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"Research on the Anti-inflammatory Activity Components and Structure-Activity Relationship of Sesquiterpene Lactones from Saussurea involucrata"

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1. Introduction

Saussurea involucrata (Asteraceae), a perennial herbaceous species of the genus Saussurea, is recognized as a rare alpine plant that has attracted substantial scientific attention due to its intricate phytochemical composition and unique pharmacological properties [1]. Although inflammation constitutes an essential physiological defense mechanism against tissue damage and pathogenic insults, growing evidence suggests that chronic inflammatory processes are pathologically associated with diverse dermatological and autoimmune conditions, such as eczema, acne, and systemic lupus erythematosus [2]. Recent advances in phytochemical research have identified sesquiterpene lactones as principal bioactive constituents in *S. involucrata*, which exhibit significant anti-inflammatory effects mediated through multiple pharmacological mechanisms [3-5]. However, despite increasing documentation of its anti-inflammatory potential [6], the specific bioactive compounds responsible for these effects and their corresponding structure-activity relationships remain to be fully elucidated.

This study systematically investigates the anti-inflammatory components and mechanisms of sesquiterpene lactones in *S. involucrata*. Through chromatographic isolation and structural identification of diverse sesquiterpene lactones coupled with in vitro bioactivity assessments using LPS-induced RAW264.7 macrophage models, we delineate their molecular mechanisms in modulating pro-inflammatory mediators, such as tumor necrosis factor (TNF)- α, Interleukin (IL)-6, IL-1β, prostaglandin (PE)G_2, via nuclear factor kappa-B (NF-κB) signaling pathways, thereby elucidating structure-activity relationship (SAR) profiles. The findings are anticipated to: Reveal the phytochemical basis underlying *S. involucrata*'s medicinal properties, advancing the functional characterization framework of sesquiterpene lactones; Establish structure-performance correlations of bioactive lactones, providing a pharmacological foundation for developing functional cosmeceutical products targeting inflammatory dermatoses including acne vulgaris and atopic dermatitis; Enhance evidence-based validation of botanical anti-inflammatory efficacy to optimize product biocompatibility and promote standardized applications of plant-derived actives in dermocosmetic formulations.

2. Materials and Methods

2.1 General experimental procedures

Optical rotations were measured with Model 343 polarimeter (Perkin-Elmer Instruments). 1D and 2D nuclear magnetic resonance (NMR) spectra were performed on JNM-EXC 400 (FT-NMR system, 400 MHz, JEOL Co.) using $CDCl_3$ as a solvent for measurement. The chemical shift values are reported in ppm relative to the solvent used. Sephadex^{TM} LH-20 (GE healthcare Co, Sweden) was used for column chromatography, and precoated silica gel 60 F-254 plates (Merck LTD., Germany) were used for thin-layer chromatography (TLC). UV spectra were recorded on a Biochrome Libra S22 spectrophotometer. The spots on TLC were detected by spraying with 3% $KMnO_4$ aqueous solution or Anisaldehyde solution with 5% H_2SO_4 and then heating on a hot plate.

2.1. Plant material

The *S. involucrata* plant material was collected in January 2024 from the Tianshan Mountains, Xinjiang, China. The specimen (No. 102) was preserved in the laboratory of Beijing Academy of TCM Beauty Supplemengts Co.

2.2. Extraction and Isolation

The aerial parts of *S. involucrata* (500 g) were extracted two times with 70% aqueous ethanol (10.0 L) under stirring for 24 h at room temperature. The extracted solution was filtered, and the filtrate was concentrated under reduced pressure and freeze drying to afford a tan powder (113.5 g). A portion of the extract (100.0 g) was suspended in water (8.0 L) and fractionated into *n*-hexane (*n*-Hex, 6.1 g), dichloromethane (CH_2Cl_2, 9.3 g), ethyl acetate (EtOAc, 8.5 g), *n*-butanol (BuOH, 14.5 g) and water (H_2O, 60.2 g) fractions.

The soluble fraction of EtOAc (8.5 g) was subjected to vacuum liquid chromatography (VLC) on silica gel using a step-gradient solvent (*n*-hexane-ethyl acetate-methanol) to give 32 fractions (Frs V1 to V32). The eluate was eluted with chloroform-methanol (15:1) and further purified by Sephadex LH-20 column chromatography to give compound 3 (10.3 mg). The *n*-Hex soluble fraction (6.1 g) was subjected to vacuum liquid chromatography (VLC) on silica gel using a step gradient solvent (hexane-ethyl acetate-methanol) to give 20 fractions (Frs VH 1 to VH 20). Compound 1 (14.2 mg) was further purified by Sephadex LH-20 column chromatography using chloroform-methanol (20:1) as eluent. Compound 4 (12.3 mg) was further purified by Sephadex LH-20 column chromatography using chloroform-methanol (10:1) as eluent. Compound 2 (4.2 mg) was further purified by Sephadex LH-20 column chromatography using chloroform-methanol (3:1) as eluent. CH_2Cl_2 soluble fraction (9.3 g) was subjected to vacuum liquid chromatography (VLC) on silica gel using a step gradient solvent (*n*-hexane-ethyl acetate-methanol) to give 28 fractions (Frs V1 to V28). The eluate was eluted with chloroform-methanol (13:1) and further purified by Sephadex LH-20 column chromatography to give compound 5 (10.3 mg).

2.3. Anti-inflammatory activities

2.3.1. Cell culture

The murine macrophage cell line RAW264.7 was purchased from American Type Cell Culture (ATCC, USA). RAW264.7 cells were cultured in Dulbecco's modified Ea-gle's medium (DMEM, GIBCO Inc., USA) supplemented with 100 U/mL of penicillin, 100 μg/mL of streptomycin and 10% fetal bovine serum (FBS, GIBCO Inc., USA). The cells were incubated in an atmosphere of 5% CO_2 at 37°C.

2.3.2. Measurement of NO production

Nitric oxide production was determined by measuring nitrite in the supernatant of cultured RAW264.7 cells. The cells were seeded in 24-well culture plates at a density of 2×10^5 cells/well for 18 h. The cells were then stimulated with LPS (100 ng/mL) and various concentrations of samples for 24 h. After, the supernatant was mixed with an equal volume of Griess reagent and was incubated at room temperature for 10 min. Absorbance was measured at 540 nm. The nitrite amounts in the test samples were calculated from $NaNO_2$ standard curve.

2.3.3. Cell viability

Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl te-trazolium bromide (MTT) assay. RAW264.7 cells (2×10^5 cells/well) were incubated in 24-well plates for 18 h. The cells were then stimulated with LPS (100 ng/mL) in the presence of various concentrations of samples and cultured for 24 h. MTT (500 μg/mL) reagent was added to the medium and left to stand for 4 h. After removing the super-natant, the formazan crystals were dissolved in dimethyl sulfoxide (DMSO) and the absorbance was measured at 570 nm.

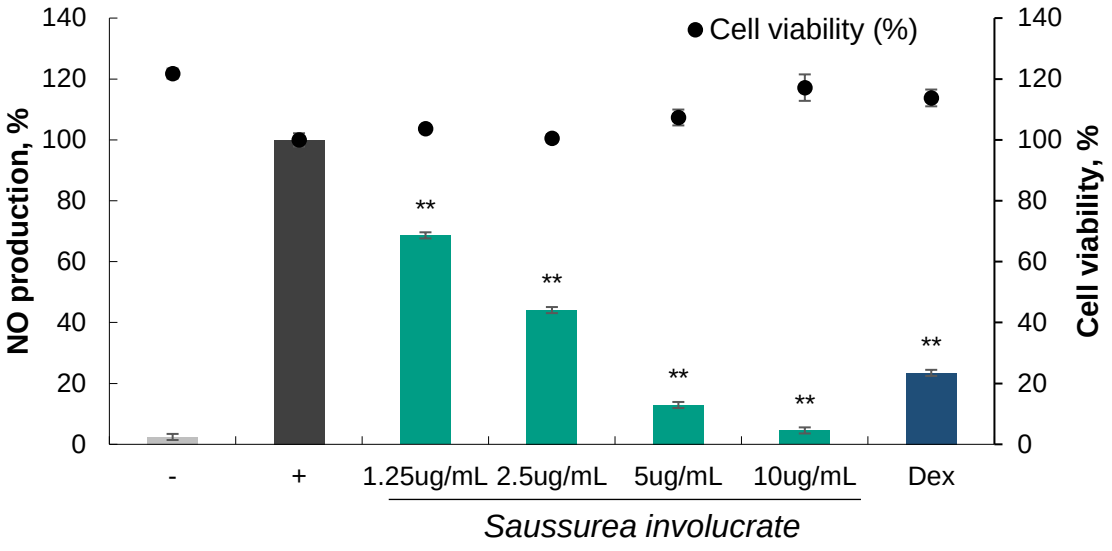
2.3.4 Detection of TNF-α, IL-6, IL-1β and PGE_2

Enzyme-linked immunosorbent assay (ELISA) was used to determine the production of TNF-α, IL-6, IL-1β, and PGE_2. RAW264.7 cells (2×10^5 cells/well) were dispensed in 24-well plates and cultured for 18 h. The cells were then stimulated with LPS (1 μg/mL) and various concentrations of samples for 24 h. After, the supernatant was harvested and were then assayed according to the ELISA kit (TNF-α; IL-6: BD Biosciences, IL-1β; PGE_2: Invitrogen).

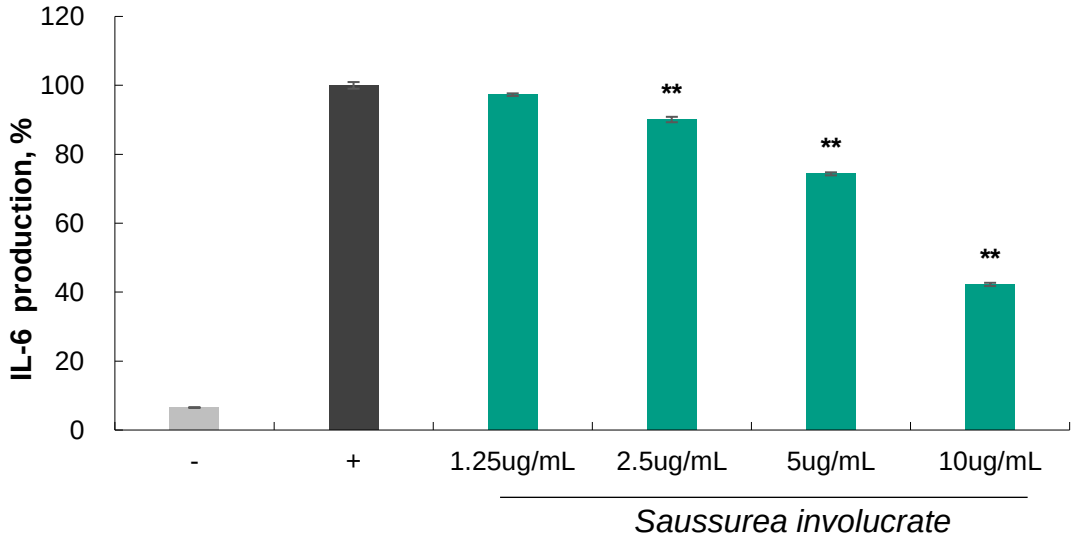
3. Results

3.1. The anti-inflammatory ability of S. involucrata extract

Lipopolysaccharide (LPS) was used to stimulate RAW264.7 cells, NO production was determined, and the anti-inflammatory activity of the 70% ethanol extract was investigated. First, we used extracts with concentrations of 1.25-10 μg/mL for cytotoxicity and NO production probing. The results, as shown in Figure 1 (a), showed that the *S. involucrata* extract did not cause cytotoxicity in the range of 1.25-10 μg/mL and inhibited NO production in a concentration gradient with an IC_{50} value of 2.35 μg/mL. In addition, in order to further elucidate the mechanism of the inhibition of inflammation, when LPS stimulated the RAW264.7 cells they secreted a variety of pro-inflammatory cytokines, such as IL-16. Therefore, the expression of IL-6 was detected using an enzyme-linked immunosorbent assay (ELISA) kit. The results, as shown in Figure 1 (b), showed that *Saussurea involucrata* extract significantly inhibited IL-6 production in the concentration range of 2.5-10 μg/mL (p<0.01).



(a)



(b)

Figure 1. (a) Effects of extract from the aerial parts of *S. involucrata* on NO production and cell viability in LPS-stimulated RAW264.7 cells.(b) Ability of *Saussurea involucrata* extract to inhibit IL-6 production. (The data represent the mean ± SD of triplicate experiments. **p* <0.05; ***p*<0.01 "-" denotes induction without inducer, "+" denotes induced by LPS, same below.)

3.2.Sesquiterpene lactones isolated from S. involucrata extracts

For further isolation and purification of *S. involucrata* extract, *n*-Hex, CH_2Cl_2, and EtOAc fractions were subjected to repetitive column chromatography on Sephadex LH-20 elution of different solvent systems, respectively, yielding five known compounds 1-5 (Figure 2): Dehydrocostus lactone (1) [7], Zaluzanin C (2) [8], 3α,8α-dihydroxy-11βH-11,13-dihydrodehydrocostus lactone (3) [9], Mokko lactone (4) [7], 11βH-11,13-dihydrodehydrocostus lactone-8-*O*-β-D-glucopyranoside (5) [10]. The chemical structures of the isolates were characterized by analyzing the ^{1}H and ^{13}C NMR spectral data of the five compounds in Table 1.

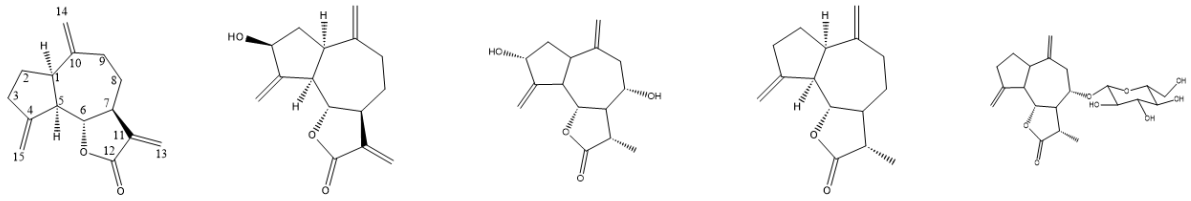
Table 1. NMR data of compounds 1-3.

No	Compound 1		Compound 2		Compound 3	
	δ_H (int, mult, *J* Hz)	δ_C	δ_H (int, mult, *J* Hz)	δ_C	δ_H (int, mult, *J* Hz)	δ_C
1	2.81-2.92 (1H, m)	45.3	2.81-2.92 (1H, m)	44.3	3.08 (1H, m)	44.2
2	1.92 (2H, m)	30.5	1.92 (2H, m)	39.1	2.53 (1H, m) 2.21 (1H, over-lapped)	39.7
3	2.42-2.58 (2H, m)	36.4	4.52 (1H, br t, *J* = 7.5)	73.6		73.8
4		149.4		153.1		154.1
5	2.81-2.92 (1H, m)	52.2	2.87 (1H, dd, *J* = 9.0, 17.5)	50.0		50.9
6	3.93 (1H, t, *J* = 9.2)	85.4	4.06 (1H, dd, *J* = 9.0, 9.0)	84.1	4.02 (1H, t, *J* = 9.6)	79.8
7	2.81-2.92 (1H, m)	47.8	2.81-2.92 (1H, m)	45.7	2.21 (1H, over-lapped)	56.2
8	2.22 (1H, m) 1.41 (1H, m)	31.1	2.22 (1H, m) 1.41 (1H, m)	30.7	3.83 (1H, dt, *J* = 8.3, 5.1)	75.4
9	2.42-2.58 (1H, m) 2.14 (1H, m)	32.8	2.42-2.58 (1H, m) 2.14 (1H, m)	34.3	2.89 (1H, m) 2.21 (1H, over-lapped)	50.2
10		139.9		148.1		143.7
11	2.47 (1H, m)	151.4	2.47 (1H, m)	139.8	2.77 (1H, m)	42.2
12		170.4		170.3		179.0
13	6.18 (1H, d, *J* = 3.2) 5.46 (1H, d, *J* = 3.2)	120.3	6.16 (1H, d, *J* = 3.0) 5.45 (1H, d, *J* = 3.0)	120.4	1.39 (3H, d, *J* = 7.3)	16.9
14	5.24 (1H, br s) 5.03 (1H, br s)	109.8	4.96 (1H, br s) 4.90 (1H, br s)	114.5	4.96 (1H, s) 4.86 (1H, s)	112.2
15	4.86 (1H, br s) 4.78 (1H, br s)	112.8	5.39 (1H, br s) 5.28 (1H, br s)	111.3	5.40 (2H, d, *J* = 1.5)	113.8

Table 2 NMR data of compounds 4 and 5.

NO	Compound 4		Compound 5	
	δ_H (int, mult, *J* Hz)	δ_C	δ_H (int, mult, *J* Hz)	δ_C
1	2.78 (1H, m)	47.3	2.97（1H,br q, *J* = 8.0）	47.0
2	2.08-2.29 (1H, m) 1.68-1.75 (1H, m)	30.4	1.92 (2H, m)	32.0
3	2.47 (2H, m)	32.7	2.52 (2H, m)	29.6
4		151.9		152.0
5	2.70 (1H, br dd, *J* = 9.5, 8.0)	52.2	2.81（1H, br t, *J* = 9.1）	52.6
6	3.90 (1H, t, *J* = 9.5)	85.5	4.02 (1H, t, *J* = 9.6)	80.7
7	2.78 (1H, m)	42.3	2.30 (1H, q, *J* = 10.0)	53.6
8	2.08-2.29 (1H, m) 1.43 (1H, m)	32.7	3.72 (1H, m)	84.1
9	2.45 (1H, ddd, *J* = 6.0, 6.0, 12.0) 2.10 (1H, m)	37.8	2.36 (1H, dd, *J* = 5.5, 13.3) 2.95 (1H, dd, *J* = 8.2, 12.9)	44.2
10		150.2		145.1
11	2.47 (1H, m)	50.1	2.76 (1H, dq, *J* = 7.0, 10.3)	40.8
12		178.9		180.5
13	1.24 (3H, d, *J* = 7.0)	13.4	1.42 (3H, d, *J* = 7.3)	15.5
14	4.86 (1H, br s) 4.76 (1H, br s)	112.1	4.90 (1H, s) 5.04 (1H, br s)	108.2
15	5.18 (1H, d, *J* = 1.8) 5.03 (1H, d, *J* = 1.8)	109.4	5.04 (1H, br s) 5.16 (1H, s)	113.5

1`	4.47 (1H, d,*J* = 4.8)	103.9
2`	3.22 (1H, br t, *J* = 9.0)	74.1
3`	3.37 (1H, br t, *J* = 9.0)	77.3
4`	3.30 (1H, br t, *J* = 9.0)	70.2
5`	3.26 (1H, br t, *J* = 7.4)	76.5
6`	3.89 (1H, d, *J* = 6.7, 13.3) 3.76 (1H, m)	61.4



Dehydrocostus lactone | Zaluzanin C | 3α,8α-dihydroxy-11*β*H-11,13-dihydrodehydrocostus lactone | Mokko lactone | 11*β*H-11,13-dihydrodehydrocostus lactone-8-*O*-β-D-glucopyranoside

Figure 2. The chemical structures of compounds 1-5.

3.3. The anti-inflammatory ability of five compounds isolated from S. involucrata

The anti-inflammatory activity of compounds **1-5** was evaluated by measuring NO molecular levels in LPS-stimulated RAW264.7 macrophages. The results, as shown in Figure 3, showed that all five compounds significantly reduced NO production at 50, 100, and 200 μM in a dose-dependent manner. Five compounds—dehydrocostus lactone, zaluzanin C, 3α,8α-dihydroxy-11βH-11,13-dihydrodehydrocostus lactone, mokko lactone, and 11βH-11,13-dihydrodehydrocostus lactone-8-*O*-β-D-glucopyranoside—with the IC_{50} for NO production being 0.8, 3.5, 126.3, 113.2, and 301.9 μM.

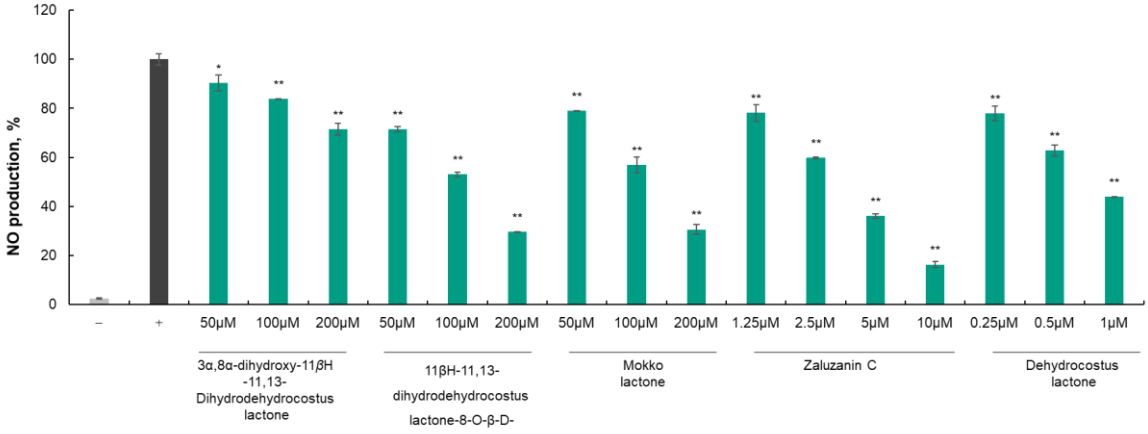
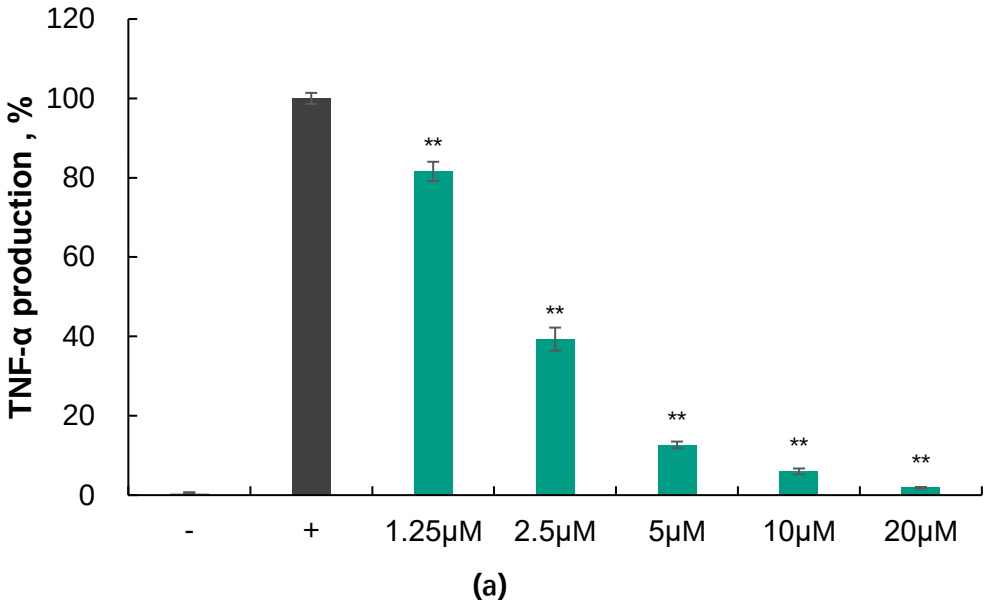


Figure 3. Effect of five compounds on NO production in LPS-stimulated RAW264.7 cells.

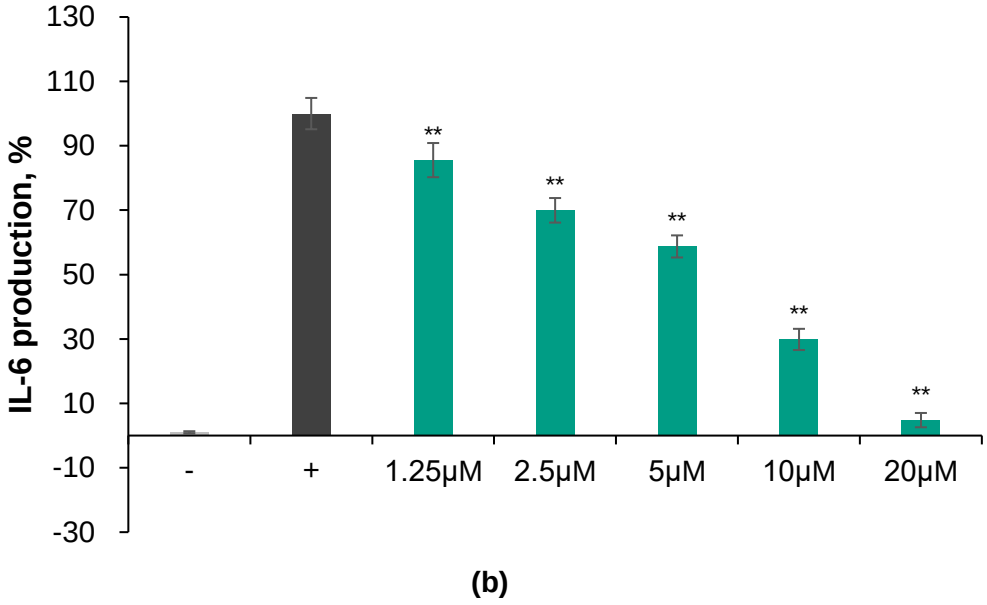
3.4. The anti-inflammatory ability of zaluzanin C

Since Zaluzanin C has the strongest ability to inhibit NO production, the content of pro-inflammatory cytokines, such as TNF-α, IL-6 and IL-1β, was further measured, and the content of

PGE_2, an important inflammatory mediator produced by cyclooxygenase (COX)-2, was also quantified [11]. The results are shown in Figure. 4(a-d), Zaluzanin C significantly ($p<0.01$) inhibited the production of TNF-α (Figure 4(a)), IL-6 (Figure 4(b)), IL-1β (Figure 4(c)) and PEG_2 (Figure 4(d)) in a concentration-dependent manner in the concentration range of 1.25-20 μM.



(a)



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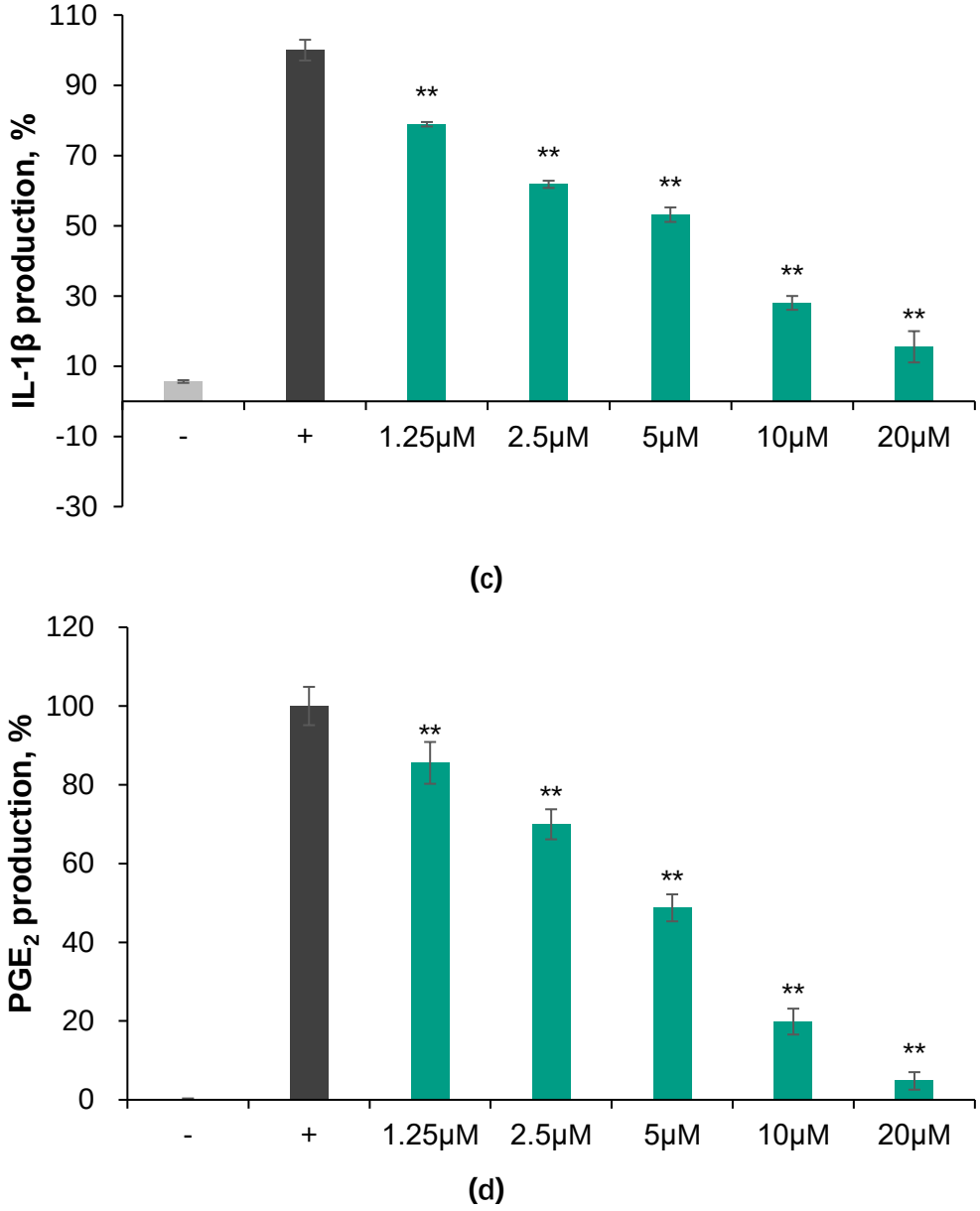

Figure 4. Effect of Zaluzanin C on TNF-α (a), IL-6 (b), IL-1β (c) and PEG_2 (d) production in LPS-stimulated RAW264.7 cells by ELISA for TNF-α, IL-6, IL-1β and PEG_2 production and release.

4. Discussion

In the present study, five sesquiterpene lactones were successfully isolated from *S. involucrata* and their anti-inflammatory activities were systematically evaluated. The results showed that the 70% ethanol extract of *S. involucrata* and its polar parts (*n*-Hex, CH_2Cl_2, and EtOAc) exhibited significant NO and IL-6 inhibitory abilities, among which Zaluzanin C showed the most prominent anti-inflammatory effect (IC_{50}=3.5 μM) and inhibited concentration-dependently the release of key inflammatory factors such as TNF-α, IL-1β, and PGE_2.

By analyzing the structure-activity relationship of the compounds, it was found that the presence of α,β-unsaturated lactone ring and free hydroxyl group may be the key moieties for the anti-inflammatory activity [12]. For example, Zaluzanin C was significantly more active than the

other compounds due to the synergistic effect of the double bond at the C-11 position and the hydroxyl group at the C-8 position[13], whereas glycosidization (e.g., Compound 5) may reduce the activity due to the spatial site barrier[3]. This finding is consistent with previous studies that the α-methylene-γ-lactone structure of sesquiterpene lactones may exert anti-inflammatory effects by inhibiting the NF-κB pathway[14].

In conclusion, *S. involucrata* sesquiterpene lactones (especially Zaluzanin C) have significant potential in anti-skin inflammation applications, and their constitutive relationships provide new ideas for natural product activity optimization.

5. Conclusion

The results indicate that the *S. involucrata* extract and its *n*-Hex, CH_2Cl_2, and EtOAc fractions all exhibit strong inhibitory effects on NO and IL-6. The *S. involucrata* extract is rich in sesquiterpene lactones. Five compounds—dehydrocostus lactone, zaluzanin C, 3α,8α-dihydroxy-11βH-11,13-dihydrodehydrocostus lactone, mokko lactone, and 11βH-11,13-dihydrodehydrocostus lactone-8-*O*-β-D-glucopyranoside—exhibited good anti-inflammatory activity, with the half-inhibitory concentrations (IC_{50}) for NO production being 0.8, 3.5, 126.3, 113.2, and 301.9 μM, respectively. Zaluzanin C has shown the strongest ability to inhibit NO production. Additionally, zaluzanin C exhibits concentration-dependent inhibitory effects on the inflammatory mediators TNF-α, IL-6, IL-1β, and PGE_2. Upon integrating the chemical structures and anti-inflammatory capabilities of the five sesquiterpene lactones, it was found that the presence of functional groups such as double bonds and hydroxyl groups, as well as their spatial arrangement, play a crucial role in determining the compounds' inhibitory potency against inflammatory mediators.

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