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Eupalinolide N, a Previously Undescribed Sesquiterpene Lactone with Anti-inflammatory Activity from *Eupatorium lindleyanum*

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Abstract: A previously undescribed sesquiterpene lactone, named eupalinolide N (**1**), was isolated from traditional Chinese medicine "Ye-Ma-Zhui" (*Eupatorium lindleyanum*) by ethanol reflux extraction, reduced pressure concentration, macroporous resin column chromatography, and C_{18} reversed-phase chromatography. Its structure was elucidated by a comprehensive interpretation of spectroscopy evidence as well as ECD calculations. **1** showed anti-inflammatory activity by inhibiting the gene expressions of pro-inflammatory factors including *IL-1β*, *TNFα COX-2*, and *iNOS* at the concentration of 7.5 μM, as well as attenuating the excretion of NO, IL-6, and TNF-α in Raw 264.7 macrophages at the concentration of 15 μM.

Keywords: *Eupatorium lindleyanum*, sesquiterpene lactone, spectroscopic analyses, anti-inflammatory activity.

1. Introduction

"Ye-Ma-Zhui", the dry aerial part of *Eupatorium lindleyanum* DC., has been used as folk medicine in China for the treatment of tracheitis, tonsillitis, hypertension, and bacillary dysentery [1-2]. It had been reported that sesquiterpene lactones, diterpenoids, triterpenoids, flavonoids, alkaloids, coumarins, and volatile oil are active constituents that partly account for the therapy effects [3-6].

As a part of our series work of searching bio-activity compounds from Chinese folk medicine resources [7-10], a phytochemical investigation of *E. lindleyanum* was performed and a previously

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undescribed sesquiterpene lactone (**1**) was obtained. The structure of **1** was confirmed by comprehensive analyses of spectroscopic evidence including HR-ESI-MS, and NMR data combined with ECD calculations. The anti-inflammatory assay also indicated that **1** inhibits the gene expression of as well as to attenuates secretion of proinflammatory factors including iNOS and COX-2 obviously in Raw 264.7 macrophages. Therefore, we report the isolation, structural elucidation, and anti-inflammatory effects of compound **1** in this manuscript.

2. Materials and Methods

2.1. Instruments and Materials

We collected the infrared spectra by a PerkinElmer one FT–IR spectrometer (Perkin Elmer, USA) with KBr disks and ultraviolet spectra by a Perkin Elmer Lambda 35 UV-VIS spectrometer (Perkin Elmer, USA). Optical rotations was measured on a Perkin Elmer Model 241 polarimeter (Perkin Elmer, USA). We obtained circular dichroism spectra on a Chirascan CD spectrometer (Applied Photophysics Lid., Leatherhead, UK). The 1D and 2D NMR data were measured by a Bruker Ascend 600-MHz spectrometer (Bruker, MA, USA), and HR-ESI-MS was recorded on a Q Exactive UHMR Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, MA, USA). Column chromatography was performed using Sephadex LH-20 (GE-Healthcare Bio-Sciences AB, Uppsala, Sweden) and RP-18 silica gel (300-400 mesh, Shanghai, China). Silica gel and TLC (GF_{254}) which we used were from Qingdao Marine Chemical Co., Ltd., (Qingdao, China). We collected the semi-preparative HPLC by an NP7000 serials instrument (Hanbang Science and Technology, China) using an Ultimate PFP-C_{18} column (4.6×250 mm, 5 μm) (Welch Tech., China).

2.2. Isolation of the Compound

The dried *E. lindleyanum* (10 kg) was powdered. We extracted 3 times under reflux for 2 hours each time using 90% ethanol which is 10 times weight of medicinal powder. Then the extract is concentrated under pressure. The fluidic extract was further solvent in 10 times of water and separated into four fractions by AB-8 macroporous adsorption resin column chromatography (eluting stepwise with a methanol-water of 0:100, 30:70, 50:50, 80:20 *v/v*. The fractions 80:20 were further fractionated into four sub-fractions using a silica gal (300-400 mesh, eluting stepwise with a petroleum ether-ethyl acetate of 30:1, 20:1, 15:1, 10:1, 8:1 6:1, 4:1 and 1:1). The sub-fraction (eluting with 10:1) was further fractionated by C_{18} reverse phase chromatography packing under high pressure, methanol: water = 58:42 *v/v* as the mobile phase, detection wavelength 210 nm. Compound **1** was isolated with a retention time of 22.3 min.

2.3. ECD Calculations

Conformational searches for **1** was performed by Conflex 8 software [11]. To generate the energy-minimized conformers, we optimized the preliminary conformers via Gaussian 16 software. The calculated spectrum of compound **1** was generated by Boltzmann weighting and adjustment according to the experimental values [12]. Then we draw the ECD curve of conformer using the SpecDis 1.71 software and simulated by the TD-DFT method. The calculation details were elaborated in the supporting information.

2.4. Cytotoxicity Assay

Raw 264.7 macrophages were cultured in a DMEM medium containing 1% antibiotics (penicillin-streptomycin) and 10% fetal bovine serum which maintained in a humidified 5% CO_2 incubator at 37 °C [13,14]. The cytotoxic activities of compound **1** against Raw 264.7 macrophages was evaluated by MTT assay to detect the decrease of metabolically active cells [15, 16].

2.5. Quantification of NO Production

Raw264.7 macrophages were seeded with the density of 8×10^3 cells/well in the 96-well plate and incubated overnight. Then, these cells were treated with 0.1% percentage DMSO as the negative control group. The others were treated with compound **1** at 7.5, 15, and 30 μM for 1 h before being stimulated with 1 μg/mL LPS for another 24 h. Finally, a Griess reagent system was applied to measure NO production [12,13].

2.6. RT-PCR

Raw 264.7 macrophages were plated in 6 well plates with the density of 1×10^5 cells/well. The cells were treated with 7.5, 15, and 30 μM **1** or DMSO for 1h when the density of cells achieved 60%. Then treated with 1 μg/mL LPS for 24 h. The next, Raw 264.7 macrophages were collected, and the total RNA was isolated using the TRIzol reagent. After the RNA concentrations was detected by Ultramicro spectrophotometer, 1 μg RNA was reverse transcribed into cDNA by HiScript II Q RT SuperMix for qPCR (+gDNA wiper). RT-PCR experiments were performed using ChamQ Universal SYBR qPCR Master Mix and Analytikjena, and cDNA was analyzed as template. The details of primers for RT-PCR can be found in our previous work [17].

2.7. Measurement of Pro-inflammatory Cytokine Production

Raw 264.7 Macrophages in a 96-well plate (8×10^3 cell/well) were treated with compound **1** (7.5, 15, 30μM) for 1 h, and then treated with LPS 1 μg/mL for 24 h. The culture media were collected and centrifuged at 12 000 rpm for 15 min. Next, the level of pro-inflammatory cytokines IL-6 and TNF-α were determined by enzyme-linked immunosorbent assay (ELISA) with 50 μL supernatant according to the instructions [18-19].

2.8. Statistical Analysis

We analyzed the date by GraphPad Prism 7.0 software (San Diego, CA, USA). The date showed as the mean ± standard error of the mean (SEM). Differences between groups is assessed using the unpaired two-tailed Student's t-test. **P*-value < 0.05 is considered statistically significant.

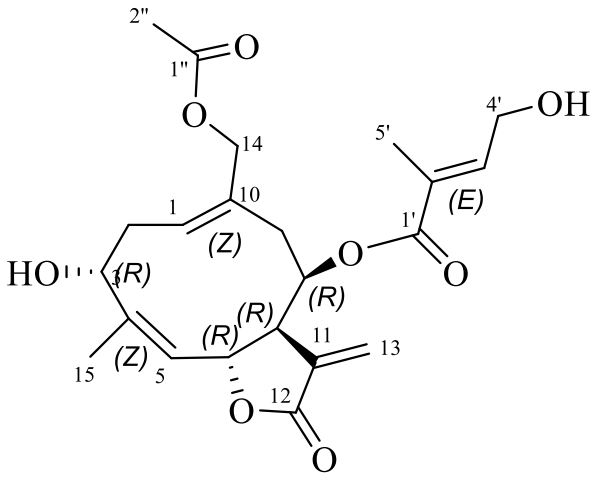

Figure 1. Chemical structure of compound **1**

Table 1. ^{1}H (600 MHz) and ^{13}C NMR (150 MHz) data of **1** (δ in ppm, J in Hz) in CD_3OD

Position	δ_H	δ_C	Position	δ_H	δ_C
1	5.44, t (8.0)	132.3	12	-	171.8
2	2.69, t (13.7)	34.5	13	6.27	125.6
	2.18, q (11.7)			5.92	
3	4.56, dd (11.4, 5.1)	70.2	14	4.92, d (12.8)	63.7
				4.62, d (12.8)	
4	-	141.8	15	1.79, s	17.7
5	5.19-5.23, m	124.8	1′	-	167.5
6	5.35, d (10.5)	76.0	2′	-	128.3
7	3.19, s	49.4	3′	6.73, t (5.1)	143.8
8	5.19-5.23, m	81.2	4′	4.24, d (5.5)	59.8
9	2.97, d (12.5)	38.6	5′	1.76, s	12.6
	2.37, d (14.1)				
10	-	133.8	1″	-	172.3
11	-	139.5	2″	1.99, s	20.7

3. Results and Discussion

3.1. Structure Elucidation

Eupalinolide N (**1**) is a powder. The molecular formula was assigned by the pseudo-molecular ion peak at *m/z* 443.1675 $[M+Na]^+$ (calculated for 433.1676, $C_{22}H_{28}O_8Na$). A comprehensive analysis of its ^{1}H-NMR, ^{13}C-NMR and HMQC spectra indicates the presence of ester [δc: 172.3 (C-1″), 171.8 (C-12), 167.5 (C-1′)]; eight olefinic moieties [δ_H: 6.73 (H-3′), 6.27 (H-13a), 5.92 (H-13b), 5.44 (H-1), 5.19-5.22 (H-5); δc: 143.8 (C-3′), 141.8 (C-4), 139.5 (C-11), 133.8 (C-10), 132.3 (C-1), 128.3 (C-2′), 125.6 (C-13), 124.8 (C-5)]; five oxy-bearing methylenes and methines [δ_H: 5.35 (H-6), 5.19-5.23 (H-8), 4.93 (H-14a), 4.58 (H-14b), 4.56 (H-3), 4.24 (H-4′); δ 81.2 (C-8), 76.0 (C-6), 70.2 (C-3), 63.7 (C-14), 59.8 (C-4′)]; two methylenes [δ_H: 2.97 (H-9a), 2.69 (H-2a), 2.37 (H-9b), 2.18 (H-2b); δc: 38.5 (C-9), 34.5 (C-2)] and three methyls [δ_H: 1.99 (H-2″), 1.79 (H-15), 1.76 (H-5′); δc: δ 20.7 (C-2″), 17.7 (C-15), 12.6 (C-5′)]. The ^{1}H-^{1}H COSY correlations of H-1/H-2/H-3, of H-5/H-6/H-7/H-8/H-9, of H-3′/H-4′ as well as the HMBC correlations from H-15 to C-3, C-4, C-5; from H-13 to C-11, C-12, C-7; from H-8 to C-1′; from H-5′ to C-1′, C-2′, C-3′from H-14 to C-1″, C-10, C-1, C-9 and from H-2″ to C-1″ established the planar structure of **1**.

The planar structure of **1** is like eupalinolide I except for a proton instead of an acetyl moiety at C-3 [4]. Its relative configuration was deduced to be 1*Z*, 3*R**, 4*Z*, 6*R**, 7*R**, 8*R**, 2″*E* by the NOESY correlations of H-14/H-2, H-15/H-5, H-4′/H-5′, H-5/H-7, H-7/H-8, H-6/H-14 as well as the coupling constants of H-3 [4]. The calculated CD spectrum of 1Z,4Z,2'Z,3R,6R,7R,8R-1 neatly matches the experimental curve. The absolute configuration of **1** was confirmed as shown in Figure 2.

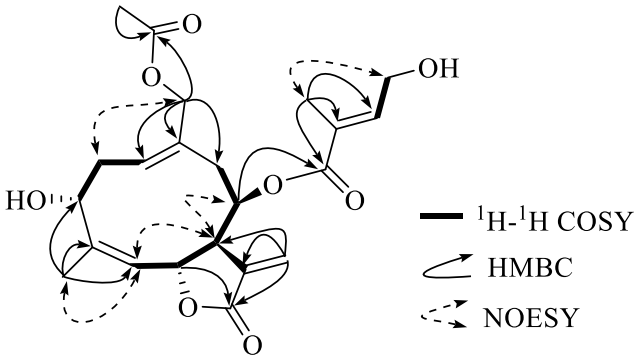

Figure 2. Key ^{1}H–^{1}H COSY, HMBC and NOESY (double arrows) correlations of **1**

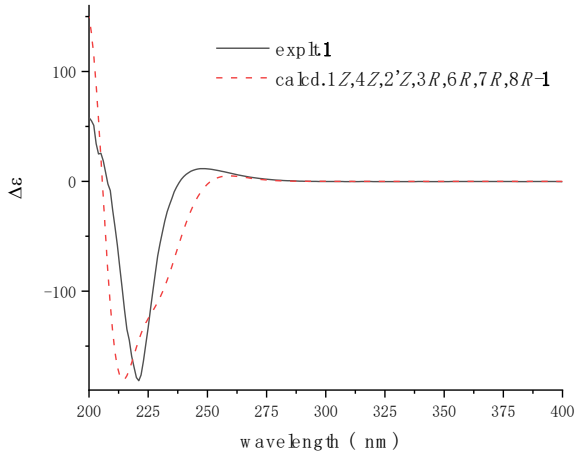

Figure 3. Calculated ECD spectra and experimental ECD curve of **1**

3.2. Anti-inflammatory Activity

As eupalinolide N did not show obvious cytotoxicity against Raw 264.7 macrophages at 60 μM (Figure 4a). Further pharmacological assays were carried out to study its anti-inflammatory activity (Figure 4b). Compound **1** can dramatically attenuate the secretion of NO at 7.5μM. RT-PCR results also displayed that **1** can significantly inhibit the gene expression of *TNF-α*, *IL-1β*, *COX-2*, and *iNOS* (Figure 4c). Besides, ELISA assay also showed that can attenuate the production of IL-1β at 7.5μM and TNF-α at 15 μM (Figure 5). Hence, compound **1** might be a hit compound for the treatment of inflammatory-related diseases.

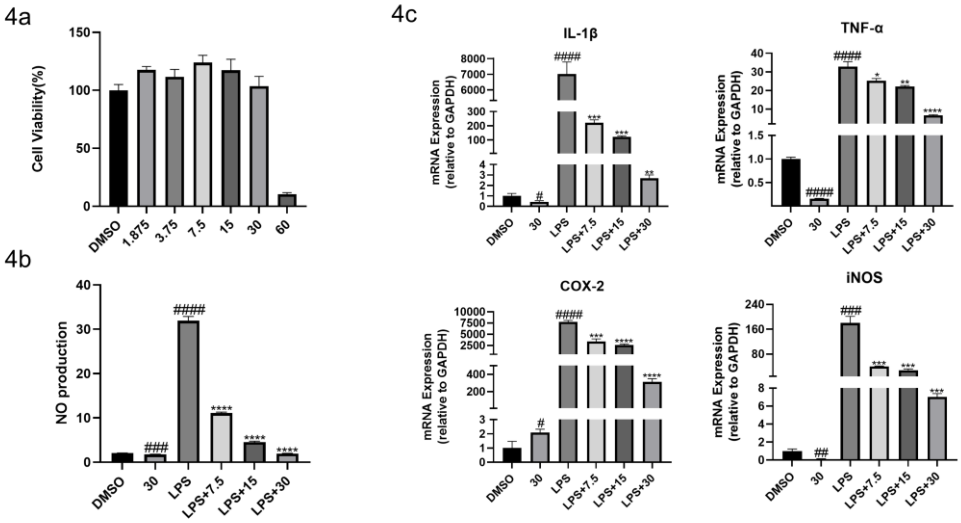

Figure 4. 4a: Cell viability of compound **1** against RAW264.7 macrophages. **4b**: NO production in LPS-stimulated RAW264.7 macrophages from compound **1**. **4c**: The expression of mRNA about IL-1β, TNF-α, COX-2, and iNOS in LPS-stimulated RAW264.7 macrophages from compound **1**

Data are expressed as mean ± SD. $^{###}p < 0.001$, compared with the normal control group. $^{***}p < 0.001$, compared with the LPS group, $^{####}p < 0.0001$, compared with the normal control group. $^{****}p < 0.0001$, compared with the LPS group.

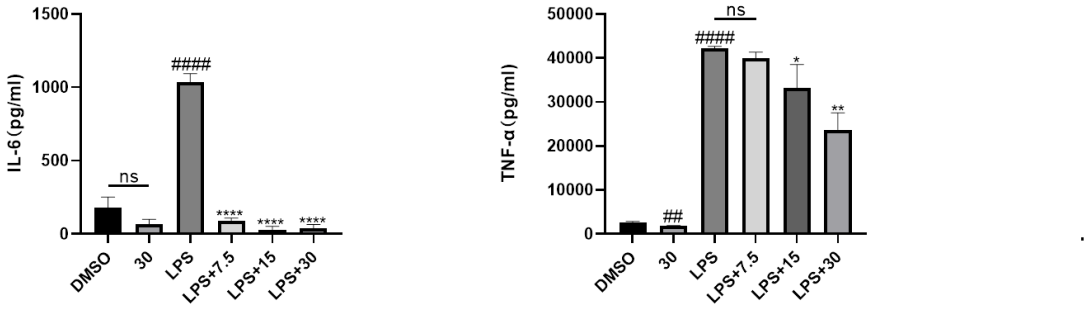

Figure 5. IL-6 and TNF-α production in LPS-stimulated RAW264.7 macrophages from compound **1**
Data are expressed as mean ± SD. $^{\#\#}p<0.01$, $^{\#\#\#\#}p<0.0001$, compared with the normal control group. $^{*}p<0.1$, $^{**}p<0.01$, $^{****}p < 0.0001$, compared with the LPS group.

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

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