0 Hz, H-14), 0.82 (3H, s, H-15). ^{13}C-NMR (150 MHz, Chloroform-d) δC: 126.2 (C-1), 25.8 (C-2), 27.1 (C-3), 40.5 (C-4), 41.2 (C-5), 37.5 (C-6), 158.2 (C-7), 106.2 (C-8), 44.0 (C-9), 136.4 (C-10), 124.6 (C-11), 172.1 (C-12), 8.2 (C-13), 15.9 (C-14), 17.9 (C-15), 58.7 (C-16), 15.4 (C-17). The data were consistent with literature reports (Wiemer et al., 1990), identifying compound **4** as 8-ethyl-palmosalide A.

Compound 5

Pale yellow solid; molecular formula $C_{15}H_{18}O_2$; ESI-MS m/z: 231.1 $[M+H]^+$. ^{1}H-NMR (600 MHz, Chloroform-d) δH: 7.26 (1H, d, J = 1.3 Hz, H-8), 6.85 (1H, s, H-4), 2.77 (2H, t, J = 7.3 Hz, H-11), 5.45–5.56 (2H, m, H-13, 14), 2.36 (3H, s, H-10), 2.15 (3H, d, J = 1.3 Hz, H-9), 1.63 (3H, d, J = 6.1 Hz, H-15). ^{13}C-NMR

(150 MHz, Chloroform-d) δC: 138.8 (C-1), 122.9 (C-2), 131.9 (C-3), 111.9 (C-4), 127.7 (C-5), 142.7 (C-6), 116.2 (C-7), 140.8 (C-8), 8.0 (C-9), 20.1 (C-10), 26.8 (C-11), 32.6 (C-12), 131.3 (C-13), 125.4 (C-14), 18.1 (C-15). The data were consistent with literature reports (Liu et al., 2007; Sun et al., 2007), identifying compound **5** as 1-hydroxy-3,7-dimethyl-2-(pent-3-enyl)benzofuran.

Compound 6

White powder; molecular formula $C_{28}H_{36}O_{13}$; ESI-MS m/z: 603.0 $[M+Na]^+$. ^{1}H-NMR (600 MHz, Pyridine-d_5) δH: 7.00 (2H, s, H-1, 1), 6.98 (2H, s, H-5, 5), 5.02 (2H, brs, H-7, 7), 4.35 (4H, m, H-9, 9), 3.86 (6H, s, H-10, 10), 3.84 (6H, s, H-11, 11), 3.24–3.31 (2H, m, H-8, 8). ^{13}C-NMR (150 MHz, Pyridine-d_5) δC: 132.1 (C-1), 105.0 (C-2), 154.0 (C-3), 138.4 (C-4), 154.0 (C-5), 105.0 (C-6), 86.6 (C-7), 55.0 (C-8), 72.3 (C-9), 56.6 (C-10), 56.8 (C-11), 130.2 (C-1), 104.8 (C-2), 149.3 (C-3), 137.3 (C-4), 149.3 (C-5), 104.8 (C-6), 86.3 (C-7), 54.9 (C-8), 72.2 (C-9), 56.6 (C-10), 56.8 (C-11), 104.9 (C-1), 76.1 (C-2), 78.4 (C-3), 71.6 (C-4), 78.7 (C-5), 62.4 (C-6). The data were consistent with literature reports (Liu et al., 2016), identifying compound **6** as syringaresinol-O-β-D-glucopyranoside.

Compound 7

White powder; molecular formula $C_{26}H_{32}O_{11}$; ESI-MS m/z: 543.0 $[M+Na]^+$. ^{1}H-NMR (600 MHz, Methanol-d_4) δH: 7.14 (1H, d, J = 8.3 Hz, H-5), 7.03 (1H, d, J = 1.8 Hz, H-2), 6.95 (1H, d, J = 1.5 Hz, H-2), 6.91 (1H, dd, J = 8.3, 1.8 Hz, H-6), 6.81 (1H, dd, J = 8.1, 1.5 Hz, H-6), 6.77 (1H, d, J = 8.1 Hz, H-5), 4.75 (1H, d, J = 4.4 Hz, H-7), 4.71 (1H, d, J = 4.0 Hz, H-7), 4.21-4.25 (2H, m, H-9, 9), 3.87 (3H, s, H-10), 3.85 (3H, s, H-10), 3.12 (2H, m, H-8, 8). ^{13}C-NMR (150 MHz, Methanol-d_4) δC: 137.4 (C-1), 111.6 (C-2), 147.5 (C-3), 150.9 (C-4), 118.0 (C-5), 120.0 (C-6), 87.1 (C-7), 55.5 (C-8), 72.7 (C-9), 56.7 (C-10), 133.7 (C-1), 111.0 (C-2), 147.3 (C-3), 149.1 (C-4), 116.1 (C-5), 119.8 (C-6), 87.5 (C-7), 55.3 (C-8), 72.7 (C-9), 56.4 (C-10), 102.8 (C-1), 74.9 (C-2), 78.0 (C-3), 71.3 (C-4), 77.8 (C-5), 62.5 (C-6). The data were consistent with literature reports (Zhang et al., 2008), identifying compound **7** as pinoresinol-O-β-D-glucopyranoside.

Compound 8

White powder; molecular formula $C_{27}H_{34}O_{12}$; ESI-MS m/z: 573.0 $[M+Na]^+$. ^{1}H-NMR (600 MHz, Methanol-d_4) δH: 7.15 (1H, d, J = 7.8 Hz, H-5), 7.04 (1H, brs, H-2), 6.93 (1H, brd, J = 7.8 Hz, H-6), 6.66 (2H, s, H-2 , 6), 4.72–4.77 (2H, overlap, H-7, H-7), 4.25–4.27 (2H, m, H-9β, 9 β), 3.88 (3H, s, H-10), 3.85 (6H, s, H-11, 12), 3.14 (2H, m, H-8, 8). ^{13}C-NMR (150 MHz, Methanol-d_4) δC: 133.1 (C-1), 104.5 (C-2), 149.3 (C-3), 137.5 (C-4), 149.3 (C-5), 104.5 (C-6), 87.6 (C-7), 55.5 (C-8), 72.7 (C-9), 56.8 (C-10), 56.8 (C-11), 56.7 (C-12), 136.2 (C-1), 111.6 (C-2), 151.0 (C-3), 147.5 (C-4), 118.0 (C-5), 119.8 (C-6), 87.1 (C-7), 55.5 (C-8), 72.8 (C-9), 102.8 (C-1), 74.9 (C-2), 77.8 (C-3), 71.3 (C-4), 78.2 (C-5), 62.5 (C-6). The data were consistent with literature reports (Nan et al., 2015), identifying compound **8** as isoeucommin A.

Compound 9

White powder; molecular formula $C_{27}H_{34}O_{12}$; ESI-MS m/z: 573.0 $[M+Na]^+$. ^{1}H-NMR (600 MHz, Methanol-d_4) δH: 6.73–6.96 (6H, overlap, H-2, 2 , 5 , 6, 6), 4.71–4.76 (2H, overlap, H-7, 7), 4.24–4.29 (2H, m, H-9β, 9 β), 3.86 (9H, s, H-10, 11, 12), 3.14–3.30 (2H, m, H-8, 8). ^{13}C-NMR (150 MHz, Methanol-d_4) δC: 135.6 (C-1), 104.8 (2C, C-2, 6), 154.4 (2C, C-3, 5), 139.6 (C-4), 87.4 (C-7), 55.4 (C-8), 72.9 (C-9), 57.1 (2C, C-10, 11), 56.4 (C-12), 133.7 (C-1), 111.0 (C-2), 149.1 (C-3), 147.3 (C-4), 116.1 (C-5), 120.1 (C-6), (C-7), 55.8 (C-8), 72.7 (C-9), 105.3 (C-1), 75.7 (C-2), 77.8 (C-3), 71.3 (C-4), 78.3 (C-5), 62.6 (C-6). The data were consistent with literature reports (Nan et al., 2015), identifying compound **9** as eucommin A.

Compound 10

Colorless needle crystals (dichloromethane); molecular formula $C_{11}H_{10}O_4$; ESI-MS m/z: 207.1 $[M+H]^+$. ^{1}H-NMR (600 MHz, Chloroform-d) δH: 7.88 (1H, d, J = 9.4 Hz, H-4), 7.13 (1H, s, H-5), 6.97 (1H, s, H-8), 6.26 (1H, d, J = 9.4 Hz, H-3), 3.92 (3H, s, H-11), 3.88 (3H, s, H-12). ^{13}C-NMR (150 MHz, Chloroform-d) δC: 163.8 (C-2), 113.5 (C-3), 145.9 (C-4), 109.9 (C-5), 148.1 (C-6), 154.7 (C-7), 100.9 (C-8), 151.2 (C-9), 113.0 (C-10), 56.9 (C-11), 56.8 (C-12). The data were consistent with literature reports (Xiao et al., 2005), identifying compound **10** as 6,7-dimethoxycoumarin.

Compound 11

Pale yellow solid; molecular formula $C_{10}H_{10}O_4$; ESI-MS m/z: 217.0 $[M+Na]^+$. ^{1}H-NMR (600 MHz, Methanol-d_4) δH: 7.60 (1H, d, J = 15.9 Hz, H-7), 7.20 (1H, d, J = 2.0 Hz, H-2), 7.07 (1H, dd, J = 8.2, 2.0 Hz, H-6), 6.81 (1H, d, J = 8.2 Hz, H-5), 6.31 (1H, d, J = 15.9 Hz, H-8), 3.90 (3H, s, H-12). ^{13}C-NMR (150 MHz, Methanol-d_4) δC: 127.8 (C-1), 116.4 (C-2), 150.5 (C-3), 149.4 (C-4), 115.9 (C-5), 124.0 (C-6), 146.9 (C-7), 111.7 (C-8), 171.0 (C-9), 56.4 (C-10). The data were consistent with literature reports (Shen et al., 2010), identifying compound **11** as ferulic acid.

Compound 12

White powder; molecular formula $C_{11}H_{12}O_4$; ESI-MS m/z: 231.0 $[M+Na]^+$. ^{1}H-NMR (600 MHz, Methanol-d_4) δH: 7.54 (1H, d, J = 15.9 Hz, H-7), 7.04 (1H, d, J = 2.0 Hz, H-2), 6.95 (1H, dd, J = 8.1, 2.0 Hz, H-6), 6.78 (1H, d, J = 8.1 Hz, H-5), 6.25 (1H, d, J = 15.9 Hz, H-8), 4.22 (2H, q, J = 7.1 Hz, H-1), 1.31 (3H, t, J = 7.1 Hz, H-2). ^{13}C-NMR (150 MHz, Methanol-d_4) δC: 127.7 (C-1), 115.1 (C-2), 146.8 (C-3), 149.5 (C-4), 116.5 (C-5), 122.9 (C-6), 146.7 (C-7), 115.2 (C-8), 169.3 (C-9), 61.4 (C-1), 14.6 (C-2). The data were consistent with literature reports (Dai et al., 2006), identifying compound **12** as ethyl caffeate.

Compound 13

White powder; molecular formula $C_{10}H_{10}O_4$; ESI-MS m/z: 217.0 $[M+Na]^+$. ^{1}H-NMR (600 MHz, Methanol-d_4) δH: 7.55 (1H, d, J = 15.9 Hz, H-7), 7.04 (1H, d, J = 2.0 Hz, H-2), 6.95 (1H, dd, J = 8.2, 2.0 Hz, H-6), 6.78 (1H, d, J = 8.2 Hz, H-5), 6.27 (1H, d, J = 15.9 Hz, H-8), 3.76 (3H, s, H-10). ^{13}C-NMR (150 MHz, Methanol-d_4) δC: 127.7 (C-1), 114.8 (C-2), 146.9 (C-3), 149.6 (C-4), 116.48 (C-5), 122.9 (C-6), 146.8 (C-7), 115.1 (C-8), 169.7 (C-9), 52.0 (C-10). The

data were consistent with literature reports (Prevost et al., 2013), identifying compound **13** as methyl caffeate.

Compound 14

White powder; molecular formula $C_{11}H_{12}O_4$; ESI-MS m/z: 231.0 $[M+Na]^+$. ^{1}H-NMR (600 MHz, Methanol-d_4) δH: 7.61 (1H, d, J = 15.8 Hz, H-7), 7.18 (1H, d, J = 2.0 Hz, H-2), 7.08 (1H, dd, J = 8.2, 2.0 Hz, H-6), 6.82 (1H, d, J = 8.2 Hz, H-5), 6.37 (1H, d, J = 15.8 Hz, H-8), 3.89 (3H, s, H-10), 3.77 (3H, s, H-11). ^{13}C-NMR (150 MHz, Methanol-d_4) δC: 126.3 (C-1), 110.3 (C-2), 147.9 (C-3), 149.2 (C-4), 115.1 (C-5), 122.7 (C-6), 145.4 (C-7), 113.8 (C-8), 168.3 (C-9), 55.0 (C-10), 50.6 (C-11). The data were consistent with literature reports (Karakousi et al., 2020), identifying compound **14** as methyl ferulate.

Compound 15

White powder; molecular formula $C_{12}H_{14}O_4$; ESI-MS m/z: 223.0 $[M+H]^+$. ^{1}H-NMR (600 MHz, Methanol-d_4) δH: 7.60 (1H, d, J = 15.9 Hz, H-7), 7.18 (1H, d, J = 2.0 Hz, H-2), 7.07 (1H, dd, J = 8.2, 2.0 Hz, H-6), 6.82 (1H, d, J = 8.2 Hz, H-5), 6.35 (1H, d, J = 15.9 Hz, H-8), 4.23 (2H, q, J = 7.1 Hz, H-10), 3.90 (3H, s, H-12), 1.32 (3H, t, J = 7.1 Hz, H-11). ^{13}C-NMR (150 MHz, Methanol-d_4) δC: 127.7 (C-1), 115.6 (C-2), 149.3 (C-3), 150.5 (C-4), 116.4 (C-5), 124.0 (C-6), 146.6 (C-7), 111.7 (C-8), 169.2 (C-9), 61.4 (C-10), 14.6 (C-11), 56.4 (C-12). The data were consistent with literature reports (Sun et al., 2018), identifying compound **15** as ethyl ferulate.

Compound 16

Light yellow solid; molecular formula $C_9H_{10}O_4$; ESI-MS m/z: 183.0 $[M+H]^+$. ^{1}H-NMR (600 MHz, Methanol-d_4) δH: 7.49 (1H, d, J = 15.8 Hz, H-7), 6.99 (1H, d, J = 2.0 Hz, H-2), 6.88 (1H, dd, J = 8.2, 2.0 Hz, H-6), 6.73 (1H, d, J = 8.2 Hz, H-5), 6.17 (1H, d, J = 15.8 Hz, H-8). ^{13}C-NMR (150 MHz, Methanol-d_4) δC: 127.8 (C-1), 115.1 (C-2), 146.8 (C-3), 149.4 (C-4), 116.5 (C-5), 122.8 (C-6), 147.0 (C-7), 115.6 (C-8), 171.1 (C-9). The data were consistent with literature reports (Lin et al., 2016), identifying compound **16** as caffeic acid.

Compound 17

Yellow oil; molecular formula $C_{18}H_{26}O_2$; ESI-MS m/z: 275.2 $[M+H]^+$. ^{1}H-NMR (600 MHz, Chloroform-d) δH: 6.58 (1H, d, J = 3.0 Hz, H-5), 6.53 (1H, d, J = 3.0 Hz, H-3), 5.30 (1H, t, J = 7.2 Hz, H-2), 5.07 (1H, t, J = 6.5 Hz, H-6), 4.80 (1H, brs, OH), 3.74 (3H, s, H-8), 3.33 (2H, d, J = 7.2 Hz, H-1), 2.22 (3H, s, H-7), 2.07-2.15 (4H, overlap, H-4 , 5), 1.78 (3H, s, H-10), 1.69 (3H, s, H-8), 1.60 (3H, s, H-9). ^{13}C-NMR (150 MHz, Chloroform-d) δC: 146.9 (C-1), 125.6 (C-2), 113.1 (C-3), 153.2 (C-4), 114.2 (C-5), 127.4 (C-6), 16.4 (C-7), 55.8 (C-8), 30.7 (C-1), 121.8 (C-2), 138.9 (C-3), 39.8 (C-4), 26.5 (C-5), 123.9 (C-6), 132.2 (C-7), 25.8 (C-8), 17.9 (C-9), 16.3 (C-10). The data were consistent with literature reports (Resch et al., 2001), identifying compound **17** as 2-[(2 E)-3 ,7 -dimethyl-2 ,6 -octadienyl]-4-methoxy-6-methylphenol.

Compound 18

Pale yellow oil; molecular formula $C_{18}H_{24}O_2$; ESI-MS m/z: 273.2 $[M+H]^+$. ^{1}H-

NMR (600 MHz, Chloroform-d) δH: 6.57 (1H, d, J = 2.9 Hz, H-7), 6.40 (1H, d, J = 2.9 Hz, H-5), 6.30 (1H, d, J = 9.8 Hz, H-3), 5.59 (1H, d, J = 9.8 Hz, H-2), 5.12 (1H, t, J = 7.2 Hz, H-3), 3.74 (3H, s, H-11), 2.18 (3H, s, H-10), 1.68 (3H, s, H-5), 1.59 (3H, s, H-6), 1.38 (3H, s, H-7). ^{13}C-NMR (150 MHz, Chloroform-d) δC: 77.8 (C-1), 130.7 (C-2), 121.2 (C-3), 123.2 (C-4), 108.9 (C-5), 153.0 (C-6), 116.2 (C-7), 126.3 (C-8), 145.1 (C-9), 15.7 (C-10), 55.7 (C-11), 40.98 (C-1), 22.8 (C-2), 124.4 (C-3), 131.7 (C-4), 25.8 (C-5), 17.7 (C-6), 26.1 (C-7). The data were consistent with literature reports (Capon et al., 1981; Resch et al., 1998), identifying compound **18** as 2,8-dimethyl-6-methoxy-2-(4 -methylpent-3 -enyl)-chromene.

Compound 19

White powder; molecular formula $C_{29}H_{50}O$; ESI-MS m/z: 415.4 $[M+H]^+$. ^{1}H-NMR (600 MHz, Chloroform-d) δH: 5.32 (1H, t, J = 2.8 Hz, H-6), 2.18–2.28 (1H, m, H-2α), 1.93–2.05 (1H, m, H-12α), 1.80–1.85 (2H, m, H-7), 1.62–1.68 (3H, overlap, H-1α, 2β, 25), 1.40–1.55 (3H, m, H-8, 15), 1.35 (5H, m, H-11, 20, 22), 1.28 (4H, m, H-16, 28), 1.25 (2H, m, H-23), 1.15 (2H, m, H-12β, 17), 0.99 (3H, s, H-19), 0.90 (3H, d, J = 6.4 Hz, H-26), 0.66 (3H, s, H-18). ^{13}C-NMR (150 MHz, Chloroform-d) δC: 37.4 (C-1), 31.7 (C-2), 71.8 (C-3), 42.3 (C-4), 140.9 (C-5), 121.7 (C-6), 32.0 (C-7), 32.0 (C-8), 50.2 (C-9), 36.3 (C-10), 21.2 (C-11), 39.9 (C-12), 42.4 (C-13), 56.9 (C-14), 24.4 (C-15), 28.4 (C-16), 56.2 (C-17), 12.1 (C-18), 19.5 (C-19), 36.26 (C-20), 18.9 (C-21), 34.0 (C-22), 26.2 (C-23), 45.9 (C-24), 29.2 (C-25), 19.2 (C-26), 19.9 (C-27), 23.2 (C-28), 12.0 (C-29). The data were consistent with literature reports (Kadowaki et al., 2003), identifying compound **19** as β-sitosterol.

Compound 20

Pale yellow oil; molecular formula $C_{28}H_{54}O_2$; ESI-MS m/z: 421.4 $[M-H]^-$. ^{1}H-NMR (600 MHz, Chloroform-d) δH: 5.33 (2H, m, H-9, 10), 4.11 (2H, t, J = 7.0 Hz, H-1), 2.27 (2H, t, J = 7.6 Hz, H-2), 2.13 (2H, m, H-8, 11), 1.62 (4H, m, H-3, 2), 1.21–1.36 (34H, m, H-4–6, 12–15, 3 -11), 0.87 (6H, t, J = 7.0 Hz, H-16, 12). ^{13}C-NMR (150 MHz, Chloroform-d) δC: 174.0 (C-1), 34.5 (C-2), 25.1 (C-3), 29.3 (C-4), 29.8 (C-5), 29.8 (C-6), 29.7 (C-7), 27.3 (C-8), 130.2 (C-9), 130.3 (C-10), 27.3 (C-11), 29.5 (C-12), 29.3 (C-13), 31.7 (C-14), 22.7 (C-15), 14.4 (C-16), 64.28 (C-1), 29.2 (C-2), 25.8 (C-3), 29.3 (C-4), 29.3 (C-5), 29.3 (C-6), 29.3 (C-7), 29.5 (C-8), 29.3 (C-9), 32.0 (C-10), 22.8 (C-11), 14.2 (C-12). The data were consistent with literature reports (Chen et al., 2021), identifying compound **20** as dodecyl(Z)-9-hexadecenoate.

Compound 21

Pale yellow oil; molecular formula $C_{26}H_{52}O$; ESI-MS m/z: 381.4 $[M+H]^+$. ^{1}H-NMR (600 MHz, Chloroform-d) δH: 9.76 (1H, s, H-1), 2.42 (2H, t, J = 7.3 Hz, H-2), 1.25–1.33 (46H, overlap, H-3–25), 0.88 (3H, t, J = 6.8 Hz, H-26). ^{13}C-NMR (150 MHz, Chloroform-d) δC: 203.0 (C-1), 43.4 (C-2), 22.7 (C-3), 29.7 (20C, C-4–23), 31.9 (C-24), 22.1 (C-25), 14.1 (C-26). The data were consistent with literature reports (Govindan et al., 2019), identifying compound **21** as hexacosanal.

[Figure 1: see original paper] Structures of compounds 1–21

3.2 Anti-inflammatory Activity Evaluation Results

Cytotoxicity assessment using the CCK-8 assay showed that compounds **1**–**3**, **6**, **11**, **12**, **17**, and **19** were non-toxic to RAW264.7 cells at concentrations up to 6.25 $mol \cdot L^{-1}$; compounds **5**, **14**–**16** were safe up to 50.00 $mol \cdot L^{-1}$; and compound **13** was safe up to 12.50 $mol \cdot L^{-1}$. Following LPS stimulation (1.00 $g \cdot mL^{-1}$) for 24 h, NO release in the model group increased significantly compared to the blank group ($P < 0.01$). Compared with the model group, compounds **1**–**3**, **6**, **11**–**16**, **17**, and **19** significantly inhibited NO release at tested concentrations (1.56–50.00 $mol \cdot L^{-1}$) ($P < 0.05$ or $P < 0.01$). Compound **5** showed no inhibitory effect at 50.00 $mol \cdot L^{-1}$ but exhibited significant inhibition at 12.50 and 25.00 $mol \cdot L^{-1}$ ($P < 0.05$). These results demonstrate that the tested compounds possess anti-inflammatory activity (Table 1).

Effects of isolated compounds on the release of NO in RAW264.7 cells ($\bar{x} \pm s$, n = 3)

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4. Conclusion and Discussion

Plants of the genus *Ligularia* are rich in sesquiterpenes, triterpenes, and phenylpropanoids, exhibiting antitumor and anti-inflammatory activities (Liao et al., 2023). This study isolated 21 compounds from the petroleum ether and n-butanol fractions of *Ligularia virgaurea*, including five sesquiterpenes (1–5), four lignans (6–9), nine phenylpropanoids (10–18), and three other compounds (19–21). Among these, compounds 1–4, 6, 11–16, 18, 20, and 21 were reported from *L. virgaurea* for the first time.

As a source plant for the Tibetan medicine "Rixiao," *L. virgaurea* has been traditionally used to clear latent heat, detoxify, heal sores, remove "yellow water," and dispel wind-dampness, yet its anti-inflammatory activity had not been previously reported. Using an LPS-induced RAW264.7 cell model, we identified 13 potential anti-inflammatory active constituents, including sesquiterpenes (1–3, 5), lignans (6), phenylpropanoids (11–16, 17), and a steroid (19). Literature reports indicate that compound **2** exerts anti-inflammatory effects via the LPS/NF- B pathway (Mora-Ramiro et al., 2020), while compound **5** lacks reported antitumor or antimicrobial activity, suggesting its pharmacological potential remains to be explored (Liu et al., 2007; Sun et al., 2007). Compound **6** inhibits 5-lipoxygenase to produce anti-inflammatory effects (Xia-Hou et al., 2022), compound **11** suppresses p38 MAPK signaling (Wei et al., 2023), compounds **12**, **13**, and **16** primarily exhibit antioxidant activity (Hu, 2013; Wang et al., 2019), compound **14** acts as a prodrug of ferulic acid with anti-inflammatory properties (Botti et al., 2022), and compound **15** (ethyl ferulate) interacts with multiple pathways and proteins, suggesting a multi-component, multi-target mechanism (Wang et al., 2023). Compound **17** inhibits 5-LOX and

COX-1 active sites (Resch et al., 2001), while compound **19** suppresses TNF-α-induced proliferation, migration, invasion, and inflammatory cytokine secretion in MH7A cells (Gu et al., 2023). This study enriches the chemical profile of *L. virgaurea*, clarifies its anti-inflammatory active constituents, and provides a foundation for further development and utilization of this medicinal plant.

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