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Research article

Anti-inflammatory effect of a triterpenoid from *Balanophora laxiflora*: results of bioactivity-guided isolation

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ABSTRACT

Balanophora laxiflora, a medicinal plant traditionally used to treat fever, pain, and inflammation in Vietnam, has been reported to possess prominent anti-inflammatory activity. This study examined the active constituents and molecular mechanisms underlying these anti-inflammatory effects using bioactivity-guided isolation in combination with cell-based assays and animal models of inflammation. Among the isolated compounds, the triterpenoid (21α)-22-hydroxyhopan-3-one (**1**) showed the most potent inhibitory effect on COX-2 expression in LPS-stimulated Raw 264.7 macrophages. Furthermore, **1** suppressed the expression of the inflammatory mediators iNOS, IL-1β, INFβ, and TNFα in activated Raw 264.7 macrophages and alleviated the inflammatory response in carrageenan-induced paw oedema and a cotton pellet-induced granuloma model. Mechanistically, the anti-inflammatory effects of **1** were mediated *via* decreasing cellular reactive oxygen species (ROS) levels by inhibiting NADPH oxidases (NOXs) and free radical scavenging activities. By downregulating ROS signalling, **1** reduced the activation of MAPK signalling pathways, leading to decreased AP-1-dependent transcription of inflammatory mediators. These findings shed light on the chemical constituents that contribute to the anti-inflammatory actions of *B. laxiflora* and suggest that **1** is a promising candidate for treating inflammation-related diseases.

1. Introduction

Inflammation is a protective physiological reaction that occurs in response to numerous endogenous and exogenous stimuli (Serhan and Savill 2005). Although the main purposes of the inflammatory response are to remove damaged tissue components or eliminate harmful pathogens, excess or persistent inflammation has been implicated in the development and progression of various diseases, including type 2 diabetes, cardiovascular diseases, neurodegenerative diseases, asthma, and cancer (Clevers 2004; Hotamisligil 2006; Amor et al., 2010). The main anti-inflammatory drugs in clinical use are nonsteroidal anti-inflammatory drugs, glucocorticoids, immunosuppressants, and some biologics. Although these classic anti-inflammatory drug classes have many serious side effects, especially when long-term treatment is required, the use of newer drugs is restricted by their cost and narrow spectra of indications (Dinarello 2010). Given the complexity of the inflammatory reaction, which often requires the selection of medicines in a context-dependent manner, the discovery of novel anti-inflammatory agents remains of interest (Tabas and Glass 2013). A large body of evidence delineates that phytochemicals can serve as an abundant source for screening and detection of new anti-inflammatory drug candidates (Tasneem et al., 2019). The advantage of these compounds is often to possess multi-mechanistic actions and well-tolerated capacity (Nunes et al., 2020).

The genus *Balanophora* (Balanophoraceae) contains about 120 species, mainly distributed in tropical and subtropical Asia and Oceania (Wang et al., 2012). These dioeciously parasitic plants have many biological functions, such as free radical scavenging and hypoglycaemic, hypouricemic, analgesic, and anti-inflammatory actions (Wang et al., 2012). *Balanophora laxiflora* Hemsl. is a well-characterized species within the Balanophoraceae (Quang et al., 2018). Chemical studies of *B. laxiflora* have identified diverse compounds, including lignans, triterpenoids, phytosteroids, tannins, and organic acids (Tanaka et al., 2005; Dai et al., 2006; Ho et al., 2010). With regards to the biological activity, previous

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studies have shown that the crude extract and compounds isolated from *B. laxiflora* have hypouricemic effects, DPPH radical-scavenging activity, cytotoxicity against some cancer cell lines, and anti-inflammatory actions (She et al., 2009; Chiou et al., 2011; Ho et al., 2012; Quang et al., 2018).

Previously, Chiou et al. evaluated the anti-inflammatory effects of 18 compounds derived from tuberous rhizomes of *B. laxiflora* and identified isolariciresinol as the most potent (Chiou et al., 2011). It was subsequently shown that lupeol acetate has antinociceptive and anti-inflammatory effects that are similar to those of the crude *B. laxiflora* extract (Chen et al., 2012). In addition, a recent study by our colleagues led to the isolation of a new *B. laxiflora* tannin that has modest anti-inflammatory effects in Raw 264.7 macrophages (Tai et al., 2019). These findings suggest that *B. laxiflora* possesses anti-inflammatory effects that can be exploited clinically. However, since earlier studies focused on a specific group of compounds isolated from *B. laxiflora*, the main bioactive constituent remains to be characterized. Furthermore, the detailed mechanisms underlying the anti-inflammatory actions of *B. laxiflora* are poorly understood. Therefore, this study sought new active constituents from *B. laxiflora* based on bioactivity-oriented isolation and identified the anti-inflammatory mechanisms of potential compounds.

2. Materials and methods

2.1. Chemicals and reagents

All the cell culture reagents were obtained from HyClone Laboratories (South Logan, UT, USA). Xanthine oxidase from bovine milk, xanthine substrate, indomethacin, N-acetylcysteine, prednisolone, 4-Nitro blue tetrazolium chloride (NBT), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), carrageenan and lipopolysaccharides (LPS) were procured from Sigma–Aldrich (St Louis, MO, USA). Diphenyleneiodonium (DPI) chloride, lucigenin, and NADPH tetrasodium was purchased from Enzo life sciences (Farmingdale, NY, USA). The primary antibodies against total and phosphorylated forms of ERK1/2, p38 MAPK and JNK1/2, and the secondary antibodies conjugated with horseradish peroxidase (HRP) were acquired from Cell Signaling Technology Inc. (Beverly, MA, USA). Anti-β-actin and anti-COX-2 were provided by BD Biosciences (San Jose, CA, USA) and Sigma–Aldrich, respectively. The anti-rabbit and anti-mouse secondary antibodies conjugated with horseradish peroxidase (HRP) were acquired from Cell Signaling Technology Inc (Beverly, MA, USA).

2.2. Plant materials

B. laxiflora were collected in Sa Pa, Lao Cai, Vietnam in November 2015. The plant was identified by botanists Nguyen The Cuong and Tran The Bach, Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology. A voucher specimen (BSP2) was deposited at the National Institute of Medicinal Materials. Voucher specimens were deposited at three herbaria located respectively at the Institute of Ecology and Biological Resources; Institute of marine biochemistry, Vietnam Academy of Science and Technology; and the Vietnam National Institute of Medicinal Materials.

2.3. Extraction and isolation of plant extract

Air-dried and pulverized *B. laxiflora* (4.0 kg) was thrice extracted with 80% aq. Ethanol by reflux for 3 h. The extracts were combined and evaporated at 50 °C under low pressure to obtain residue (650.12 g). The total residue was suspended in water and successively extracted with *n*-hexane and ethyl acetate. Solvent was then evaporated *in vaccuo* to obtain the *n*-hexane (**BLH**, 60.2 g), ethyl acetate (**BLEA**, 124.1 g), and water layer (**BLW**, 180.7 g) fractions.

The **BLEA** fraction was chromatographed on a silica gel column and eluted with a gradient solvent system of *n*-hexane-ethyl acetate (100/1-1/10) to afford 15 fractions E1-E15, respectively. The fraction E9 (2.95 g) was crystallized by *n*-hexane-ethyl acetate (3/1) to give compound **1** (2.26 g**).** The fraction E8 (7.27 g) was separated by CC eluting with DCM-MeOH (100/1-1/10) to give five smaller fractions. Compound **2** (50 mg) was yielded after crystallized in acetone from E8.2 fraction. The E8.3 fraction (3.25 g) was chromatographed on a silica gel column and eluted with a gradient solvent system of DCM-MeOH (100/1-1/10) to afford 4 fractions E8.3A-E8.3D. Fraction E8.3A (605.4 mg) was continuously separated on a RP-C18 column chromatography eluting with MeOH/water (1/2-1/1) to give compound **3** (77.7 mg). Fraction E6 (1.35 g) was chromatographed on a RP-C18 column chromatography eluting with MeOH/water/Aa (acid acetic) (1/1/0.1) to give smaller fractions E6.1-E6.4. Fraction E6.3 (142.4 mg) was continuously separated on a RP-C18 column chromatography eluting with MeOH/water/Aa (acid acetic) (1/1/0.1) to give compound **4** (59.5 mg). The E11 fraction (2.6 g) was chromatographed on a silica gel column and eluted with a gradient solvent system of DCM-MeOH (8/1-1/10) to afford 8 fractions E11.1-E11.8, respectively. Fraction E11.4 (105.4 mg) was continuously separated on a RP-C18 column chromatography eluting with acetone/water (1/2-1/1) to give compound **15** (15.0 mg).

The water layer was passed through a Diaion HP-20 column eluting with methanol-water gradients (0%, 25%, 50%, 75%, 100% methanol) to give four fractions, W1–W4. Fraction W2 (40.0 g) was separated on a *silica gel* column chromatography eluting with DCM/MeOH (0–100% methanol) to give four smaller fractions, 1A-1D. Fraction 1B (10.0 g) was continuously separated on a RP-C18 column chromatography, eluting with methanol/water (1/7-1/0) to give 4 fractions 2A1-2A4. Fraction 2A1 was separated on a *silica gel* column chromatography, eluting with DCM/MeOH/water (4/1/0.1, v/v/v) to yield compound **5** (25 mg). Fraction 2A2 (1.3 g) was separated on a *silica gel* column chromatography, eluting with DCM/MeOH/water (5/1/0.1, v/v/v) to yield compound **13** (26 mg) and compound **14** (17 mg). The 2A1 fraction (1.2 g) was chromatographed on a silica gel column and eluted with a gradient solvent system of DCM/MeOH/H_2O (5/1/0.1/0.05, v/v/v) to afford 6 fractions smaller 3A1-3A6. Fraction 3A2 was continuously separated on a RP-C18 column chromatography eluting with MeOH/H_2O (1/2, v/v) to give compound **12** (9 mg). The 3A5 fraction (1.2 g) was chromatographed on a sephadex LH-20 and eluted with solvent system of MeOH/H_2O (1/1, v/v) to afford 8 fractions smaller 3B1–3B8. Fraction 3B2 was continuously separated on a RP-C18 column chromatography eluting with MeOH/H_2O (1/2.5, v/v) to give compound **6** (22.0 mg). The 3B3 fraction was chromatographed on a a RP-C18 column and eluted with solvent system of MeOH/H_2O (1/2, v/v) to afford 4 fractions smaller 4A1-4A4. Fraction 4A3 was continuously separated on a a silica gel column chromatography eluting with DCM/MeOH/H_2O (5/1/0.1, v/v/v) to give compound **8** (21.0 mg) and **10** (27.0 mg). Fraction 4A4 was purified by CC on sephadex LH-20 (MeOH/water: 1/1) to give compound **9** (18 mg).

Fraction W3 was separated on a RP-C18 column chromatography eluting with MeOH/water (1/7-1/10, v/v) to give 6 fractions, 5A1-5A6. Fraction 5A2 (8.3 g) was continuously separated on a *silica gel* column chromatography eluting with DCM/MeOH/water (5/1/0.1 v/v/v) to give 3 fractions, 6A1-6A3. Compound **7** (18 mg) was yielded from 6A1 fraction using a *silica gel* chromatography column and EtOAc/MeOH/water (7/1/0.1:v/v/v) as an eluent. Fraction 5A3 was purified by CC on sephadex LH-20 (MeOH/water: 1/1) to give 2 fractions 7A1 and 7A2. Fraction 7A1 and 7A2 was continuously separated on a RP-C18 column chromatography eluting with MeOH/water (1/2, v/v) to yield compound **11** (22 mg).

2.4. Cell culture and treatment

The Raw 264.7 macrophage cell line was procured from American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% FBS and 1% penicillin–streptomycin. Cells were routinely cultured in an 37 °C incubator under a humidified atmosphere of 95% O_2 and 5% CO_2.

2.5. RNA isolation and quantitative RT-qPCR

For evaluating the effect of *B. laxiflora* derived extracts or purified compounds on COX-2 mRNA, cells were seeded in 35-mm dishes at the density of 1×10^6 cells/dish. After overnight incubation, cells were treated with 5 μg/mL LPS with or without sample solution, followed by further incubation for 18 h. Finally, cells were collected and subjected to qRT-PCR according to a previously described protocol (Pham et al., 2020a, b). Total RNA was isolated and purified using miRNeasy Mini Kit (Qiagen, MD, USA) according to the manufacturer's instructions. Quantitative reverse transcription PCR assay (RT-qPCR) was then performed with the QuantiFast SYBR Green RT-PCR Kit based on StepOnePlus Real-Time PCR System (Applied Biosystems, US). The comparative Ct ($^{\Delta\Delta}$Ct) method was employed to calculate the relative messenger RNA (mRNA) expression. The primers used for amplification of target genes were provided by Qiagen (QuantiTect primer assays).

2.6. Western blot analysis

Western blot analysis was performed essentially as previously described (Pham et al., 2020a, b). Cells were lysed in a lysis buffer containing 120 mM NaCl, 40 mM Tris (pH 8), and 0,1% NP40 for 30 min on ice. Total lysates were subsequently centrifuged to discard cell debris and protein concentration was quantified using protein assay kit (Pierce, Rockford, IL). Equal amounts (30 μg) of protein lysates were separated in a 10% sodium dodecyl sulfate polyacrylamide gel by electrophoresis, transferred to PVDF membranes, and blocked with 3% bovine serum albumin for alleviating non-specific binding. Membranes were then sequentially incubated with a primary antibody and an appropriate HRP-conjugated secondary antibody. Finally, the immunocomplexes were detected by enhanced chemiluminescence (ECL) detection system (Thermo Scientific, UK).

2.7. Cellular reactive oxygen species (ROS) measurement

Raw 264.7 were seeded in 35-mm dishes at the density of 1×10^6 cells/dish. After overnight incubation, cells were pretreated with tested compounds for 2 h, followed by stimulation with LPS for further 18 h. Cells were then incubated with 5 μM of 5-chloromethyl-2,7-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) (Thermo Scientific, Waltham, MA, USA) in the dark for 30 min for labeling ROS. Cells were subsequently washed with PBS, collected by scraping and centrifugation, and resuspended in the flow cytometry buffer. Mean fluorescence intensity (MFI) values were acquired using BD FACSCalibur™ (BD Biosciences, San Jose, CA, USA).

2.8. Cell viability assay

Cell viability assay was carried out as described previously (Pham and Park 2022). In brief, Raw 264.7 cells were seeded in 96-well plates at the density of 5×10^4 cells/well. After overnight incubation, cells were treated with compound **1** with or without LPS for 24 h. Finally, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfopheny)-2H-tetrazolium (MTS) solution (Promega Corporation, Madison, WI, USA) was added into culture media and cells were then incubated for 2 h. The number of viable cells was determined based on amount of resultant formazan dye by measuring the absorbance of each well at 490 nm using Spark™ 10M multimode microplate reader (Tecan, Mannedorf, Switzerland).

2.9. Bioluminescent firefly luciferase assays

Raw 264.7 macrophages were seeded in 24-well plates at the density of 3×10^5 cells/well. After overnight incubation, cells were co-transfected with an AP-1-dependent luciferase expressing plasmid and a control vector (pRL-TK) expressing wild type Renilla luciferase using Fugene HD transfection reagent (Promega Corporation) according to manufacturer's instructions. After 6 h of transfection, cells were treated with **1** for 2 h, followed by stimulation with LPS for further 6 h. The Firefly and Renilla luciferase activity were determined using the Dual Luciferase Reporter Assay System (Promega Corporation) in comply with manufacturer's recommendations. The relative AP-1 promoter activity was calculated based on ratio of Firefly to Renilla luciferase activity.

2.10. Measurement of the NADPH oxidases (NOXs) activity

Cells were seeded in 96-well plates at the density of 5×10^4 cells/well. After treatments as indicated, cells were completely lysed using a lysis reagent containing 0.1 N NaOH and 0.1% Triton X-100 in Hank's balanced salt solution (HBSS). The cell lysates were then incubated with NADPH substrate (400 μM) and lucigenin (200 μM) in dark for 30 min. NOXs activity was examined by measurement of the luminescence emitted upon the cleavage of luminogenic substrate lucigenin using a luminometer (Spark™ 10M multimode microplate reader; Tecan, Mannedorf, Switzerland).

2.11. Evaluation of in vivo anti-inflammatory activities

2.11.1. Animals

The adult male *Wistar* rats (10 weeks old, weighing 160–180 g) were provided by the Centre of Experimental Animals, Vietnam Military Medical University (Hanoi, Vietnam). All the animal experiments were handled in compliance with the guidelines of Hanoi University of Pharmacy for the care of laboratory animals. The experimental protocols were reviewed and approved by the Scientific and Ethical Committee of Hanoi University of Pharmacy (No 1213/QD-DHN). Rats were housed in 612 mm × 345 mm × 216 mm cages (Tecniplast, 2000P) in an animal room (Department of Pharmacology, Hanoi University of Pharmacy) with a temperature of (25 ± 1) °C, humidity of 55%–60%, regular 12/12 h light/dark cycle, and have free access to foods and tap water. The animals were acclimated for at least one week before any experimental manipulation.

2.11.2. Drug administration

Crude ethanol extract and purified compounds were suspended in 0.5% sodium carboxymethylcellulose (CMC-Na) and orally given to rats at indicated doses (once daily). The control rats were administered with 0.5% CMC-Na in the same way. For the carrageenan-induced paw edema model, all treatments were started 5 days prior to inflammatory induction. For the chronic inflammation model, the treatments were applied immediately after inflammatory induction and maintained thorough the experiment.

2.11.3. Carrageenan-induced paw edema model

The acute anti-inflammatory activity was assessed using carrageenan-induced paw edema model as previously described (Nguyen et al., 2017). Briefly, 1 h after the final treatment, paw edema was induced by injection of 1% (w/v) carrageenan (100 μl per paw) in normal saline into the plantar surface of the right hind paw. The volume of the rat paws was then determined by a Plethysmometer (Ugo Bisile, Italy) shortly before and at 1, 3, 5 or 7 h after edema induction. Rat paws were pre-marked in order to immerse it to the same extent in the measurement chamber and all measurements were performed in double blind by the same investigator. Indomethacin was used as a positive control in this model.

2.11.4. Cotton pellet–induced chronic inflammation model

The effect of treatments on chronic inflammation was examined in the rat model of cotton pellet induced granuloma. Cotton pellets (20 ± 1 mg) were immersed in 1% carrageenan solution followed by drying and sterilizing in a hot air oven at 120° for 2 h. After taking the dried weight, the sterilized cotton pellets containing carrageenan were implanted subcutaneously in the flank region under mild ether anesthetic condition.

Then, rats bearing granuloma were applied with appropriate treatments for consecutive 7 days. On day 8, the rats were sacrificed before the granulomas were carefully dissected out and dried at 60 °C for 18 h. Finally, the weight of dried granulomas was recorded and the difference between initial and post-implantation dry weight was considered as the real weight of the granuloma tissues.

2.12. Statistical analysis

Data were presented as mean ± standard error of mean (SEM). All statistical analyses were carried out using Graphpad Prism software version 8.02. Difference among groups was determined by the one-way analysis of variance (ANOVA) followed by LSD post-hoc test. P values of below 0.05 are considered statistically significant.

3. Results

3.1. Screening the COX-2 inhibitory activity of a crude ethanol extract and its fractions from Balanophora laxiflora

B. laxiflora has *in vitro* anti-inflammatory activity mediated by downregulation of COX-2. Hence, we examined the effect of a crude ethanol extract and its fractions on LPS-induced COX-2 expression in Raw 264.7 macrophages. The results confirmed that *B. laxiflora* significantly decreases COX-2 mRNA expression in LPS-stimulated Raw 264.7 macrophages (Figure 1). Furthermore, of the fractions tested, the ethyl acetate fraction had the greatest COX-2-suppressive effect. Therefore, this fraction and the aqueous residue were subjected to further chemical characterisation.

3.2. Structural identification of the isolated compounds

To elucidate the active components of *B. laxiflora*, the ethyl acetate fraction and aqueous residue were subjected to chromatographic separation, which led to the purification of 15 compounds. Based on MS and NMR spectral data (supplementary data, Fig. S1), these compounds were identified as (21α)-22-hydroxyhopan-3-on (**1**) (Tanaka and Matsunaga 1992), pinoresinol (**2**) (Páska et al., 2002), salicifoliol (González et al., 1989), ethyl caffeate (**4**) (Etzenhouser et al. 2001a, 2001b), 1-*O*-*E*-caffeoyl-*β*-D-glucopyranose (**5**) (Chiou et al., 2011), (8S,7′R,8′S)-isolariciresinol 4-O-β-D-glucopyranoside (**6**) (Yoshikawa et al., 1997), coniferin (**7**) (Chen et al., 2005), (8R,7′S,8′R)-lariciresinol 4′-O-β-D-glucopyranoside (**8**) (Kikuchi and Sugiyama 1993; Sugiyama and Kikuchi 1993), (8S,8′S)-secoisolariciresinol 9′-O-β-D-glucopyranoside (**9**) (Yuan et al., 2002), (8R,8′R)-secoisolariciresinol 4-O-β-D-glucopyranoside (**10**) (Yuan et al., 2002), (8S,7′R,8′S)-isolariciresinol 9-O-β-D-glucopyranoside (**11**) (Cai et al., 2009), coniferyl aldehyde β-D-glucopyranoside (**12**) (Kanchanapoom et al., 2001), 4-O-galloyl-1-O-E-caffeoyl-β-D-glucopyranose (**13**) (Jiang et al., 2001a, b), 6-O-galloyl-1-O-E-caffeoyl-β-D-glucopyranose (**14**) (Aliotta et al., 1992; Kamiya et al., 2002), and deacetyl asperulosidic acid (**15**) (Satake et al., 2002) (Figure 2).

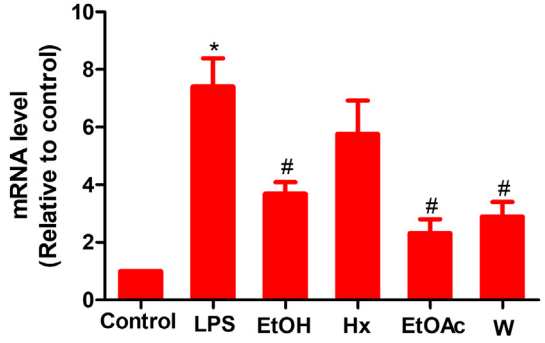

Figure 1. Effects of different fractions from *B. laxiflora* extract on the COX-2 mRNA expression. Raw 264.7 macrophages were stimulated with 100 ng/ml LPS in presence or absence of testing samples (30 μg/ml). The messenger RNA levels of COX-2 were determined as described in Materials and Methods. * denotes $p < 0.05$ compared to control; # denotes $p < 0.05$ compared to cells treated with LPS alone ($n = 3$).

3.3. Characterization of bioactive compounds from Balanophora laxiflora

Fifteen compounds isolated from the ethyl acetate fraction and aqueous residue, plus three previously identified compounds (compounds **16**, **17**, and **18** with chemical structures as shown in Supplementary data Fig. S2) (Tai et al., 2019)) derived from the aqueous fraction of *B. laxiflora*, were next screened for inhibition of LPS-induced COX-2 expression in Raw 264.7 macrophages. Of these, only **1** and 5 suppressed COX-2 mRNA expression upon LPS stimulation, with the reduction induced by **1** being more significant (Figure 3A). The COX-2-suppressive effect of **1** was confirmed by immunoblotting analysis, and **1** mitigated the induction of COX-2 protein expression by LPS in a dose-dependent manner (Figure 3B). We also examined the effect of **1** on the mRNA levels of other inflammatory intermediates and found that it suppressed the expression of pro-inflammatory iNOS, TNFα, IL-1β, and INF-β genes in macrophages, but not IL-6 (Figure 3C). Finally, MTS assay demonstrated that different concentrations of **1** had no significant impact on the viability of Raw 264.7 macrophages regardless of the presence of LPS (Figure 3D), excluding potential cytotoxic effects of **1**. Together, these findings reveal that **1** is an active constituent of *B. laxiflora* and has marked anti-inflammatory activity in Raw 264.7 macrophages.

3.4. The anti-inflammatory effect of 1 is mediated via suppression of ROS production

Given **1** as the most potent active compound from *B. laxiflora*, we sought to unveil the molecular mechanisms underlying its anti-inflammatory effects. First, we observed that **1** decreased cellular ROS levels in Raw 264.7 macrophages, similar to DPI, a pharmacological inhibitor of NADPH oxidases (NOXs), and N-acetylcysteine (NAC), a ROS scavenger, indicating that **1** suppresses LPS-stimulated ROS production (Figure 4A). Previous studies suggested that NOXs contribute to ROS production in response to LPS. Therefore, we examined whether NOXs are implicated in the suppression of ROS generation by **1**. As expected, stimulation of Raw 264.7 macrophages with LPS led to a dramatic increase in NOX activity, whereas pre-treatment with **1** or DPI reduced NOX activation by LPS (Figure 4B), strongly suggesting that **1** alleviates cellular ROS levels by inhibiting NOXs. Furthermore, the ROS inhibitors DPI and NAC also decreased LPS-induced COX-2 expression (Figure 4C), confirming a positive correlation between cellular ROS levels and the inflammatory response. Collectively, these results support the notion that downregulation of NOX activity mediates the anti-inflammatory effect of **1** by suppressing NOX-dependent ROS production in macrophages.

3.5. ROS suppression by 1 mitigates LPS-induced inflammation via inactivation of MAPK signaling pathways

It has been previously documented that MAPK signaling pathways are activated upon inflammatory stimulation by LPS in a ROS-dependent manner (Noguchi et al., 2008; Lee et al., 2022). We next examined the effects of **1** on MAPK activation and its role in modulating the inflammatory response caused by this compound. As shown in Figure 5A, **1** suppressed MAPK pathway activation, as evidenced by a drastic reduction in the levels of phosphorylated ERK 1/2, JNK 1/2, and p38 MAPK in LPS-stimulated Raw 264.7 macrophages. Notably, the suppression of ROS production by DPI or NAC also resulted in decreased phosphorylation of these MAPKs, suggesting that modulation of MAPK activation by **1** is mediated by blockade of ROS signaling. Given the role of activator protein 1 (AP-1) as a key effector of MAPK signaling in the inflammatory cascade, we speculated that **1** suppresses the expression of

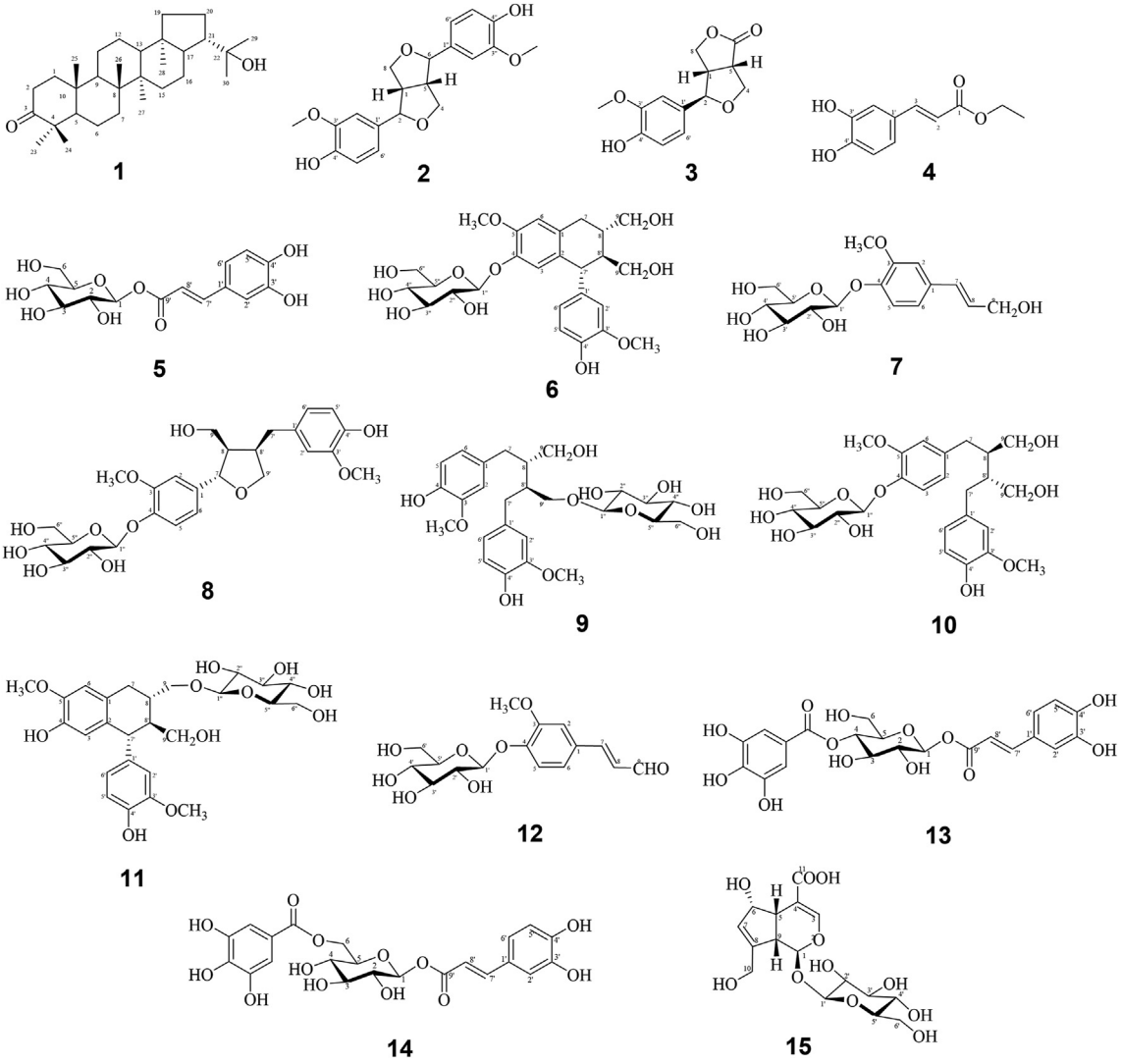

Figure 2. Chemical structure of compounds from *Balanophora laxiflora* (1–15).

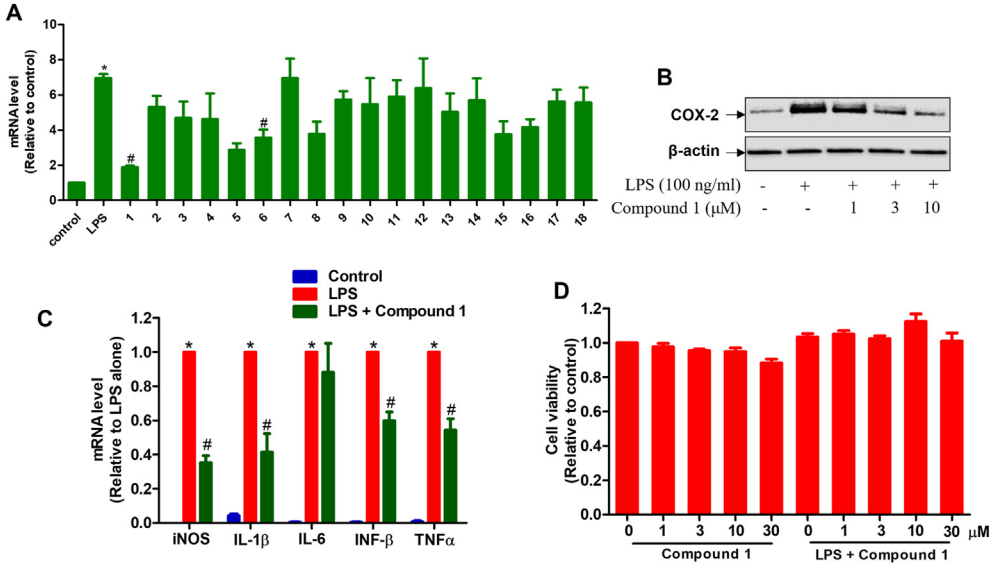

Figure 3. The anti-inflammatory effect of compounds isolated from *B. laxiflora*. (A) Raw 264.7 macrophages were pretreated with various compounds isolated from *B. laxiflora* (10 μM), followed by incubation with LPS (100 ng/ml) for further 6 h. COX-2 mRNA expression was analyzed as previously described. (B) Raw 264.7 macrophages were pretreated with indicated concentrations of 1 for 2 h, followed by stimulation with 100 ng/ml of LPS for further 8 h. Total protein lysates were immunoblotted for COX-2 and β-actin (served as a loading control). Representative images from three independent experiments were presented. Uncropped gel images were shown in Supplementary data, Fig. S4. (C) Raw 264.7 macrophages were pretreated with 1 (10 μM), followed by incubation with LPS (100 ng/ml) for further 6 h. The messenger RNA levels of target genes were examined by RT-qPCR assay. (D) Raw 264.7 macrophages were treated with indicated concentrations of 1 with or without 100 ng/ml of LPS for 24 h. Cell viability was measured using MTS assay. * denotes $p < 0.05$ compared to control; # denotes $p < 0.05$ compared to cells treated with LPS alone ($n = 3$).

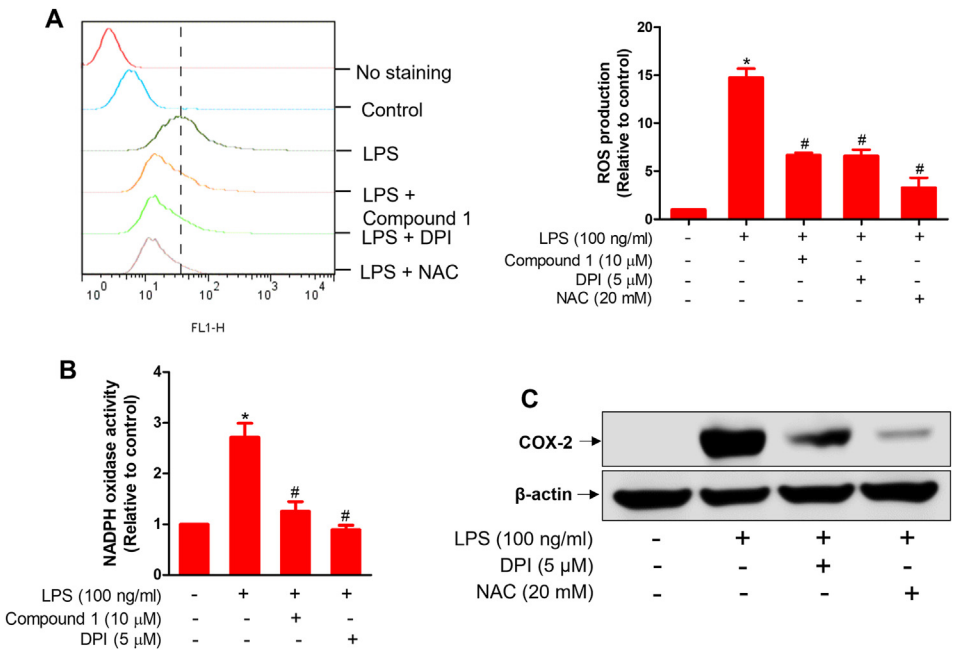

Figure 4. Suppression of ROS production critically contributes to the anti-inflammatory effects of 1. (A) Raw 264.7 macrophages were pretreated with 1, DPI, and N-acetylcysteine (NAC) at indicated concentrations for 2 h, followed by stimulation with LPS (100 ng/ml) for further 18 h. The cellular ROS level was measured by the flow cytometry analysis using 5-chloromethyl-2,7-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) as a ROS indicator. (B) Raw 264.7 macrophages were pretreated with 1 and DPI at indicated concentrations for 2 h, followed by stimulation with LPS (100 ng/ml) for further 6 h. The NOX activity was examined at the end of treatment period. (C) Raw 264.7 macrophages were pretreated with DPI and NAC for 2 h followed by stimulation with LPS (100 ng/ml) for 8 h. The COX-2 expression level was measured by Western blot. β-actin was served as a loading control. Uncropped gel images for Western blot experiments were presented in Supplementary data, Fig. S4 * denotes $p < 0.05$ compared to control; # denotes $p < 0.05$ compared to cells treated with LPS alone ($n = 3$).

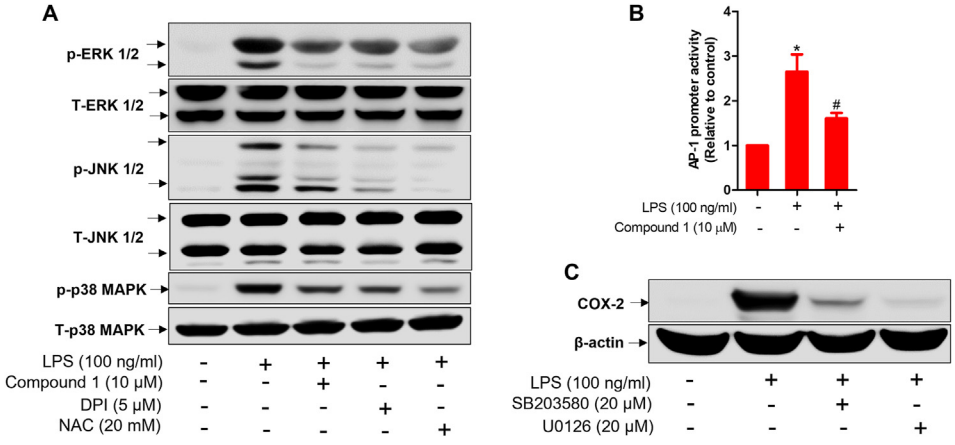

Figure 5. The role of MAPK signaling pathways in the anti-inflammatory effect of 1. (A) Raw 264.7 macrophages were pretreated with 1, DPI, and N-acetylcysteine (NAC) at indicated concentrations for 2 h, followed by stimulation with LPS (100 ng/ml) for 30 min. The phosphorylated and total levels of ERK 1/2, JNK 1/2, and p38 MAPK were determined by Western blot. (B) Raw 264.7 macrophages were pretreated with SB203580, a pharmacological inhibitor of p38 MAPK, or U0126, a pharmacological inhibitor of MEK/ERK, for 2 h followed by stimulation with LPS (100 ng/ml) for 6 h. The COX-2 expression level was measured by Western blot. β-actin was served as a loading control. Representative images from three independent experiments were presented. Original gel images were further shown in Supplementary data, Fig. S4.

pro-inflammatory genes by modulating the transcriptional activity of AP-1. Using the luciferase reporter assay, we demonstrated that **1** significantly decreased AP-1-dependent transcriptional activity in LPS-activated macrophages (Figure 5B). Finally, we found that the MAPK pathway inhibitors U0126 (MEK/ERK inhibitor) and SB203580 (p38 MAPK inhibitor) mitigated LPS-induced COX-2 expression (Figure 5C), verifying the involvement of MAPK signaling pathways in the inflammatory response of Raw 264.7 macrophages. Combined, these data clearly implicate the modulation of MAPK phosphorylation in the anti-inflammatory activities of **1**.

3.6. Compound 1 modulates the inflammatory response in animal models of acute and chronic inflammation

Having demonstrated that **1** modulates the inflammatory responses of macrophages by suppressing ROS- and MAPK-mediated signaling pathways, we examined whether **1** has anti-inflammatory activity in vivo. Using the carrageenan-induced paw oedema model, we found that **1** had a dose-dependent anti-inflammatory effect, as evidenced by the reduced rat paw volume over time (Figure 6A). Compared with the control, the total inflammatory response represented by the paw swelling decreased by 35.4% and 45.0% after treatment with 1 at 40 and 80 mg/kg, respectively (Figure 6B). A similar result was observed in the cotton pellet-induced chronic inflammation model, in which oral administration of 1 at 40 and 80 mg/kg significantly reduced granuloma size (Figure 6C). Notably, the suppression of inflammatory tissue formation by 80 mg/kg of **1** was comparable with that by 5 mg/kg of prednisolone, clearly indicating that **1** has glucocorticoid-like chronic anti-inflammatory activity.

4. Discussion

B. laxiflora and some of its isolated compounds have shown anti-inflammatory activities in previous studies. However, the mechanisms underlying these anti-inflammatory effects and the main bioactive constituents of *B. laxiflora* remain unclear. Using bioactivity-guided isolation, we identified 15 compounds in the ethyl acetate and aqueous fractions of *B. laxiflora*, including one new lignan and 14 known compounds. Of the known compounds, nine were characterized from *B. laxiflora* for the first time. An additional three compounds separated from the aqueous fraction, which were previously reported to possess weak anti-inflammatory activities, were included in the bioactivity assays to identify the most active compound. Based on COX-2 suppression, we found that (21α)-22-hydroxyhopan-3-one (compound **1**), a

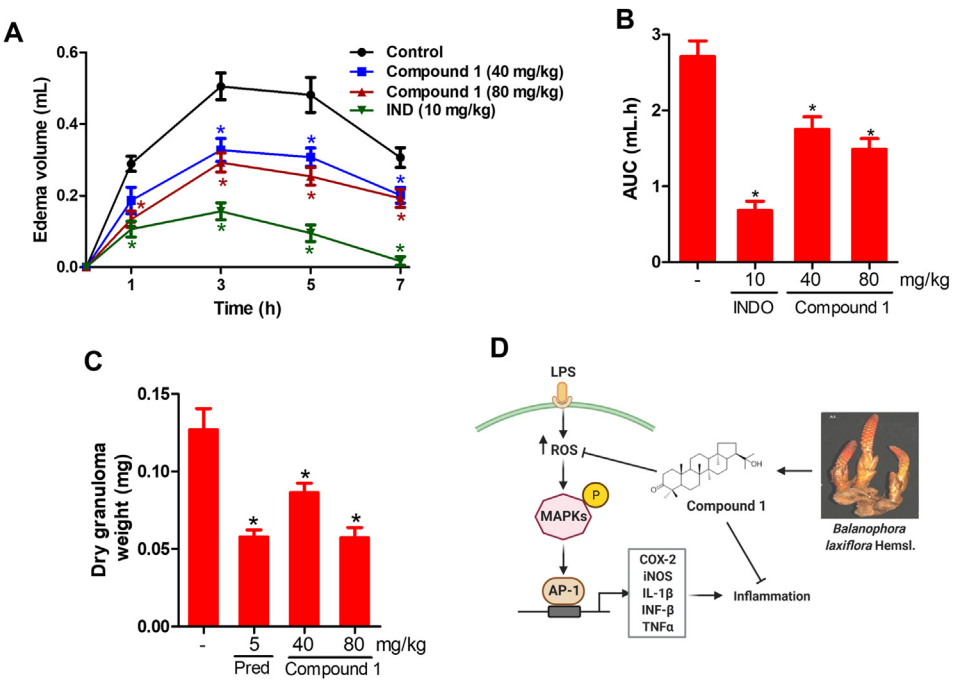

Figure 6. The anti-inflammatory effect of 1 in animal models of acute and chronic inflammation. (A-B) (A-B) Rats were pretreated with 1 at doses of 40 and 80 mg/kg, Indomethacin (IND) (10 mg/kg) or 0.5% CMC-Na (control group). Paw edema was induced in rats by injection of 1% (w/v) carrageenan into the plantar surface of the hind paw. The paw volume was monitored at 0, 1, 3, 5, and 7 h upon edema induction (A) and the area under the curve (AUC) of paw swelling was calculated (B). (C) Granulomas were generated in rats as previously described. Indicated doses of 1 and prednisolone were applied for 1 week followed by separating, drying, and weighing the granulomas. Dry weights of the granulomas were shown. * denotes $p < 0.05$ compared to control ($n = 8$). (D) The mechanistic model was proposed for the anti-inflammatory effect of 1. The compound 1 suppresses ROS production promoted by pro-inflammatory agents such as LPS and thereby inhibits the phosphorylation of MAPKs including ERK 1/2, JNK 1/2, and p38 MAPK. Alleviated phosphorylation of MAPKs in turn leads to decrease in the promoter activity of AP-1, a transcriptional factor responsible for regulating expression of inflammatory mediators such as COX-2, iNOS, IL-1β, INF-β and TNFα, and reduction in the inflammatory response as a result.

triterpenoid first isolated from a species in the genus *Balanophora*, had the most promising anti-inflammatory activity. Intriguingly, while this compound was first recognized almost 50 years ago (Haan Hui et al., 1975), little was known about its pharmacological actions. The antifungal effects and cytotoxicity of compound **1** against cancer cells have been evaluated previously, although this compound failed to show any significant effect in these assays (Kashiwada et al., 2007; Luciano et al., 2010). Therefore, to our knowledge, this is the first report on the anti-inflammatory activity of **1** that makes it interesting to get a further insight into the modulation of inflammatory response by this compound. Furthermore, since **1** was the most abundant among the isolated compounds (with an extraction yield of 0.56 mg/g), this compound can serve as an excellent marker for anti-inflammatory potential of *B. laxiflora*.

There is accumulating evidence that ROS act as intracellular signaling molecules that contribute to the pathogenesis of various disorders, including inflammation-associated diseases. Excess ROS production causes oxidative stress, a critical pathological event observed in acute and chronic inflammation (Mittal et al., 2014; Bar-Or et al., 2015; Forrester Steven et al., 2018). ROS signaling is also essential for the activation of inflammasomes, molecular platforms playing a key role in the processing of pro-inflammatory cytokines, such as IL-1β, IL-18, and IL-33 (Harijith et al., 2014; Pham and Park 2020). Unsurprisingly, ROS inhibitors may ameliorate the inflammatory conditions triggered by diverse stimuli and prevent the development and progression of inflammation-linked complications (Yang et al., 2018; Baek et al., 2020). In line with previous reports, we observed that blockade of ROS signaling by either suppressing NOX-mediated ROS production with DPI or scavenging ROS using NAC leads to decreased COX-2 expression in macrophages, confirming the involvement of ROS in modulating COX-2 in our experimental models. Furthermore, compound **1** reduced the ROS levels in activated macrophages, suggesting that it modulates the inflammatory response by impairing ROS signaling. Given the role of NOXs as enzymes responsible for cytoplasmic ROS production (Magnani and Mattevi 2019), we examined whether **1** affects NOX activity. Herein, we showed that NOXs are activated in response to inflammatory stimuli, and **1** decreased NOX activity in LPS-stimulated macrophages, demonstrating that NOX inhibition contributes, at least in part, to the suppression of ROS generation by **1**. While previous studies suggested that some triterpenoids possess antioxidant activity by scavenging free radicals (Gülçin et al., 2006), our study provides evidence that natural triterpenoids such as **1** inhibit NOX enzymatic activity and counteract oxidative stress and inflammation. Moreover, **1** scavenged free radicals, as determined by the decreased formation of DPPH and superoxide radicals (Supplementary data, Fig. S3), indicating a multifaceted role of **1** in modulating cellular ROS levels.

MAPKs are evolutionarily conserved protein kinases, belonging to three major classes (ERK, JNK, and p38) with diverse physiological functions (Lawrence et al., 2008). In response to pro-inflammatory stimuli, MAPKs are activated to trigger the inflammatory cascade by modulating pro-inflammatory mediators at both the transcriptional and translational levels. For example, phosphorylation by MAPKs enhances AP-1 transcription, which increases the expression of numerous cytokines (Kaminska 2005). Notably, while both ROS production and the MAPK signalling pathways are upregulated in the inflammatory response, there is evidence that ROS and MAPKs modulate each other mutually to promote inflammation (Millar et al., 2007; Reddy and Reddanna 2009). Since **1** reduces ROS production, the role of MAPKs in the modulatory effects of **1** on oxidative stress and inflammation should be investigated. In this study, **1** decreased the phosphorylation of ERK, JNK, and p38 by LPS in macrophages. Furthermore, the ROS inhibitors DPI and NAC also inhibited MAPK activation, strongly suggesting that **1** downregulates MAPK signalling pathways in inflammation via suppression of ROS production. The downregulation of MAPKs, in turn, alleviates the inflammatory response by decreasing the activity of transcription factors such as AP-1, resulting in reduced pro-inflammatory mediator expression, including, but not limited to, TNFα, IL-1β, INFβ, iNOS, and COX-2.

The *in vitro* anti-inflammatory activities observed with **1** are essentially similar to those of total extract of *B. laxiflora* which has been reported in a previous study (Chen et al., 2012). It has been also shown that total extract of this plant exhibits *in vivo* efficacy in some animal models of inflammation such as carrageenan- and serotonin-induced paw edema (Chen et al., 2012). In the present study, we confirmed the anti-inflammatory effect of 1 using the carrageenan-induced paw oedema model, the most widely used method for investigating the acute anti-inflammatory actions of plant extracts and natural products. Although the positive control indomethacin and 1 both had significant oedema-suppressive effects

during the late phase of carrageenan-induced inflammation (3 h after inducing inflammation), characterized by the production and secretion of inflammatory mediators, including kinins, prostaglandins, nitric oxide, and cytokines [48, 1 was less efficacious than indomethacin. This difference may arise because indomethacin directly inhibits the enzymatic activity of COXs, whereas 1 modulates the expression of COX-2 and other inflammatory mediators. While carrageenan-induced inflammation is sensitive to NSAIDs, the cotton-pellet-induced granuloma model recapitulates the features of chronic inflammation and responds best to steroids (Spector 1969). Surprisingly, the higher dose of 1 (80 mg/kg) strongly inhibited the formation of cotton pellet granulomas and was comparable with 5 mg/kg prednisolone, suggesting that **1** has steroid-like effects. Interestingly, a recent report attributed the anti-inflammatory effects of pentacyclic triterpenoids, such as bartogenic acid, to their structure similarity to steroids (Patil and Patil 2017). Further insights into the anti-inflammatory properties of 1 would help explain its role in different inflammatory contexts.

5. Conclusions

In summary, our bioactivity-guided isolation led to the identification of (21α)-22-hydroxyhopan-3-one (**1**) as the main constituent responsible for the anti-inflammatory activities of *B. laxiflora*. For the first time, we demonstrated that this triterpenoid possesses anti-inflammatory effects *in vitro* and *in vivo*. Furthermore, our data suggest a mechanistic model for the anti-inflammatory effects of **1** (Figure 6D), which alleviates cellular oxidative stress during inflammation by inhibiting NOX-dependent ROS production or acting as a ROS scavenger. Impaired ROS signalling blocks the MAPK signalling pathway and decreases the transcription of inflammatory mediators promoted by MAPK-regulated transcription factors. Our results suggest that **1** is a promising candidate for anti-inflammatory therapy. Future work should examine its potential clinical applications in inflammatory diseases.

Declarations

Author contribution statement

Thuy-Duong Nguyen: Conceived and designed the experiments; performed the experiments; Contributed reagents and materials.

Thi-Hong-Anh Nguyen; Van Thi-Hong Tran: Performed the experiments; Analyzed and interpreted the data.

Thi-Ha Do: Contributed reagents and materials; Analyzed and interpreted the data.

Hoang-Anh Nguyen: Analyzed and interpreted the data; Wrote the paper.

Duc-Vinh Pham: Conceived and designed the experiments; Wrote the paper.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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