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Anti-inflammatory Properties of an Active Sesquiterpene Lactone and its Structure-Activity Relationship

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Abstract

A sesequiterpenoid, 2α-hydroxyl-3β-angeloylcinnamolide (HAC) was isolated from the Chinese medicinal herb *Polygonum jucundum* Lindex. (Polygonaceae) with anti-inflammatory activities *in vivo*. In the present study, we investigated the anti-inflammation effects of HAC on lipopolysaacharide (LPS)-induced murine RAW264.7 cells. As the results, we found that HAC dose-dependently decreased NO over-production with IC_{50} value of 17.68 μM but showed very weak inhibition on TNF-α release with IC_{50} value of 98.66 μM. Meanwhile, eight novel derivatives modified at C-2 position of HAC were synthesized to further explore the structure-activity relationships (SARs) of HAC on anti-inflammation effects. Compound PJH-1, an acetyl easer of HAC, showed better inhibition on over-production of NO and TNF-α (IC_{50}, 7.31 and 3.38 μM, respectively). Furthermore, we demonstrated that HAC and PJH-1 attenuates the mitogen-activated protein kinases (MAPK) signaling pathways through blocking the phosphorylation of ERK, p38, JNK/MAPK. We also found that the structure of PJH-1 are more stable than that of HAC in cell medium, these finding are useful to develop *in vitro* molecular mechanism research of HAC. In a conclusion, our studies enhance the understanding of anti-inflammation activities of HAC and lead to the discovery of novel derivatives as potential anti-inflammation agents.

Keywords: 2α-hydroxyl-3β-angeloylcinnamolide (HAC); Anti-inflammation effects; iNOS expression; Mitogen-activated protein kinases (MAPK); Chemical-structural modification.

Introduction

Inflammation is the first response of a tissue to injury, it can be classed as both acute and chronic inflammations. Chronic inflammation is a persistent one, which cause progressive damage to the body [1]. Macrophages play a key role in the specific and non-specific immune responses during the inflammation process, after macrophages are activated by LPS, large amounts of the cytokines and inflammatory mediators will be released [2-4]. Among the many pro-inflammatory mediators, NO is a key one in inflammation reactors [5] which is a free radical produced from L-argine by nitric oxide synthases (NOS) and is known to regulate various physiological functions in many tissues [6], however, excessive NO has been implicated in various pathological processes. The inhibition of NO overproduction has been suggested to be an important therapeutic approach for treatment of inflammation [7,8]. Expression of the iNOS in macrophages is regulated mainly at the induction of transcription factors through mitogen-activated protein kinases (MAPKs).

The aerial parts of *Polygonum jucundum* Lindex. (Polygonaceae) is used as traditional Chinese herbs for inhibiting inflammation, lowering serum cholesterol levels, and treating rheumatism [9-11]. In our previous study, a drimane-type sesquiterpenoid, 2α- hydroxyl - 3β – angeloyl -cinnamolide (HAC) from *P. jucundum*, was identified with anti-inflammatory effects by oral administration effects by oral administration at dose of 50-200mg/kg in mouse, and a sensitive and rapid LC-MS method was developed to study its pharmacokinetics and distribution in rats [12-14]. Up to now, some natural sesquiterpenoids were shown to possess significant inhibitions on pro-inflammation mediator production [15-17]. The drimane-type sesquiterpenoids has been identified with anti-inflammatory properties [18]. Therefore, in this study, we investigated the effects of HAC on the release of LPS-induced pro-inflammatory mediators and explored the molecular mechanism in terms of inflammatory signaling pathways. Meanwhile, eight novel derivatives modified at carbon-2 position of HAC were synthesized (Scheme 1) to further explore structure-activity relationships (SARs) of HAC on LPS-activated RAW264.7 cells. These studies also lead to a better understanding of the structure-activity relationship for the sesquiterpene lactones family and the discovery of novel derivatives as potential anti-inflammation agents.

Materials and Method

Cell culture

RAW 264.7 cell line was obtained from the Cell Bank of Chinese Academic of Sciences, Shanghai, China. The cells were cultured in DMEM, supplemented with 10% fetal bovine serum, 100 units/mL of penicillin, 100 μg/mL of streptomycin, 2 mM L-glutamine, and 1 mM nonessential amino acids, incubated at 37°C in a humidified atmosphere containing 5% CO_2.

Anti-inflammatory effect on RAW264.7 cells

Cytotoxicity assay: Cell viability was assessed by the MTT staining method [23]. All samples were firstly dissolved in DMSO and then diluted with DMEM, the final concentration of DMSO in tested samples is lee than 0.1%. Briefly, cells at 1×10^5 cells/mL were seeded into 96-well microplates and treated with various samples at 100 μM for 24 h. The culture medium was eliminated and 100 μL/well of 5 mg/mL solution of MTT with PBS buffer (10 mM Na_2HP0_4, 1 mM KH_2PO_4, 137 mM NaCl and 2.7 mM KCl, PH 7.4) was added to the cells which were then incubated at 37°C for 4 h. The supernatant was eliminated and the colored metabolite was dissolved in DMSO (100 μL/well). Absorbance was measured at 570 nm with the aid of a microplate reader.

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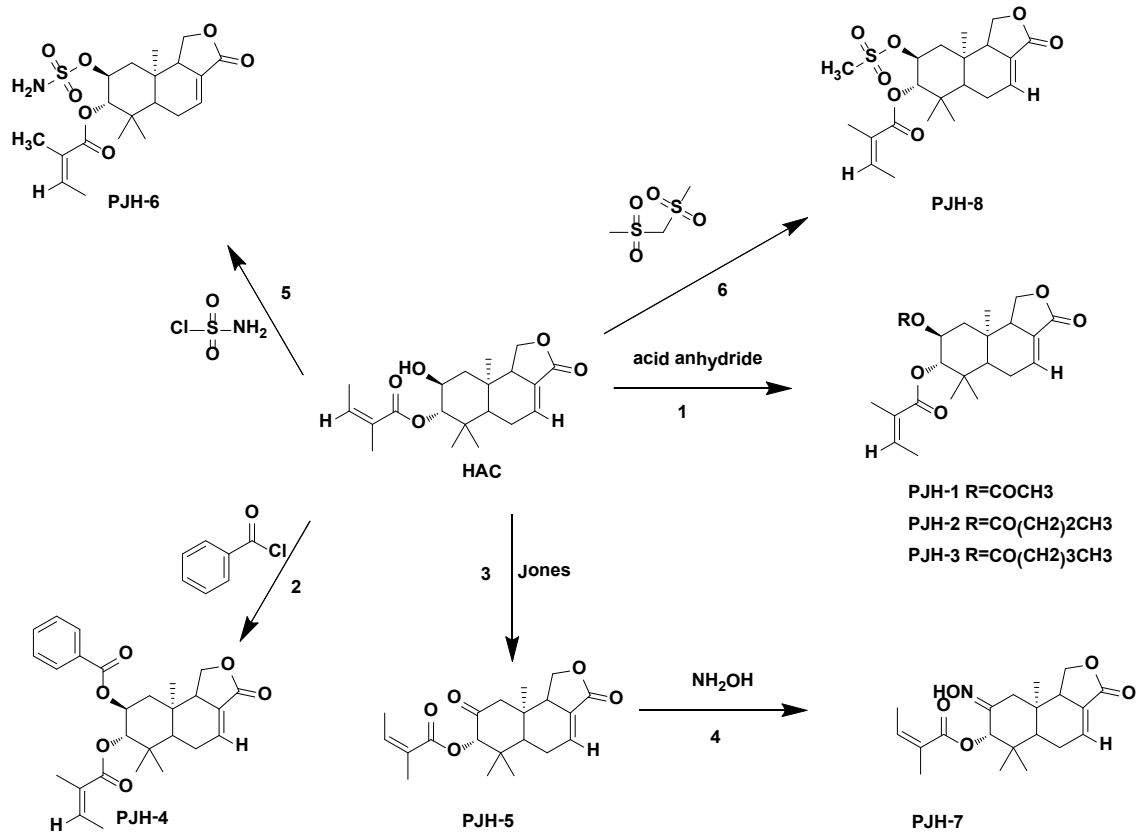

Scheme 1: Structures of **HAC** and chemical derivatives (**PJH-1~8**) of **HAC.**

Nitrite and TNF-α measurement: Nitrite was measured by adding 50 μL of the Griess reagent to 50μL of medium for 5min [20]. The optical density at 540 nm (OD540) was measured with a microplate reader (Epoch, Bio-Tek, USA). Concentrations were calculated by comparison with the OD540 of a standard solution of sodium nitrite prepared in culture medium. The levels of TNF-α in the RAW264.7 cell culture medium were measured by ELISA assay kits according to the manufacturer's instructions [21].

Western blot analysis: After indicated treatment, the cells were harvested and then lysed immediated by sonication in cold PBS containing 1% phenylmethanesulfonyl fluoride (PMSF). The lysate was centrifuged at 12,000 rpm for 5 min, and the supernatant was collected and the total protein concentration was determined with a Bradford protein assay kit. After dissolved in SDS/PAGE loading buffer and boiled for 3 min at 100°C, 25 μg of proteins were resolved on SDS/PAGE and then electrotransfered onto a nitrocellulose membrane. The membrane was washed with Tris-buffered saline Tween (TBST). Non-specific sites on the membrane were blocked by incubating the membrane in the blocking solution containing 5% non-fat dry milk in TBST for 60 min. The membrane was washed and incubated in diluted respective primary antibody at 4°C overnight. The membrane was washed and incubated in HRP-conjugated secondary antibody solution for 1 h. The final washed membrane was reacted with an enhanced chemiluminescence reagent (ECL, Beyotime) and exposed to Kodak Scientific Film to detect the immunoblots [22].

Structural determination of HAC and its derivatives

General (Chemical): Optical rotation were determined with a JASCO P-1030 polarimeter; Silica gel (Qingdao Haiyang Chemical Co. Ltd., Qingdao, China) was used for column chromatography; ^{1}H and ^{13}C NMR spectra: Bruker ACF-300, 500 NMR spectrometer, chemical shifts δ in ppm with $SiMe_4$ as an internal standard (=0 ppm), coupling constants J in Hz. HPLC: Agilent 1260 high performance liquid chromatograph.

Structural modification of HAC: PJH-1, PJH-2 and PJH-3 were prepared by the condensation of HAC with the appropriate acid anhydride. Treatment of HAC with Jones oxidation afforded PJH-5 in 78% yield. Then condensation of PJH-5 with NH_2OH gave PJH-7 as a sole product with low yield. Treatment of HAC with Benzoyl chloride, sulfamoyl chloride, and methyl sulfonic anhydride afforded PJH-4, PJH-6, and PJH-8 accordingly. Their structures were determined by NMR spectral analysis.

2α- acetoxy- 3β- angeloylcinnamolide (PJH-1 $C_{22}H_{30}O_6$): To a solution of HAC (200 mg, 0.574 mmol) in di-chloromethane (20 mL) was added DMAP (60 mg) and acetic anhydride (0.54 mL, 1.7 mmol). The reaction mixture was stirred at room temperature for 3 h (TLC monitoring). The crude product was chromatographed on a silica gel column using the elution (petroleum ether: acetyl acetate=1:1) to afford PJH-1 (83% yield) as a white needle crystal. Yield: 83%; white needle crystal. $[\alpha]^{20}_{D}$=-0.73° (c 0.3, CH_2Cl_2). ^{1}H-NMR ($CDCl_3$, 500 MHz): δ2.02 (1H, dd, *J*=4.5, 12.5 Hz, H-1β), 1.46 (1H, t, *J*=12.1, 12.1 Hz, H-1α), 5.16 (1H, m, H-2), 4.94 (1H, d, *J*=9.0 Hz, H-3), 1.62 (1H, q, *J*=5.5 Hz, H-5), 2.47 (1H, m, H-6α), 2.25 (1H, m, H-6β), 6.90 (1H, m, H-7), 2.90 (1H, m, H-9), 4.39 (1H, t, *J*=9.0 Hz, H-11α), 4.05 (1H, t, *J*=9.0 Hz, H-11β), 1.26 (3H, s, H-13), 1.08 (3H, s, H-14), 0.97 (3H,

s, H-15), angelica acyl: δ6.09 (1H, ddd, *J*=1.5, 1.5, 14.5 Hz, H-3'), 1.98 (3H, dd, *J*=1.5, 7.5 Hz, 3'-CH_3), 1.89 (3H, t, *J*=1.5 Hz, 2'-CH_3), Acetyl: 1.96 (3H, s, H-2'). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ42.6 (C-1), 66.7 (C-2), 79.2(C-3), 39.2(C-4), 48.9(C-5), 24.7(C-6), 135.7(C-7), 126.9(C-8), 50.6(C-9), 35.1(C-10), 68.6(C-11), 167.4(C-12), 17.1(C-13), 28.1(C-14), 14.3(C-15), angelica acyl: 167.4(C-1'), 127.7(C-2'), 138.4(C-3'), 20.9(2'-CH_3), 15.7(3'-CH_3), acetyl: 167.4(C-1''), 20.5(2'-CH_3).

2α- butanoic acid- 3β- angeloylcinnamolide (PJH-2 $C_{24}H_{32}O_8$): To a solution of HAC (200 mg, 0.574 mmol) in dichloromethane (20 mL) was added DMAP (60 mg) and succinic anhydride (225 mg, 2.25 mmol). The reaction mixture was stirred at 50°C for 3 h (TLC monitoring), the mixture was extracted with 10% hydrochloric acid. The CH_2Cl_2 layer was concentrated to dryness and crystallized from methanol to give PJH-2 (71% in yield) as a white needle. White needle crystal. $[\alpha]^{20}_D$=-1.5° (c 0.14, CH_2Cl_2). ^{1}H-NMR ($CDCl_3$, 300 MHz): δ2.05 (1H, dd, J=4.2 Hz, H-1β), 1.46 (1H, t, J=12.3, 12.0 Hz, H-1α), 5.18 (1H, m, H-2), 4.95 (1H, d, J=10.5 Hz, H-3), 1.62 (1H, q, J=5.4, 11.4 Hz, H-5), 2.44 (1H, m, H-6α), 2.32 (1H, m, H-6β), 6.90 (1H, m, H-7), 2.91 (1H, m, H-9), 4.40 (1H, t, J=9.3 Hz, H-11α), 4.05 (1H, t, J=9.3 Hz, H-11β), 1.08 (3H, s, H-13), 0.98 (3H, s, H-14), 0.96 (3H, s, H-15), angelica acyl: 6.12 (1H, ddd, J=1.5, 1.5, 14.5 Hz, H-3'), 1.99 (3H, dd, J=1.5, 5.7 Hz, 3'-CH_3), 1.89 (3H, t, J=1.5 Hz, 2'-CH_3), butyryl: 2.60(2H, m, H-2), 2.52 (2H, m, H-3'). ^{13}C-NMR ($CDCl_3$, 125 MHz): δ42.4 (C-1), 66.7 (C-2), 79.0 (C-3), 39.2 (C-4), 48.9 (C-5), 24.6 (C-6), 135.6 (C-7), 126.9 (C-8), 50.6 (C-9), 35.1 (C-10), 69.2 (C-11), 169.4 (C-12), 17.1 (C-13), 28.1 (C-14), 14.2 (C-15), angelica acyl : 167.3 (C-1'), 127.5 (C-2'), 139.0 (C-3'), 20.5 (2'-CH3), 15.7 (3'-CH_3), butyryl: 171.5 (C-1''), 29.0 (2''-CH2), 28.6 (3''-CH2), 176.9 (C-4'').

2α- pentanoic acid- 3β- angeloylcinnamolide (PJH-3 $C_{25}H_{34}O_8$): To a solution of HAC (100 mg, 0.287 mmol) in di-chloromethane (20 mL) was added DMAP (60 mg) and glutaric anhydride (327 mg, 2.87 mmol). The reaction mixture was stirred at room temperature for 3 h (TLC monitoring), the mixture was extracted with 10% hydrochloric acid. The CH_2Cl_2 layer was concentrated to dryness and crystallized from methanol to give PJH-3 (36% in yield) as a white needle. Amorphous solid. $[\alpha]^{20}_D$=-2.5° (c 0.12, CH_2Cl_2). ^{1}H-NMR ($CDCl_3$, 300 MHz): δ2.05(1H, dd, *J*=4.5 Hz, H-1β), 1.41 (1H, t, *J*=12.3, 12.0 Hz, H-1α), 3.9 (1H, m, H-2), 4.68 (1H, d, *J*=10.5 Hz, H-3), 1.58 (1H, q, *J*=5.4 Hz, H-5), 2.46 (1H, m, H-6α), 2.23 (1H, m, H-6β), 6.90 (1H, m, H-7), 2.92 (1H, m, H-9), 4.43 (1H, t, *J*=9.3 Hz, H-11α), 4.07 (1H, t, *J*=9.3 Hz, H-11β), 1.05 (3H, s, H-13), 0.98 (3H, s, H-14), 0.90 (3H, s, H-15); angelica acyl: 6.16 (1H, q, *J*=1.5 Hz, H-3'), 1.96 (3H, dd, *J*=1.5, 5.7 Hz, 3'-CH_3), 1.89 (3H, brs, 2'-CH_3). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ42.5 (C-1), 66.7 (C-2), 79.1 (C-3), 39.2 (C-4), 48.8 (C-5), 24.6 (C-6), 135.7 (C-7), 126.8 (C-8), 50.5 (C-9), 35.0 (C-10), 68.7 (C-11), 169.4 (C-12), 17.1 (C-13), 28.0 (C-14), 14.2 (C-15); angelica acyl: 167.2 (C-1'), 127.4 (C-2'), 139.2 (C-3'), 20.5 (2'-CH_3), 15.8 (3'-CH_3); valery: 169.4 (C-1''), 32.8 (2'-CH_2), 19.8 (3''-CH_2), 33.2 (4''-CH_2), 172.2 (C-5'').

2α- benzoate- 3β- angeloylcinnamolide (PJH-4 $C_{27}H_{32}O_6$): To a solution of HAC (200 mg, 0.574 mmol) in anhydrous pyridine (10 mL) was added benzoyl chloride (0.33 mL, 2.3 mmol). The reaction mixture was stirred at room temperature for 5 h (TLC monitoring). The crude product was chromatographed on a silica gel column using the elution (petroleum ether: acetyl acetate=5:1) to afford PJH-4 (76% yield) as a white needle. White needle crystal. $[\alpha]^{20}_D$=-6.5°(c 0.13, CH_2Cl_2)$_0$ ^{1}H-NMR ($CDCl_3$, 300 MHz): δ2.22 (1H, dd, J=4.5, 12.5 Hz, H-1β), 1.59 (1H, t, J=12.3, 12.0 Hz, H-1α), 5.17 (1H, m, H-2), 5.15 (1H, d, J=10.2 Hz, H-3), 1.70 (1H, dd, J=5.4, 11.4 Hz, H-5), 2.46 (1H, m, H-6α), 2.32 (1H, m, H-6β), 6.93 (1H, m, H-7), 2.94 (1H, m, H-9), 4.39 (1H, t, J=9.3 Hz, H-11α), 4.07 (1H, t, J=9.3 Hz, H-11β), 1.14 (3H, s, H-13), 1.04 (3H, s, H-14), 1.03 (3H, s, H-15); angelica acyl: 5.95 (1H, ddd, J=1.5, 1.5, 14.5 Hz, H-3'), 1.87 (3H, dd, J=1.5, 7.5, 14.5 Hz, 3'-CH_3), 1.79 (3H, t, J=1.5Hz, 2'-CH_3), benzoyl: 7.97 (2H, t, J=1.8,5.1 Hz, H-2', H-3'), 7.54(1H, m, H-5'), 7.41(2H, t, J=1.2, 1.5Hz, H-4', H-6'). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ42.6 (C-1), 66.7 (C-2), 79.1 (C-3), 39.2 (C-4), 49.0 (C-5), 24.7 (C-6), 135.6 (C-7), 126.9 (C-8), 50.6 (C-9), 35.2 (C-10), 68.5 (C-11), 169.4 (C-12), 17.2 (C-13), 28.1 (C-14), 14.3 (C-15); angelica acyl : 167.5 (C-1'), 127.6 (C-2'), 138.4 (C-3'), 20.5 (2'-CH_3), 15.6 (3'-CH_3); benzoyl: 166.0 (C-1''), 133.2, 129.8, 129.7, 128.4.

2α - oxours - 3β - angeloylcinnamolide (PJH-5 $C_{20}H_{26}O_5$): To a solution of HAC (800 mg, 2.3 mmol) in acetone (4 mL) was added Jones (5 mL). After stirring for 4 h at room temperature (TLC monitoring), the mixture was diluted with H_2O and extracted with $CHCl_3$.The $CHCl_3$ layer was concentrated to dryness and crystallized from methanol to give PJH-5 (78% in yield) as a white needle. White needle crystal. $[\alpha]^{20}_D$=4.7° (c 0.14, CH_2Cl_2). ^{1}H-NMR ($CDCl_3$, 300 MHz): δ1.58 (1H, s, H-1β), 1.26 (1H, s, H-1α), 5.09 (1H, s, H-3), 2.15 (1H, q, J=5.4 Hz, H-5), 2.52 (1H, m, H-6α), 2.28 (1H, m, H-6β), 6.94 (1H, m, H-7), 3.16 (1H, m, H-9), 4.43 (1H, t, J=9.3 Hz, H-11α), 4.05 (1H, t, J=9.3 Hz, H-11β), 1.18 (3H, s, H-13), 1.01 (3H, s, H-14), 0.85 (3H, s, H-15), angelica acyl: 6.17 (1H, q, J=1.5 Hz, H-3), 2.03 (dd, J=1.5, 5.7 Hz, 3'-CH_3), 1.96(3H, t, J=1.5 Hz, 2'-CH_3). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ49.2 (C-1), 201.5 (C-2), 83.3 (C-3), 43.1 (C-4), 50.6 (C-5), 24.9 (C-6), 135.6 (C-7), 127.0 (C-8), 51.5 (C-9), 39.8 (C-10), 66.3 (C-11), 169.0 (C-12), 17.1 (C-13), 28.1 (C-14), 14.1 (C-15); angelica acyl: 167.0 (C-1'), 127.2 (C-2'), 139.5 (C-3'), 20.5 (2'-CH_3), 15.9 (3'-CH_3).

2α– sulfamoyloxy- 3β- angeloylcinnamolide (PJH-6 $C_{20}H_{29}NO_7S$): To a solution of HAC (60 mg, 0.17 mmol) in anhydrous pyridine (4 mL) was added sulfamoyl chloride (40 mg, 0.34 mmol). The reaction mixture was stirred at ice for 4 h (TLC monitoring). The mixture was extracted with 10% hydrochloric acid and dichloromethane, the CH_2Cl_2 layer was concentrated to dryness and crystallized from methanol to give PJH-6 (78% in yield) as a white power. Amorphous solid. $[\alpha]^{20}_{D=}$-4.4° (c 0.06, CH_2Cl_2). ^{1}H-NMR ($CDCl_3$, 300 MHz): δ2.03(1H, s, H-1β), 1.60 (1H, s, H-1α), 4.94 (1H, d, *J*=9.9 Hz, H-2), 4.47 (1H, d, *J*=8.4 Hz, H-3), 1.71 (1H, q, *J*=5.4 Hz, H-5), 2.46 (1H, m, H-6α), 2.32 (1H, m, H-6β), 6.89 (1H, m,H-7), 2.92 (1H, m, H-9), 4.42 (1H, t, *J*=9.3 Hz, H-11α), 4.05 (1H, t, *J*=9.3 Hz, H-11β), 1.07 (3H, s, H-13), 0.97 (3H, s, H-14), 0.94 (3H, s, H-15); angelica acyl: 6.16 (1H, ddd, *J*=1.2, 1.2, 14.4 Hz, H-3'), 2.03 (dd, *J*=1.5, 5.7 Hz, 3'-CH_3), 1.96 (3H, t, *J*=1.5 Hz, 2'-CH_3). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ43.4 (C-1), 76.6 (C-2), 78.5 (C-3), 39.7 (C-4), 48.6 (C-5), 24.6 (C-6), 135.6 (C-7), 126.8 (C-8), 50.5 (C-9), 35.3 (C-10), 66.6 (C-11), 169.3 (C-12), 17.2 (C-13), 28.1 (C-14), 14.3 (C-15); angelica acyl: 167.0 (C-1'), 127.4 (C-2'), 139.8 (C-3'), 20.5 (2'-CH_3), 15.9 (3'-CH_3).

2α- hydroxyimino- 3β- angeloylcinnamolide (PJH-7 $C_{20}H_{27}NO_5$): To a solution of HAC (60 mg, 0.17 mmol) in anhydrous pyridine (4 mL) was added hydroxylaminel·HCl (60 mg). The reaction mixture was stirred at 50°C for 4 h (TLC monitoring), the mixture was diluted with CH_2Cl_2 and extracted with 10% hydrochloric acid. The crude product was chromatographed on a silica gel column using the elution (petroleum ether: acetyl acetate=2:1) to afford PJH-7 (67% yield) as a white needle crystal. White needle crystal. $[\alpha]^{20}_D$=9.5° (c 0.12, CH_2Cl_2). ^{1}H-NMR ($CDCl_3$, 300 MHz): δ1.58 (1H, s, H-1β), 1.26 (1H, s, H-1α), 5. 08 (1H, s, H-3), 1.81 (1H, q, *J*=5.4 Hz, H-5), 2.52 (1H, m, H-6α), 2.28 (1H, m, H-6β), 6.91 (1H, m, H-7), 3.02 (1H, m, H-9), 4.47 (1H, t, *J*=9.3 Hz, H-11α), 4.12 (1H, t, *J*=9.3 Hz, H-11β), 1.11 (3H, s, H-13), 1.04 (3H, s, H-14), 0.81 (3H, s, H-15); angelica acyl : 6.09 (1H, ddd, *J*=1.2, 1.2, 14.4 Hz, H-3'), 2.03 (dd, *J*=1.5, 5.7 Hz, 3'-CH_3), 1.96 (3H, t, *J*=1.5 Hz, 2'-CH_3). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ152.9 (C-2), 78.7 (C-3), 37.5 (C-4), 49.5 (C-5), 24.8 (C-6), 135.6 (C-7), 127.5 (C-8), 49.9 (C-9), 35.8 (C-10),

66.7 (C-11), 16.5 (C-13), 27.7 (C-14), 13.6 (C-15); angelica acyl: 127.5 (C-2'), 139.1 (C-3'), 20.6 (2'-CH_3), 15.9 (3'-CH_3).

2α-methylsulfonyl- 3β- angeloylcinnamolide (PJH-8 $C_{21}H_{30}O_7S$): To a solution of HAC (150 mg) in 50 mL di-chloromethane, was added DMAP (63 mg) and methanesulfonic anhydride (82.5 mg). The reaction mixture was stirred at room temperature for 18 h (TLC monitoring). The crude product was chromatographed on a silica gel column using the elution (petroleum ether: acetyl acetate=4:1) to afford PJH-8 (71% yield) as a white needle crystal. White needle crystal. $[\alpha]^{20}_D$=-9.2° (c 0.4, CH_2Cl_2). ^{1}H-NMR($CDCl_3$, 400 MHz): δ6.91 (1H, H-4), 6.19 (1H, H-15), 4.97 (1H, H-7), 4.90 (1H, H-8), 4.44 (1H, H-1α), 4.07 (1H, H-1β), 2.94 (1H, H-9b), 2.94 (3H, H-17), 2.48 (1H, H-5α), 2.27 (1H, H-5β), 2.22 (1H, H-9α), 2.05 (3H, H-16), 1.96 (3H, H-14α), 1.74 (1H, H-5α), 1.66 (1H, H-9β), 1.10 (3H, H-10), 1.00 (3H, H-11), 0.96 (3H, H-12); ^{13}C NMR($CDCl_3$, 100MHz): δ169.28 (C-3), 166.75 (C-13), 140.30 (C-15), 135.59 (C-4), 127.17 (C-14), 126.75 (C-3a), 78.15 (C-7), 76.44 (C-8), 66.64 (C-1), 50.45 (C-9b), 48.51 (C-5a), 44.09 (C-9), 39.75 (C-6), 38.60 (C-17), 35.37 (C-9a), 28.09 (C-12), 24.62 (C-5), 20.60 (C-14a), 17.22 (C-11), 15.97 (C-16), 14.30 (C-10).

Determination of absolute structure of HAC: The crystal of HAC was established by slow evaporation of solvent (methanol: water=2:1) at room temperature (20°C). The X-ray structural data were collected with CAD4 EXPRESS (Enraf-Nonius, 1994) at 293.0 ± 0.1 K using graphite monochromatised MoKα radiation (λ=0.71073 Å). Cell refinement were processed with CAD4 EXPRESS, data reduction used XCAD4 (Harms & Wocadlo, 1995), structure solved by SHELXS-97 (Sheldrick, 1990) and refined by SHELXL-97 (Sheldrick, 1997). Molecular graphics employed DIAMOND and MERCURY.

HAC crystallized in monoclinic space group P21 with unit cell parameters a=6.8640 (14) Å, b=25.676 (5) Å, c=11.190 (2) Å, β=106.14 (3) Å, V=1894.4 (7) Å3, Z=2, Dx=1.222 g/cm3, T=293 K, λ (Mo Kα)=0.71073 Å, the final R1=0.1116, wR2=0.1735 (w=1/σ/ F/ 2), and S=1.003 observed reflections with I > 2σ(I). The deposition number CCDC 906633 for HAC contains the supplementary crystallographic data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif or Cam bridge crystallographic Data Centre, 12, Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033.

Stability of HAC and its derivatives in the culture medium: The test compounds was exposed to DMEM (final concentration 100 μM; final volume 200 μL) in 96-well microplates for 24 h, subsequently drew 100 μL supernatant to centrifuged and freeze-dried, add 1mL of methanol preparation chromatography. Then selected each compound to dissolve in 100μL DMSO with 1 mL methanol as a standard reference. The HPLC analysis of samples were performed on an Agilent 1260 HPLC system with Agilent C_{18} chromatographic column (4.6 mm ×250 mm, 5 μm). The column temperature was maintained at 30°C.The detection wavelength was set to 222 nm. The mobile phase consisted of MEOH (A) and water (B) with a flow rate of 1.0 mL/min. The linear gradient was 50% A to 90% A in 25 min. Each sample analysis was repeated in triplicate.

Statistical analysis

All value were obtained from measurements performed in triplicates. For determination of IC_{50} values, log concentrations and linear response data were analyzed by non-linear curve fitting using Prism soft package (GraphPad Software Inc.)

Results

Cytotoxicity of HAC and its inhibitions on LPS-induced pro-inflammatory mediators

The potential cytotoxicity of HAC was evaluated by the MTT assay after incubating cells for 24h in the absence of LPS, the results showed cell viabilities were not affected by HAC at indicated concentrations (1~100 μM, Figure 1A). Thus, HAC did not display significant cytotoxicity against RAW264.7 cells.

To determine the effects of HAC on the pro-inflammatory mediators in RAW264.7cells, the concentrations of NO and TNF-α in the cell supernatants were examined. Compound HAC showed higher 50% inhibition at 17.68 ± 2.99 μM in the suppression of LPS-induced NO production, however, the lower inhibitory effect (IC_{50}, 98.66 ± 13.55 μM) of HAC was obtained on TNF-α content by LPS-induced RAW 264.7 cells. So, HAC was found to inhibit LPS-induced over production of NO and TNF-α in a dose-dependent manner compared to the LPS group (Figure 1B–C). In present paper, Compounds in which the NO, TNF-α inhibition rate value exceed 50% were detected by the IC_{50} values.

Chemical-structural modification of HAC at C-2.

In order to evaluate the importance of hydroxyl group at position of

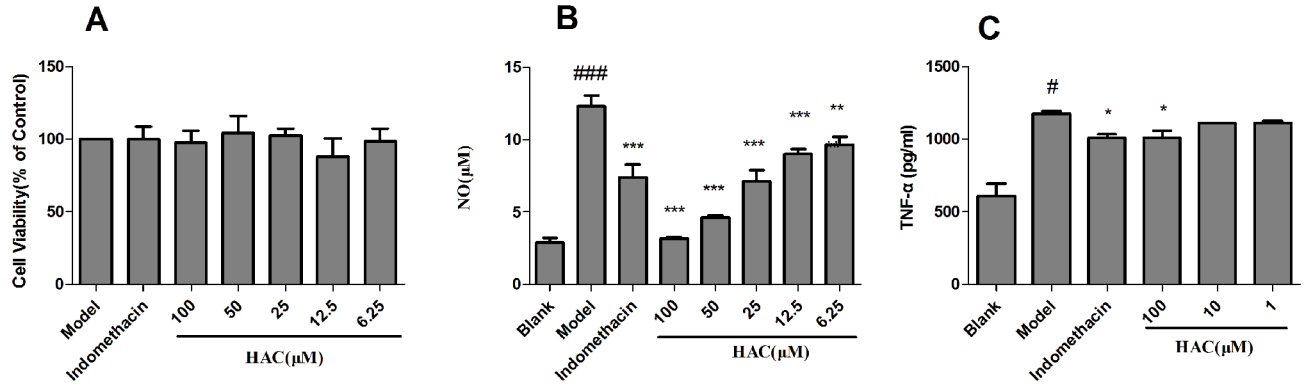

Figure 1: Effects of HAC on the cell viability, the production of NO and TNF-α in LPS-induced RAW264.7 cells (**A–C**). (**A**) The cells were treated with HAC (1~100 μM) in the absence of LPS (1 μg/ml) for 24 h. Cell viability was assessed by MTT reduction assays. (**B**) The cells were treated with different concentrations (1~100 μM) of HAC for 1 h before stimulation with 1 μg/ml of LPS for 24 h, measured NO production by Griess reaction. (**C**) The concentrations of TNF-α were determined after incubation for 12 h with commercially available ELISA kits.

C-2 in HAC, eight novel compounds (named as PJH-1 to PJH-8) were obtained aimed at its hydroxyl group at C-2 position (Scheme 1).

Absolute configuration of HAC

The relative stereo-structure of HAC has been determined in our previous paper [13]. In present day, the crystal of HAC was established by slow evaporation of solvent and analyzed by X-ray diffraction method (Figure 2).

Cytotoxicity of HAC derivatives and their inhibitions on LPS-induced pro-inflammatory mediators

All derivatives (PJH-1~PJH-8, 1-100 μM) were tested for inhibitory activities against NO production in LPS-induced macrophages and for their cytotoxic effects. The model group was established as a negative control, and the indomethacin (100 μM) group was established as a positive control, the NO concentration was assessed with Griess reagent. TNF-α concentration were measured by ELISA assay. The data are presented as the means ± S.D of three independent experiments. As the results, compounds PJH-1 and PJH-6 showed higher inhibition effects in NO production with IC_{50} value of 7.31 μM or 9.28 μM, respectively, however, compounds PJH-1 and PJH-7 showed better inhibition effects on TNF-α levels. Therefore, the effect of PJH-1, as a derivative acetylated at C-2 hydroxyl group of HAC, on macrophages my come from mainly the suppression of TNF-α/NO pathways in a dose-dependent manner (Figure 3A-C).

Influence of HAC and PJH-1 on iNOS protein and MAPKs signaling pathways in LPS-induced RAW264.7 by western blotting assay

To address whether the inhibition of NO production was associated with decreased levels of iNOS, the effects of HAC and its most active derivative PJH-1 on LPS-induced expression of iNOS were investigated by Western blot analysis. The expression levels of iNOS were strongly induced by LPS, HAC and PJH-1 (1-100 μM) inhibited the LPS-induced iNOS protein induction in a dose-dependent manner (Figure 4). These results are consistent with the inhibitory effects of them on NO production. This result indicates that HAC and its derivatives suppress LPS-induced expression of iNOS at the transcriptional level.

The MAPKs pathways are known to be important for the expression of iNOS and COX-2. Therefore, MAP kinases act as specific targets for inflammatory responses. To test whether the inhibition of inflammation by HAC is regulated through the MAP kinase pathways, we examined the effect of HAC and PJH-1 on LPS induced phosphorylation of ERK, p38 and JNK in Raw264.7 cells using Western blot analysis. As shown in Figure 5, HAC and PJH-1 attenuated the LPS-stimulated phosphorylation of ERK, p38 and JNK in a concentration-dependent manner. These results suggested that the MAPKs pathways were involved during HAC suppressed LPS-mediated expression of inflammatory mediators.

Stability of HAC and derivatives in cell medium by HPLC method

As above results, HAC and its derivatives, PJH-1 inhibited NO and TNF-α production in LPS-activated RAW264.7 macrophage cells without significant cytotoxicity. As we known, the stability of target structure in cell medium is important for its in vitro activity evaluation. So HAC and PJH-1 were determined followed by incubation in cell medium for 24h. HAC in medium is unstable and easy to decompose a new peak ($t_{R=}$12.059 min) in HPLC chromatogram, the concentration of active derivative PJH-1 is unchanged (Figure 6).

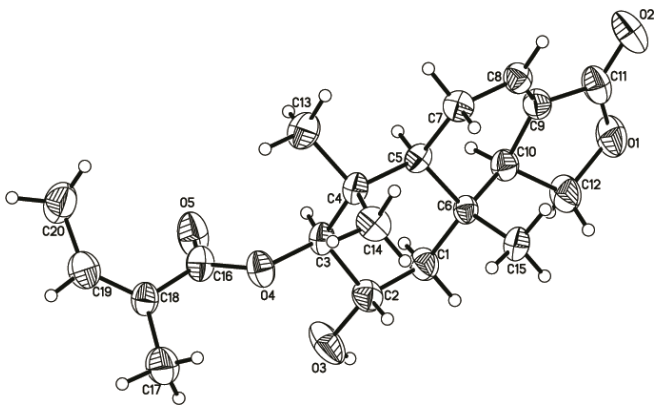

Figure 2: The single crystal structures of HAC obtained in methanol-water (2:1) solvent.

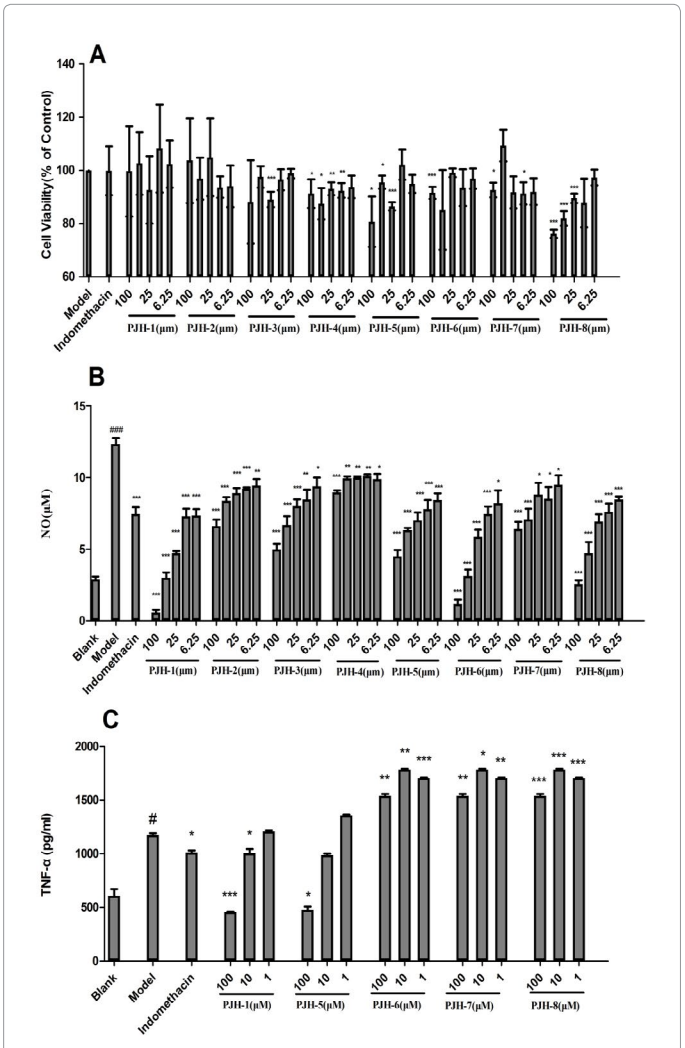

Figure 3: Effects of HAC derivatives on the cell viability, the production of NO and TNF-α in LPS-induced RAW264.7 cells (**A–C**). (**A**) The cells were treated with HAC derivatives (1~100 μM) in the absence of LPS (1 μg/ml) for 24h. Cell viability was assessed by MTT reduction assays. (**B**) The cells were treated with different concentrations (1-100 μM) of HAC for 1h before stimulation with 1 μg/ml of LPS for 24 h, measured NO production by Griess reaction. (**C**) The concentrations of TNF-α were determined after incubation for 12 h with commercially available ELISA kits.

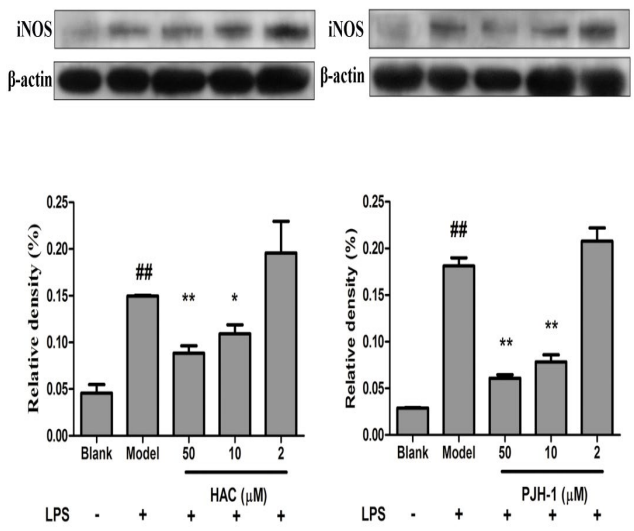

Figure 4: The Influence of HAC and PJH- on iNOs protein expression in LPS-induced RAW264.7 by western blotting assay. The cells were treated with different concentrations (2, 10, 50 μM) of HAC and PJH-1 for 1h followed by the addition of 1 μg/ml of LPS for 24 h. Total cellular proteins (20 μg/μg) were resolved by SDS-PAGE, then transferred to PVDF membrane and detected with specific antibodies as described in Materials and methods. Quantification of iNOS protein expression was normalized to β-actin using a densitometer. The data are representative of three independent experiments and expressed as mean ± S.D. ##P<0.01vs.control, **P<0.01vs.LPS group.

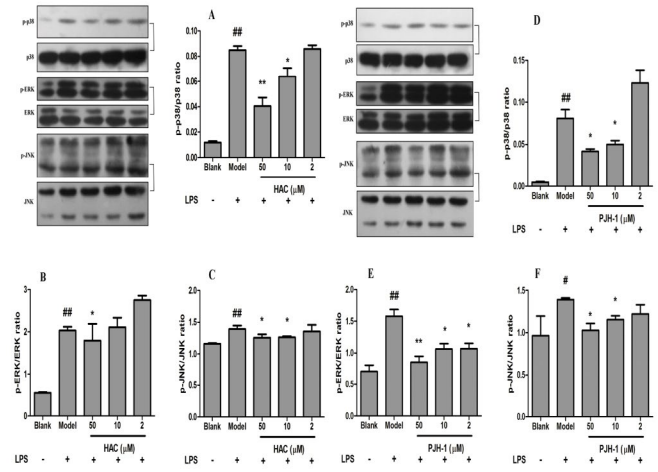

Figure 5: The Influence of HAC (A, B and C) and PJH-1 (D, E and F) on MAPKs (p-p38/p38, p-ERK/ERK and P-JNK/JNK) signaling pathways in LPS-induced RAW264.7 by western blotting assay. The cells were treated with different concentrations (2, 10, 50 μM) of HAC and PJH-1 for 1h followed by the addition of 1 μg/ml of LPS for 24 h. Total cellular proteins (20 μg/μg) were resolved by SDS-PAGE, then transferred to PVDF membrane and detected with specific antibodies as described in Materials and methods. These data are representative of three independent experiments and expressed as mean ± S.D. ##P<0.01vs. control, **P<0.01vs.LPS group.

Discussion

Sesquiterpenoids are a large group of secondary metabolites of many medicinal plants and exhibit a variety of biological activities.

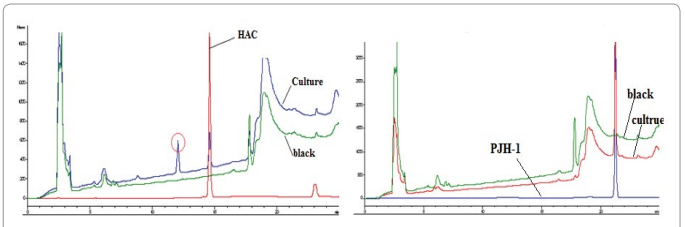

Figure 6: HPLC charts of compounds HAC and PJH-1 incubated in RAW264.7cells medium for 24h.

Macrophages play a key role in the specific and non-specific immune response during the inflammation process, large amount of the inflammatory mediators such as nitric oxide (NO), prostanoids and pro-inflammatory cytokines will be released in LPS-activated macrophages [23].

Until now, some sesquiterpenoids has been evaluated on their anti-inflammation effects focused on the activation of NF-kB or inhibition of iNOS-dependent NO synthesis [24], only few investigation on structure-activity relationship of drimane-type sesuiterpenoids have been performed. Drimane sesquiterpenes are frequently occurring metabolites in plants, which exhibit a variety of biological activities [25]. In our previous paper, a drimane sesquiterpene lactone, 2α-hydroxyl - 3β - angeloylcinnamolide (HAC) from the Chinese folk medicinal herb *Polygonum jucundum* Lindx, has been reported as a new anti-inflammatory remedy by using xylene-induced ear edema and acetic acid-induced vascular permeability mouse inflammation models [13], HAC can be translated in vivo into another new drimane sesquiterpenoid, 2α, 3β- dihydroxylcinnamolide [14].

In present day, we found that pretreatment with HAC (1-100 μM) significantly inhibited the production of NO in LPS-induced RAW264.7 cells at IC_{50} concentrations 17.68 μM. NO is a free radical produced from L-arginine by nitric oxide synthases (NOS), the high level of NO might cause inflammatory damage to target tissue during an infection [26,27]. The inhibition of NO release may be effective as a therapeutic agent in the inflammatory disease [28]. Therefore, the regulation of NO release via inhibiting iNOS is helpful to alleviate the inflammatory destruction. As we presumed, HAC can significantly decreased iNOS expression levels induced by LPS in a dose-dependent manner. TNF-α and IL-1 are regulated by NF-KB but are at the same time potent activators of NF-kB themselves, a series of structurally different sesquiterpenoids has been evaluated for their inhibition activities of inflammatory cytokine production and correlation with NF-kB pathway, however HAC show no effect on TNF-α production in LPS-induced RAW264.7 cells.

Sesquiterpene lactones with α, β-unsaturated carbonyl moieties are reactive to cysteine thiol group in the Michael-type addition and have been identified to inhibit the NF-kB signaling pathway. α,β-unsaturated carbonyl moieties in HAC partly account for its inflammatory activities, but the importance of hydroxyl group on anti-inflammation effects are unclear. Therefore, a series of closely related compounds PJH-1 ~ PJH-8 were obtained by chemical procedures and investigated for their inhibitions on NO and TNF-α production in LPS-induced RAW 264.7 cells. As the results, the NO production was significantly inhibited in a dose-dependent manner with IC_{50} -values of 11.8 μM PJH-1. In accordance with the TNF-α assay, compound PJH-1 also inhibited significantly TNF-α production with IC_{50}-value of 3.38μM. In addition, compound PJH-6, another sulfated derivative of HAC, with sulfate group combined to the hydroxyl group at C-2, also significantly inhibited the NO production with IC_{50} value of 9.28 μM in a dose-dependent manner, but show no effect on TNF-α production in LPS-induced RAW264.7 cells.

These results suggested that the anti-inflammatory effects of HAC were dramatically improved after being acetylated at C-2 position (compound PJH-1). Expression of the iNOS in macrophages is regulated mainly at the induction of transcription factors through mitogen-activated protein kinases (MAPKs). MAPKs important to macrophage cells include p38, c-jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK). This process activates transcription factors such as NF- κB, which in turn upregulates production of cytokines, such as TNF-α and NO. Accordingly, we investigated the effect of HAC and PJH-1 on the LPS-induced ERK, p38 and JNK activation. They caused a dose-dependent inhibition of the phosphorylation of ERK, p38 and JNK. Interestingly, HAC and PJH-1 suppressed activated of all three MAPKs significantly. Taken together, our results provide evidence that HAC and PJH-1 suppressed LPS-induced iNOS expression through the blockage of MAPKs signaling cascade activation. In addition, it should be noted that a high possibility that sesequiterpenoids form adducts in the Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS before their application to the cells. Hence, in the cell viability assay, the pre-incubation of HAC and PJH-1 (200 M, respectively) in the 10% FBS-DMEM was examined for 24h. It showed that a novel peak (tR=12.059 min) can be detected in HPLC chart of HAC, on the contrary, PJH-1 had no change in the culture.

Conclusion

In a conclusion, we proposed to take HAC as the lead compound, designed 8 new compounds, the structure of the compounds was confirmed by spectroscopic methods. Meanwhile, absolute stereo-structure of HAC was determined by X-ray crystallography analysis. Inhibitory effects of these compounds on the production of NO and TNF-α induced by LPS on RAW264.7 cell were examined. HAC and PJH-1 may be attributed to their roles in down-regulation of MAPKs pathways by Western blot assay, and the stabilities of PJH-1 were more stable in cell culture medium than that of HAC by HPLC methods.

These results indicated that purposefully modified compounds and the stability of compounds in the medium should be considered to explore the convictive structure-activity relationships of sesquiterpene lactones. Hence, compound PJH-1 can be selected as the candidate drug for HAC in vitro pharmacological mechanism research of anti-inflammation. So, HAC derivatives might potentially constitute a novel class of anti-inflammatory agents, which require further studies.

Acknowledgements

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