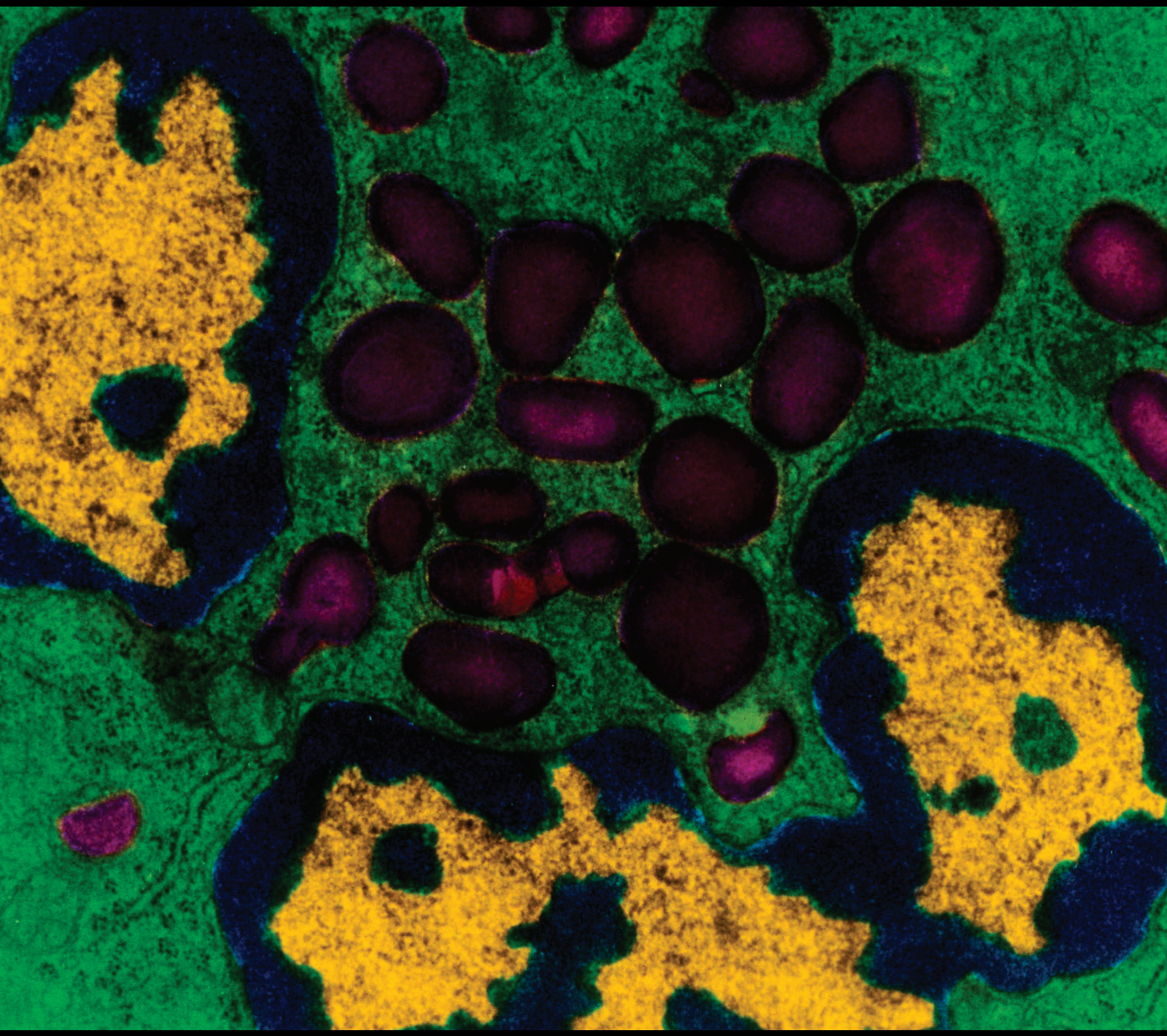
Mediators of Inflammation

Natural Anti-Inflammatory Products/ Compounds: Hopes and Reality

Guest Editors: Barbara Romano, Asif J. Iqbal, and Francesco Maione



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Editorial

Natural Anti-Inflammatory Products/Compounds: Hopes and Reality

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Inflammation is a complex biological response to injury as a result of different *stimuli* such as pathogens, damaged cells, or irritants. Nowadays, the commercially approved anti-inflammatory drugs are represented by nonsteroidal anti-inflammatory drugs (NSAID), glucocorticoids (SAID), and in some cases immunosuppressant and/or biological drugs. These agents are effective for the relief of the main inflammatory symptoms. However, they induce severe side effects, whereas most of them are inadequate for chronic use.

Starting from these premises, the demand of new effective and safely anti-inflammatory drugs has furthered research into new therapeutic approaches. The recent and emerging scientific community slant is oriented to the herbal medicines that could represent a treasure for the discovery of new active compounds and for the development of new drugs and potentially useful therapeutic agents.

The articles contained in this special issue include both reviews and basic scientific studies focused on characterizing the molecular mechanisms of the inflammatory process in humans and also in animal models.

In particular, on this special issue the attention on the role of proinflammatory mediators and pathways such as IL-1, IL-6, mPGES-1, and NF-B had been pointed out ("*Moringa oleifera* Flower Extract Suppresses the Activation of Inflammatory Mediators in Lipopolysaccharide-Stimulated RAW 264.7 Macrophages *via* NF-κB Pathway" by W. S. Tan et al. and "Downregulation of mPGES-1 Expression *via* EGR1 Plays an Important Role in Inhibition of Caffeine on PGE_2 Synthesis of HBx(+) Hepatocytes" by Y. Ma et al. and "Anti-Inflammatory Effect of 1,3,5,7-Tetrahydroxy-8-isoprenylxanthone Isolated from Twigs of *Garcinia esculenta* on Stimulated Macrophage" by D.-D. Zhang et al.).

Moreover, this special issue has highlighted the importance of some mechanism and strategy involved in inflammatory bowel disease and experimental model of osteoarthritis ("Botanical Drugs as an Emerging Strategy in Inflammatory Bowel Disease: A Review" by F. Algieri et al. and "Therapeutic Effect of Chenodeoxycholic Acid in an Experimental Rabbit Model of Osteoarthritis" by Z. Yan et al.). Finally, interesting contributions regarding the anti-inflammatory activity of marine diterpenoids and leishmaniasis inflammatory response have been successfully submitted to this special issue ("Marine Diterpenoids as Potential Anti-Inflammatory Agents" by Y. González et al. and "Natural Products: Insights into Leishmaniasis Inflammatory Response" by I. A. Rodrigues et al.).

We hope that this special issue will stimulate the interest of the scientific community involved in studying the effects of natural products/compounds on different fields of interests such as inflammation and pain. The papers published here will surely contribute to proposing new additional insights into the mechanism of several conditions as well as to suggesting new diagnostic alternatives and therapeutic targets in widespread pathologies. The discovery of the new is, as always, anchored to the recourse of the old.

Acknowledgments

We would like to express our thanks to all the authors for their contributions and to all the reviewers for their

support, commitment, and constructive critiques in making this special issue possible.

Barbara Romano
Asif Jilani Iqbal
Francesco Maione

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Research Article

Moringa oleifera Flower Extract Suppresses the Activation of Inflammatory Mediators in Lipopolysaccharide-Stimulated RAW 264.7 Macrophages via NF-κB Pathway

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Aim of Study. *Moringa oleifera* Lam. (*M. oleifera*) possess highest concentration of antioxidant bioactive compounds and is anticipated to be used as an alternative medicine for inflammation. In the present study, we investigated the anti-inflammatory activity of 80% hydroethanolic extract of *M. oleifera* flower on proinflammatory mediators and cytokines produced in lipopolysaccharide- (LPS-) induced RAW 264.7 macrophages. *Materials and Methods*. Cell cytotoxicity was conducted by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Nitric oxide (NO) production was quantified through Griess reaction while proinflammatory cytokines and other key inflammatory markers were assessed through enzyme-linked immunosorbent assay (ELISA) and immunoblotting. *Results*. Hydroethanolic extract of *M. oleifera* flower significantly suppressed the secretion and expression of NO, prostaglandin E_2 (PGE_2), interleukin- (IL-) 6, IL-1β, tumor necrosis factor-alpha (TNF-α), nuclear factor-kappa B (NF-κB), inducible NO synthase (iNOS), and cyclooxygenase-2 (COX-2). However, it significantly increased the production of IL-10 and IκB-α (inhibitor of κB) in a concentration dependent manner (100 μg/mL and 200 μg/mL). *Conclusion*. These results suggest that 80% hydroethanolic extract of *M. oleifera* flower has anti-inflammatory action related to its inhibition of NO, PGE_2, proinflammatory cytokines, and inflammatory mediator's production in LPS-stimulated macrophages through preventing degradation of IκB-α in NF-κB signaling pathway.

1. Introduction

The inflammatory process is consecutive and well-regulated mechanisms which respond to the stimulation and activation of the defense systems. The target cells such as macrophages have been stimulated by physical, chemical, microbial, and immunological reaction which produce inflammatory responses [1]. Inflammation is the central features of many chronic diseases which cause morbidity and mortality. The occurrence of chronic diseases has triggered prolonged inflammation that induced the expression of robust proinflammatory mediators and cytokines, which are harmful, which leads to the pathogenesis of inflammation associated chronic diseases [2].

Moringa oleifera Lam. (*M. oleifera*) family of Moringaceae is indigenous to India, Pakistan, Bangladesh, and Afghanistan, which is now widely distributed in many countries of the tropics and subtropics over the world [3]. *M. oleifera* is a perennial angiosperm plant, and it is one among thirteen species belonging to the monogeneric family [4]. The bioactive compounds from various parts of the plant including leaves, roots, bark, gum, flowers, fruits, seeds, and seed oil have been attributed to high nutrition value and prophylactic and medicinal virtue [5]. Edible parts of

M. oleifera have shown various pharmacological properties: mainly, antimicrobial, antihypercholesterolemic, antitumor, antidiabetic, and antioxidant properties [6, 7]. The medicinal importance of different parts of the plant including leaves, roots, seeds, and fruits has long been used as folkloric medicine to treat various ailments related to inflammation [8, 9]. Currently, *M. oleifera* have been interesting for many biomedical researchers due to the presence of bioactive compounds which are responsible for various biomedical applications. However, only few scientific findings have reported the biomedical application of *M. oleifera* flower extract; thus, we are interested in exploring its therapeutic potential as anti-inflammatory agents.

Lipopolysaccharide (LPS) is a principal component of the outer membrane of Gram-negative bacteria that can activate immunological responses in cells [10]. LPS activates the inflammatory mechanisms through three pathways which are mitogen-activated protein kinases (MAPKs), nuclear factor-kappa B (NF-κB) signaling, and janus kinase/signal transducers and activators of transcription (JAK/STAT) pathways [11, 12]. NF-κB signaling pathway is one of the highly expressed pathways among all other pathways, which enhanced various inflammatory genes expression (NF-κB), inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and production proinflammatory mediators (interleukin- (IL-) 6, IL-1β, and tumor necrosis factor-alpha (TNF-α)) [13–16]. Therefore, in the present study, we have investigated and reported the anti-inflammatory potential of 80% hydroethanolic extract of *M. oleifera* flower on producing various inflammatory mediators, NO, PGE_2, IL-6, IL-1β, TNF-α, IL-10, NF-κB, IκB-α, COX-2, and iNOS, in LPS-stimulated murine macrophages through NF-κB signaling pathway.

2. Materials and Methods

2.1. Chemical Reagents. Dulbecco's Modified Eagle's medium (DMEM), Fetal Bovine Serum (FBS), Penicillin/Streptomycin for cell culture, and Bovine Serum Albumin (BSA) and RIPA buffer were purchased from Nacalai (Kyoto, Japan). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), lipopolysaccharides from *Escherichia coli* 0111:B4 (LPS), and N-1-naphthylethylendiamide-dihydrochloride (NED) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Bicinchoninic acid (BCA) assay and sulphanilamide were obtained from Thermo Scientific (Waltham, MA, USA) and Friendemann Schmidt (CT Parkwood, WA, Australia), respectively. Primary antibodies specific to iNOS, COX-2, NF-κB, IκB-α, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and, in addition, anti-rabbit and/or anti-mouse secondary antibodies conjugated to horseradish peroxidase were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Plant Material Collection and Extraction. The *M. oleifera* flowers were obtained from Garden No. 2 at Universiti Putra Malaysia and have been confirmed with the voucher specimen (SK 1561/08) that has been deposited in the IBS Herbarium unit. The flowers were washed, air-dried at room temperature for 12 h and oven-dried for two consecutive days at 45°C, grounded to powder form, and stored in vacuum bags. *M. oleifera* flower powder was macerated in hydroethanolic solvent (ethanol : distilled water, 80 : 20 [80%]) for 3 days under rotary shaker at room temperature. Further, the residue was filtered, solvent-evaporated, freeze-dried, weighed, and stored at 4°C until further investigation.

2.3. Chromatographic Analysis and Instrumentation. The analysis was carried out using a HPLC-UV system (Agilent 1100 series, USA) equipped with a binary pump, array detector (diode array detector [DAD]) (200 to 600 nm range; 5 nm bandwidth), and an autosampler. A LUNA C18 (4 × 250 mm, 5 μm) Phenomenex column (Torrance, CA, USA) maintained at room temperature (25°C) was used in the chromatographic analysis. The separation was carried out in a gradient system with its mobile phase consisting of solvent A, distilled water, and solvent B, methanol : distilled water 70 : 30 (v/v). The gradient program profile was a combination of solvents A and B as follows: 0 to 10 min, 30% solvent B; 10 to 20 min, 40% solvent B; 20 to 35 min, 50% solvent B; 35 to 40 min, 60% solvent B; 40 to 45 min, 70% solvent B; and 45 to 50 min, 0% solvent B. The detection was made at 254 nm and the injection volume and flow rate were 20 μL and 1.0 mL/min, respectively. The compounds in the hydroethanolic *M. oleifera* flower extracts were separated using a C18 column (4 × 250 mm, 5 μm, Phenomenex) with a gradient mobile phase consisting of water (solvent A) and methanol with 1% acetonitrile (solvent B), each containing 0.1% formic acid and 5 mM ammonium format, using the gradient program of 40% solvent B to 50% solvent B over 11.00 min at a flow rate of 1.0 mL/min, and were identified with accurate mass detection using an AB Sciex 3200 QTrap LCMS/MS with a Perkin Elmer FX 15 UHPLC system (MA, USA). The sample injection volume was 20 μL and the negative ion mass spectra were obtained with a LC QTrap MS/MS detector in full ion scan mode (100 to 1200 *m/z* for full scan and 50–1200 *m/z* for MS/MS scan) at a scan rate of 0.5 Hz. The system was supported with mass spectrometry software and a spectral library provided by ACD Labs (Toronto, ON, Canada). All chromatographic procedures were performed at ambient temperature, and the corresponding peaks from the QTrap LC MS/MS analysis of the compounds were identified by comparison with the literature/ACD Labs Mass Spectral Library.

2.4. Cell Culture. The murine macrophage cell line, RAW 264.7, was obtained from the American Type Culture Collection (ATCC, VA, USA) and maintained in DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin at 37°C in a humidified incubator with 5% CO_2. The cell's media were changed every 2-3 days and passaged in 70–90% confluent condition by trypsinization to maintain cells exponential growth stage.

2.5. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Colorimetric Assays. MTT assay was performed to determine the cytotoxicity and cell viability

of 80% hydroethanolic *M. oleifera* flower extract on RAW 264.7 macrophages. The 100 μL of RAW 264.7 macrophages was seeded in triplicate into 96-well plates (1×10^5 cells/well) and incubated for 24 h. The macrophages were treated with various gradient concentration hydroethanolic flower extract with serial dilutions at 15.625, 31.25, 62.5, 125, 250, 500, and 1000 μg/mL and then incubated for 24 h. Briefly, thereafter, 20 μL of MTT solution (5 mg/mL) in phosphate-buffered solution (PBS) was added to each well and then followed by incubation for another 3 h. The medium was removed and the purple formazan crystals formed were dissolved by adding 100 μL dimethyl sulfoxide (DMSO). The plate was swirled gently to mix well and kept in dark condition at room temperature for 30 min. The absorbance was determined by using ELx800 Absorbance Microplate Reader (BioTek Instruments Inc., VT, USA) at 570 nm wavelength. The results were expressed as a percentage of surviving cells over control cells.

2.6. Nitrite Quantification Assay. The NO was determined through the indication of nitrite level in the cell culture media. The macrophages were seeded in 6-well plates (1×10^6 cells/well) with 2 mL of cell culture media and incubated for 24 h. This was followed by discarding the old culture media and replacing them with the new media to maintain the cells. Different concentrations of hydroethanolic *M. oleifera* flower extract (100 μg/mL and 200 μg/mL) and the positive control dexamethasone (0.5 μg/mL) were pretreated with the RAW 264.7 macrophages. Induction of RAW 264.7 macrophages with LPS (1 μg/mL) for all samples was conducted except in control for another 24 h. Then, 100 μL of the collected supernatants was added with 100 μL of Griess reagent (0.1% NED, 1% sulphanilamide, and 2.5% phosphoric acid) and incubated in room temperature for 10 min in dark condition. The absorbance was determined by using microplate reader at 540 nm wavelength. The NO concentration was determined by comparison to the standard curve.

2.7. Enzyme-Linked Immunosorbent Assay (ELISA). RAW 264.7 macrophages with or without hydroethanolic *M. oleifera* bioactive flower extract and dexamethasone (0.5 μg/mL) in the presence of LPS (1 μg/mL) were seeded in 6-well plates (1×10^6 cells/well) for 24 h. RAW 264.7 macrophages untreated with LPS which act as control were included for comparison. The concentrations of PGE_2 and cytokine mediators such as IL-6, IL-1β, TNF-α, and IL-10 were assayed in cultured media of macrophages using mouse ELISA kits (R&D Systems Inc., MN, USA), according to the manufacturer's instructions.

2.8. Immunofluorescence Staining. Macrophages (RAW 264.7 cells) were cultured in glass coverslips in 6-well plate (1×10^6 cells/well) and inflammation induced by LPS with presence or absence of flower extract for 24 h and then fixed with methanol/acetone fixation. After that, fixed cells were permeabilized with 0.2% 10x Triton in PBS for 2 min at room temperature (RT). The macrophages in coverslips were then rinsed with PBS and incubated with (1% BSA in PBS) blocking buffer for 30 min at RT. The cells then incubated with NF-κB primary antibody (1 : 250) and anti-rabbit secondary antibodies conjugated to fluorophores (1 : 1000) in blocking buffer for 1 h, respectively. Nuclear macrophages were stained with Hoechst (1 : 5000) from Thermo Scientific (Waltham, MA, USA) in PBS for 15 min. The macrophages were ready to view and photographs were taken through fluorescent microscope at 200x magnification (Olympus, Tokyo, Japan).

2.9. Immunoblot Analysis. Protein extracts were harvested and prepared by using RIPA buffer for Western blot analyses from treated macrophages. The concentration of protein was determined by using the BCA. Equal amounts of cellular proteins were loaded on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions for separation. The separated protein was then transferred to polyvinylidene difluoride (PVDF; GE Healthcare) membranes for 1 h. The membrane underwent blocking step for minimum 1 h with blocking solution (5% of BSA in phosphate-buffered saline containing 1% Tween-20 (PBST)) at room temperature prior to incubation of specific primary antibodies such as NF-κB, IκB-α, iNOS, COX-2, and β-actin at 4°C overnight. The membrane was washed 5 times with PBST followed by incubation with respective anti-rabbit or anti-mouse secondary antibodies conjugated to horseradish peroxidase for 1 h and washed 5 times with PBST for 10 min each. The bands were visualized using chemiluminescence system (Chemi Doc, Bio Rad, USA). The bands were followed by analysis using Image J software (Bio Techniques, New York, USA).

2.10. Statistical Analysis. The results were summarized from three independent experiments and data expressed as the mean ± standard deviation (SD). The significant differences were examined using IBM with SPSS 20.0 software (SPSS Inc., Chicago, USA). One-way analysis of variance (ANOVA) and Turkey's post hoc test were used for pairwise comparisons. p value of 0.05 or less was considered as statistically significant.

3. Results

3.1. Phytochemical Analysis of M. oleifera Flower Extract. To further interpret the observed effects of the *M. oleifera* flower extract, it is important to understand the molecular composition of the extract. In this regard, the HPLC fingerprint of 80% hydroethanolic *M. oleifera* flower extract (Figure 1(a)) was obtained to screen its peaks, followed by identification of compounds by LC-MS analysis (Figure 1(b)). Among the seven identified compounds, majority of the compounds were documented as phenolic compounds. Tentatively, these compounds have been identified and reported as quinic acid, 4-*p*-coumaroylquinic acid, quercetin-3-*O*-acetyl glucoside, kaempferol-3-*O*-acetyl hexoside, octadecenoic acid, heneicosanoic acid, and docosanoic acid and inclusive of other details such as m/z values and retention time, which were reported in (Table 1), based on the literature [7, 17–21]/ACD Labs Mass spectral Library.

Table 1: Retention times, MS, and MS fragments of the major bioactive constituents present in hydroethanolic *M. oleifera* crude flower extract by HPLC–DAD–ESI–MS/MS.

Peak	Retention time (RT)	Molecular ion peak $(M-H)^-$	MS^2 fragment ions intensity	Tentative compounds identified
1	0.53	191	173, 127, 93 (100), 85	Quinic acid
2	1.05	337	191, 163, 119 (100)	4-*p*-Coumaroylquinic acid
3	2.24	506	300 (100), 271, 255, 179, 151	Quercetin-3-*O*-acetyl glucoside
4	2.63	490	284/286, 255 (100), 227	Kaempferol-3-*O*-acetyl hexoside
5	3.57	329	229, 211 (100), 171, 99	Octadecenoic acid
6	5.67	325	281, 253, 225, 183 (100)	Heneicosanoic acid
7	6.07	339	275, 239, 199, 183 (100)	Behenic (docosanoic) acid

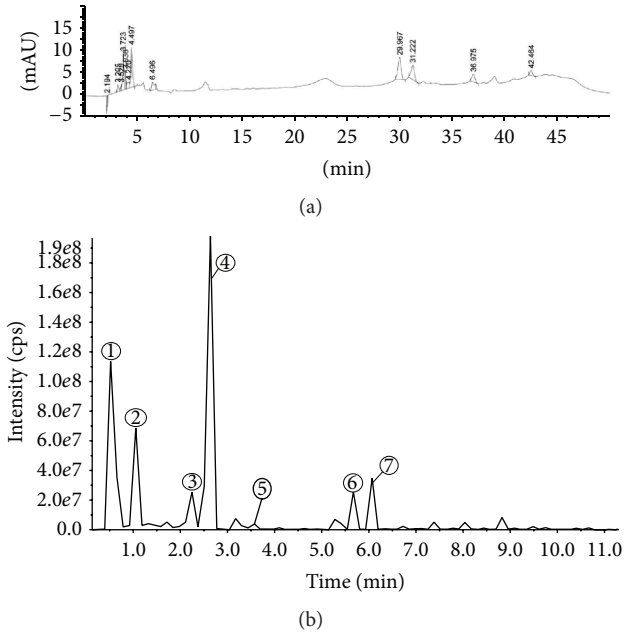

Figure 1: (a) HPLC-DAD (254 nm) fingerprints and (b) LC-MS/MS (254 nm) chromatogram of *M. oleifera* hydroethanolic flower extract.

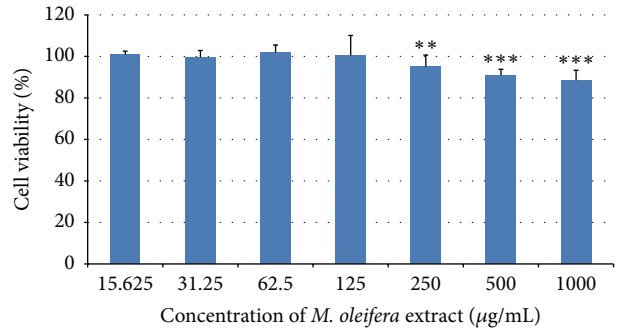

Figure 2: Effects of 80% hydroethanolic *M. oleifera* bioactive flower extract on the viability of RAW 264.7 macrophages. A density of 1×10^5 cells/well of macrophages were seeded in 96-well plate and incubated with various concentrations of flower extract for 24 h. Cell viability was determined by MTT assay. The data are presented as mean ± SD of three independent experiments. $^{***}p < 0.001$, $^{**}p < 0.01$ versus culture media without flower extract which act as control.

3.2. Effect of M. oleifera on Cell Viability. MTT reduction assay was used to access the cytotoxicity effect of 80% hydroethanolic *M. oleifera* flower extract at concentration ranging from the lowest to highest (15.625–1000 μg/mL) on RAW 264.7 macrophages. The cytotoxicity potential of flower extract on macrophages was presented in Figure 2. The results showed that increasing concentrations of hydroethanolic *M. oleifera* flower extract have caused reduction of cell viability. However, hydroethanolic *M. oleifera* flower extract did not exhibit any toxicity to macrophages at concentrations ranging from 15.625 to 125 μg/mL. According to the cytotoxicity investigations, the concentrations at 100 μg/mL and 200 μg/mL were chosen for further anti-inflammatory experiments.

3.3. Effect of M. oleifera on NO Production. The effect of 80% hydroethanolic *M. oleifera* bioactive flower extract on NO production in LPS-induced RAW 264.7 macrophages was tested with NO assay. Griess reagent was used to determine nitrite (NO_2^-) released in the cell culture supernatant. Result from Figure 3 showed that the untreated control group released low level of nitrite (2.21 ± 0.016 μM), while treated LPS group promoted nitrite production (6.120 ± 0.110 μM) in inflammatory nature. The two different concentrations (at concentrations 100 μg/mL and 200 μg/mL) of 80% hydroethanolic flower extract gave good inhibitory effect on nitrite production. Dexamethasone, which was used as positive control, has also reduced the nitrite production (5.316 ± 0.106 μM). *M. oleifera* extract treatment with 100 μg/mL has decreased the nitrite secretion into 4.098 ± 0.133 μM while 200 μg/mL induced more attenuation effect on nitrite production (1.051 ± 0.149 μM).

3.4. Effect of M. oleifera on PGE_2 and Proinflammatory Cytokines Production. LPS-induced RAW 264.7 macrophages were used to determine the inhibitory action of 80% hydroethanolic *M. oleifera* flower extract on the production of PGE_2 and proinflammatory enhancement of anti-inflammatory cytokines which was shown in Figures 4(a)–4(d): proinflammatory cytokines include IL-6, IL-1β, and TNF-α, while anti-inflammatory cytokine includes IL-10. Figure 4(e) showed increased production of PGE_2 in macrophages whereas these levels were suppressed while being treated with *M. oleifera*

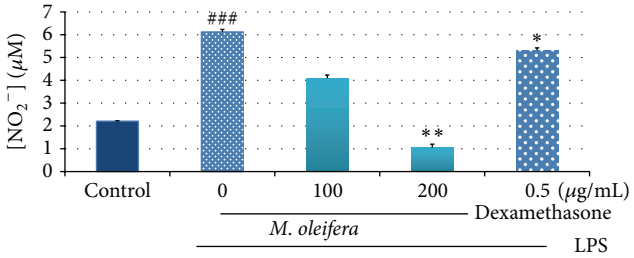

Figure 3: Effects of 80% hydroethanolic *M. oleifera* bioactive flower extract on NO production by LPS-induced RAW 264.7 macrophages. A density of 1×10^6 cells/well of macrophages in the presence or absence of LPS were seeded in 6-well plate and treated with indicated concentrations of flower extract and dexamethasone for 24 h. The supernatants were collected and investigated by Griess assay. The data are presented as mean ± SD of three independent experiments. Control; basal level of nitrite released without LPS induction. $^{###}p < 0.001$: LPS-treated group versus control; $^{**}p < 0.01$ and $^{*}p < 0.05$: treated group significantly different from LPS-treated group.

flower extract. LPS induction had trigged the production of all types of proinflammatory cytokines in macrophages. *M. oleifera* flower extract at concentration 200 μg/mL treatment significantly reduced the production of IL-6 (19.083 ± 0.003 pg/μL), IL-1β (116.889 ± 0.002 pg/μL), and TNF-α (6840.5 ± 0.016 pg/μL) but slightly increased production of IL-10 (1036 ± 0.002 pg/μL) from 436 ± 0.0067 pg/μL at concentration of 100 μg/mL flower extract in the LPS-stimulated macrophages.

3.5. Effect of M. oleifera on NF-κB p65 Expression. Immunofluorescence staining and fluorescence microscopy were used to examine the effect of *M. oleifera* flower extract on NF-κB activation. As Figure 5 shows, the higher expression of NF-κB activation was observed in LPS-stimulated macrophages; NF-κB p65 were translocated from cytoplasm into nucleus. However, pretreatment with flower extract with concentrations of 100 and 200 μg/mL suppressed/inhibited the LPS-induced NF-κB p65 activation. These investigations were consistent with Western blot results indicating that *M. oleifera* flower extract effectively suppressed LPS-induced NF-κB p65 expression in a concentration dependent manner.

3.6. Effect of M. oleifera on Expression of Inflammatory Mediators. Immunoblotting was conducted to evaluate the expression of inflammatory mediators which included NF-κB, IκB-α, iNOS, and COX-2 in LPS-stimulated RAW 264.7 macrophages treated with the 80% hydroethanolic *M. oleifera* flower extract at concentrations 100 and 200 μg/mL. As illustrated in Figure 6, the NF-κB, iNOS, and COX-2 target markers are significantly expressed in the LPS-treated group compared to the control untreated group. However, the treatment of *M. oleifera* flower extract concentration dependently downregulated the target molecule expressions in LPS-stimulated macrophages. On the other hand, IκB-α expression is increased with the presence of flower extract.

4. Discussion

In recent years, utilization of plant-derived constituents in the field of pharmaceutical research arena has been increased abundantly, due to its wide array of medicinal properties and minimal or null toxicity compared with the synthetic drugs. Among traditional medicine, *M. oleifera* is well known for its impressive range of medicinal and nutritional value. Edible parts of this plant contain a high content of essential minerals, proteins, nutrients, and also various phenolic compounds stands for its medicinal properties. The leaves of this plant have been extensively investigated and certainly reported for its therapeutic potential and mechanism of action against various clinical complications, due to presence of rich bioactive candidates. Currently, *M. oleifera* flower has also been in the pipeline of investigation against hepatotoxicity, microbial infection, and other medical complications, which revealed positive reports [22–24]. However, only a few reports exist on the therapeutic potential of *M. oleifera* flower extract. Thus, in this study, we intended to evaluate the anti-inflammatory potential of *M. oleifera* flower extract and identify its liable active candidates through various chromatographic techniques.

Previously, our research team has reported that *M. oleifera* leaves are enriched with flavonoids such as kaempferol and quercetin [7] and also reported the presence of high flavonol contents in *M. oleifera* flowers grown at South Africa [25]. Accordingly, the results of this study also indicated that *M. oleifera* flower extract is enriched with major phenolic compounds such as quercetin and kaempferol. Hämäläinen et al. [26] and García-Mediavilla et al. [27] reported the anti-inflammatory potential of quercetin and kaempferol by inhibition of signal transducer and activator of transcription 1 (STAT-1) and NF-κB pathway. These reports strongly suggested that the presence of quercetin and kaempferol in *M. oleifera* flower extract is supposedly responsible for its elevated anti-inflammatory activity. Despite other phenolic compounds such as quinic acid, 4-*p*-coumaroylquinic acid which has been previously reported *in M. oleifera* leaves is recently found to be present as of GC-MS/MS results on *M. oleifera* flower [28]. Accordingly, we identified the existence of quinic acid and 4-p-coumaroylquinic acid in *M. oleifera* flower extract, also evidently involved in its anti-inflammatory potential [29]. Apart from the phenolic compounds, few fatty acids/their derivatives have also been identified in *M. oleifera* flower extract. Fatty acids such as α-linolenic acid, oleic acid, octadecenoic acid, palmitic acid, heneicosanoic acid, capric acid, and behenic acid have already been reported to exist in *M. oleifera* leaves, root, and seed. However, to the best of our knowledge, we report here for the first time the presence of octadecenoic acid, heneicosanoic acid, and behenic acid in *M. oleifera* flower extract. Thus, from these reports, it can be concluded that the coexistence of major phenolic compounds and essential fatty acids is supposedly responsible for the enhanced anti-inflammatory potential of *M. oleifera* flower extract.

Raw 264.7 macrophages have been used as model to evaluate the effects of 80% hydroethanolic *M. oleifera* flower extract in anti-inflammatory activity due to phagocytic

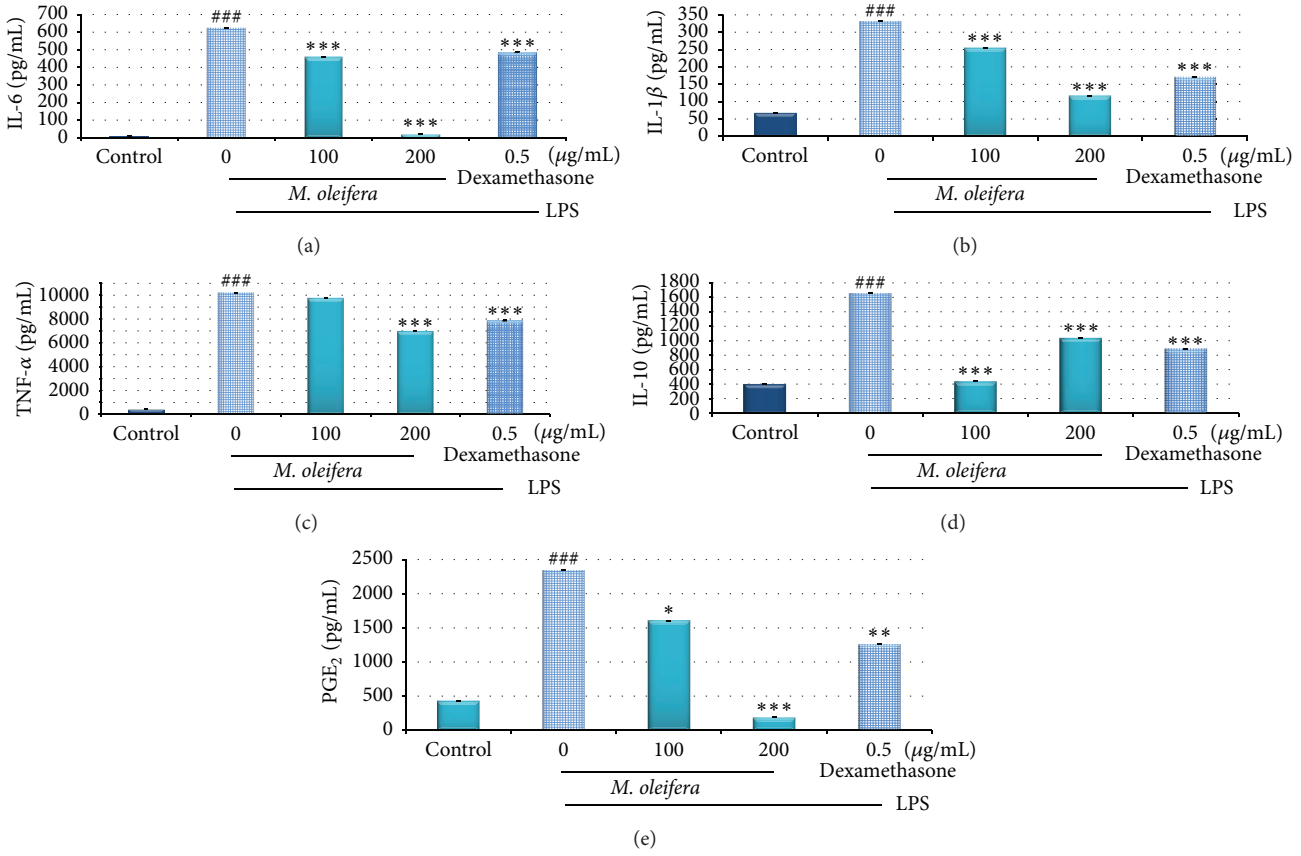

Figure 4: Effect of 80% hydroethanolic *M. oleifera* bioactive flower extracts on the production of cytokines IL-6, IL-1β, TNF-α, IL-10, and PGE_2 by LPS-induced RAW 264.7 macrophages. A density of 1×10^6 cells/well of macrophages induced by LPS were seeded in 6-well plate and treated with indicated concentrations of flower extract and dexamethasone for 24 h. The supernatants were collected and analysed by ELISA kits. The data are presented as mean ± SD of three independent experiments. $^{###}p < 0.001$: LPS-treated group versus control; $^{***}p < 0.001$, $^{**}p < 0.01$, and $^{*}p < 0.05$: treated group significantly different from LPS-treated group. Control: basal level of cytokines released without LPS induction.

activities for immunological defence. Bacterial, viral, and fungal infection and tissue damage have caused the activation of proinflammatory signaling proteins especially toll-like receptors (TLRs). Macrophages produced various highly active proinflammatory mediators including the cytokines and chemokines like monocytes chemoattractant protein-1 (MCP1) and other inflammatory active molecules upon activation of TLRs [30]. Besides, inflammation involves induction of transcriptional mediators NF-κB and activator protein 1 (AP-1), downstream from protein tyrosine kinases such as Syk and Src, serine/threonine kinases such as Akt, IKK, and TBK1, and mitogen-activated protein kinases [MAPKs: ERK (extracellular signal-related kinase), p38, and JNK (c-Jun N-terminal kinase)] [31].

LPS was bonded to toll-like receptor 4 (TLR-4) of macrophages and activated the downstream pathways which is signal transduction pathway kinases to induce inflammation via TLR-NF-κB signaling pathways [10]. As shown in Figure 5, phosphorylation and degradation of IκB-α in cytosol activated transcription factors and transferred NF-κB into nucleus which caused increase in activity after stimulation with LPS. NF-κB bonded to its response element and enhanced gene expression to produce proinflammatory cytokines and enzymes [32–34]. On activation, the level of cytokines (IL-6, IL-1β, TNF-α, and PGE_2) (Figure 4) production in the culture supernatants was increased in response to LPS stimulation which showed the successful *in vitro* inflammation experimental model.

Mitochondrial dependent reduction of MTT colorimetric assay is one of *in vitro* assays to determine the potential cytotoxicity effect of flower extract. As the concentration of extract increased, the number of viable cells reduced. However, as shown in Figure 2, *M. oleifera* bioactive flower extract does not possess cytotoxicity effect on macrophages up to concentration 1000 μg/mL since the cell viability is more than 80%. In this study, flower extract with concentrations 100 and 200 μg/mL within the range of concentrations which give better cell viability percentages has been used for further *in vitro* anti-inflammatory investigations.

NO, a labile free radical gas, is an important mediator and regulator of inflammatory response and excessively generated during inflammation reaction [35]. The three types of isoforms of NO synthase (NOS) include neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS

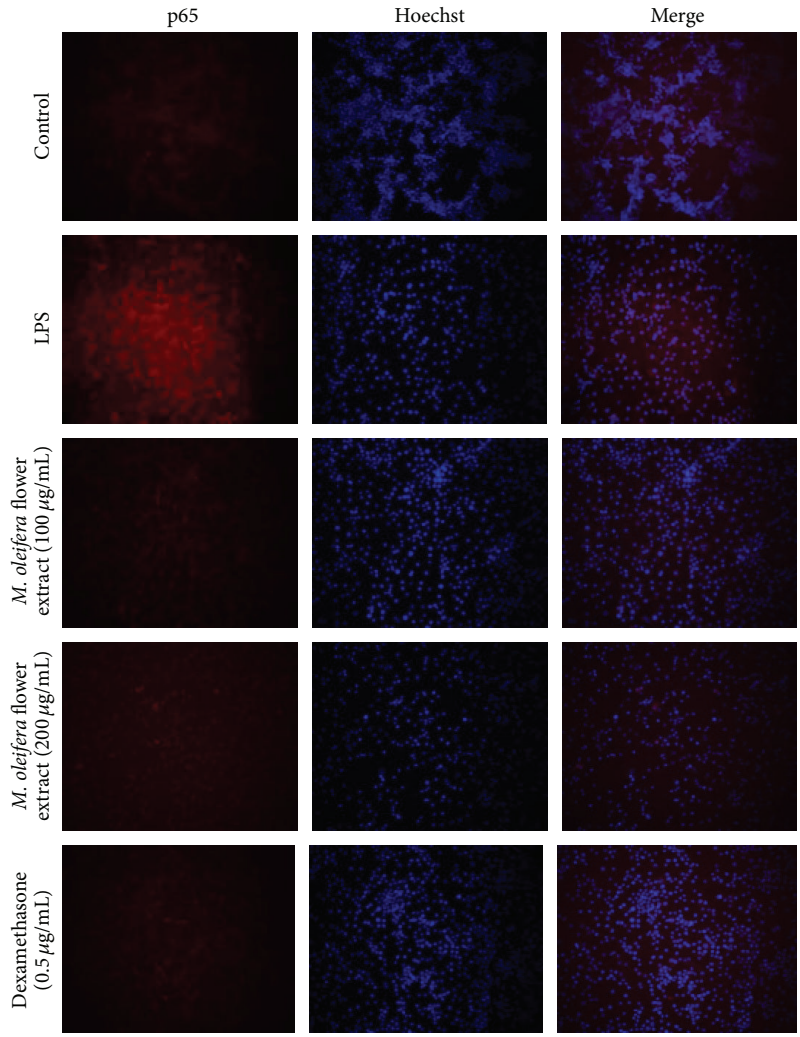

Figure 5: The effect of *M. oleifera* flower extract on NF-κB p65 expression in LPS-stimulated RAW 264.7 macrophages. Macrophages were treated with extract (100 and 200 μg/mL) and dexamethasone (0.5 μg/mL) in the presence of LPS (1 μg/mL) for 24 hours. Expression of NF-κB p65 was observed by fluorescence microscope after immunofluorescence staining with anti-NF-κB p65 antibody and fluorescein labeled anti-rabbit IgG (red). Nuclei of the cells were stained with Hoechst 33342 (blue) and images were captured (original magnification, ×200).

(iNOS). NO production in macrophages upon exposure to LPS is due to the oxidation of L-arginine into L-citrulline via the action iNOS in animal tissue [36, 37]. NO plays a role in vasodilatation, neurotransmission, and inhibition of platelet aggregation inflammation and induced cell apoptosis [38–40]. However, oversecretion of NO reacts with superoxide leading to tissues damage and contributes to pathological development of chronic inflammatory illnesses [41]. According to [29], licochalcone E (Lic E) suppressed the expression of iNOS and reduced the production of NO in dependent dose and showed it possesses potential anti-inflammatory effect. In present study, LPS-induced NO production (Figure 3) was significantly reduced by treatment with hydroethanolic *M. oleifera* flower extract via inhibiting iNOS expression (Figure 5) in a concentration dependent manner. Suppression of the iNOS and NO was observed after dexamethasone treatment in LPS-induced macrophages.

According to Makarov [42], increased production of proinflammatory cytokines such as TNF-α, IL-6, and IL-1β has resulted in adverse effect of inflammatory responses. Production of TNF-α mainly in macrophages via NF-κB activation also stimulated the production of IL-1β, IL-6, and NO, thus acting as factor amplifying the inflammation and its associated complications [43]. According to [44], IL-6 is a B-cell differential factor which acts as multifunctional cytokine to regulate the immune and inflammatory response. Overproduction of IL-6 is often correlated with chronic diseases in inflammatory autoimmune diseases. However, IL-10 is an immunosuppressive, anti-inflammatory, and pleiotropic cytokine that modulates functions of immune cells. Treatments with hydroethanolic *M. oleifera* flower extract have suppressed the LPS-induced production of IL-6, IL-1β, and TNF-α but enhanced IL-10 by concentration dependently (Figures 4(a)–4(d)). Treatment with dexamethasone also

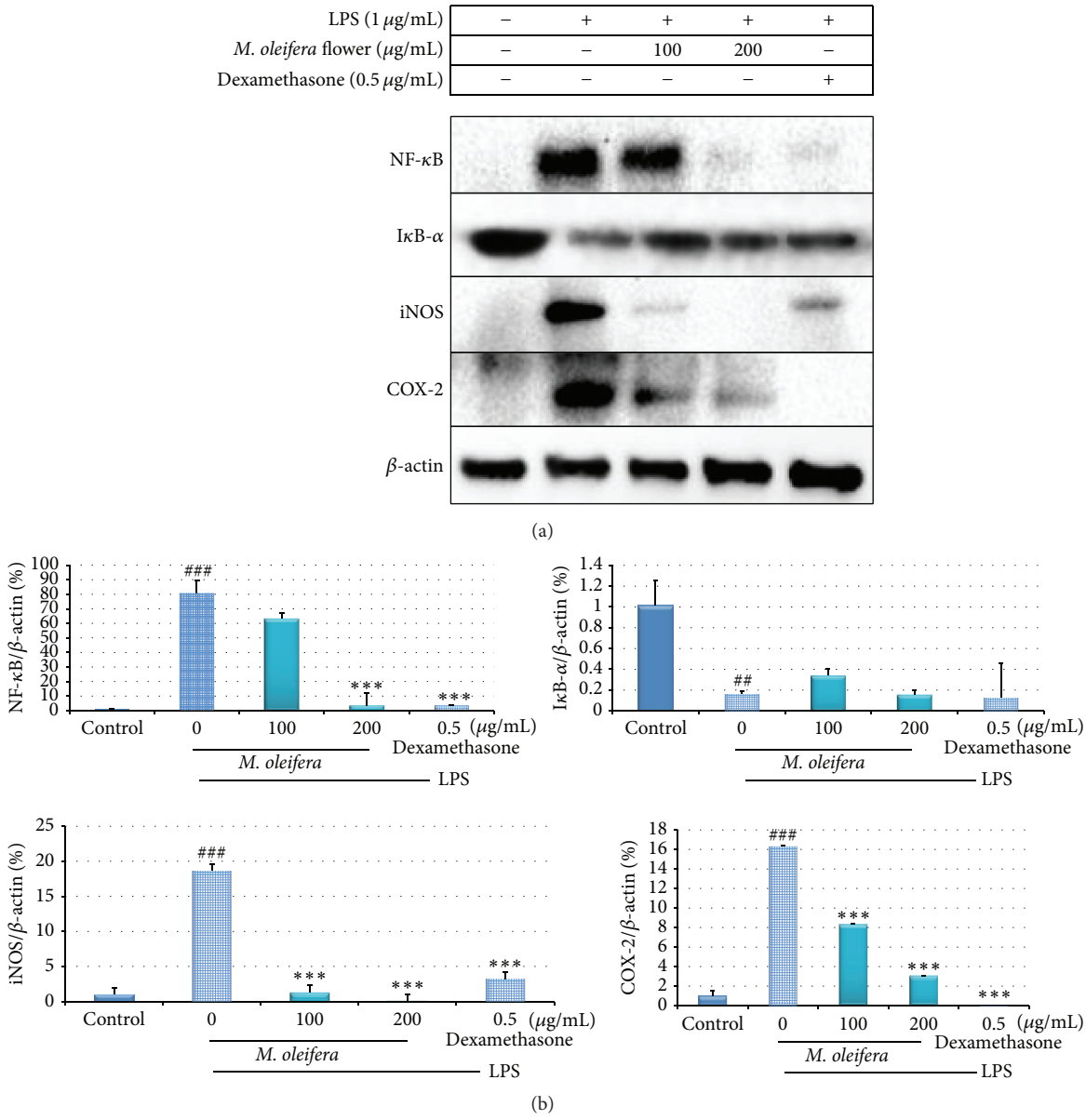

FIGURE 6: (a) Anti-inflammatory effect of 80% hydroethanolic *M. oleifera* bioactive flower extract on the expression of NF-κB, IκB-α, iNOS, and COX-2 in LPS-induced RAW 264.7 macrophages. A density of 1×10^6 cells/well of macrophages in the presence or absence of LPS were seeded in 6-well plate and treated with indicated concentrations of flower extract for 24 h. The protein of cells was collected through RIPA buffer and analysed by Western blotting. β-actin acts as a loading control and also standard for target proteins in quantitative determination. (b) Densitometry analysis results of the effect of *M. oleifera* flower extract on proteins expression. $^{###}p < 0.001$ and $^{##}p < 0.01$ were LPS-treated group versus control; $^{***}p < 0.001$: treated group significantly different from LPS-treated group. Control: basal level of cytokines released without LPS induction. The data are presented as mean ± SD of three independent experiments.

revealed the inhibition on proinflammatory cytokines production but enhancement in IL-10 level in LPS-induced macrophages ($^{***}p < 0.001$).

NF-κB is critical regulator mediator for iNOS, COX-2 transcription, and the production cytokines in LPS-induced macrophages. Inactive NF-κB is located in cytoplasm as part of complex but activated NF-κB upon LPS translocated to nucleus and bonded to its cognate DNA-binding sites to stimulate several intracellular signaling pathways [36]. This increases the expression of iNOS and COX-2 during inflammation [45]. Overexpressed iNOS in macrophages caused overproduced NO which induced inflammatory response. High expression of COX-2, an inducible enzyme which induced excessive production of PGE_2, which act as proinflammatory mediators in inflammatory state [46]. The production of cytokines is regulated by NF-κB expression through IκB-α phosphorylation by IκB kinase complex (IKK) [10, 47, 48]. Immunoblot results have (Figure 6) shown that LPS induces the degradation of IκB-α expression by IKK complex, while *M. oleifera* flower extract and positive

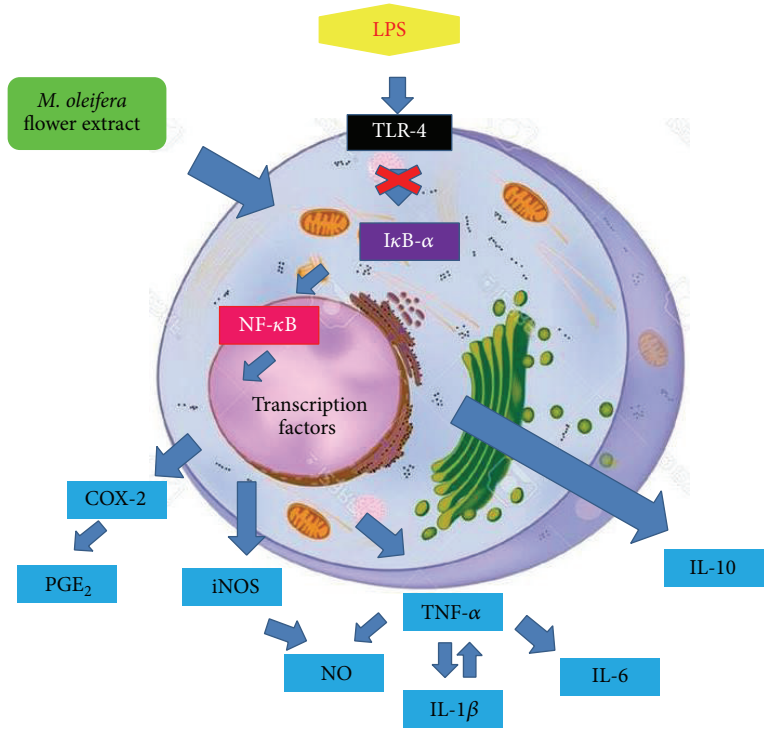

Figure 7: Mechanism blockade of NF-κB activation in RAW 264.7 macrophages by 80% hydroethanolic *M. oleifera* flower extract.

control treatment showed significantly enhanced expression of IκB-α. Hydroethanolic *M. oleifera* flower extract and dexamethasone have exhibited anti-inflammatory properties in a concentration dependent fashion in suppressing LPS-induced production of proinflammatory mediators including IL-6, IL-1β, and TNF-α, as well as NF-κB, iNOS, and COX-2 expression. However, they enhanced production of IL-10 and expression of IκB-α. These results have proven that hydroethanolic *M. oleifera* flower extract exerted its activity on upstream signaling pathway. *M. oleifera* flower extract might inhibit NF-κB activation activity by blocking the degradation of IκB-α and retained NF-κB in cytoplasm from further activation. Proinflammatory genes expressions from downstream targets of NF-κB have been downregulated [8]. In this study, blockade of NF-κB activation by inhibiting LPS-induced IκB-α phosphorylation is an effective molecular target to prevent elevation of proinflammatory mediators as the mechanism shown in Figure 7.

5. Conclusion

In conclusion, we demonstrated that 80% hydroethanolic *M. oleifera* flower extract has significant effect on inhibiting the production of NO and downregulated the expression of inflammatory mediators (NF-κB, iNOS, and COX-2) and proinflammatory cytokines (TNF-α, IL-1β, IL-6, and PGE_2) whereas it increased expression of anti-inflammatory cytokines, IL-10 and IκB-α, in LPS-stimulated macrophages. These findings suggest that 80% hydroethanolic *M. oleifera* flower extract can be a potent inhibitor of inflammation through NF-κB signaling pathway. Further studies are needed to understand the precise molecular mechanisms regulating the anti-inflammatory activity in animal model and validate it as a modulator of macrophage activation.

Conflict of Interests

The authors declare that there is no conflict of interests.

Authors' Contribution

Woan Sean Tan and Palanisamy Arulselvan started the investigation, planned and performed all the scientific experiments, and wrote the paper. Govindarajan Karthivashan analysed the chromatographic results (HPLC and LC-MS analysis). Sharida Fakurazi and Palanisamy Arulselvan established the key experimental approaches and contributed all the research materials to complete the research and finalized the final paper. Woan Sean Tan and Palanisamy Arulselvan contributed equally to this work.

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Review Article

Botanical Drugs as an Emerging Strategy in Inflammatory Bowel Disease: A Review

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Crohn's disease and ulcerative colitis are the two most common categories of inflammatory bowel disease (IBD), which are characterized by chronic inflammation of the intestine that comprises the patients' life quality and requires sustained pharmacological and surgical treatments. Since their aetiology is not completely understood, nonfully efficient drugs have been developed and those that show effectiveness are not devoid of quite important adverse effects that impair their long-term use. Therefore, many patients try with some botanical drugs, which are safe and efficient after many years of use. However, it is necessary to properly evaluate these therapies to consider a new strategy for human IBD. In this report we have reviewed the main botanical drugs that have been assessed in clinical trials in human IBD and the mechanisms and the active compounds proposed for their beneficial effects.

1. Introduction

Inflammatory bowel disease (IBD) is a chronic gastrointestinal inflammatory disorder characterized by alternating relapses and remissions. The two most common types of IBD are Crohn's disease (CD) and ulcerative colitis (UC), which are characterized by exacerbated uncontrolled intestinal inflammation that contributes to worsening of the life quality of the patients and require prolonged medical and/or surgical interventions. The inflammation associated with CD can discontinuously affect all the gastrointestinal tract, from the mouth to the anus, but it is more often localized to the distal small bowel and/or colon. Samples of inflamed bowel obtained from patients with active CD show transmural inflammation with an important accumulation of acute and chronic inflammatory cells within the mucosa, submucosa, and muscularis propia. On the other hand, UC is characterized by a nontransmural inflammation, just localized within the rectum and the large bowel. Typically, the inflammation is restricted to the mucosa and submucosa, with cryptitis and crypt abscesses, although the inflammatory cell composition is similar to CD. The clinical presentation in these intestinal conditions mostly depends on disease location and is characterized by diarrhoea, abdominal pain, fever, bowel obstruction, passage of blood, and/or mucus [1, 2]. Unfortunately, the aetiology of IBD is not fully understood [3], although there is a general agreement that IBD is the result of a complex combination of four main factors: multiple genetic variations, alterations in the composition of the intestinal microbiota, changes in the surrounding environment, and overreactivity of the intestinal mucosal immune response [4]. Thus, genetically susceptible patients build up an exaggerated and uncontrolled immune response in the gastrointestinal tract towards an altered intestinal microbiota that turns into a chronic intestinal inflammation. Similarly to other inflammatory conditions, a broad spectrum of inflammatory mediators is responsible of the pathophysiology of IBD, including cytokines, chemokines, leukotrienes, and prostaglandins, together with reactive oxygen and nitrogen species. Their synthesis and release are severely altered, which participate in the different phases of the inflammatory process that take place in the gut [5].

Considering all the above, IBD treatment pursues two clear goals: firstly, to promote the symptom remission during the acute flare, and secondly, to maintain the remission and control the chronic inflammation to prevent or hold up the reactivation of the intestinal inflammatory process. It is evident that suppression of the exaggerated immune response is crucial for the management of IBD patients. Actually, this is the major aim of the pharmacological therapy, which includes aminosalicylates (sulfasalazine or mesalamine), immunosuppressants (glucocorticoids, azathioprine, methotrexate, and cyclosporine A), and biologicals (infliximab or adalimumab) [6]. Nevertheless, despite the efficacy shown by these drugs, the important rate of side effects may even limit their necessary long-term use [7]. Therefore, the development of new therapies that combine efficacy and safety in human IBD therapy is needed.

In this regard, the use of alternative therapies has emerged as a common approach in gastrointestinal diseases [8]; actually, a study described that almost half of IBD patients have ever taken or currently use complementary remedies [9]. Different factors may contribute to this situation, including the lack of a complete response to standard therapy and the general feeling about a better safety profile of traditional remedies, in combination with the appreciation of an improved control of their disease [10–12]. There are many different types of alternative and/or complementary therapies, although the botanical drugs are very relevant for the treatment of the intestinal inflammation [13]. This can be mainly related to their safety, since they have been taken from ancient times, in addition to their reputed efficacy, most probably due to the presence of different active components that can concurrently target several pathways or mediators of the inflammatory response. However, most of these uses have an empirical basis, and in consequence, it is necessary to properly evaluate these botanical drugs to consider them as an adequate strategy to treat IBD.

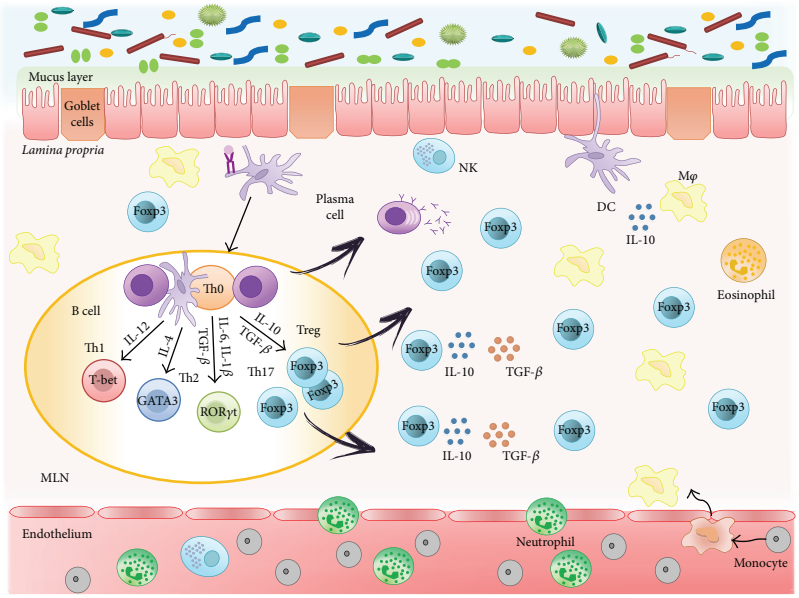
The aim of the present review is to provide scientific arguments that would support the use of medicinal plants as alternative and/or complementary therapy in human IBD. For this purpose, we have focused our attention on those botanical drugs evaluated in human IBD by clinical trials, most of them based on preclinical studies performed in experimental models of colitis. In addition, the mechanisms that may be involved in their intestinal anti-inflammatory effects will be analysed, as well as the main components that can account for the reputed beneficial effects, with a special consideration to polyphenols, including flavonoids, phenylpropanoids, and stilbenes. In fact, these compounds have been well characterized by their antioxidant properties that may prevent the damage caused by reactive oxygen and nitrogen species [26], which have been proposed to be key for the pathogenesis of these intestinal diseases [27].

2. Cellular and Molecular Mechanisms Involved in the Inflammatory Response in the Intestine (Figure 1)

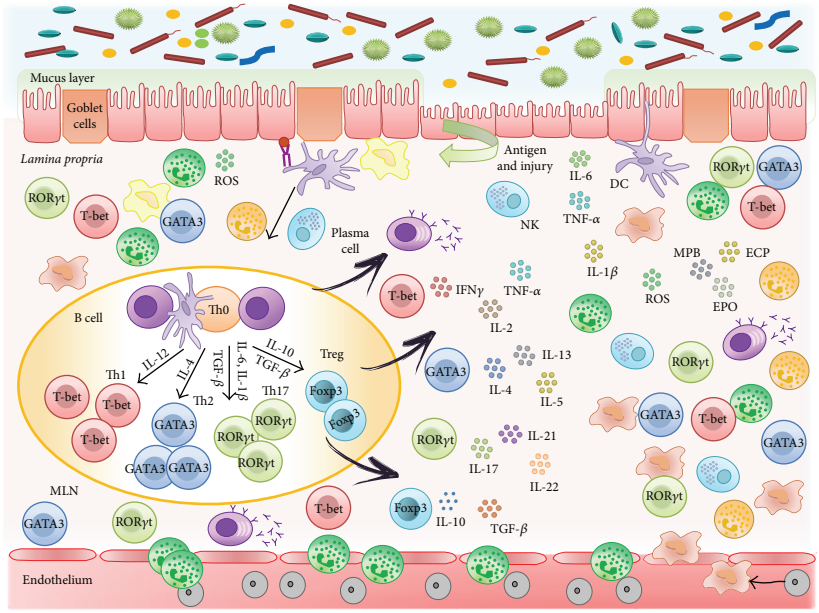
The physical barrier of the intestinal epithelium is complemented by a well-evolved mucosal innate immune system, which is poised to defend against pathogenic incursions, and limits inflammatory responses to maintain a state of hyporesponsiveness to commensal bacteria. However, it is also the effector arm that mediates intestinal inflammation. The epithelial-cell layer is comprised of absorptive and secretory cells, goblet cells, and Paneth cells. Goblet cells contribute to the formation of the protective mucus layer [28]. Under physiological conditions, *lamina propria* hosts a large number of different immune cells, including macrophages (Mφ), dendritic cells (DCs), mast cells, neutrophils, eosinophils, natural killer (NK), NKT cells, and T and B cells. All of these cells coexist in perfect equilibrium that confers tolerance and protection at the same time (Figure 1).

The presence of either pathogenic bacteria or the disruption of the epithelial-cell barrier may result in inflammation and dysregulation of mucosal homeostasis: cells of the innate immunity, such as macrophages and dendritic cells, are specialized in identifying microorganism's molecular patterns by using the pattern recognition receptors (PRR), such as toll-like receptors (TLR) [29]. Moreover, newly recruited monocyte-derived macrophages (and other innate host defense cells) generate cytokines and chemokines to recruit monocytes and other leukocyte populations to contain the inflammation [30]. When innate immunity is no longer able to counteract the pathogen aggression, the adaptive immune response is triggered: DCs migrate to the mesenteric lymph nodes, where they present the antigen to naive T cells and, depending on the factors released by DCs, induce the T cell differentiation [31]. T cells are key players of adaptive immune response that cooperate with other cells and molecules from innate immune system to generate an effective response in order to eliminate the invading pathogens. Upon contact with APCs, naive CD4+ cells have the potential to differentiate into different T helper (Th) subtypes; this process is controlled by the effector cytokines produced by APCs: in presence of IL-12 into Th1 and in presence of IL-4 into Th2, with IL-10 and TGF-β, they induce regulatory T cells (iTreg) and with IL-6, IL-1β, and TGF-β, they induce Th17 cells [32–35]. Each Th subtype exerts specific functions: Th1 cells are essential to eliminate intracellular pathogens, Th2 cells mediate allergic reaction and they confer protection against parasites; and Th17 cells contribute to removing extracellular bacteria and fungi [36, 37]. Then, the interaction between T and B leads to the production of antibodies upon the contact with T cell or DC [38, 39]. Although their main function is the antibody production, B cells can also act as antigen presenting cell and, moreover, they are able to produce cytokines and are involved in maintaining mucosal immune homeostasis [40].

Furthermore, activated leukocytes, such as neutrophils, which are infiltrated in the inflamed mucosa not only generate different proinflammatory cytokines, but also induce oxidative reactions, which markedly alter the redox equilibrium within the gut mucosa, and maintain inflammation by inducing redox-sensitive signalling pathways and transcription factors [41]. Moreover, several inflammatory molecules generate further oxidation products, leading to a self-sustaining and autoamplifying vicious circle, which eventually impairs the gut barrier.



(a)



(b)

Figure 1: Physiopathology of IBD. (a) The intestine is the largest mucosal surface exposed to the external environment. It constitutes an interface between the host and the luminal contents, which include nutrients and the highest count of resident microbes. Thus, the intestinal immune system meets more antigens than any other part of the body and it must discriminate between invasive organisms and harmless antigens, such as food, proteins, and commensal bacteria, to prevent infections or preserve the homeostasis. This intestinal homeostasis depends on the dynamic interaction between the microbiota, the intestinal epithelial cells, and the resident immune cells, which coordinate a response that keeps the balance between immunity and tolerance. (b) A breakdown of this balance triggers the chronic inflammatory process that characterizes inflammatory bowel disease. There are often several preexisting conditions that lead to the disease: first of all, a genetic susceptibility of the intestinal immune system to distinguish an environmental antigen presented within the gastrointestinal tract; secondly, the contact with the antigen; finally, usually due to an alteration of the permeability, the antigen is presented to the gastrointestinal mucosal immune system through its paracellular passage, which triggers the inflammatory cascade. During early inflammation, luminal antigens activate the different innate immune cells located in the intestine, including natural killer cells, mast cells, neutrophils, macrophages, and dendritic cells, and maintained inflammatory reaction promotes the activation of the adaptive immune response. Abnormally activated effector CD4+ T helper (Th) cells synthesize and release different inflammatory mediators that generate an amplified inflammation that originates from chronic tissue injury and epithelial damage.

In consequence, and as a result of this complex immune cell activity, IBD, similarly to other inflammatory conditions, is characterized by the involvement of a broad spectrum of inflammatory mediators, including cytokines, chemokines, leukotrienes, and prostaglandins, which actively participate in all the phases of the inflammatory process: initiation, progression, and resolution, when it occurs.

During the active phases of IBD, some chemokines are consistently increased: IL-8 and its receptor, monocyte chemoattractant protein- (MCP-) 1 and MCP-3, and macrophage inflammatory proteins (MIP). Chemokines mediate the recruitment of leucocyte effector populations to the sites of immune reaction and tissue injury since they tightly control leukocyte adhesion and migration across the endothelium, being also able to trigger multiple inflammatory actions including leukocyte activation, granule exocytosis, production of metalloproteinases for matrix degradation, and upregulation of the oxidative burst [42]. Similarly, the upregulated expression of different adhesion molecules in IBD, such as the intercellular adhesion molecule- (ICAM-) 1, the lymphocyte function-associated antigen- (LFA-) 1, the macrophage 1 antigen (Mac-1), the vascular cell adhesion molecule- (VCAM-) 1, the very late antigen- (VLA-) 4, and P- and E-selectins, collaborates in the recruitment of granulocytes and lymphocytes through blood vessels [43].

The roles of cytokines in IBD are very diverse and complex. The fact that these mediators control T-cell differentiation and regulation has made them to be considered as central points of potential intervention to control the inflammatory response. IL-12, IL-18, and IL-23 have a crucial function in Th1 differentiation and chronic activation, whereas other cytokines, such as TNF-α, IL-1β, and IL-6, augment the inflammatory response by recruiting other cells and enhancing the production of inflammatory mediators [44]. Moreover, IL-23 is induced by pattern recognition receptors (PRRs), whose sustained activation drives chronic intestinal inflammation [45]. Although it was initially linked to the preferential expression of Th17 responses, it can promote a wide range of pathological responses in the intestine, mediated either by T cells or by excessive innate immune activation. IL-23-mediated enhancement of Th1 and Th17 responses is consistent with the increased levels of IFN-γ, IL-17, and IL-22 observed in the chronically inflamed intestine [46, 47]. When considering the IL-17 cytokine family, a group of cytokines that includes at least six members, IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (or IL-25), and IL-17F, acts both in vitro and in vivo as potent proinflammatory cytokines [48]. IL-17 can induce the expression of proinflammatory cytokines (like IL-6 and TNF-α), chemokines (including keratinocyte chemoattractant (KC), MCP-1, and MIP-2), and matrix metalloproteases, which mediate tissue infiltration and tissue destruction [49], thus playing a key role in human IBD [50]. Finally, it has been reported that an altered production of anti-inflammatory cytokines, including IL-10 and TGF-β, can account to the pathogenesis of IBD, because they are considered as key regulators of immunological homeostasis and inflammatory responses in the gut [51, 52].

3. Intestinal Anti-inflammatory Effects of Botanical Drugs: Preclinical and Clinical Studies

The growing interest about the potential role that medicinal plant extracts may play in intestinal inflammatory conditions has promoted the development of different clinical studies, thus trying to evaluate their potential efficacy and safety. It is important to note that nowadays most botanical drugs go through a similar rigorous testing as pharmaceutical medicines, in an attempt to avoid inconsistent conclusions. Unfortunately, different factors associated with the design, execution, and interpretation of these clinical trials still make it difficult to easily get clear conclusions with the different strategies to be evaluated against these pathologies [53]. Among these factors, the clinical heterogeneity of both intestinal conditions, UC and particularly CD, can be highlighted, as well as the selection of appropriate therapeutic end points to evaluate the efficacy. In spite of all these concerns, there are positive examples of successful human-controlled trials within the literature of botanical drugs. Although the preclinical studies reporting the beneficial effects of plant extracts on experimental models of colitis are numerous, only a few plant extracts have been used in different clinical assays, which will be described in the present review (Table 1). The main botanical drugs were *Aloe vera*, *Andrographis paniculata*, *Artemisia absinthium*, *Boswellia serrata*, *Cannabis sativa,* and *Curcuma longa*.

3.1. Aloe vera. *Aloe vera* (Xanthorrhoeaceae) is a tropical plant used in traditional medicine all over the world, mainly for its gel, which is the leaf pulp mucilaginous, aqueous extract. The *Aloe vera* juice has been reported to exert anti-inflammatory activity; therefore, it has been empirically used for the treatment of UC patients [14] (Table 1), being considered as the most popular botanical drug [10]. These beneficial effects have been related to the immunomodulatory properties ascribed to this gel. This was confirmed when the gel was tested in the dextran sulphate sodium (DSS) model of experimental colitis in rats, since it produced an amelioration of the colonic tissue injury induced by DSS, being related to a downregulation of the inflammatory mediators, including cytokines, and attenuation of the immune cell recruitment [54]. Among the different components of the gel, which comprise acetylated mannans, polymannans, anthraquinone C-glycosides, anthrones, anthraquinones (emodin), and lectins, with reputed biological activities, in the same study it was proposed that the chromone aloesin seemed to be essential for the control of the intestinal inflammatory process [54]. It was reported that aloesin, mainly, but also aloin and aloe-emodin (Figure 2) were able to reduce myeloperoxidase (MPO) activity, an enzyme involved in neutrophil activity, an effect that can account in inhibiting the progression of IBD. Moreover, aloesin is a strong inhibitor of leukotriene B_4 (LTB_4) that can activate and recruit the inflammatory cells in the injured tissue [55]. Also, these compounds significantly decreased the expression of proinflammatory cytokines, like TNF-α and IL-1β, in the colonic segment in a dose-dependent manner, being aloesin again the most effective. However, the

Table 1: Clinical trials of botanical drugs in patients with inflammatory bowel disease.

Herbal preparation	Study design	Number of patients	IBD type	Dose	Comparator	Frequency	Endpoint	Reference
Aloe vera	Randomized, double-blind controlled study	44	UC	100 mL twice/day	Placebo	4 weeks	*Aloe vera* produced a significantly better clinical response than in those receiving placebo. The Simple Clinical Colitis Activity Index and histological scores decreased significantly during treatment with *Aloe vera* but not with placebo	[14]
Andrographis paniculata (HMPL-004)	Randomized, double-blind multicentre study	120	UC	1.2 g/day	Mesalazine (4.5 mg/day)	8 weeks	There were no significant differences between the two treated groups when considering the clinical efficacy rates or the safety profile	[15]
	Randomized, double-blind placebo-controlled study	224	UC	1.2 g/day and 1.8 g/day	Placebo	8 weeks	Patients treated with the extract, mainly at the highest doses, were more likely to achieve clinical response than those receiving placebo, whereas the incidence of adverse events was similar among groups, although the occurrence of rash was higher in the HMPL-004 extract groups	[16]
Artemisia absinthium	Randomized, double-blind multicentre study	40	CD	3 × 500 mg/day	Placebo	10 weeks	After 8 weeks of treatment with wormwood, there was almost complete remission of symptoms in 65% of the patients, whereas no beneficial effect was observed in those receiving the placebo	[17]
	Randomized, double-blind multicentre study	20	CD	3 × 750 mg/day (in addition to standard therapy)	Standard therapy + placebo	6 weeks	Wormwood administration promoted the clinical improvement of the symptoms in all the patients. The beneficial effect was associated with a significant decrease in TNFα serum levels in comparison with those obtained in the placebo group, where no amelioration in the disease was observed	[18]
Boswellia serrata (Gum resin)	—	?	UC	750 mg (3 × 250 mg)	Sulfasalazine 3 g (3 × 1 g)	6 weeks	All parameters tested improved after treatment with *Boswellia serrata* gum resin, with the results being similar compared to controls: 82% out of treated patients went into remission; in case of sulfasalazine remission rate was 75%	[19]
(Gum resin)	—	30	UC?	900 mg (3 × 300 mg)	Sulfasalazine 3 g (3 × 1 g)	6 weeks	Patients showed an improvement in several parameters: stool properties, histopathology, and scanning electron microscopy, besides haemoglobin, serum iron, calcium, phosphorus, proteins, total leukocytes, and eosinophils. The remission was higher in patients treated with *Boswellia serrata*	[20]
(Boswelan)	Randomized, double-blind, multicentre placebo-controlled study	82	CD	2.4 g/day	Placebo	12 months (52 weeks)	Boswelan showed a safety profile during the long-term therapy but the results obtained did not show a higher efficacy when compared with placebo	[21]

Table 1: Continued.

Herbal preparation	Study design	Number of patients	IBD type	Dose	Comparator	Frequency	Endpoint	Reference
Cannabis sativa	Retrospective observation study	30	CD	—	—	—	Cannabis administration was associated with an improvement in disease activity and a reduction in the need of other medications, as well as a reduced risk of surgery	[22]
	Prospective Placebo-controlled study	21	CD	2 cigarettes containing 115 mg of THC/day	Placebo	8 weeks	A significant amelioration of the CD activity index has been reported in the majority of the subjects after cannabis treatment in comparison with placebo administration; in fact, complete remission was achieved in half of the subjects in the cannabis group, whereas it only occurred in 10% of the placebo group patients	[23]
Curcuma longa	Open-label pilot study	5	UC	1.100 g/day (550 mg × 2) for 1 month, then 1.650 g/day (550 mg × 3) for 1 month and	—	2 months	The results from this study revealed that the treatment of these patients with curcumin for two months resulted in an overall improvement in all the patients, as evidenced by amelioration of the serological parameters evaluated (erythrocyte sedimentation rate and C-reactive protein) as well as the disease activity index followed, together with a reduction in the dose of medication, or even suppression. In the CD group, all patients also reported fewer bowel movements, less diarrhoea, and less abdominal pain and cramping	[24]
	Open-label pilot study	5	CD	1.080 g/day (360 mg × 3) for 1 month, and then 1.440 g/day for two months	—	3 months		
	Randomized, double-blind multicentre placebo-controlled study	89	UC	2 g/day plus sulfasalazine or mesalazine	Placebo plus sulfasalazine or mesalazine	6 months	The relapse rate was significantly higher in the placebo group, receiving only the aminosalicylate (20.5%), than in the curcumin-treated cohorts (4.7%). During the period of the study, a marked reduction of the disease-associated clinical activity index and the endoscopic index scores was reported	[25]

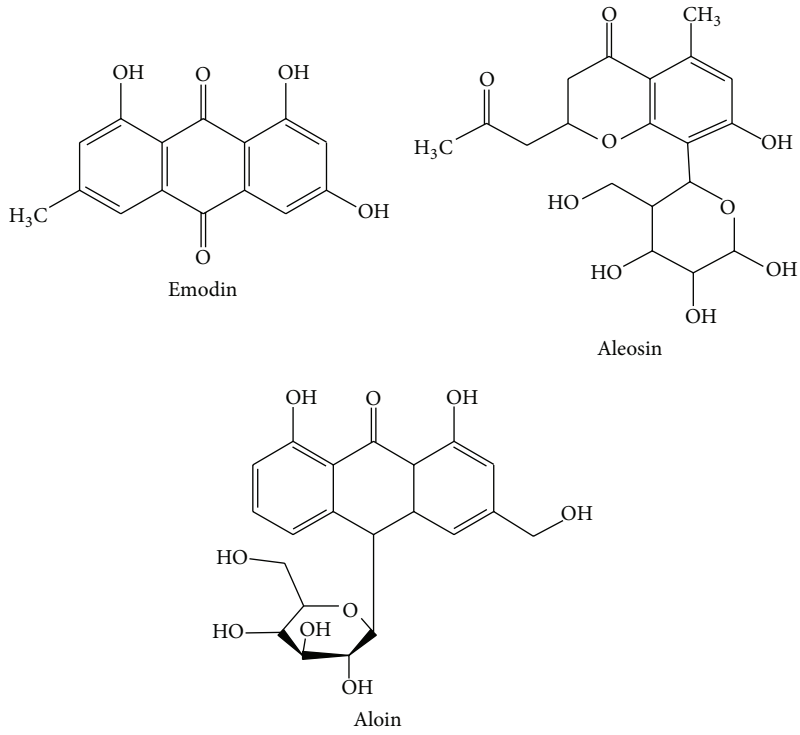


Figure 2: Chemical structures of *Aloe vera* compounds.

mechanism through which it exerts this capacity remains unidentified. It is known that this chromone derivative blocks the activation of the NF-κB pathway, thus inhibiting the expression of related proinflammatory genes, including TNF-α [56]. Based on the effect observed in retina ganglion cells [57], the anti-inflammatory effect of aloe-emodin and aloin metabolite, in DSS rat colitis, maybe is due to MAP kinase pathways phosphorylation inhibition.

3.2. Andrographis paniculata. *Andrographis paniculata* (Acanthaceae) can be mainly found in India and Sri Lanka, as well as in South and South-Eastern Asia, where its extracts are used as anti-inflammatory remedies [58]. HMPL-004 is a proprietary extract from this plant that has been evaluated for its intestinal anti-inflammatory effects in human trials [15, 16] (Table 1). The phytochemical analyses of the extracts from *Andrographis paniculata* reveal that the main known components are diterpene lactones, principally andrographolide (Figure 3) and its derivatives, which have been reported to exert anti-inflammatory properties through inhibition of the transcription factor NF-κB [59, 60]. Particularly, andrographolide reacts with reduced cysteine 62 of p50 subunit forming a covalent adduct blocking the bond of NF-κB oligonucleotide to nuclear proteins. NF-κB activation promoted the increased expression and synthesis of different proinflammatory mediators involved in the inflammatory response associated with IBD, including chemokines, cytokines, and adhesion molecules, in the different cell types that participate in the altered immune response in these intestinal conditions [61]. For instance, when stimulated-endothelial cells were treated with andrographolide, the reduction of adhesion molecule E-selectin was observed preventing the E-selectin-mediated leukocyte migration [59]. Andrographolide is also able to suppress the expression of inducible nitric oxide synthase (iNOS) as observed in RAW 264.7 cells and, as a consequence, there is a reduction of NO production. This inhibition of NO is due to the blockage of the synthesis and also to the reduction of the stability via a posttranscriptional mechanism [62]. Andrographolide prevents the reactive oxygen species (ROS) production by neutrophils through the modulation of a protein kinase C- (PKC-) pathway. This confers to andrographolide the capacity to downregulate leukocyte integrin Mac-1 ($\alpha_M\beta_2$ CD11bCD18) that it has been reported to be upregulated by ROS. This reduction leads to reducing neutrophil infiltration and transmigration [63]. Besides, andrographolide exerts immunomodulatory properties, most likely affecting the innate immune cells, including macrophages and dendritic cells, but also T cells, by downregulating the production of proinflammatory cytokines [64–66]. It has been observed that andrographolide reduced significantly, in a dose-dependent manner, the IFN-γ production in concanavalin A-stimulated murine T cell in vitro, whereas its effects on IL-2 inhibition were partial. Moreover, it can reduce the ERK1/2 phosphorylation that is associated with a reduction of IFNγ production [64]. In another study, the ability of andrographolide to interfere with the DCs maturation and with their capacity to present antigen to T cells has been showed. When the DCs were treated with the compound and then were mixed with lymphocytes for allogeneic stimulation, IL-2 release and proliferation were reduced [65]. The extract of *A. paniculata*

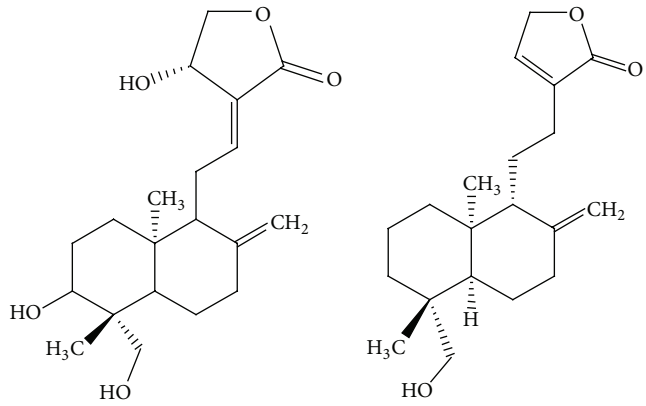


Figure 3: Chemical structures of *Andrographis paniculata* compounds.

also contains andrograpanin (Figure 3), a hydrolysate of neoandrographolide (another bicyclic diterpenoid lactone), which also showed anti-inflammatory activity. It was able to reduce the mRNA expression of several genes, including TNF-α, IL-16, IL-12p35, and IL-12p40, in a dose-dependent manner. In particular, the reduction of IL-12p35 and IL-12p40 proteins was lower than their mRNA levels, suggesting that andrograpanin applies the major changes at a posttranscriptional level for these two genes [66].

All these studies could justify the inhibitory effects that the extracts of *Andrographis paniculata* may exert on multiple immune cells (DC, macrophages, and T cells) that are implicated in disease development and progression in UC and CD. Supporting this, Michelsen et al. reported the intestinal anti-inflammatory effects of the extract HMPL-004 in a T cell driven experimental model of chronic colitis, by inhibiting the proliferation and/or differentiation of naïve T cells, as well as the Th1/Th17 responses that are activated in intestinal inflammation, being these effects associated with reduced expression of the different proinflammatory cytokines, including TNFα, IL-1β, IFNγ, and IL-22 [67].

3.3. Artemisia absinthium. *Artemisia absinthium* (Compositae), commonly known as wormwood, is widely distributed all over the world and it is described in different pharmacopoeias, with leaves and stems being used for medicinal purposes. This botanical drug is usually standardised based on its content in the dimeric guaianolides absinthins [68], being considered as a high-quality wormwood when it contains at least 0.2% of absinthin.

Two different clinical trials have reported the beneficial effect of this botanical drug in CD [17, 18] (Table 1). TNF-α is considered to play a key role in the pathogenesis of CD, which supports the high efficacy obtained with the biologicals acting as TNF-α inhibitors, like infliximab and adalimumab, for severe cases of CD [69]. The results obtained in these clinical trials showed that wormwood administration promoted the clinical improvement of the symptoms in all the patients, whereas no amelioration in the disease was observed in the placebo group. The beneficial effect induced by wormwood was associated with a significant decrease in TNF-α serum levels in comparison with those obtained in the placebo group. The suppression of TNF-α, as well as of others proinflammatory cytokines like IL-1β or IL-6, by wormwood extracts has been reported in vitro as well [70]. Among the components of *Artemisia absinthium*, the flavonoid 5,6,3′,5′-tetramethoxy 7,4′-hydroxyflavone (p7F) (Figure 4) has been isolated that has been shown to exert anti-inflammatory effects. In fact, p7F was able to inhibit the expression of COX-2 and iNOS in LPS-stimulated RAW 264.7 cells. As a consequence of this inhibition, a reduced production of PGE_2 and NO in the same cells has been observed. Moreover, p7F decreased the activation of NF-κB, induced by LPS, probably through its antioxidant properties [70]. p7F also suppressed the serum levels of TNF-α and inhibited NF-κB activation in vivo [70]. Another compound also isolated from this plant, 20,40-dihydroxy-60-methoxychalcone, known as cardamonin (Figure 4), has shown to dose-dependently inhibit NO release and iNOS expression in LPS-stimulated RAW 264.7, as well as NF-κB activation [71]. All these results would support the use of *Artemisia absinthium*, at least as complementary therapy, in IBD.

3.4. Boswellia serrata. The oleo-gum resin from *Boswellia serrata* (Burseraceae), or Indian frankincense, is a traditional Ayurvedic remedy used to treat inflammatory diseases, including UC. As claimed by a survey performed in Germany, approximately 36% of IBD patients have been administered with *Boswellia serrata* extracts to treat their intestinal condition, reporting positive therapeutic effects [72], and they have been assayed in different clinical trials [19–21] (Table 1).

Among the different chemical compounds of this resin, triterpenes are the most abundant (30–60, depending on its origin), with boswellic acids being the major constituents (Figure 5), which are thought to largely contribute to the pharmacological activities such as anti-inflammatory and antiarthritic effects ascribed to this crude drug [73]. In vivo experiments performed in the acetic acid model of rat colitis have proposed that the antioxidant properties of the extracts from *Boswellia serrata* may also account for their intestinal

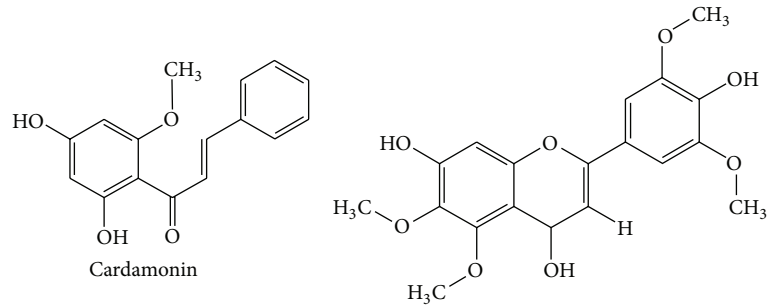


Figure 4: Chemical structures of *Artemisia absinthium* compounds.

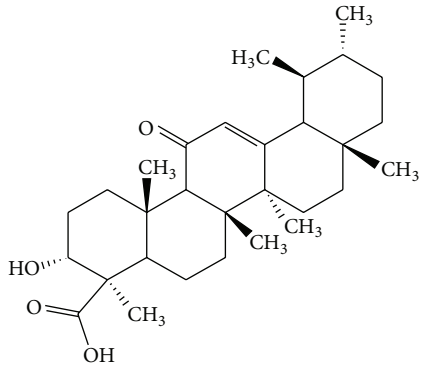


Figure 5: Chemical structure of boswellic acid.

anti-inflammatory effect [74, 75]. It has been shown that *Boswellia serrata* extract reduced the lipid peroxidation, while it increased the levels of superoxide dismutase (SOD), thus ameliorating the oxidative stress associated with intestinal inflammation.

Additional mechanisms can also account for their beneficial effects. Thus, in vitro experiments have shown that these compounds decreased the leukotriene formation by blocking the 5-lipoxygenase pathway which can account in the beneficial effect showed by this botanical drug since leukotrienes have been clearly involved in the pathogenesis of IBD [76]. Similarly, the anti-inflammatory effects of boswellic acids seem to involve the inhibition of different cellular pathways including those related to the transcription factor NF-κB activation, which has been described to induce the expression and/or to activate the function of many proinflammatory cytokines, like TNFα, IL-1β, and IL-6, that are crucial for the development and the maintenance of intestinal inflammation [77, 78]. Surprisingly, contradictory results have been reported when the effectiveness of *Boswellia* extracts was assayed in DSS- or trinitrobenzene sulfonic acid- (TNBS-) induced experimental models, since no efficacy was demonstrated in ameliorating colitis in these models [79]. Furthermore, in the same study, the ability of different boswellic acids to enhance the basal and the IL-1β-stimulated NF-κB activity in intestinal epithelial cells was demonstrated, as well as the potential hepatotoxic effect of *Boswellia*, claiming for an special attention with the use of this botanical drug in IBD and other inflammatory disorders [79]. On the contrary, a semisynthetic form of acetyl-11-keto-β-boswellic acid lessened the disease activity index in DSS colitis, and it managed to diminish the recruitment of leucocytes and platelets, maybe due to its ability to prevent P-selectin upregulation, thus protecting colonic mucosa from DSS insult. The beneficial effects showed by this derivative were similar to those obtained with the standard corticosteroid dexamethasone [80].

3.5. Cannabis sativa. *Cannabis sativa* (Cannabaceae) has been long employed for the treatment of different diseases, especially for chronic pain and different neurological conditions [81, 82]. Moreover, this botanical drug has treated different gastrointestinal conditions including anorexia, emesis, abdominal pain, diarrhea, and diabetic gastroparesis [83]. It has been reported to contain over 60 different cannabinoid compounds, which are responsible for the biological activities reported for *Cannabis sativa* [84]. In addition, experimental evidence suggests that the endogenous cannabinoid system is involved in most of the major immune events, including those located in the gastrointestinal tract [85, 86]. For this reason, it was proposed that the activation of this system by cannabinoids might have a therapeutic role in human IBD [87]. However, and although its use is common in IBD patients, there are few controlled studies that evaluate the exact role of cannabis in IBD [22, 23, 88] (Table 1).

The mechanisms involved in the intestinal anti-inflammatory effects of cannabis can be related to the capacity of cannabinoids to downregulate the production and release of different proinflammatory mediators including TNFα, IL-1β, and nitric oxide, thus restoring the altered immune response that occurs in IBD [89]. Most probably, these effects would be related to cannabinoid receptors type 1 (CB1) activation that mediates essential protective signals and counteracts proinflammatory pathways, since it has been reported that the severity of two different experimental models of colitis, induced by the intrarectal infusion of 2,4-dinitrobenzene sulfonic acid (DNBS) or by oral administration of DSS, are higher in CB1-deficient mice (CB1(−/−)) than in wild-type [90]. Lack of CB1 receptors rendered mice more sensitive to inflammatory insults, indicating a protective role of the

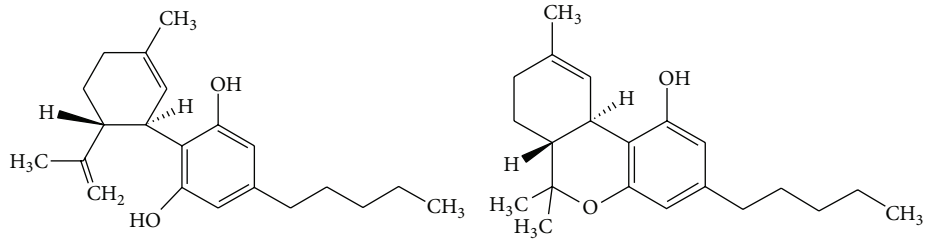


Figure 6: Chemical structures of *Cannabis sativa* compounds.

CB1 receptors during inflammation induction. Consistently, the administration of a specific CB1 antagonist to these wild-type mice before colitis induction resulted in a similar degree of intestinal damage to that seen in CB1(−/−) mice, whereas the administration of a cannabinoid receptor agonist protected from DNBS-induced colitis in mice [90]. Supporting these observations, both Δ-tetrahydrocannabinol (THC) and cannabidiol (CBD) (Figure 6), two of the main components of cannabis, were able to exert beneficial effects in the TNBS model of acute colitis in rats, with a similar efficacy to that shown by sulphasalazine, which was used as a positive control [91]. Particularly, treatment with THC and combined treatment with CBD was able to reduce macroscopic damage score. Both alone and in combination, THC and CBD reduced the MPO activity similarly to treatment with sulphasalazine [91].

Unfortunately, the main concern with the use of cannabis in IBD can be derived from the indiscriminate binding of the cannabinoids to the receptors in the central nervous system, which may result in serious side effects including dizziness, dry mouth, nausea, fatigue, somnolence, euphoria, vomiting, disorientation, drowsiness, confusion, loss of balance, and even hallucination [92]. It is evident that the manipulation of the endocannabinoid system can be helpful for the management of human IBD; however, additional research needs to be carried out to consider cannabis (and cannabinoids) as a suitable medicine in these gastrointestinal conditions. Maybe, the use of specific cannabinoids can constitute an attractive alternative. This may be the case of cannabidiol, which possesses anti-inflammatory and immunomodulatory properties, as demonstrated in experimental models of rodent colitis, although it lacks of any cognitive or psychoactive effects [93].

3.6. Curcuma longa. Curcuma longa (Zingiberaceae) is commonly known as Turmeric. It is an Indian spice obtained from the rhizomes of the plant, which has been long used in Ayurvedic medicine for the handling of different inflammatory diseases [94]. Curcumin (Figure 7) is the main active component of turmeric that also provides its vibrant yellow colour [95]. Numerous pharmacological activities have been reported for curcumin, including antioxidant, antimicrobial, anticancer, and anti-inflammatory properties. Regarding the latter, curcumin has been suggested to exert positive effects in IBD, although just a few clinical studies have shown the power of curcumin to prevent and/or ameliorate this condition.

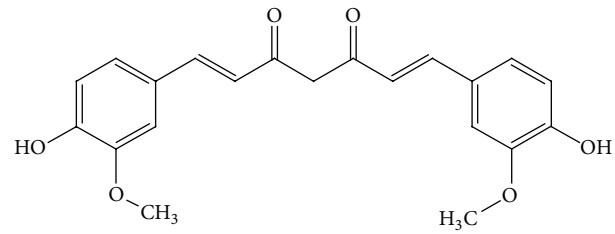


Figure 7: Chemical structure of curcumin.

Clinical studies showed that the concurrent administration of curcumin and standard drugs improved their efficacy [24, 25] (Table 1) and seemed to be well tolerated after a prolonged administration, which can be considered as a secure medication for maintaining remission and preventing relapse [96].

The preclinical studies performed with curcumin have shown its efficacy in different experimental models of colitis, either chemically induced or in knockout mice, and following different protocols of administration, that is, preventative or curative [97–102]. Besides, all these studies have rendered important information about the possible mechanisms implicated in these beneficial effects. Among these, the antioxidant properties of curcumin, derived from its ability to scavenge free radicals, may play a prominent role, given the oxidative stress that characterizes these intestinal conditions [103]. In addition, curcumin can also modify multiple signaling pathways, especially the kinases MAPK and ERK, thus affecting the expression of different proteins implicated in the intestinal inflammatory cascade, like MPO, COX-2, iNOS, or LOX [104]. Additionally, a modulation of the altered immune response has been proposed to occur after curcumin administration in experimental colitis, by attenuating the production of proinflammatory cytokines, like TNFα, IL-1β, IL-12, or IFNγ, but increasing of the expression of anti-inflammatory cytokines [98, 101]. Other mechanisms involved in the beneficial effects of curcumin can be related to an inhibitory effect of NF-κB activity [105]. In fact, it has been reported that elevated levels of NF-κB in IBD proportionately amplified the production of inflammatory cytokines and resulted in mucosal damage, which in turn can upregulate the production of this transcription factor, promoting a recurring feedback loop of inflammation [106].

In consequence, the ability of curcumin to modulate NF-κB activation may prevent the inflammatory response, thus preventing colonic mucosa damage.

In conclusion, the results reviewed show that botanical drugs might prompt clinical remission and a clinical response in IBD patients. In particular, botanical drugs significantly induce clinical remission in CD patients and clinical response in UC patients; however, there was not a significant induction of clinical remission in UC patients and an obvious clinical response in CD patients. The results of subanalyses taking into account the plant type demonstrate that only *Artemisia absinthium* and *Boswellia serrata* were able to induce clinical remission, while *Aloe vera* induced a clinical response. However, none of the plants confirmed endoscopic or histological efficacy. On the other hand, none of the plants produced any adverse effects in comparison with placebo. So, although some of the botanical drugs may have clinical efficacy in patients with IBD, there is too limited evidence to make any strong conclusions. However, botanical drugs could still be safer than synthetic drugs, despite the fact that they are not completely devoid of risk.

4. Perspectives

The potential additional benefits of botanical drugs could be that patients accept them, besides their efficacy, acceptable safety, and comparatively low cost. Patients all over the world seem to be prone to use botanical drugs, and their efficacy has been now evaluated in multitude of clinical trials in the management of UC. However, the evidences are still partial, intricate, and puzzling and unquestionably related to both benefits and side effects.

First of all, there should be a deeper knowledge of their composition, the active compounds that are responsible of their properties, and their side effects or toxicity. It is also important to control the harvest of the right plants, their quality, and the later processing to ensure the stability of the active components. Therefore, there is a need for a regulation that establishes the quality standards of the botanical drugs that are sold.

Secondly, further controlled clinical trials, with larger number of patients, are required to evaluate the potential efficacy and toxicity of botanical drugs in the treatment of UC. In this regard, it is also important to let the doctors know any evidence so they can prescribe these botanical drugs with the maximal guarantee.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

M. Angeles Ocete and Julio Galvez contributed equally to the supervision of the study.

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Review Article

Natural Products: Insights into Leishmaniasis Inflammatory Response

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Leishmaniasis is a vector-borne disease that affects several populations worldwide, against which there are no vaccines available and the chemotherapy is highly toxic. Depending on the species causing the infection, the disease is characterized by commitment of tissues, including the skin, mucous membranes, and internal organs. Despite the relevance of host inflammatory mediators on parasite burden control, *Leishmania* and host immune cells interaction may generate an exacerbated proinflammatory response that plays an important role in the development of leishmaniasis clinical manifestations. Plant-derived natural products have been recognized as bioactive agents with several properties, including anti-protozoal and anti-inflammatory activities. The present review focuses on the antileishmanial activity of plant-derived natural products that are able to modulate the inflammatory response *in vitro* and *in vivo*. The capability of crude extracts and some isolated substances in promoting an anti-inflammatory response during *Leishmania* infection may be used as part of an effective strategy to fight the disease.

1. Introduction

Human leishmaniasis is an infectious disease caused by 20 different *Leishmania* species reported in 98 countries and territories spread across four continents (Africa, Americas, Asia, and Europe). Leishmaniasis is considered a major public health issue as it currently affects 12 million people [1]. The anthroponotic and zoonotic forms of transmission may occur. In the last case, the primary reservoirs of *Leishmania* are sylvatic mammals such as forest rodents, hyraxes, and wild canids. However, urban or domestic dogs are the most relevant species in the epidemiology of this disease [2].

Leishmania infection occurs during the hematophagy of female sand flies belonging to *Phlebotomus* (Old World) and *Lutzomyia* (New World) genus. The metacyclic promastigote forms present in the foregut of the sand flies are inoculated in the dermis-epidermis junction of the vertebrate host, infecting cells of the mononuclear phagocyte system [3]. The interaction between parasites and host immune cells leads to an inflammatory response essential for parasite control. However, an exacerbated proinflammatory response may cause tissue damage, such as those easily observed in cutaneous leishmaniasis cases [4, 5]. On the other hand, the lack of an effective inflammatory response may promote increased

parasite burden. In this scenario, a moderate inflammatory response would be ideal for an effective control of the disease.

Plants have been long recognized as a rich source of biologically active extracts, essential oils, and isolated substances. In fact, research laboratories around the world search in plants for active substances against diverse illnesses such as microbial and protozoal infections, cancer, diabetes, and inflammatory processes [6]. Indeed, plant-derived natural products such as phenolic compounds, steroids, quinones, coumarins, terpenoids, and alkaloids have been widely investigated for their antileishmanial potential [7, 8].

In the present review, we start with an introduction about the current scenario of leishmaniasis epidemiology and treatment, followed by some highlights on the inflammatory response generated by *Leishmania* infection. The last part of this work focuses on the modulatory effects of plant-derived natural products over inflammatory mediators and their impact on parasite burden *in vivo* and *in vitro*.

2. Leishmaniasis: A Global Threat

It is estimated that leishmaniasis has about 1.6 million new cases per year. However, only 600,000 cases are reported annually. Socioeconomic conditions such as poverty and malnutrition, environmental changes such as atmospheric temperature and humidity, ecological conditions affecting the vector, parasite, and its reservoir, and population movements caused by migration and tourism are all risk factors that directly interfere with the world's distribution of leishmaniasis [9–11]. In addition, the ecology of sand fly species also plays a significant role in the spread of the disease [12].

According to geographical criteria, leishmaniasis can be divided into two main syndromes: (1) Old World Leishmaniasis, which includes two clinical manifestations: cutaneous leishmaniasis (CL), a disease confined to the skin, and visceral leishmaniasis (VL), involving the bloodstream and inner organs; (2) New World Leishmaniasis, which includes CL and mucocutaneous leishmaniasis (MCL). The latter involves mucous membranes in addition to the skin. Currently, new terminology regarding leishmaniasis forms was introduced such as mucosal leishmaniasis (ML). ML involves mucosal tissues, particularly those of the upper respiratory tract and oral cavity. It is typically a consequence of infection by New World *Leishmania* species, such as *L. braziliensis, L. panamensis, L. amazonensis*, and *L. guyanensis* [10].

Cutaneous leishmaniasis is found in South America, Asia, Europe, and Africa. Latin America is the most important endemic area, particularly the Amazon. Different *Leishmania* species cause Old World (Eastern hemisphere) *versus* New World (America) CL: in the Old World, the etiologic agents include *L. tropica, L. major*, *L. aethiopica*, *L. infantum*, and *L. donovani;* the main species in the New World are either those of the *L. mexicana* complex (*L. mexicana, L. amazonensis*, and *L. venezuelensis*) or the ones of the subgenus *Viannia* (*L.* (*V.*) *braziliensis, L.* (*V.*) *guyanensis, L.* (*V.*) *panamensis,* and *L.* (*V.*) *peruviana*).

The general term visceral leishmaniasis can refer to different degrees of disease severity, including chronic, subacute, or acute, affecting internal organs, particularly spleen, liver, and bone marrow. The two most important causative agents of VL are *L. donovani*, which shows anthroponotic transmission (human to human), and *L. infantum*, with zoonotic transmission (canine to human). Together, they cause 40,000 deaths *per* year [13]. *L. donovani* is only found in the Old World, being responsible for VL cases in East Africa and the northeast of India. On the other hand, *L. infantum* is found in the Mediterranean and in Latin American regions [14]. Over 90% of VL cases occur in Bangladesh, Brazil, Ethiopia, India, South Sudan, and Sudan [11].

3. Available Chemotherapy for Leishmaniasis

Chemotherapy is the current method for human leishmaniasis treatment since there are no vaccines available. Usually, the therapeutic approach starts with the use of pentavalent antimonials such as sodium stibogluconate and meglumine antimoniate. However, when these drugs exhibit low efficacy or simply cannot be prescribed for leishmaniasis treatment, second-line drugs are indicated [12, 15, 16].

Several *Leishmania*-killing mechanisms have been attributed to pentavalent antimonials including apoptosis, disturbance of fatty acids β-oxidation, adenosine diphosphate phosphorylation, and redox balance. In addition, antimonials inhibit the glycolysis pathway and are able to directly act on infected macrophages eliciting an oxidative/nitrosative stress against internalized parasites [17, 18]. Despite the variety of antileishmanial targets, the use of pentavalent antimonials has been extensively discussed due to their toxic effects to liver and heart tissues. Regarding the use of amphotericin B, this drug targets ergosterol, an essential plasma membrane sterol found in *Leishmania* spp. Also, amphotericin B recognizes cholesterol in mammalian cells, which leads to high toxicity and severe side effects, including kidney failure, anemia, fever, and hypokalemia [19].

Miltefosine and paromomycin are two other drugs that have been introduced for the treatment of leishmaniasis. Miltefosine was the first orally administered drug effective against VL. The mechanism of antileishmanial action of miltefosine remains unclear but apoptosis preceded by drug intracellular accumulation has been described. Other possible mechanisms include cytochrome c oxidase inhibition, which leads to mitochondrial dysfunction and immunomodulation [20]. The recommended dose of miltefosine for VL treatment is approximately 2.5 mg/kg/day for 4 weeks. The long term therapy in conjunction with miltefosine long half-life (about 150 h) can accelerate the onset of drug resistance. Moreover, recent studies have pointed out that miltefosine has a potential teratogenic and abortifacient effect, preventing its prescription during pregnancy [18, 21]. Paromomycin is an aminoglycoside antibiotic that has shown important results in leishmaniasis treatment, mainly for the cutaneous form of the disease [22]. However, *in vitro* studies have already reported the emergence of paromomycin-resistant parasites, compromising its use as a wide antileishmanial agent in the future [23]. In addition, the toxicity of miltefosine and paromomycin has also been described [12].

In summary, the current chemotherapy scenario urges for more efficient and secure antileishmanial treatments, encouraging the search for new bioactive compounds such as those from natural origin. In fact, plant-derived natural products represent a promising class of drug candidates against leishmaniasis.

4. Inflammatory Response to *Leishmania* Infection

Parasite-host interaction is a complex process that modulates *Leishmania* infection and the immunological response to it, including inflammation. Several molecules are involved in inflammation during leishmaniasis, such as cytokines and the lipid mediator leukotriene B4 (LTB4). Many of the molecules that promote inflammation also activate phagocytes leading to the production of nitric oxide (NO), the main effector molecule in parasite killing. However, an exacerbated production of these molecules may also lead to tissue damage.

Tumor necrosis factor (TNF) and interleukin-1 (IL-1) are cytokines produced by macrophages after the recognition of pathogens, including *Leishmania*. They promote inflammation by inducing the expression of adhesion molecules (selectin and integrin ligands) on the endothelial surface. TNF- or TNF-receptor 1- (TNFR1-) deficient mice are able to control *L. major* replication but develop larger lesions [24, 25]. The role of IL-1 in leishmaniasis is controversial, as IL-1 contributes to Th1 priming at early infection but worsens the disease outcome in established infection [26].

IL-10 is an important anti-inflammatory cytokine responsible for peripheral tolerance to self-antigens and preventing exacerbated immune responses to foreign antigens. However, when expressed in large quantities, IL-10 may have deleterious effects during leishmaniasis, leading to an early suppression of innate and acquired immune responses, pathogen proliferation, and aggravation of the disease [27]. In leishmaniasis, phagocytes are stimulated to produce IL-10, which leads to a reduced production of cytokines related to the Th1 profile, such as IL-12 and interferon gamma (IFN-γ) [28]. This causes a reduction in NO production that consequently reduces the microbicidal capacity of macrophages. IL-10 may be secreted by numerous cells, including macrophages, T cells, and B cells.

The cytokines IL-12 and IL-4 also play an important role during *Leishmania* infection. They define the cell profile through the polarization of CD4+ T cells and modulate the response from other cells [29, 30]. IL-12 activates NK cells and CD8+ T cells, leading to IFN-γ production [31]. In addition, IL-12 induces the differentiation of CD4+ T cells to the Th1 profile, which also produces IFN-γ, a potent inducer of NO production in macrophages. Thus, IL-12 possesses an indirect microbicidal action. In contrast, IL-4 induces the differentiation of CD4+ T cells to a Th2 profile, which produces IL-4, IL-5, and IL-13. This profile suppresses NO production and leads to an increase in eosinophils [32].

LTB4 is an eicosanoid with chemotactic function synthesized from leukotriene A4 by leukotriene-A4 hydrolase. *In vitro*, LTB4 contributes to the microbicidal action of macrophages through the production of NO and reactive oxygen species while, *in vivo*, LTB4 reduces the parasite load and the footpad swelling [33, 34].

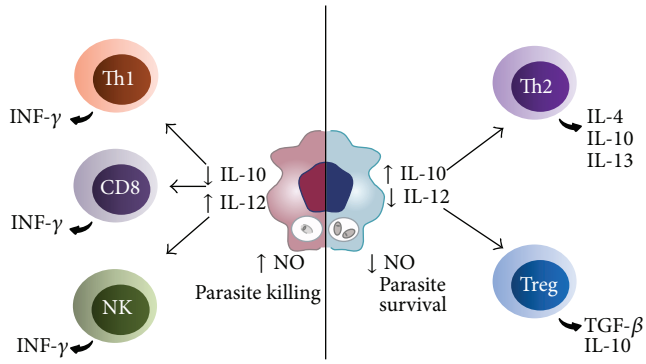


Figure 1: Cytokine profile regulates the type of immune response to *Leishmania* infection. The balance between IL-10 and IL-12 produced by macrophages regulates the parasitic load by controlling NO production, CD4 + T lymphocytes profile, and IFN-y production by NK and CD8 + cells.

The importance of the type of immune response, if Th1 or Th2, lies in the fact that Th1 immune response characterizes the resistance mechanism to *Leishmania* infection, while Th2 response has been associated with susceptibility to parasite infection. The Th1 immune response is associated with production of proinflammatory cytokines such as IFN-γ, TNF-α, and IL-12, while the susceptibility profile of Th2 response is characterized by anti-inflammatory cytokines expression such as IL-10 and IL-4 (Figure 1) [35].

In humans, protection against VL is mediated by Th1 immune response whereas pathogenesis is associated with Th2 response. Most studies suggest that poor Th1-type responses are associated with severe clinical forms of leishmaniasis [36]. Some studies have demonstrated the importance of proinflammatory cytokines IFN-γ, TNF-α, and IL-12 in *L. donovani* infection. Depletion of these cytokines aggravated the disease progression or made hosts susceptible to infection by *L. donovani* [37].

However, studies about CL showed that higher frequency of proinflammatory cytokine production leads to larger lesions. Some studies pointed that high production of IFN-γ, TNF, and NO is not always beneficial [38]. Thus, inadequately controlled immune responses could potentially lead to pathological manifestations and tissue damage. This is contradictory since many studies pointed out that the Th1-mediated response is important for disease control. The activation of type effector cells that produce the macrophage-activating cytokines (i.e., IFN-γ) is necessary for host control over parasite replication [39]. Increasing evidence suggests that the paradigm established about the necessity of a Th1 response for a better prognosis of leishmaniasis is not a rigid concept and the balance between proinflammatory and anti-inflammatory cytokines determines the outcome of the infection [40–42].

5. Natural Products Effects on Host Immunological Response

As mentioned earlier, leishmaniasis treatment is primarily based on antimonial compounds followed by amphotericin B as a second choice drug. However, high toxicity, severe side effects, and elevated costs hinder the use of these drugs in countries where leishmaniasis is endemic. In many instances, traditional medicines are the alternative for accessible treatments against parasitic diseases [41]. Unfortunately, most of them are hardly explored and their mechanisms of action are mainly unknown. Plants possess a large repertoire of secondary metabolites that display a wide variety of pharmacological activities. Indeed, numerous plant-derived bioactive compounds have been described, such as terpenoids, flavonoids, alkynes, alkaloids, saponins, sterols, phenylpropanoyl esters, lactones, tannins, and coumarins [43–45].

Traditional herbal medicines are gaining increased attention as they can reduce the risk of chronic diseases and act as antibiotics, antioxidants, and/or immunomodulators. Several studies have described the effects of plant extracts or isolated compounds in immune cells and cytokine production [43]. Thus, the study of active compounds obtained from plants used in traditional medicine plays a pivotal role in the search for new antileishmanial molecules [39, 41].

Several raw extracts from different plants have been shown to exhibit antileishmanial activity, which may not only be due to their direct action on the parasite, but also due to a concomitant effect on the host immune response [41]. Therefore, the search for plant extracts with a wide spectrum of antileishmanial and immunomodulatory activities may enable the discovery of substances suitable for the disease control. Some studies have focused on the effects of leishmanicidal essential oils and plant extracts in the production of pro- and anti-inflammatory soluble mediators. Altogether, these studies suggest that the induction or inhibition of cytokine production is a critical factor for effective parasite destruction without producing excessive tissue damage. Table 1 summarizes the currently known plant extracts and their effects on inflammatory mediators.

The plant popularly known as Evanta (*Angostura longiflora* (*Krause*) *Kallunki*) is used for the treatment of leishmaniasis and other parasitic diseases in Bolivia [41]. In addition to having direct activity against *L. braziliensis*, Evanta extracts also interfere with the activation of both mouse and human T cells. Calla-Magarinos et al. (2009) [41] showed that the alkaloid-rich extract from Evanta barks (AEE) reduced INF-γ expression in J774 and spleen cells, despite its lack of effect on TNF-α and NO production. Similar effects were observed in human peripheral blood mononuclear cells (PBMCs). The major compound in the alkaloid-rich extract from Evanta barks is 2-phenylquinoline. Interestingly, the isolated substance (Figure 2) showed a similar effect to that observed for AEE. Moreover, 2-phenylquinoline reduced INF-γ production and cell proliferation *in vitro*, suggesting that it may contribute to the control of the chronic inflammatory reaction that characterizes *Leishmania* infection.

Recently, Calla-Magariños et al. (2013) demonstrated that the alkaloid-rich Evanta extract interferes with *in vitro* antigen-specific lymphocyte activation [40]. When spleen cells from *L. braziliensis*-immunized mice were pretreated with AEE and stimulated with *Leishmania* lysate or *Leishmania*-infected bone marrow macrophages (L-BMM), the levels of IFN-γ decreased. In addition, *in vivo* treatment with the Evanta extract affected reactivation of primed lymphocytes, reducing the production of IFN-γ, IL-12, and TNF-α by spleen cells induced with L-BMM. AEE treatment also affected the kinetics of infection. Mice infected with *L. braziliensis* promastigotes in the left hind footpad showed a more effective decrease in the footpad thickness when treated with AEE than those treated with meglumine antimoniate. These results suggest that AEE can control both *Leishmania* infection and the inflammatory reaction against it.

The leaf methanol extract and the essential oil from *Xylopia discreta* display antileishmanial activity and immune stimulatory effects over infected murine macrophages [42]. To evaluate the effects of the methanol extract and the essential oil from *X. discreta*, López et al. (2009) infected J774 cells with *L. panamensis* and measured the levels of proinflammatory mediators. IL-12, IL-10, IL-6, MCP-1, and TNF-α were quantified after treatment with different concentrations of *X. discreta* extract or essential oil. No statistical differences in the production of interleukins and TNF-α were observed between treated and untreated cells. However, a significant increase in MCP-1 production was observed after cell treatment. Surprisingly, no differences in cytokine production were detected when pentamidine was used as antileishmanial drug [42].

The extract produced from the leaf of Neem (*Azadirachta indica*) presents antileishmanial and immunomodulatory activities [46]. The leaf and seed extracts of *A. indica* were shown to possess immunomodulatory, insecticidal, antiseptic, anticancer, antiviral, antifungal, and antiprotozoal properties. Its oil, bark, and leaf extracts have therapeutic efficacy against leprosy, intestinal helminthiasis, and respiratory disorders in children [47]. Similar to the *X. discreta* extract [42], the ethyl acetate extract fraction of Neem also induces a Th1 response. Cytokine production was evaluated by real time quantitative PCR (RT-qPCR) on THP-1 and PBMCs infected with *L. donovani* strain Dd8. Cells treated with Neem extract showed a significant increase in TNF-α, IL-8, and IL-1β production, while IL-10 expression was unaltered, indicating a strong Th1 response. However, the expression of TNF-α and IFN-γ was unaltered in spleen tissue (*in vivo* analysis), whereas the expression of Th2 cytokines (IL-10, IL-4, and TGF-β) was significantly reduced [46]. These results suggest that the leaf extract of Neem induces a protective immune polarization during leishmaniasis.

Chouhan et al. (2015) evaluated the antileishmanial and immunomodulatory activities of the ethanol extract of leaves (ALE), seeds (ASE), and bark (ABH) from *A. indica*. In contrast to Dayakar et al. (2015) [46], they used other parts of the plant and different extraction methods. ABH is not effective against *L. donovani* promastigotes, while ALE and ASE exhibited leishmanicidal activity in both promastigote and amastigote cells. Sera of treated mice infected with *L.*

Table 1: Plant extracts and isolated compounds with antileishmanial and immunomodulatory activities.

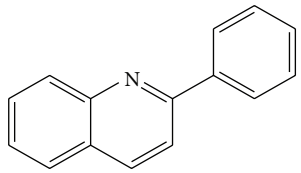
Plant species	Substance or extract	*In vitro* activity (IC_{50})	*In vivo* in mice	Substance cytokines activity	Reference
Glycyrrhiza glabra L.	18β-glycyrrhetinic acid	*L. donovani* 4.6 μg/mL	*L. donovani* 50 mg/kg/day	Reduces levels of IL-10 and IL-4, but increases levels of IL-12, IFN-γ, TNF-α, and inducible NO synthase	[57]
Tanacetum parthenium	Parthenolide	*L. amazonensis* 0.37 μg/mL	—	Inhibits IB kinase β	[58]
Baccharis uncinella	Oleanolic acid and ursolic acid	—	*L. amazonensis* 1 and 5 mg/kg/day	Increases IL-12 and IFN-γ cytokines	[51, 59]
Dictyota pfaffii	Dolabelladienetriol	*L. amazonensis* 43.9 μM	—	Diminishes TNF-α and TGF-β production in uninfected and *Leishmania*-infected macrophages	[60]
Artemisia indica	Artemisinin	*L. donovani*, *L. infantum*, *L. tropica*, *L. braziliensis*, *L. mexicana,* and *L. amazonensis* 100 μM to 120 μM	*L. donovani* 10 mg/kg and 25 mg/kg body weight	Restores Th1 cytokines (interferon-gamma and interleukin-2)	[61]
Glycyrrhiza glabra	Glycyrrhizic acid	—	*L. donovani* 1, 10, 25, 50, 75, or 100 mg/kg body weight/day	Enhances the expression of IL-12 and TNF-α, in parallel with a downregulation of IL-10 and TGF-β	[37]
Nectandra leucantha	(a) Dehydrodieugenol B, (b) 1-(8-propenyl)-3-[3′-methoxy-1′-(8-propenyl)phenoxy]-4,5-dimethoxybenzene, and (c) 1-(7R-hydroxy-8-propenyl)-3-[3′-methoxy-1′-(8′-propenyl)-phenoxy]-4-hydroxy-5-methoxybenzene	(a) 26.7 μM (*L. donovani*), (b) 17.8 μM (*L. donovani*), and (c) 101.9 μM (*L. donovani*)	—	((a) to (c)) Reduced production of IL-6 and IL-10. Minimal effect on nitric oxide production in *L. donovani*-infected macrophages	[56]
Quassia amara	Quassin	—	—	Upregulating proinflammatory cytokines such as TNF-α and IL-12	[62]
Vitis vinifera	Resveratrol	*L. amazonensis* Antipromastigote activity (27 ± 0.59 μM) Antiamastigote activity (42 ± 7.18 μM)	—	Decreases the levels of the proinflammatory cytokine TNF-α in infected macrophages stimulated with IFN-γ	[63]
Raputia heptaphylla	11α,19β-dihydroxy-7-acetoxy-7-deoxoichangin	*L.* (V) *panamensis* J774.2 EC_{50} = 7.9 μM; hDCs EC_{50} = 25.5 μM	—	Increases on the production of IL-12p70, TNF-α, and NO, as also, in the number of hDCs HLA-DR-positive in treated infected hDCs	[64]
Galipea longiflora	Crude extract containing 13 different quinolinic alkaloids and 2-phenylquinoline as major compounds	*L. braziliensis* IC_{90} = 20 μg/mL	*L. braziliensis* 6.25 and 12.5 mg/kg/day	Reduced production of IFN-γ, IL-12, and TNF-α by spleen cells. Reduced the inflammatory reaction in mice infected with *L. braziliensis* promastigotes	[40, 41]

Table 1: Continued.

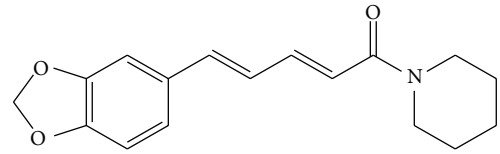
Plant species	Substance or extract	*In vitro* activity (IC_{50})	*In vivo* in mice	Substance cytokines activity	Reference
Xylopia discreta	(a) Methanol extracts containing ~50% of alkaloids and terpenes (β-pyrenes, camphene, β-myrcene, and 1,8 cineol) and (b) essential oil	(a) *L. panamensis* in J774 LC_{50} = 598.37 μg/mL and EC_{50} = 9.32 μg/mL, (b) *L. panamensis* in J774 LC_{50} = 857.7 μg/mL and EC_{50} = 37.5 μg/mL, (a) *L. panamensis* in U937 LC50 = 698,45 μg/mL and EC50 = 6,35 μg/mL, (b) *L. panamensis* in U937 LC50 = 160 μg/mL and EC50 = 6.25 μg/mL	—	Increased the secretion of MCP-1 by U937 and J774 cell lines	[42]
Galium mexicanum	Hexane fraction (HE 5)	*L. donovani* MIC = 333 μg/mL	—	Reduced production of IL-6 in THP-1 cells	[49]
Laennecia confusa	(a) Aqueous extract and chloroform extracts (antileishmanicidal), (b) methanol, and (c) chloroform extracts (anti-inflammatory)	*L. donovani* (a) IC_{50} = 20 μg/mL and IC_{50} = 20 μg/mL	—	(b) Reduced IL-6 production in THP-1 cells	[48]
Azadirachta indica	Ethyl acetate extract fraction	*L. donovani* IC_{50} = 52.4 μg/mL	*L. donovani* 100 mg/kg body weight	Increased the expression of TNF-α, IL-8, and IL-1β in THP-1 cells and TNF-α, IFN-γ in PBMCs	[46]
Azadirachta indica	Leaves ethanol extract (ALE) and seeds ethanol extract (ASE)	Antipromastigote IC_{50} = 34 and 77,66 μg/mL (ALE and ASE). Antiamastigote IC_{50} = 17,66 and 24,66 μg/mL (ALE and ASE)	—	Increased the expression of INF-γ, TNF-α, and IL-2 and declined in IL-4 and IL-10 levels in spleen cells	[47]
Lopezia racemosa	Hexane extract fractions (HE11–14b)	IC_{50} = 30,66 μg/mL	—	Reduced IL-6 production in THP-1 cells	[50]
Croton caudatus	Semipurified hexane extract (JDHex)	*L. donovani* Antipromastigote IC_{50} = 10 μg/mL. Antiamastigote IC_{50} = 2.5 μg/mL	*L. donovani* 1.25, 2.5, 3.75 or 5 mg/kg body weight for five days	Increased the production of IL-12 and TNF-α in murine peritoneal macrophages *in vitro*. Increased the intracellular IFN-γ and decreased the IL-10 production in $CD4^+$ T cells *in vivo*	[39]
Baccharis uncinella	Triterpenic purified fraction containing oleanolic and ursolic acids	—	*L. amazonensis* 1.0 mg/kg and 5.0 mg/kg for five days	Increased the IL-12 and IFN-γ production in mice	[51]
Sambucus nigra	Commercial preparation (Sambucol)	—	*L. major* 25 μL twice a day	Increased the production of IL-1β, IL-6, IL-8, and TNF-α by human monocytes	[65]

Table 1: Continued.

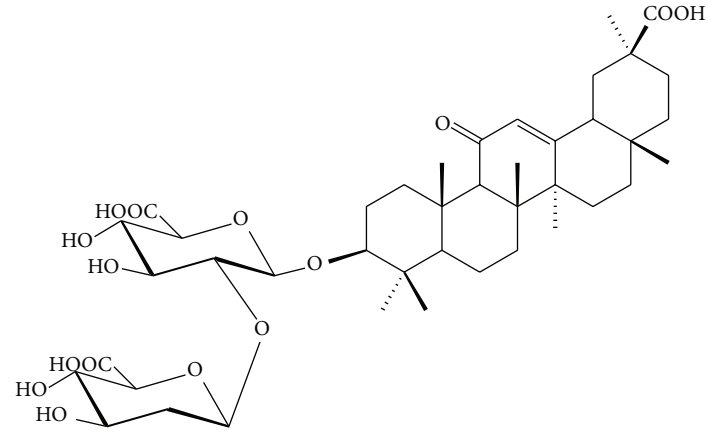
Plant species	Substance or extract	*In vitro* activity (IC_{50})	*In vivo* in mice	Substance cytokines activity	Reference
Syzygium cumini	Essential oil (ScEO) containing as major component α-pinene	*L. amazonensis* Antipromastigote IC_{50} = 19.7 μg/mL	—	Increased in lysosomal volume, phagocytosis, and NO production by peritoneal macrophages	[66]
Artemisia annua	Hexane extract	—	*L. donovani* 50, 100 and 200 mg/kg body weight daily for ten days	Increased in levels of IFN-γ and reduction of levels of IL-4 and IL-10 in serum and culture supernatant of lymphocytes from mice	[53]
Piper nigrum	Alkaloids (piperine and analogue phenylamide)	*L. amazonensis* Antipromastigote IC_{50} = 14.2 μM. Antiamastigote IC_{50} = 28 μM	—	Suppressed MCP-1, TNF-α, NF-KB activation, and NO production *in vitro* and *in vivo* and showed anti-inflammatory properties	[55, 67]
Tinospora cordifolia	Pure herb extract (tablet form)	—	*Leishmania donovani* 100 mg/kg b.wt. for 15 days daily	Enhanced proliferation and differentiation of lymphocytes and induced Th1 immune response and IFN-γ and IL-2, but declined IL-4 and IL-2 levels	[68]
Withania somnifera	Aqueous extract	—	*L. donovani* 5 mg/kg/day	Increased antileishmanial efficacy of cisplatin. Increased in the levels of IFN-γ and IL-2 (Th1-type immunity) and the levels of IgG2 over IgG1. Decreased in levels of IL-4 and IL-10	[69]
Lophanthera lactescens	6α,7α,15β,16β,24-pentacetoxy-22α-carbomethoxy-21β, 22β-epoxy-18β–hydroxy-27,30-bisnor-3,4-secofriedela-1,20 (29)-dien-3,4 R-olide (LLD-3)	*L. amazonensis* . Antiamastigote IC_{50} = 0.41 μg/mL	—	Affected proliferation of naïve or activated B and T cells, as well as the B cells immunoglobulin synthesis	[54]



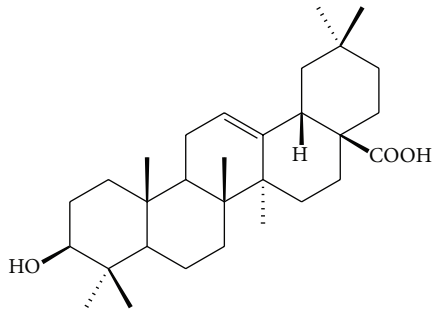
2-Phenylquinoline



Piperine



Glycyrrhizic acid



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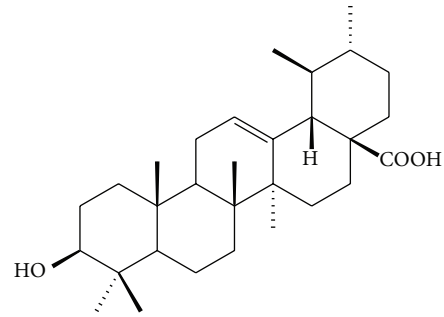
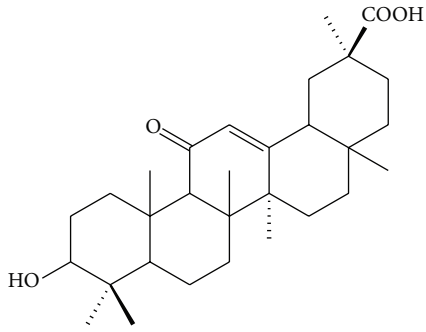
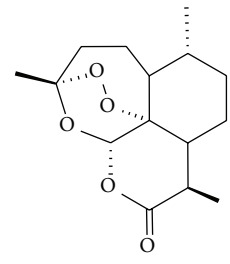


Ursolic acid



18β-Glycyrrhetinic



Artemisinin

Figure 2: Structures of the compounds assayed *in vitro* and *in vivo* against *Leishmania* species and cytokines activity.

donovani were analyzed for IgG2a (induced by INF-γ) and IgG1 (induced by IL-4) levels. Highest levels of IgG2a are indicative of Th1 response, while IgG1 indicate Th2 activation. ALE and ASE stimulated the production of high levels of IgG2a and low levels of IgG1. As expected, ALE and ASE treatment induced NO generation by macrophages primed with SLA. Confirming these results, Th1/Th2 cytokine levels were quantified in culture supernatants of spleen cells from animals treated with ALE and ASE. The extracts significantly increased the levels of Th1 cytokines, such as INF-γ, TNF-α, and IL-2, and decreased the IL-10 and IL-4 levels [47]. Although Chouhan et al. (2015) and Dayakar et al. (2015) have used different parts of *A. indica*, both of them showed the proinflammatory effects of bioactive molecules derived from this plant.

The genus *Laennecia* and the correlated genus *Conyza* are known to produce bioactive substances displaying antimicrobial, antiparasitic, antidiarrhoeal, antinociceptive, antioxidant, and anti-inflammatory activities. Aiming to evaluate the potential of *L. confusa*, Ruiz et al. (2012) investigated the inhibitory effect of different extracts from its stems against several pathogenic microorganisms. In addition, the anti-inflammatory activity of these extracts was evaluated. The aqueous and chloroform extracts, as well as a chloroform fraction, named, CE2, presented antiparasitic activity against *L. donovani*. However, these extracts and fractions did not affect the production of proinflammatory cytokines (IL-6) in THP-1 cells [48].

A similar approach was conducted by Bolivar et al. (2011) with *Galium mexicanum* and by Paredes et al. (2013) with *Lopezia racemosa*, with both of them being traditional medicinal plants used in Mexico [49, 50]. Flavonoids, iridoid glycosides, iridoid acids, triterpene saponins, and anthraquinones have been isolated from the *Galium* genus. Among the *G. mexicanum* extracts and fractions analyzed, the hexane fractions HE 5 and HE 14b presented anti-*L. donovani* promastigotes activity, while the hexane fraction HE 5 and methanol fractions ME 13–15 reduced the LPS-induced macrophage production of IL-6, suggesting an anti-inflammatory character of these samples [49].

The aerial parts of *L. racemosa* were submitted to extraction with various solvents and the extracts were fractionated. The hexane fractions HF 11–14b, methanol fractions MF 28–36, and the chloroform extract were able to inhibit *L. donovani* growth. In relation to the reduction of IL-6 production by macrophages exposed to LPS, the fractions HF 11–14b showed significant anti-inflammatory activity by reducing the secretion of the aforementioned cytokine [50].

Croton caudatus leaves extract is a promising extract against visceral leishmaniasis. Stems and leaves of *C. caudatus* have been used for the treatment of rheumatic arthritis, malaria, convulsions, ardent fever, numbness, worm-infested animals, vomiting, and dysentery in India [39]. Terpenes as crotocaudin, isocrotocaudin, crotoncaudatin, and crocaudatol have been isolated from this extract. Dey et al. (2015) demonstrated that the semipurified hexane extract of *C. caudatus* leaves (JDHex) inhibited the proliferation of *L. donovani* promastigotes (IC_{50} = 10 μg/mL) and intracellular amastigotes (IC_{50} = 2.5 μg/mL). To evaluate the immunomodulatory activity of JDHex, the production of proinflammatory cytokines, such as IL-12 and TNF-α, as well as anti-inflammatory cytokines, IL-10 and TGF-β, was investigated *in vitro* and *in vivo*. *L. donovani*-infected murine peritoneal macrophages treated with JDHex showed an increase in intracellular IL-12 (p70 fraction) and a reduction in TGF-β and IL-10 production. In addition, JDHex induced an increase in NO that could be directly correlated with the induction of TNF-α expression in infected macrophages. These results suggest that the immunomodulatory activity of JDHex occurs via a Th1 response. *In vivo* experiments performed with mice infected with *L. donovani* and treated orally with different concentrations of JDHex for 5 days after 1 month of infection showed that treated mice had an induction in IFN-γ production. In addition, the parasite load in spleen was reduced dose-dependently. As JDHex was efficient against *L. donovani* intracellular amastigotes, the authors suggested that the proinflammatory activity of JDHex may be useful for antileishmanial therapy [39]. Using a similar *in vivo* model, Bhattacharjee et al. (2012) and Chouhan et al. (2015) found comparable results for treatment of *L. donovani* with glycyrrhizic acid (Figure 1) extract from liquorice (*Glycyrrhiza glabra*) and ethanol extract of *A. indica*, respectively [37, 47].

In accordance with Dey et al. (2015) [39], Yamamoto et al. (2014) also described an antileishmanial compound that induces Th1 response. *L. amazonensis*-infected mice were treated with a triterpene-rich fraction of *Bacchari suncinella* during five days. The analysis of immune response revealed that treated mice presented higher levels of IL-12 and IFN-γ than the control group. Treatment with the triterpenic fraction reduced the size of lesions, as well as the parasitism and the parasite load [51]. It is worth noting that the triterpenic fraction of *B. suncinella* stimulated the inflammatory process while reducing the size of mice lesions.

The flavonoid-rich *Artemisia annua* L. extract has been shown to possess antioxidant, antimicrobial, and anti-inflammatory activities [52]. Studies carried out with the leaves and seeds of *A. annua* against *L. donovani*-infected mice caused increased production of Th1 cytokines (IFN-γ) and a simultaneous decrease in Th2 cytokines (IL-4 and IL-10). Moreover, *A. annua* extracts resulted in higher CD4+ and CD8+ T cell numbers, lymphoproliferation, upregulation of costimulatory molecules (CD80 and CD86) on APCs, and generation of NO [53].

The nor-triterpene 6α,7α,15β,16β,24-pentacetoxy-22α-carbometoxy-21β,22β-epoxy-18β-hydroxy-27,30-bisnor-3,4-secofriedela-1,20(29)-dien-3,4 R-olide (LLD-3), extracted from *Lophanthera lactescens* Ducke, showed a remarkable antileishmanial activity against intracellular amastigotes (IC_{50} = 0.41 μg/mL) but no cytotoxicity to mouse peritoneal macrophages or B cells, which makes it a promising drug candidate for leishmaniasis treatment [54]. In addition, piperine (Figure 1), the main alkaloid of *Piper nigrum*, and its analogue phenylamide are active against *L. amazonensis* promastigotes and amastigotes. They act synergistically to boost the leishmanicidal effect and reduce the NO production in infected macrophages [55]. The hexane extract of the twigs of *Nectandra leucantha* Nees and Mart displayed

activity against the promastigote forms of *L. donovani*. Isolated phenylpropanoid dimers suppressed the production of disease exacerbatory cytokines IL-6 and IL-10 but had minimal effect on NO production in *L. donovani*-infected macrophages. Thus, the antileishmanial activities appear to be mediated by molecular mechanisms that are independent of NO production [56].

6. Conclusion

Promising drug candidates for leishmaniasis treatment should be able to eliminate the parasite but also elicit an appropriate immune response. Plant-derived natural products such as crude extracts, purified fractions, or isolated substances have demonstrated their effectiveness as immunomodulatory agents. The anti-inflammatory activity of the natural products pointed here could be useful for the control of an exacerbated proinflammatory response, ameliorating leishmaniasis clinical symptoms, such as tissue damage.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Marine Diterpenoids as Potential Anti-Inflammatory Agents

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The inflammatory response is a highly regulated process, and its dysregulation can lead to the establishment of chronic inflammation and, in some cases, to death. Inflammation is the cause of several diseases, including rheumatoid arthritis, inflammatory bowel diseases, multiple sclerosis, and asthma. The search for agents inhibiting inflammation is a great challenge as the inflammatory response plays an important role in the defense of the host to infections. Marine invertebrates are exceptional sources of new natural products, and among those diterpenoids secondary metabolites exhibit notable anti-inflammatory properties. Novel anti-inflammatory diterpenoids, exclusively produced by marine organisms, have been identified and synthetic molecules based on those structures have been obtained. The anti-inflammatory activity of marine diterpenoids has been attributed to the inhibition of Nuclear Factor-κB activation and to the modulation of arachidonic acid metabolism. However, more research is necessary to describe the mechanisms of action of these secondary metabolites. This review is a compilation of marine diterpenoids, mainly isolated from corals, which have been described as potential anti-inflammatory molecules.

1. Introduction

Inflammation is a complex biological response against pathogens or tissue damage characterized by vasodilation, increased blood flow, vascular permeability, and cellular extravasation [1]. Macrophages, mast cells, and dendritic cells, resident in the tissues, are the first cells of innate immunity that detect and recognize the pathogen and initiate the inflammatory response [1]. Acute inflammation is an early response in which innate immune cells such as polymorphonuclear cells and monocytes are recruited to the site of irritation and secrete inflammatory mediators (e.g., cytokines, chemokines, and free radicals), which amplify the response [2]. Chronic inflammation, in turn, is the long-term inflammatory process that occurs as a dysregulation of acute inflammation often due to extended exposure to the initial irritant, persistent injury, or autoimmune disease. Chronic inflammation is associated with many pathological diseases including cancer, autoimmune diseases, atherosclerosis, rheumatoid arthritis, asthma, and cardiovascular diseases [3–5].

The search for new anti-inflammatory agents is challenging due to the complexity of the inflammatory process and its role in host defense. However, the progress attained in understanding the mechanisms involved in inflammation has made the identification of new targets possible, opening the range of search for new compounds with potential therapeutic effects on acute or chronic inflammatory diseases. Several drug discovery and development programs are focused on the search for bioactive compounds obtained from natural sources. Many drugs used today for the treatment of several diseases have been developed from natural products. The studies in terrestrial organisms have been extended to the marine environment, a resource with an enormous potential for drug discovery [6–8].

In the world of natural products, terpenoids are one of the largest and most studied groups of molecules. Terpenoids are secondary metabolites containing a C5 isoprene unit derived from a biosynthetic pathway based on mevalonate, which is essential for diverse cellular functions [9]. Terpenoids can be classified into hemi, mono, sesqui, di, sester, or tri based

on the number of isoprene C5 units. These compounds are found largely in higher plants, but also in lower invertebrates including marine organisms.

Diterpenoids, in particular, are a promising class of mol ecules of secondary metabolites with a range of activity including antiviral, antibacterial, antiparasite, anticancer, and anti-inflammatory [10]. Diterpenes are comprised of four isoprene units with the chemical structure $C_{20}H_{32}$. Several studies have demonstrated a variety of diterpenoid structures presenting anti-inflammatory capacity. This review discusses the potential anti-inflammatory role of several diterpenoids derived from marine organisms.

2. Current Anti-Inflammatory Drugs

There are several classes of anti-inflammatory drugs available today, including nonsteroidal anti-inflammatory drugs (NSAIDs), glucocorticoids, and immunomodulatory drugs. NSAIDs, including aspirin, ibuprofen, and naproxen, are a widely administered class of drugs used for anti-inflammatory and analgesic purposes. Drugs in the NSAID category differ significantly in structure, but all share common mechanisms of action. These drugs prevent the release of prostaglandins through the inhibition of cyclooxygenase (COX) by covalently modifying the enzyme or by competing with the substrate for the active site [11, 12]. Side effects of these engineered drugs, however, are often severe and range from gastric ulcers to kidney damage and death.

Two COX isozymes are encoded in the human genome, COX-1 and COX-2. COX-1 is expressed in nearly all organs and cells but is most prominent in the stomach and in platelets, whereas COX-2 is an inducible, inflammation-specific isoform and regulates the synthesis of prostaglandins during inflammation [13–15]. Prostaglandins modulate different immune cell types including macrophages, dendritic cells, and T and B lymphocytes, leading to pro- and anti-inflammatory effects. Prostaglandins have several functions, including augmenting the blood flow and vascular permeability, regulating the expression of cytokines by innate immune cells, and inducing the expression of costimulatory molecules [16].

When it was discovered that the gross reduction or elimination of prostaglandins achieved by nonselective inhibition of COX enzymes often resulted in gastric ulcers [17, 18], researchers sought drugs that selectively inhibited COX-2. The resulting set of COX-2 selective drugs are collectively called coxibs and exhibit much lower rates of gastric ulcers than nonselective COX inhibitors [19, 20]. Unfortunately, it was then discovered that many coxibs also carry a higher risk of cardiovascular events such as coronary heart disease, heart attack, and stroke [21].

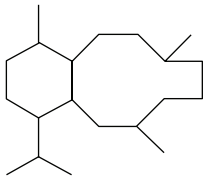
Glucocorticoids (GCs) are steroidal hormones that are naturally produced by vertebrates and function to control inflammation [22]. Because of their native role, synthetic GCs have been produced and used to treat a variety of inflammatory-related diseases including asthma [23], inflammatory bowel disease [24], rheumatoid arthritis [25], and systemic lupus erythematous [26]. Both synthetic and natural GCs act by binding to and activating the glucocorticoid receptor (GR), a transcription factor that acts as an activator or repressor of several genes by direct binding with specific DNA sequences or by interfering with the transcriptional activity of other transcription factors [27]. Several mechanisms have been proposed to explain the inhibitory effect of GCs on the transcription of inflammatory genes. Many of them are related to the inhibition of Nuclear Factor-κB (NFκB) activation at different levels, including a direct physical association of GR with NFκB and the induction of the expression of the regulatory protein IκBα [28].

NFκB is a constitutively expressed protein present in nearly all cell types. It has been implicated in the regulation of apoptosis genes, cell adhesion molecules, stress responses, cancer, immune system, and inflammatory responses [29, 30]. In inflammation, NFκB regulates the transcription of inflammatory genes induced by a variety of intra- and extracellular stimuli. The activation of NFκB and its translocation to the nucleus depends on the phosphorylation and degradation of the IκB proteins [31, 32]. It has been shown that several NSAIDs also inhibit NFκB activation independently of their effect on COX inhibition [33–36]. These agents include aspirin, salicylates, sulindac, and sulphasalazine. However, until now inhibitors of NFκB with a comparable anti-inflammatory capacity as glucocorticoids have not been identified. Although glucocorticoids, like dexamethasone, prednisone, and hydrocortisone, are successful at treating many inflammatory based diseases, continued use may lead to adverse events such as bruising, cataracts, muscle weakness, skin changes, sleep disturbances, weight gain, or more severe side effects such as type II diabetes mellitus, osteoporosis, and psychiatric symptoms [37–39].

Immunomodulatory drugs, such as thalidomide and its analogs, are also inhibitors of NFκB activation [40]. These drugs have anticancer, anti-inflammatory, and antiangiogenic actions by modulating the secretion of cytokines such as Tumor Necrosis Factor Alpha (TNF-α) and interleukins IL-6 and IL-12 [41–43]. It has been proposed that the anti-inflammatory effect of these drugs occurs by inhibition of IκB degradation and downregulation of NFκB DNA-binding activity. Inhibitors of TNF-α are also being used currently for the treatment of inflammatory diseases such as rheumatoid arthritis, Crohn's disease, and asthma [44]. These molecules act by inhibiting the binding of TNF-α to its receptor or by neutralizing the soluble and the membrane-bound forms of TNF-α [44, 45]. However, several adverse effects for these drugs have been described, such as heart failure, increased predisposition to infection, and exacerbation of latent tuberculosis [44, 46].

There are many natural remedies for inflammation and pain, such as curcumin and green tea, which act via similar mechanisms but exhibit limited, if any, unwanted side effects [47]. Curcumin, a compound found in turmeric, has also been described to confer anti-inflammatory effects through a combination of mechanisms including inhibition of COX-2, lipoxygenase, and the NFκB pathway [48–51]. In addition to anti-inflammatory effects, curcumin has also been attributed with antitumor [52, 53], antiviral [54], and antibacterial [55] effects. Curcumin is being tested for efficacy in patients with ulcerative colitis [56–58].

Table 1: Anti-inflammatory effect of eunicellane diterpenoids.

	Families name	Biological source	COX-2	iNOS	Superoxide anion generation	Elastase release	Other
Eunicellane diterpenoids 	Krempfielins	*Cladiella krempfi*		B-C, D* [73] E*, G, I [77]		K, M [82] N, P [84]	
	Hirsutalins	*C. hirsuta*	B [71]	B-D, H [71] K [78]		N [86] S [87]	
	Cladieunicellins	*Cladiella* sp.			C-E [80] 6-*epi* F [81]	A, C-D [80] 6-*epi* F [81]	
	Klymollins	*Klyxum molle*	F-G [74]	C-H [74]	M [83]	M [83]	X [79]
	Klysimplexins	*K. simplex*	R-S [72]	J-N, R-S [75]			
	Klysimplexin sulfoxides	*K. simplex*	C [72]	A-C [72]			
	Simplexin	*K. simplex*	E [70]	A, D-E [70]			

Data refer to compounds with percentage of inhibition > 50% for COX-2 and iNOS and >25% for superoxide anion generation and elastase release.
*Percentage of inhibition 40–50%.

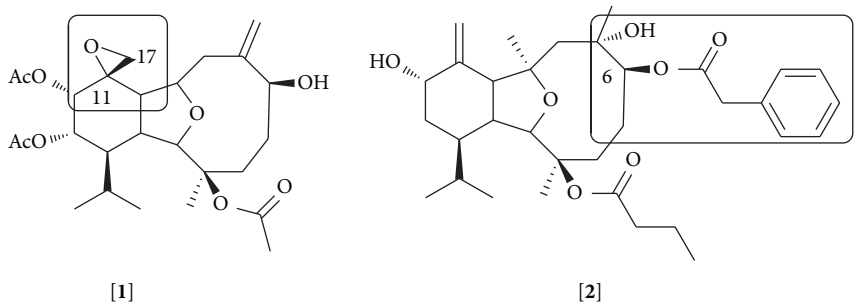
Epigallocatechin-3-gallate (EGCG) is the main component in green tea that is responsible for conferring not only anti-inflammatory effects but also antiviral [59, 60], antibacterial [61], and anticancer effects [62, 63]. The anti-inflammatory effects are achieved most notably through COX-2 inhibition at the RNA and protein level [64]. Interestingly, EGCG has not been found to have an effect on COX-1 expression.

3. Marine-Derived Diterpenoids as Anti-Inflammatory Compounds

De las Heras and Hortelano in 2009 compiled a comprehensive list of the most promising anti-inflammatory diterpenoids, almost all of which were extracted from plants [10]. In their compilation, they describe the mechanisms of action associated with inhibition of the NFκB signaling pathway of most families of diterpenoids. Bioactive diterpenoids act in the NFκB pathway by blocking a range of activities including DNA-binding, IKK complex activation, and IκB phosphorylation. Clinical studies have shown that commercial extracts from medicinal plants that contain large concentrations of diterpenoids that inhibit the NFκB pathway are effective in reducing symptoms of rheumatoid arthritis [65, 66]. These extracts have also been tested in the treatment of other autoimmune and inflammatory diseases showing efficacy with variable mild side effects [67–69]. In this regard we discuss different families of diterpenoids isolated from marine organisms with anti-inflammatory capacity. Several of those molecules are promising candidates for further anti-inflammatory drug development.

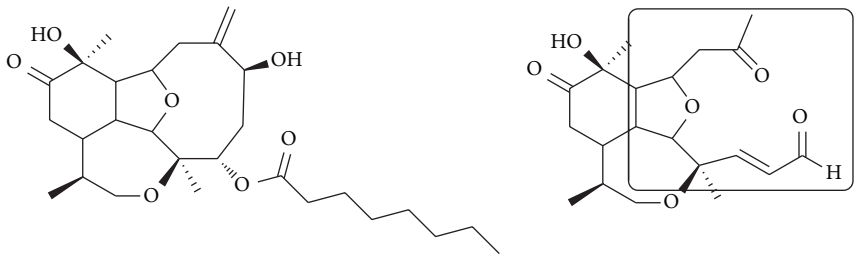
3.1. Eunicellane Diterpenoids. Anti-inflammatory activity for eunicellin-based diterpenoids has been reported in the last few years. This class of compounds is secondary metabolites that present the cladiellane skeleton with a C2-C9 or C2-C6 oxygen bridge. Eunicellin-based diterpenes include krempfielins, hirsutalins, klymollins, klysimplexin, klysimplexin sulfoxide, simplexin, and cladieunicellin and have been isolated and identified from soft corals belonging to the genera *Cladiella* or *Klyxum*. Some of these compounds have shown the capacity to inhibit the upregulation of inducible nitric oxide synthases (iNOS), COX-2, or IL-6 proteins in RAW 246.7 macrophages stimulated with lipopolysaccharide (LPS) [70–79]. Other compounds of these types were identified as inhibitors of superoxide generation and elastase release by N-formyl-methionyl-leucyl-phenylalanine/cytochalasin B (FMLP/CB) induced human neutrophils [80–87]. The release of superoxide and elastase by immune cells, mainly neutrophils, is important for the killing of host invading microorganisms but also contributes to host tissue damage during a chronic inflammatory disease. The mechanisms of action by which these compounds exert their anti-inflammatory effect have not been elucidated yet. Interestingly, most of these compounds selectively influenced certain inflammatory responses without affecting others (Table 1 and Supplementary Material available online at http://dx.doi.org/10.1155/2015/263543).

Reports have proposed that an epoxy group on C-11/C-17 present in some members of the klymollins is important for the inhibitory activity on iNOS expression [74]. However, some compounds of this family, the klymollins F and G (Figure 1, e.g., **1**), were significant inhibitors of both iNOS and COX-2, suggesting that the modulation of these enzymes might be due to the inhibition of a common molecule upstream in the signaling pathway that governs their expression. These two compounds, in addition to the epoxy, present a fatty acid residue at C-6 position attributing a micelle-like feature to the structure that might be important for membrane diffusion. Other authors have attributed the capacity of inhibiting elastase release and superoxide generation of klymollin M (**2**) to the presence of a phenylacetate group at C-6 (Figure 1) [83]. Comparing the structure of klymollin M with other eunicellin-based diterpenoid inhibitors of elastase release, it appears that the presence of a butyric acid at C-3, a common feature of these molecules, might be also important for this activity (Figure 1



[**1**] [**2**]

Figure 1: Structures of klymollin G [**1**] and klymollin M [**2**]. The epoxy group at C-17 for klymollin G and phenylacetate group at C-6 are labeled.



[**3**] [**4**]

Figure 2: Structures of briarellin S [**3**] and *seco*-briarellinone [**4**]. The opening of the 10-member ring and the presence of carbonyl groups in the *seco*-briarellinone that have been suggested as being responsible for the higher anti-inflammatory effect than briarellin S are labeled.

and Supplementary Material). Further studies are necessary to identify the structural components that play a role in the anti-inflammatory effect of these molecules and to describe their mechanisms of action.

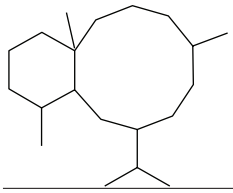
Briarellins are another class of eunicellane diterpenoid. Most of the briarellins have been isolated from corals of the genera *Briareum* and *Pachyclavularia*. The anti-inflammatory activity of this family has been little explored. Our group recently showed that briarellin S (**3**) inhibits the production of nitric oxide (NO) by primary murine macrophages stimulated with LPS. This effect was smaller than the one observed when cells were exposed to LPS in the presence of *seco*-briarellinone (**4**). Differences in the IC_{50} of briarellin S (20.3 μM) and *seco*-briarellinone (4.7 μM) might be due to the opening of the 10-member ring and the presence of carbonyl groups in the *seco*-briarellinone, which is the main structural difference with the briarellin S [88] (Figure 2). The ester moiety present in the molecule of briarellin S could also be interfering with the activity of this compound. Structural modifications of these molecules would give a clue about the groups responsible for the anti-inflammatory effect.

3.2. Briarane Diterpenoids. Briarane diterpenoids form a family of compounds that present a basic chemical structure of a [8.4.0] bicycle carbon skeleton with most members containing a γ-lactone moiety. These compounds have been exclusively isolated from soft corals belonging to the order Gorgonacea (reviewed by [89]) and genera including *Briareum*, *Dichotella*, *Junceella*, and *Verrucella*. Around 600 briarane diterpenoids have been identified with a variety of bioactivities including antimicrobial, cytotoxic, and in some cases anti-inflammatory effects. Briaranes, such as frajunolides, juncenolides, and the briarenolides, are inhibitors of the superoxide generation and elastase released by human neutrophils stimulated with FMLP/CB [90–98].

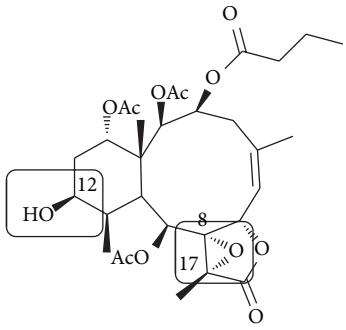
Compounds isolated from *Junceella juncea*, the juncenolides, have shown moderate inhibition in the release of elastase [99] and junceol has presented weak inhibitory effects on neutrophil superoxide generation [100, 101]. However, neither the mechanisms of action nor the structural components involved in these differences in anti-inflammatory activity have been described. The inhibitory effect of briarane compounds on COX-2 and iNOS expression induced by LPS in macrophages has been also reported [102, 103]. Table 2 shows a compilation of briarane diterpenoids with anti-inflammatory properties.

Excavatolide B (BrD1) (**5**), a briarane diterpenoid isolated from the coral *Briareum excavatum*, demonstrates *in vitro* and *in vivo* anti-inflammatory activity [104]. This compound inhibited vascular permeability and edema and decreased the expression of iNOS, COX-2, and matrix metallopeptidase (MMP-9) when topically applied in the skin of mice with 12-O-tetradecanoylphorbol-13-acetate- (TPA-) induced dermatitis. This effect might occur by a mechanism involving the inhibition of NFκB and Akt activation observed in the skin of the animals. Comparing the effect on IL-6 secretion induced by LPS in bone marrow derived dendritic cells (BMDC) of different briarane diterpenoids isolated from the same coral and semisynthetic analogs of BrD1, the authors concluded that 8,17-epoxide and 12-hydroxyl groups are essential for the inhibition of IL-6 secretion by BrD1 [104] (Figure 3).

Table 2: Anti-inflammatory effect of briarane diterpenoids.

	Families name	Biological source	iNOS	Superoxide anion generation	Elastase release
Briarane diterpenoids 	Frajunolide	*Junceella fragilis*		P-Q [97]	P-Q [97]
	Juncenolide	*J. juncea*		H [92] O [99]	N-O [99]
	Junceol	*J. juncea*		A-C [101] E [100]	
	Briarenolides	*Briareum* sp.	K-L [103]	F [95] I [96] J [98]	E [94] F [95] J [98]

Data refer to compounds with percentage of inhibition > 50% for iNOS and >25% superoxide anion generation and elastase release.



[**5**]

Figure 3: Structure of excavatolide B [**5**]. The 8,17-epoxide and 12-hydroxyl groups that have been suggested as being responsible for the anti-inflammatory effect are marked.

3.3. Cembrane Diterpenoids. Cembranes are a large family of diterpenoids isolated from terrestrial and marine organisms that exhibit a range of biological activities including antibacterial, antitumor, anti-inflammatory, and antiviral effects [105]. The basic structure of cembrane diterpenoids is constituted by a common 14-membered carbocyclic skeleton and usually presents cyclic ether, lactone, or furan moieties around this nucleus (reviewed by [106]). Unconventional cembranoids with 12-, 13-, or 14-membered variants have also been described [107, 108]. Cembranoids from marine organisms are mainly isolated from corals of the genera *Sinularia*, *Lobophytum, Eunicea*, and *Sarcophyton*.

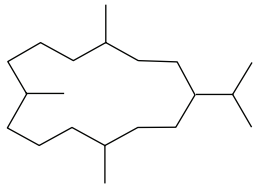
Anti-inflammatory activity for different groups of cembrane diterpenoids has been reported. Cembranoids such as gibberosenes, grandilobatin, querciformolides, sarcocrassocolides, crassumolides, crassarines, sinularolides, durumolides, and columnariols have shown a capacity to inhibit the expression of iNOS and/or COX-2 by LPS-stimulated RAW 264.7 cells [109–121] (Table 3 and Supplementary Material). The presence of a α-methylene-γ-lactone in cembranolides has been suggested to be essential for the inhibition of iNOS expression [119] (Figure 4, e.g., **6**).

Some cembranoids have been identified as modulators of NFκB signaling pathway [122–126]. Compounds from the crassumolide and laevigatol groups have shown dose-dependent inhibitory effects on the mRNA expression of iNOS and COX-2 induced by TNF-α in HepG2 cells by a mechanism that involved the inhibition of NFκB transcriptional activation [123, 124]. The cembrane lobohedleolide (**6**) isolated from *Sarcophyton* sp. showed inhibitory activity on the production of TNF-α in LPS-stimulated RAW 264.7 cells [127]. This effect was later attributed to the ability of this compound to inhibit the degradation of IκBα and the binding of NFκB to the DNA [122]. However, lobohedleolide also induced an increase in the production of IL-8 in LPS-stimulated THP-1 cells through the activation of the IL-8 promoter region [122]. High levels of IL-8 have been found in some human cancers and have been associated with tumor progression and metastasis [128–130]. Thus, the identification of new anti-inflammatory molecules must be accompanied by a rigorous description of the mechanisms involved in the effect. Considering the pharmacological properties of lobohedleolide in the inhibition of NFκB pathway, synthetic analogs could be produced with structural modifications that might favor the anti-inflammatory properties.

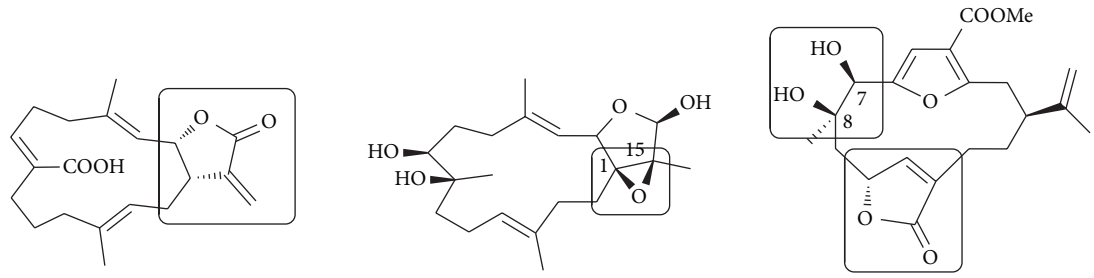
Members of the cembrane diterpenoids, lobocrasols isolated from *Lobophytum crassum*, have also shown inhibitory activity on NFκB activation in TNF-α stimulated HepG2 with consequent decreases in COX-2 and iNOS gene expression [125]. The presence of an epoxy group at C-1/C-15 in the active compounds appears to be essential for the anti-inflammatory effect (Figure 4, e.g., **7**). Cembrane sinumaximols B and C isolated from *Sinularia maxima* were identified as potent inhibitors of IL-12 secretion by dendritic cells stimulated with LPS [131]. This activity could be attributed to the lactone moiety present in these molecules. Later, it was demonstrated that the sinumaximols A, B, and G inhibited the transcriptional activity of NFκB induced by TNF-α in HepG2 cells and the expression of the intracellular adhesion molecule (ICAM-1) and iNOS [126]. Authors suggested that hydroxyl groups at C-7 and/or C-8 are responsible for the anti-inflammatory activity of these compounds. One of those compounds, sinumaximol B (**8**), exhibited inhibitory activity in both dendritic and HepG2 cells (Figure 4). It is important to note that only sinumaximol B contains the lactone and the hydroxyl at C-7 and C-8.

3.4. Diterpene Glycosides. Marine diterpene glycosides are derivatives exclusively produced by Gorgonian corals [132].

Table 3: Anti-inflammatory effect of cembrane diterpenoids.

	Families name	Biological source	COX-2	iNOS	NFκB
Cembrane diterpenoids 	Crassarines	*Sinularia crassa*		H [118]	
	Grandilobatins	*S. grandilobata*		D [110]	
	Querciformolides	*S. querciformis*	C [111]	C [111] E [115]	
	Sinumaximols	*S. maxima*			A-C, G, I [126]
	Sarcocrassocolides	*Sarcophyton crassocaule*	I [117] Q [120]	A-D [116] F-L [117] M-O [119] P-R [120]	
	Crassocolides	*S. crassocaule*	A, E [120]	A-B, D-E [120]	
	Crassumolides	*Lobophytum crassum*	A, C [112]	A-C, F [112]	
	Crassumols	*L. crassum*			E [124]
	Lobocrasols	*L. crassum*			A-C [125]
	Durumolides	*L. durum*	C [113] F [114]	A-E [113] F-L [114]	
	Laevigatols	*L. laevigatum*			A-B [123]
	Columnariols	*Nephthea columnaris* (cultured coral)	A-B [121]	A-B [121]	

Data refer to compounds with percentage of inhibition > 50% for COX-2 and iNOS and IC_{50} values < 50 μM in NFκB.



[**6**] [**7**] [**8**]

Figure 4: Cembrane diterpenoids: lobohedleolide [**6**], lobocrasol A [**7**], and sinumaximol B [**8**]. The presence of a α-methylene-γ-lactone in lobohedleolide (and cembranolides); an epoxy group at C-1/C-15 in lobocrasol A and a hydroxyl group at C-7 and/or C-8 in sinumaximol B are labeled.

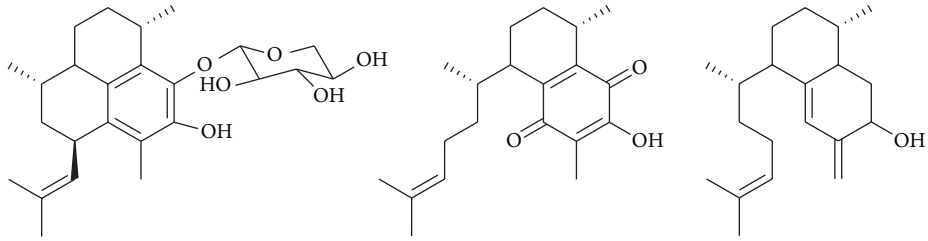
A diterpene aglycone core and a carbohydrate moiety characterize this class of compounds. Among the marine diterpenes glycosides, eleutherobins, fuscosides, and pseudopterosins are the most studied compounds [132]. The pseudopterosins (Ps) have been described as molecules with important anti-inflammatory and analgesic properties and were the first to be isolated from *Pseudopterogorgia elisabethae* [133, 134]. Pseudopterosin A (**9**) was identified as a potent anti-inflammatory agent, with a greater effect than the NSAID indomethacin, in the phorbol myristate acetate- (PMA-) induced topical inflammation animal model [133]. Pseudopterosin A also inhibited prostaglandin E_2 and leukotriene C_4 secretion in zymosan-stimulated murine peritoneal macrophages [135]. This molecule inhibited phagosome formation and triggered intracellular calcium release by a mechanism that involved its binding to a G protein coupled receptor [136]. Other pseudopterosins with exceptional anti-inflammatory activity also have been identified [137, 138] and are suggested to inhibit the synthesis of leukotrienes and the degranulation of human neutrophils [135, 137] (Table 4 and Supplementary Material). Several analogs of Ps such as *seco*-pseudopterosins and amphilectosins reduced the mouse ear edema induced by different inflammatory stimuli [138, 139] and the levels of myeloperoxidase at the inflammation site [139].

Due to the relevant anti-inflammatory properties of Ps, they have attracted great attention from the organic chemistry community and new synthetic pseudopterosins have been obtained. Discussions of Ps syntheses are out of the scope of this review but they can be found elsewhere [reviewed by [132]]. It appears that the location and identity of carbohydrate moiety are not relevant for the anti-inflammatory activity; instead, the intact diterpene glycoside is needed for the Ps biological effect [140]. However, nonglycosylated compounds structurally related to the aglycone component of Ps, such as elisabethadione (**10**) and elisabethatrienol (**11**), have shown anti-inflammatory activity

Table 4: Glycosides diterpenes.

Families name	Biological source	Bioactive compounds
Pseudopterosins	*Pseudopterogorgia elisabethae*	Pseudopterosins A [133], E [137], Q [134], P, T, U [139]
Fuscosides	*Eunicea fusca*	Fuscosides A-B [145]
Eleutherobins	*Eunicea* sp.	Calyculaglycoside B [149]

Data refer to glycosides diterpenoids with anti-inflammatory activity.



[**9**] [**10**] [**11**]

Figure 5: Pseudopterosin A [**9**], elisabethadione [**10**], and elisabethatrienol [**11**]. Glycoside diterpene, pseudopterosin A, nonglycoside diterpenes, elisabethadione, and elisabethatrienol.

[138, 139] (Figure 5). Simplified structural analogs of the Ps and *seco*-Ps have been synthesized, which conserve the anti-inflammatory effect, suggesting that a more accessible aglycone would be sufficient for the activity [141, 142]. A semisynthetic derivative of pseudopterosin A maintaining the anti-inflammatory capacity has been obtained [143]. Due to their anti-inflammatory properties natural extracts from *P. elisabethae* rich in pseudopterosins are used in commercial skin care products [144].

Fuscosides have been isolated from the coral *Eunicea fusca*. Fuscosides A and B exhibit anti-inflammatory activity [145, 146]. Both compounds, when topically applied, reduce PMA-induced edema in mouse ears by inhibiting neutrophil infiltration. Fuscoside B inhibits the synthesis of leukotriene C_4 in calcium ionophore-activated murine macrophages [145, 146]. It was demonstrated using cultures of human leukocytes that fuscoside B is a selective inhibitor of 5-lipoxygenase [147] (Table 4 and Supplementary Material). The aglycone precursor of fuscoside B, the fuscol, and other compounds as eunicol and the analogous eunicidiol, isolated from *E. fusca*, have also shown anti-inflammatory activity by reducing the edema induced by PMA in mouse ear [148]. Different approaches for the synthesis of naturally occurring fuscosides, conserving the anti-inflammatory capacity, have been attempted unsuccessfully.

Other members of the diterpene glycosides compounds have also shown anti-inflammatory activity. A calyculaglycoside isolated from *Eunicea* sp. exhibited topical anti-inflammatory activity in two *in vivo* assays, and it was suggested as a nonselective inhibitor of the 5-lipoxygenase and COX pathways [149]. It is relevant to note that compounds belonging to this family have the same aglycone (dilophol) and only differ in the identity of the carbohydrate moieties. Anti-inflammatory activity has not been reported for the eleutherobin compounds; however, two nonglycoside compounds, the valdivones A and B, which are related to the eleutherobin aglycone, inhibited chemically induced inflammation in mouse ear [150]. These findings question the relevance of carbohydrate moiety for the biological activity of glycoside compounds.

3.5. Other Diterpenoids. Pseudopteranes are only found in corals of the genera *Pseudopterogorgia*. Their ring system could be originated from a ring contraction reaction of a cembrane precursor [151]. Pseudopterolide 1 was the first compound identified and isolated from *Pseudopterogorgia acerosa* [152]. Other pseudopterane compounds include kallolides and isogorgiacerodiol isolated from *P. kallos* and *P. acerosa*, respectively [153, 154]. Pseudopterolide 1 and some kallolides have shown anti-inflammatory capacity in topical skin inflammation induced by PMA [152, 153]. Our group has recently demonstrated that pseudopterolide 1 derivative (**12**) also exhibits anti-inflammatory capacity. This compound inhibited the secretion and/or mRNA expression of a variety of inflammatory mediators (TNF-α, IL-6, NO, IP-10, iNOS, COX-2, and MCP-1) induced by TNF-α and ligands of TLRs in mouse peritoneal macrophages [155]. This effect was due to the capacity of this compound to inhibit IκBα phosphorylation and the subsequent activation of NFκB. The compound also inhibited the expression of macrophages activation markers such as CD80 and CD86 suggesting a role in the modulation of a variety of processes occurring during macrophage activation. The methoxyl group at C-9 appears to be important in the anti-inflammatory effect as it was more potent than the isogorgiacerodiol pseudopterane (**13**), which has a hydroxyl group at the same position [155] (Figure 6). It is notable how subtle structural differences in small molecules are essential for modifying their immune modulation activities.

Smaller groups of diterpenoids called verticillane-based and norditerpenoids, isolated from coral of the genera

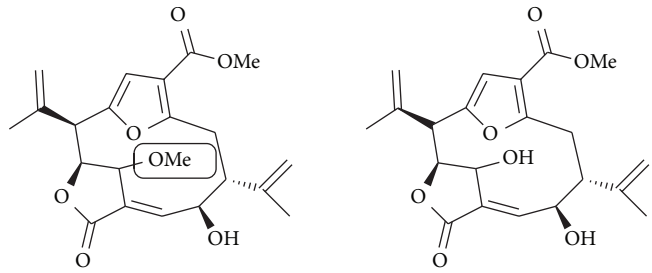


Figure 6: Pseudopterolide derivative [**12**] and isogorgiacerodiol [**13**]. The methoxyl group at C-9 in pseudopterolide derivative that has been suggested as being responsible for the higher anti-inflammatory effect than isogorgiacerodiol is labeled.

Cespitularia and *Sinularia*, respectively, have been recently identified to have anti-inflammatory capacity [156, 157]. It has been reported that members of these families, for example, cespitularin (verticillane-based diterpenoid), isolated from *C. hypotentaculata* and a series of norcembranolides, gyrosanolides, and other norditerpenoids isolated from *S. gyrosa*, inhibit the expression of iNOS in LPS-stimulated RAW 264.7 cells [156, 157].

The neorogioltriol, a tricyclic brominated diterpenoid isolated from the red algae *Laurencia glandulifera*, showed anti-inflammatory effects *in vitro* and *in vivo* [158]. This compound inhibited the activation of NFκB and the production of TNF-α, COX-2, and NO in RAW 264.7 macrophages stimulated with LPS. The systemic administration of neorogioltriol reduced the edema formation in an animal model of carrageenan-induced local inflammation.

Dolabellane diterpenoids have been isolated mainly from plants but are also present in marine organisms. These compounds have a 5,11-bicyclic skeleton and exhibit antiviral, antiprotozoa, and antibacterial properties [159]. Recently, it has been suggested that the dolabelladienetriol, isolated from the brown marine alga *Dictyota pfaffii*, downregulates the production of TNF-α and NO through the inhibition of NFκB activation in *Leishmania amazonensis* infected and uninfected macrophages, conferring an anti-inflammatory activity to this compound [160]. To our knowledge, this is the first report of anti-inflammatory capacity described for a marine-derived dolabellane diterpenoid.

4. Conclusions

Many efforts have been made to identify new anti-inflammatory molecules from natural sources. Terrestrial organisms are commonly used in traditional medicine to treat inflammatory diseases and have often been ascribed diterpenoid compounds to the anti-inflammatory effects. Marine invertebrates are exceptional sources of new molecules with therapeutic potential including diterpenoids secondary metabolites, which exhibit notable anti-inflammatory properties.

The anti-inflammatory capacity of some diterpenoids isolated from marine organisms is due to the inhibition of the NFκB signaling pathway at different levels [122, 155]. NFκB plays a crucial role in regulating the inflammatory responses and in the development of various human pathological conditions. Hence, this transcription factor constitutes a suitable target for the development of new anti-inflammatory drugs. Moreover, some marine diterpenoids have been shown to be inhibitors of prostaglandins and leukotrienes secretion and in some cases found to be selective inhibitors of 5-lipoxygenase and COX enzymes [147, 149]. Together, this evidence demonstrates that marine diterpenoids show a capacity of inhibiting different pathways involved in inflammation, supporting their potential for anti-inflammatory drugs development. However, little is known about the molecular mechanisms involved in the anti-inflammatory characteristics of marine diterpenoids. Thus, further studies are necessary to better understand their mechanisms of action.

The largest limitation for the study of natural products is the small amount of compounds that are obtained and the variations on their production that are influenced by the environmental changes to which marine organisms are exposed. Due to the potential applications of coral-derived compounds, coral aquaculture has been proposed as a way to establish a stable supply of bioactive materials for the extraction of natural products [161]. Some laboratories use this approach for the production of marine invertebrates with bioprospecting purposes. Importantly, natural growth rate of these organisms is not enough to sustain pharmaceutical exploitation. Many researchers have developed new strategies for the synthesis of compounds that conserve the biological activities of their natural analogs; nonetheless, it remains a challenging area.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Therapeutic Effect of Chenodeoxycholic Acid in an Experimental Rabbit Model of Osteoarthritis

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Osteoarthritis (OA) is a slowly progressive joint disease typically seen in middle-age to elderly people. At present, there is no ideal agent to treat OA. Chenodeoxycholic acid (CDCA) was a principal active constituent from animal bile. However, the therapeutic effect of CDCA on OA severity was largely unknown. The purpose of this study was to evaluate the therapeutic effect of intra-articular injection of CDCA in a rabbit OA model. OA was induced in experimental rabbits by anterior cruciate ligament transection (ACLT) and then rabbits were intra-articularly injected with CDCA (10 mg/kg or 50 mg/kg) once per week for 5 weeks. The results showed that CDCA significantly decreased cartilage degradation on the surface of femoral condyles, reducing the pathological changes of articular cartilage and synovial membrane by macroscopic and histological analysis. CDCA also significantly decreased bone destruction and erosion of joint evaluated by micro-CT. Furthermore, CDCA could markedly reduce the release of matrix metalloproteinase-1 (MMP-1), matrix metalloproteinase-3 (MMP-3), interleukin-1β (IL-1β), and prostaglandin E_2 (PGE_2) in synovial fluid. These observations highlight CDCA might be a potential therapeutic agent for OA.

1. Introduction

Osteoarthritis (OA) is a prevalent form of arthritic disease and a leading cause of physical disability in adult population [1–3]. It was generally considered a whole joint disease characterized mainly by cartilage destruction, subchondral bone sclerosis, osteophyte formation, and joint synovitis [4, 5]. Though much research has been performed, the concrete causes of OA remain unclear.

At present, OA progression is considered to be regulated largely by an excess of matrix metalloproteinases (MMPs), which contribute to the degradation of the extracellular matrix. Among these enzymes, MMP-1 and MMP-3 play important roles in OA progression by degrading the extracellular matrix [6–8]. Moreover, inflammatory mediators such as IL-1β and PGE_2, have been implicated in the synovial inflammation and cartilage degradation in OA [9, 10]. Levels of IL-1β and PGE_2 are increased in the synovial fluid and cartilage of OA patients, implying a role for IL-1β and PGE_2 in the pathogenesis of OA [11].

The currently available pharmacological treatments for OA are effective only temporarily and might result in undesirable gastrointestinal, renal, and cardiovascular side effects [12–14]. There is an increasing interest in the use of natural compounds extracted from Traditional Chinese Medicine (TCM) for the treatment of OA because they are reported to demonstrate satisfactory clinical efficacy with minimal side effects, compared to routine pharmacological strategies [15]. Our group has previously reported that chenodeoxycholic acid (CDCA) was an important active constituent from animal bile and exhibited obviously inhibitory effect on MMPs in vitro [16, 17]. However, the effect of CDCA on OA severity in vivo has not yet been studied at present. In continuation of our previous study and development of a

novel small molecule drug to treat OA, we elucidated the therapeutic effect of CDCA in an experimental rabbit model of OA for the first time in this paper.

2. Materials and Methods

2.1. Reagents. Chenodeoxycholic acid (CDCA, with its purity above 98%) and celecoxib (with its purity above 98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Experimental Animal Model and Drug Treatment. Forty SPF adult male white New Zealand rabbits weighing 3.0–3.5 kg were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China). This experiment was carried out in accordance with the Chinese Guidelines for Animal Welfare and Experimental Protocol and approved by the Animal Care and Use Committee of Soochow University. The rabbits were placed in a left lateral position and the right operated legs were shaved and disinfected with betadine solution. Thirty-two of the 40 rabbits underwent anterior cruciate ligament transection (ACLT) on the right knee joints to induce OA [18] and were randomly divided into 4 groups ($n = 8$ per group). The other 8 rabbits (sham-operation group) received sham operations on the right knee joints, which involved opening the articular cavity and resuturing it without cutting the short anterior cruciate ligament. Postoperatively, the animals were permitted cage activity without immobilization. The rabbits were closely monitored for infections and other complications. Treatments began 4 weeks after surgery. Drugs were given once per week for 5 weeks. The rabbits in CDCA-treated groups were given intra-articular injections of CDCA (10 mg/kg or 50 mg/kg) in the knees operated on. The rabbits in celecoxib-treated group were intra-articularly injected with celecoxib (15 mg/kg) in the knees operated on. The rabbits in model group were intra-articularly injected with 0.5 mL vehicle alone in the knees operated on. In sham-operation group, no other procedures were conducted. All rabbits were sacrificed 7 days after the last injection.

2.3. Macroscopic Observations. After the treatment, the rabbits were sacrificed and femoral condyles were collected for macroscopic observation. The cartilage degradation on the surface of femoral condyles was observed under dissecting microscope and the degree of degradation was graded on a scale of 0–4 as follows: 0 = surface smooth with normal color; 1 = surface rough with minimal fibrillation or a slight yellowish discoloration; 2 = cartilage erosion extending into superficial or middle layers; 3 = cartilage ulceration extending into deep layers; 4 = cartilage depletion with subchondral bone exposed [18, 19]. The examination was performed by two independent observers who were kept unaware of the treatment groups.

2.4. Histological Examination. After macroscopic observations, isolated specimens were prepared for further histological analysis. The specimens were decalcified (10% EDTA), embedded in paraffin, cut into 5 μm sections, and stained with hematoxylin and eosin (H&E). The histological evidence of articular cartilage was assessed according to the scoring system by Kikuchi et al. and eight parameters [20], namely, loss of superficial layer, erosion of cartilage, fibrillation and/or fissures, loss of proteoglycan, disorganization of chondrocytes, loss of chondrocytes, exposure of subchondral bone, and cluster formation. In addition, the histological evidence of synovial membrane was also assessed based on the four parameters [21, 22], namely, intimal hyperplasia, lymphocytic/plasmocytic infiltration, subintimal fibrosis, and vascularity. All sections were graded by two independent observers that were kept unaware of the treatment groups.

2.5. Micro-Computed Tomography (Micro-CT) Scanning. At the end of study, the right knee joints of experimental rabbits were subject to analysis using a SkyScan micro-CT apparatus, operating at a resolution of 35 μm voxel size. Three-dimensional reconstructions were performed using NRECON software, and the images were further processed with CT-analyzer software. The projection images were reconstructed into three-dimensional images using NRECON software and CT-analyzer (both from SkyScan).

2.6. ELISA for Measurement of MMP-1, MMP-3, IL-1β, and PGE_2 in Synovial Fluid. Synovial fluids were obtained from anesthetized animals before sacrifice. The samples were stored at −70°C until assayed. The levels of MMP-1, MMP-3, and IL-1β in synovial fluid were measured by ELISA kits (TSZ Scientific LLC, Framingham, MA, USA). The levels of PGE_2 in synovial fluid were measured by ELISA kits (R&D Systems, Minneapolis, MN, USA). All the assays were performed in accordance with the manufacturer's instructions. All samples from animals in each experimental group were assayed in duplicate.

2.7. Statistics. Data were presented as the mean ± SEM. Statistical analyses were performed using SPSS 16.0. Statistical comparisons were performed using the Mann-Whitney U test. $P < 0.05$ was considered significant.

3. Results

3.1. Macroscopic Observations. As shown in Figure 1(a), the cartilage on the femoral condyles in the sham-operation group was macroscopically normal, with a smooth, glistening surface, and no cartilage defect or osteophyte was observed. In the model group, general characteristics of OA, including erosion and osteophyte formation, were seen on the side of the femoral condyles after surgery. In the treatment groups, less bone wear was observed after CDCA or celecoxib treatment, compared with the model group. Accordingly, as scored by macroscopic observations, CDCA or celecoxib treatment could significantly decrease the degree of cartilage degradation (Figure 1(b)).

3.2. Histological Findings. As shown in Figure 2(a), the sham operation of rabbits revealed no significant histological changes in the articular cartilages, whereas the model group

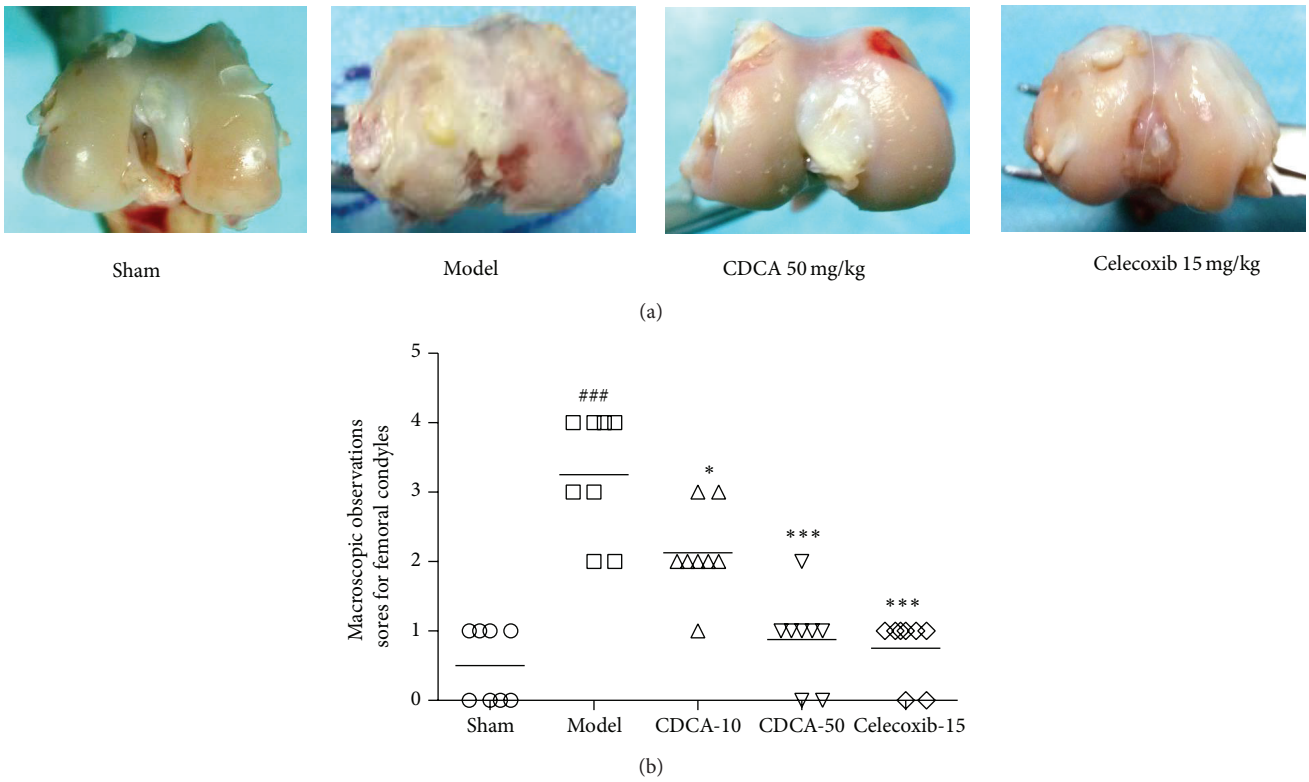

FIGURE 1: (a) Representative macroscopic observations images of femoral condyles are shown. (b) Macroscopic observations sores for femoral condyles in the five groups of experimental rabbits. Results are presented as individual data points and the median for each group is indicated by a horizontal bar. $^{###}P < 0.001$, compared with the sham-operation group; $^{*}P < 0.05$, $^{***}P < 0.001$, compared with the model group. Similar results were observed in two separated experiments.

and other treated groups developed different degrees of OA-like degenerative changes. A significant decrease in the severity of cartilage degradation was observed in CDCA-treated groups, particularly in the 50 mg/kg group. Histological sores in the CDCA-treated and celecoxib-treated group were significantly lower than in the model group (Figure 2(b)).

In synovial membrane tissues, the model group showed a similar degree of thickened synovium associated with hyperplasia, hypotrophy, inflammatory cell infiltration, and vascularization (Figure 3(a)). The synovium changes in the CDCA-treated groups showed significant alleviation compared to model groups (Figure 3(b)).

3.3. Micro-CT Analysis. Micro-computed tomography (micro-CT) analysis is a powerful tool for the evaluation of bone tissue because it provides access to the 3D microarchitecture of the bone. It is a nondestructive imaging technique that can be widely used to track changes in subchondral bone structure in drug intervention studies designed to treat the progression of OA [23, 24].

In order to observe the bone changes of knee joints after CDCA treatment, micro-CT analysis was performed on OA rabbit treated with CDCA, celecoxib, and vehicle in this study. As shown in Figure 4, the bone contact was high and the surface of knee joints was smooth in the sham-operation group. In contrast, obvious bone destruction and erosion of joint was observed in the model group. The CDCA- (50 mg/kg) and celecoxib- (15 mg/kg) treated groups markedly prevented bone erosions at the knee joints; no obvious osteophyte formation was observed. The results suggested that CDCA treatment was an effective therapeutic approach to reduced joint destruction in OA rabbits.

3.4. Effects of CDCA on the Levels of MMP-1, MMP-3, IL-1β, and PGE_2 in Synovial Fluid from Knee Joints Were Measured by ELISA. Compared to sham-operation group, model group showed higher level of MMP-1 and MMP-3 in synovial fluid ($P < 0.001$) (Figures 5(a) and 5(b)). The levels of MMP-1 and MMP-3 in synovial fluid were significantly decreased by CDCA treatment in a dose-dependent manner compared with those in model group. Moreover, the inflammatory mediators involved in cartilage destruction, such as IL-1β and PGE_2, were also significantly inhibited by CDCA treatment (Figures 5(c) and 5(d)). The capability of CDCA to reduce the release of MMP-1, MMP-3, IL-1β, and PGE_2 in synovial fluid was similar to the effect of celecoxib.

4. Discussion

Osteoarthritis (OA) is a prevalent form of arthritic disease and a leading cause of physical disability in adult population [1–3]. It was generally considered a whole joint disease

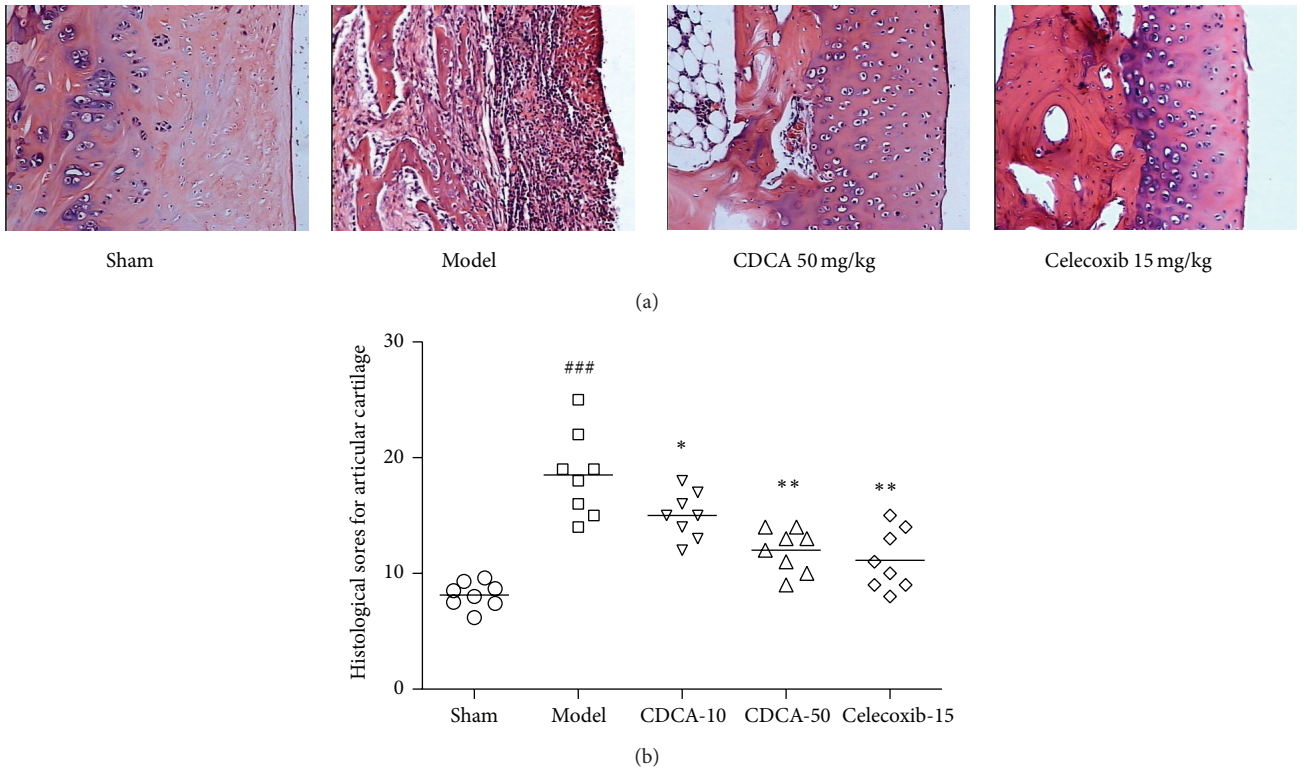

Figure 2: (a) Representative hematoxylin-eosin (HE) stained sections of articular cartilage are shown. Magnification 100x. (b) Histological sores for articular cartilage in the five groups of experimental rabbits. Results are presented as individual data points and the median for each group is indicated by a horizontal bar. $^{###}P < 0.001$, compared with the sham-operation group; $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the model group. Similar results were observed in two separated experiments.

characterized by cartilage destruction, subchondral bone sclerosis, osteophyte formation, and joint synovitis [4, 5]. At present, nonsteroidal anti-inflammatory drugs (NSAIDs), corticosteroids, and hyaluronan have been clinically used for the treatment of OA in the clinic. However, they fail to reverse cartilage damage, always resulting in undesirable cardiovascular, renal, and gastrointestinal side effects [14]. Thus, there is a continuing need for novel better agents with which to treat OA.

CDCA was an important active constituent from animal bile, which has been reported for the treatment of cardiovascular, cancer, respiratory, liver, and gallbladder diseases [25, 26]. However, little is known about its possible use in the treatment of OA. Our group has previously reported that CDCA exhibited obviously inhibitory effect on MMPs in vitro [16, 17]. In continuation of our previous study and search for a novel small molecule drug to treat OA, we elucidated the beneficial effect of CDCA on an OA model in rabbits for the first time in this paper.

In our study, we investigated the protective effect of CDCA against cartilage degradation in a rabbit OA model induced by ACLT, which is the most commonly used method of identifying OA disease-modifying therapies by intra-articular administration. The results showed that CDCA attenuated the severity of OA by reducing macroscopic observations sores for femoral condyles and histological sores for articular cartilage and synovial membrane (Figures 1–3). CDCA also significantly decreased bone destruction and erosion of joint evaluated by micro-CT (Figure 4). Notably, CDCA significantly reduced the degree of OA-like lesions at a dose of 50 mg/kg.

The matrix metalloproteinases (MMPs) have been considered the important enzymes responsible for degradation of aggrecan and collagens in cartilage [6, 7]. Many research groups have demonstrated that expression of several MMPs was elevated in synovial tissues and cartilage of OA patients [27]. MMP-1 belongs to collagenase subgroup in MMP family. It is able to cleave the triple helical chains of type II collagen in articular cartilage and play an important role in abnormal collagen turnover in OA [28–30]. MMP-3 also may have utility as a prognostic biomarker of OA progression. Patients with higher MMP-3 levels are more likely to suffer from progression of OA over a 30-month period than patients in the lower tertile [31]. Therefore, effects of CDCA on the levels of MMP-1 and MMP-3 in synovial fluid from rabbit knee joints were measured by ELISA in this paper. As shown in Figures 5(a) and 5(b), CDCA significantly decreased the levels of MMP-1 and MMP-3 in synovial fluid in a dose-dependent manner.

It is well established that many inflammatory mediators, such as IL-1β and PGE_2, have been implicated in the synovial inflammation and cartilage degradation in OA [9, 10]. Levels

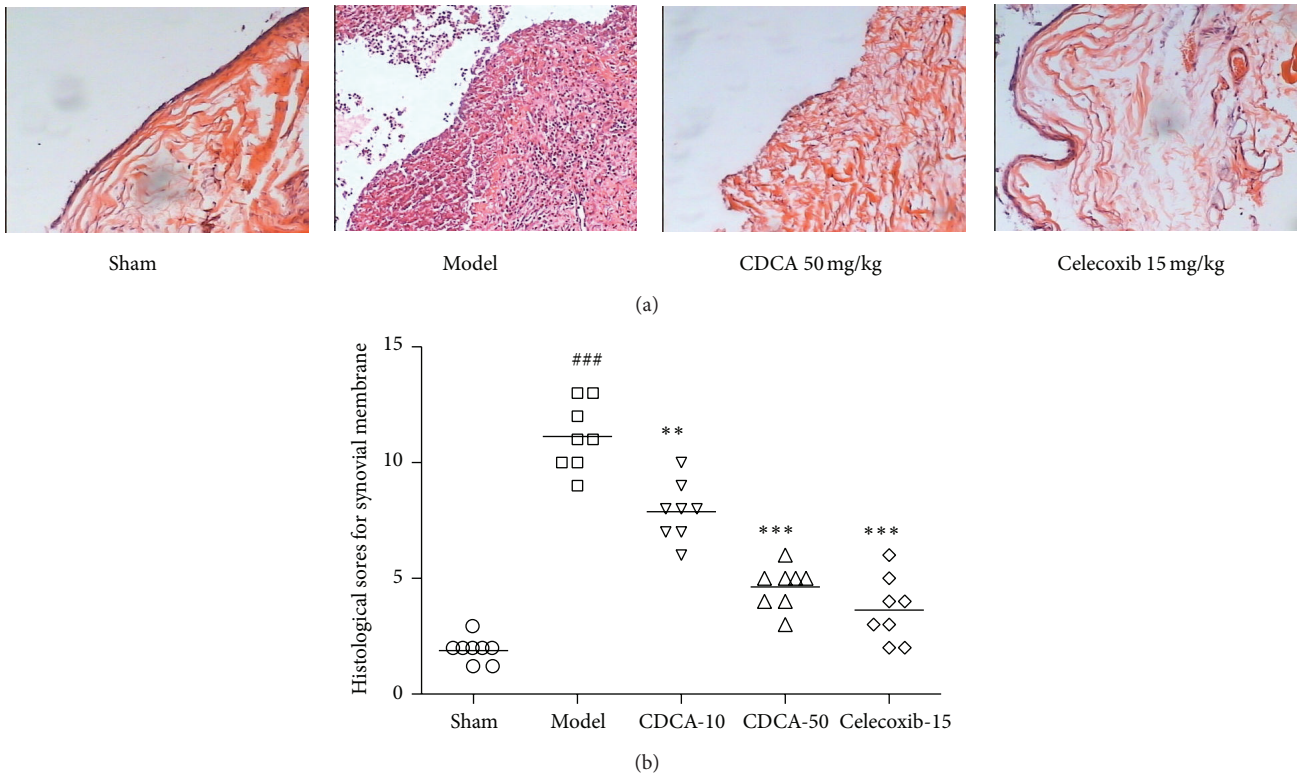

Figure 3: (a) Representative hematoxylin-eosin (HE) stained sections of synovial membrane are shown. Magnification 100x. (b) Histological sores for synovial membrane in the five groups of experimental rabbits. Results are presented as individual data points and the median for each group is indicated by a horizontal bar. $^{###}P < 0.001$, compared with the sham-operation group; $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the model group. Similar results were observed in two separated experiments.

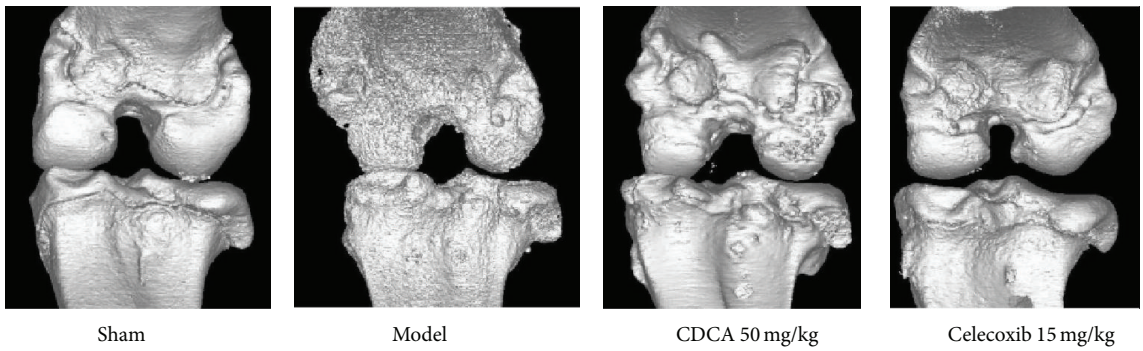

Figure 4: Representative micro-CT images of rabbit knee joints are shown.

of IL-1β and PGE_2 are increased in the synovial fluid and cartilage of OA patients, implying a role for IL-1β and PGE_2 in the pathogenesis of OA [11]. In this study, the levels of IL-1β and PGE_2 in synovial fluid were also measured. Our results indicate that both IL-1β and PGE_2 were significantly inhibited in the CDCA-treated group (Figures 5(c) and 5(d)). The capability of CDCA to reduce the release of MMP-1, MMP-3, IL-1β, and PGE_2 in synovial fluid was similar to the effect of celecoxib.

Taken together, these results confirm the therapeutic potential of CDCA for treatment of OA. However, further studies are needed to clarify the exact mechanism of action of CDCA in the future.

5. Conclusions

In this study, we evaluated the therapeutic effect of intra-articular injection of CDCA in a rabbit OA model for the first time. The results showed that CDCA significantly attenuated the severity of OA as determined by macroscopic, histological, and micro-CT analysis. Furthermore, CDCA could markedly reduce the release of MMP-1, MMP-3, IL-1β,

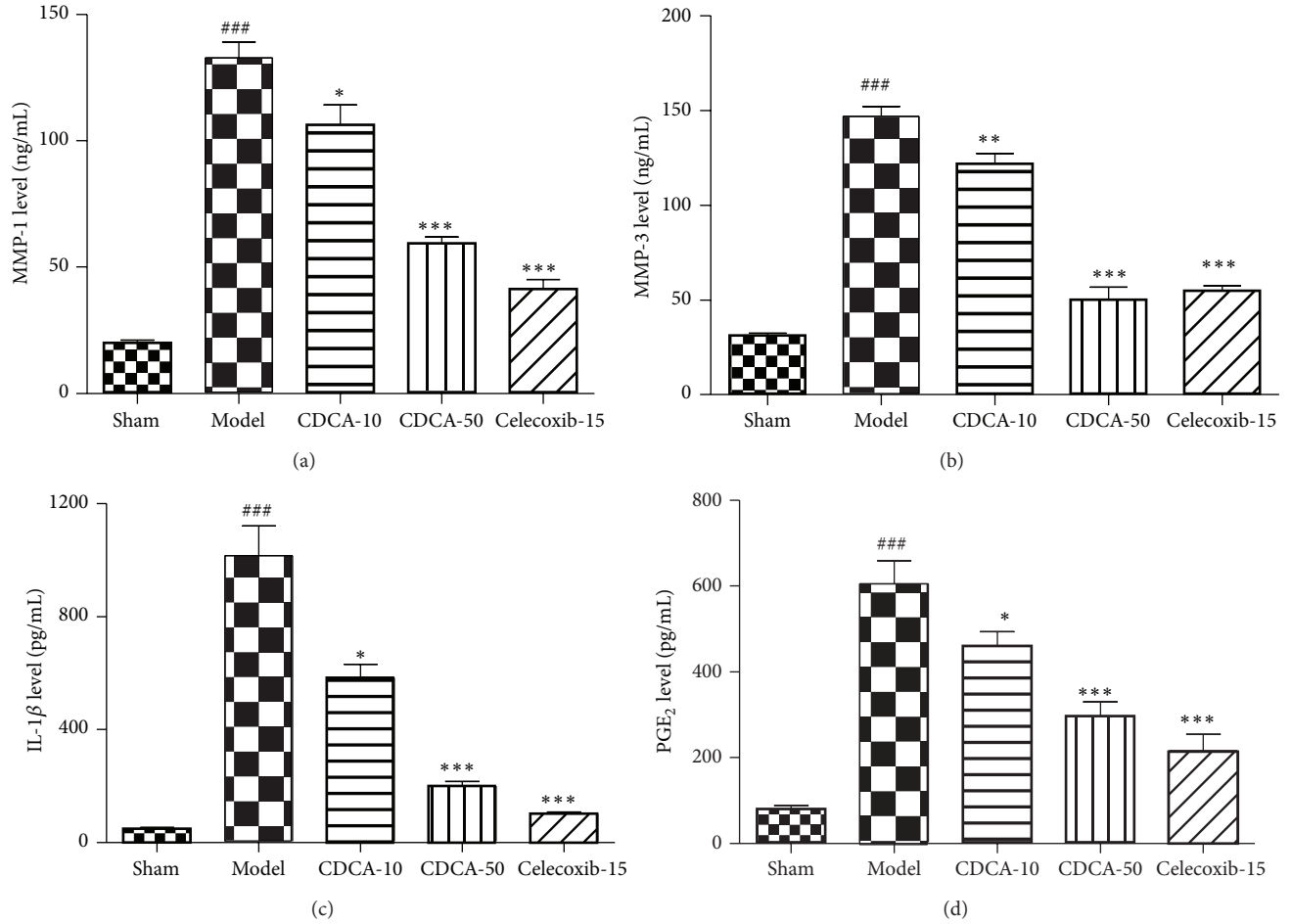

FIGURE 5: The levels of MMP-1 (a), MMP-3 (b), IL-1β (c), and PGE_2 (d) in synovial fluid from knee joints were measured by ELISA. Each reported value is a mean ± SEM ($n = 8$); $^{###}P < 0.001$, compared with the sham-operation group; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, compared with the model group. Similar results were observed in two separated experiments.

and PGE_2 in synovial fluid. Therefore, highlighting CDCA might be a potential therapeutic agent for OA.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Anti-Inflammatory Effect of 1,3,5,7-Tetrahydroxy-8-isoprenylxanthone Isolated from Twigs of *Garcinia esculenta* on Stimulated Macrophage

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Garcinia Linn. plants having rich natural xanthones and benzophenones with anti-inflammatory activity attracted a great deal of attention to discover and develop them as potential drug candidates. Through screening targeting nitric oxide accumulation in stimulated macrophage, we found that 1,3,5,7-tetrahydroxy-8-isoprenylxanthone (TIE) had potential anti-inflammatory effect. To understand how TIE elicits its anti-inflammatory activity, we uncovered that it significantly inhibits the production of nitric oxide (NO) and prostaglandin E2 (PGE2) in LPS/IFNγ-stimulated RAW264.7 cells. In further study, we showed that TIE reduced the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), two key molecules responsible for the production of NO and PGE2 during inflammation progress. Additionally, TIE also suppressed the expression of inflammatory cytokines IL-6, IL-12, and TNF-α. TIE-led suppression in iNOS, COX-2, and cytokines production were probably the consequence of TIE's capability to block ERK and p38MAPK signaling pathway. Moreover, TIE blocked activation of nuclear factor-kappa B (NF-κB) as well as NF-κB regulation of miR155 expression. Our study suggests that TIE may represent as a potential therapeutic agent for the treatment of inflammatory diseases.

1. Introduction

Acute inflammatory response represents an initial protective mechanism in the body. However, excessive and chronic inflammation results in severe damage of cells and tissues. Emerging evidences support the hypothesis that chronic inflammation plays a critical role in various pathological conditions, including atherosclerosis, autoimmune disorders, neurodegenerative diseases, and inflammation related various human cancers [1].

Nitric oxide (NO) is a free radical that is synthesized from L-arginine by nitric oxide synthase (NOS). There are three types of NOS: two constitutive NOS, eNOS and nNOS, and one inducible NOS (iNOS). Constitutive NOS generate nanomolar concentration of NO and are known to mediate various physiological functions. Contrarily, iNOS produces NO at the level of micromolar concentration that often results in pathological consequences such as chronic inflammation [2]. PGE2 are synthesized from arachidonic acid by cyclooxygenase (COX) during the inflammatory reaction. Two COX isoenzymes are known as COX-1 and COX-2. COX-1 is expressed constitutively in most cells and involved in homeostasis, whereas COX-2 is not produced in normal tissues until being induced by chemical and physical stimulations and enhanced by oncogenes, growth factors, and cytokines [3].

LPS triggers a series of signal transduction events which lead to the activation of NF-κB, mitogen-activated protein

kinase (MAPK) signaling pathway, and differential expression of miRNAs that contribute to the inflammatory response [4].

NF-κB is an essential transcription factor that regulates proinflammatory gene expression such as iNOS, COX-2, and interleukin-6 (IL-6) [5]. In mammals, three major MAPKs subfamilies have been described such as ERK, JNK, and p38 MAPK. Secretion of several macrophage factors such as IL-6 and NO requires MAPK activity [6]. miR155 represents a typical multifunctional miRNA and contributed to the progressive inflammatory diseases that expression in macrophages was correlated positively with proinflammatory cytokine expression [7, 8].

There are some cross talks between these classical inflammatory pathways. MAPKs can be activated by Toll-like receptor 4 (TLR4) leading to the activation of nuclear translocation of NF-κB and finally initiate proinflammatory responses [9]. NF-κB is activated by phosphorylation of IκBα via activation of MAPKs and then migrates into the nucleus and activates the expression of inflammatory cytokines and mediators [10]. miR155 induction requires NF-κB signaling to upregulate fos/jun transcription factors during the responses to infection [11].

Garcinia esculenta Y. H. Li (Clusiaceae) is one of *Garcinia* Linn. species, the fruit of which is edible and juicy with sweet and sour taste; meanwhile it is a well-known Chinese traditional medicine with multiple pharmacological functions in treating inflammation and tumor, distributed in the western and northwestern part of Yunnan province in China [12]. However, mechanisms associated with its anti-inflammatory effect are not clear. During the previous course of bioassay-guided screening compounds from twig of *Garcinia esculenta* Y. H. Li, we found 1,3,5,7-tetrahydroxy-8-isoprenylxanthone (TIE) suppressed NO accumulation in stimulated macrophage [13]. In the present paper, we further study its mechanism on LPS/IFNγ-induced inflammatory responses in murine macrophages RAW264.7 cells.

2. Materials and Methods

2.1. Materials and Reagents. TIE (molecular weight 328 Da) was isolated from twig of *Garcinia esculenta* Y. H. Li (Clusiaceae), and the collection, identification, and specimen restoration were described in the previous report [13]. The chemical structure of TIE is shown in Figure 1. The purity of TIE was detected by HPLC and the results suggested a purity of above 98%.

Murine recombinant IFNγ and NF-κB p50/p65 EZ-TFA transcription factor assay kit were purchased from Millipore (Bedford, MA, USA); mouse PGE2 and IL-6 ELISA kit were purchased from Cayman Chemical Company (Ann Arbor, MI, USA); lipopolysaccharide (LPS; *Escherichia coli* O111:B4), dimethyl sulfonamide (DMSO), *N*-(1-naphthyl)-enthylendiaminedihydrochloride, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoleum (MTT), and L-N^6-(1-iminoethyl)lysine hydrochloride (L-NIL, iNOS selective inhibitor) were obtained from Sigma Chemical Co. (St. Louis, MO, USA); RPMI-1640 was purchased from Gibco Invitrogen Corporation (Grand Island, NY, USA); fetal bovine serum

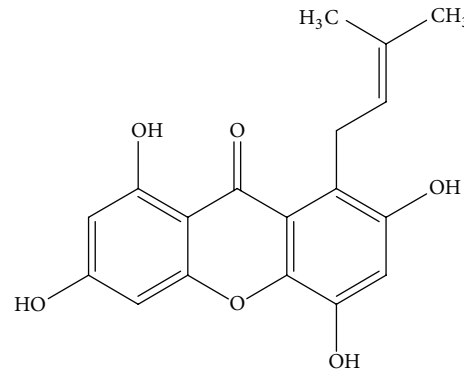

Figure 1: Chemical structure of TIE.

(FBS) was purchased from Hyclone (Logan, UT, USA); TRIzol Reagent, lipofectamine TM 2000, Reverse Transcription Kit, and SYBR Green PCR Master Mix regents were obtained from Invitrogen (Carlsbad, CA, USA). The promoter-luciferase plasmid for NF-κB was kindly provided by Professor Jin-wen Xu; Dual Luciferase Reporter reagents were purchased from Promega (Madison, WI, USA). Antibodies used in this study include the following: iNOS monoclonal antibody from Abcam (Cambridge, MA, USA); p-p38, T-p38, p-JNK, T-JNK, p-ERK1/2, and T-ERK1/2 and β-actin antibodies from Cell Signaling Technology (Beverly, MA, USA); TaqMan MicroRNA Reverse Transcription Kit and TaqMan Universal Master Mix purchased from Applied Biosystems (Waltham, MA, USA); ECL regent kit purchased from GE Healthcare Life Sciences (Buckinghamshire, UK).

2.2. Cell Culture. RAW264.7 cells were originally obtained from the American Tissue Culture Collection. Cells were maintained in RPMI 1640 medium supplemented with 10% FBS at 37°C in a humidified 5% CO_2 atmosphere.

2.3. Assay for Cell Viability. Cell viability was assessed by MTT assay. RAW264.7 cells were cultured in a 96-well plate (5000 cells/well) in an incubator at 37°C, 5% CO_2, and 95% humidity. TIE was dissolved in DMSO and added after dilution with culture media to 3.125, 6.25, 12.5, and 25 μM at final concentration of DMSO never exceeding 0.1%. The cells were incubated with 10 μL MTT (5 mg/mL in phosphate-buffered saline, pH = 7.4) for 4 h at 37°C and discarded the supernatant followed by adding 150 μL DMSO. Absorbance was measured at 490 nm in a microplate reader (Molecular Devices, Sunnyvale, CA). The absorbance of control (untreated) cells was considered as 100% of viability.

2.4. Measurement of NO Production. RAW264.7 cells were plated in a 96-well plate (1×10^5 cells/well) overnight, followed by the addition of 10 U/mL IFNγ and 100 ng/mL LPS for 24 h in the presence or absence of different dosage of TIE with increasing concentration at 3.125, 6.25, 12.5, and 25 μM, and L-NIL (50 μM), a selected iNOS inhibitor. To analyze NO production, 100 μL of supernatant was incubated with equal volume of Griess solution (1% sulfanilamide in 5% phosphoric acid and 1% α-naphthylamine in distilled water)

Table 1: Primer sets for qRT-PCR.

Gene name	Forward primer	Reverse primer
iNOS	GGAGCGAGTTGTGGATTGTC	GTGAGGGCTTGGCTGAGTGAG
COX-2	TGCCTGGTCTGATGATGTATG	AGTAGTCGCACACTCTGTTGT
IL-6	CCACTTCACAAGTCGGAGGCTTA	GTGCATCATCGCTGTTCATACAATC
IL-12p35	ACCTGCTGAAGACCACAGATGACA	TAGCCAGGCAACTCTCGTTCTTGT
IL-12p40	ACCTGTGACACGCCTGAAGAAGAT	TCTTGTGGAGCAGCAGATGTGAGT
TNF-α	ATGGGAAGGGAATGAATCCACC	GTCCACATCCTGTAGGGCGTCT
TBK1	ACTGGTGATCTCTATGCTGTCA	TTCTGGAAGTCCATACGCATTG
β-actin	GCTACAGCTTCACCACCACAG	GGTCTTTACGGATGTCAACGTC

at room temperature for 10 min and absorbance was then read at 540 nm. Since NO content was reflected by the amount of nitrite, a calibration curve was generated using sodium nitrite. The amount of nitrite in the supernatants was calculated based on the calibration curve. The percentage inhibition of NO production is evaluated using the formula {1-[(nitrite amount of fraction-treated)/(nitrite amount of vehicle)]}× 100.

2.5. Detection of PGE2 and IL-6 in Supernatant. Inhibitory effects of TIE on the PGE2 and cytokine IL-6 production from LPS plus IFNγ treated RAW264.7 cells were detected by sandwich ELISA KIT according to the manufacturer's instruction. After incubation with different dosage of TIE and stimulation with LPS plus IFNγ on RAW264.7 cells for 24 h, supernatants were harvested and assayed for PGE2 and IL-6. Results of three independent experiments were used for statistical analysis. L-NIL (50 μM) was used as the positive control.

2.6. RNA Isolation and Quantitative qRT-PCR. Total RNA was extracted using TRIzol Reagent according to manufacturer's instruction. The concentration and integrity of purified RNA were measured by absorption of light at 260 and 280 nm (A260/280). From each sample, 2.0 μg of total RNA was then reverse transcribed to single-stranded cDNA by Invitrogen Reverse Transcription Reagents. Then qPCR analyses were performed on the aliquots of the cDNA preparations with SYBR Green PCR Master Mix to detect quantitatively the gene expression of iNOS, COX-2, IL-6, IL-12p35, IL-12p40, TNF-α, TBK1, and β-actin (an internal standard) using Applied Biosystems 7500HT Fast Real-Time PCR System (Applied Biosystems Inc., Foster City, CA, USA). The $2^{-\Delta\Delta CT}$ method was utilized to analyze the fold increase. The primers used (Sangon, shanghai, China) are listed in Table 1.

2.7. TaqMan MicroRNA Real-Time RT-PCR Assays. The reactions were set according to the manufacturer's protocol. Briefly, total RNA was purified by TRIzol. For each reaction, 10 ng of total RNA was used for reverse transcription using TaqMan MicroRNA Reverse Transcription Kit and reverse transcription primers for mmu-miR155 and the housekeeping gene RNU6B. Real-time PCR quantification was performed using TaqMan PCR primers and TaqMan Universal Master Mix using the following conditions: 16°C for 30 min, 42°C for 30 min, and 85°C for 5 min on Applied Biosystems 7500HT Fast Real-Time PCR System. The samples were measured in triplicate cases. RNU6B endogenous control was used for normalization, and expression levels were presented as $2^{-\Delta\Delta CT}$ with standard deviation.

2.8. Transient Transfection and Dual Luciferase Reporter Assay. For the reporter assay, briefly, cells were seeded into 24-well plates at a density of 5×10^5 cells/well in 500 μL of DMEM without antibiotics and incubated overnight. The cells in each well were transiently transfected with NF-κB luciferase reporter construct and internal control Renilla luciferase vector using lipofectamine TM 2000 reagent according to the manufacturer's procedures. Five hours after transfection, the cells were washed with phosphate-buffered saline and then supplied with fresh medium with FBS and treated with TIE (3.125, 6.25, 12.5, and 25 μM) and L-NIL (50 μM) as the positive control for 1 h before stimulation with LPS and IFNγ for 18 h. Subsequently, luciferase activities were measured in cell lysates placed in opaque 96-well plates using Dual Luciferase Reporter reagents following manufacturer's instruction. Luciferase activity was normalized to transfection efficiency as monitored by Renilla luciferase expression. The level of luciferase activity was determined compared to control cells with no stimulation.

2.9. The DNA-Binding Activity of NF-κB Assay. The DNA-binding activity of NF-κB in nuclear extracts was measured using the NF-κB p50/p65 EZ-TFA transcription factor assay kit according to the manufacturer's instructions. Briefly, the cells were pretreated with different-concentrations of TIE and stimulated for 30 min with LPS plus IFNγ. The nuclear protein extracts of each sample were added to each well after measuring its concentration. The plates were incubated for 1 h at room temperature. After washing each well with wash buffer, 100 μL of diluted NF-κB antibody was added to each well, and then the plates were incubated further for 1 h. After washing wells with wash buffer, 100 μL of diluted HRP-conjugated antibody was added to each well, followed by 1 h incubation. 100 μL of developing solution was added to each well for 5 min, followed by the addition of stop solution. Finally, the absorbance of each sample at 450 nm was determined with a spectrophotometer within 5 min, and the final p50/p65 binding activity of each treatment group was normalization by protein concentration.

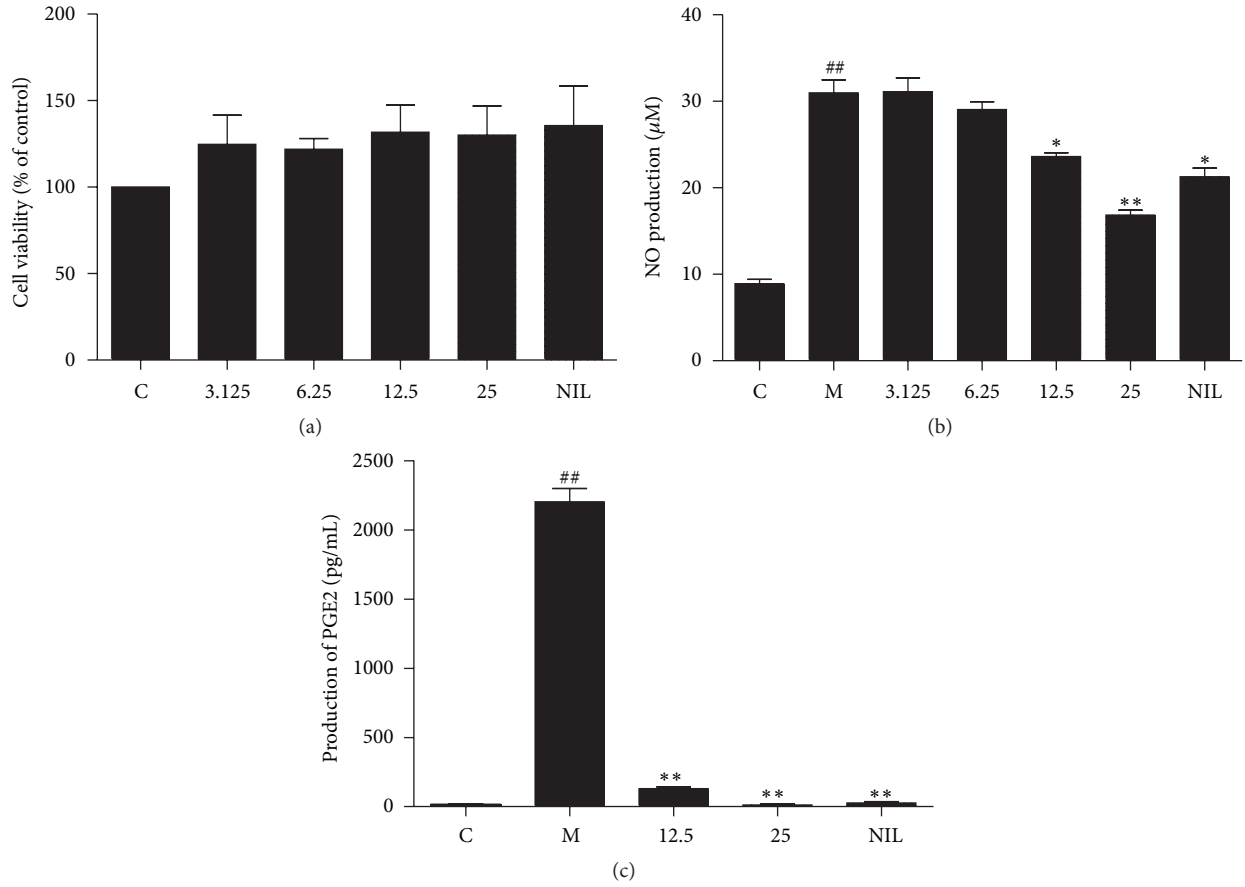

Figure 2: Effect of TIE on NO and PGE2 production in LPS/IFNγ-stimulated RAW264.7 macrophages. (a) RAW264.7 cells were treated with TIE (3.125–25 μM) or 50 μM L-NIL for 24 h. Cell viability was measured by MTT assay. Changes in survival are represented as percentages of the control group. (b) Cells were plated at a density of 1×10^5 cells/well in a 96-well plate and allowed to attach overnight. Cells were treated with the various concentrations of TIE (3.125–25 μM) or 50 μM L-NIL and incubated with LPS (100 ng/mL) and IFNγ (10 U/mL) in fresh FBS-free medium for 24 h. The nitrite production was measured by the Griess reaction. (c) Cells were treated with the indicated concentrations of TIE (12.5, 25 μM) and incubated with LPS (100 ng/mL) and IFNγ (10 U/mL) in fresh FBS-free medium for 24 h. The PGE2 concentration in cell supernatant was determined by ELISA kit. The values were presented as mean ± SEM of three independent experiments. $^{\#\#}P < 0.01$; $^{\#}P < 0.05$ versus control group; $^{**}P < 0.01$; $^{*}P < 0.05$ versus model group.

2.10. Protein Extraction and Western Blotting Analysis. Protein samples (25–50 μg/mL) were mixed with loading buffer, boiled for 5 min, and then separated through 7.5% or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to nylon membranes by electrophoretic transfer. The membranes were blocked in 10% nonfat dry milk (1 h), rinsed, and incubated with primary antibodies in TBST overnight at 4°C. Primary antibody was removed, membranes were washed three times in TBST, and peroxidase-labeled secondary antibody was added for 1 h at room temperature. Following three washes in TBST, bands were visualized by ECL regent kit and exposure to X-ray film.

2.11. Statistical Analysis. The results are presented as means ± standard error of the mean (SEM). Student's test was used to analyze the difference between treated and untreated groups. Statistically significant differences between multiple groups were calculated by the application of an analysis of variance (ANOVA) test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Concentration-Dependent Inhibition of TIE on LPS/IFNγ-Induced NO and PGE2 Production with Nontoxic Effects. To determine the effects of TIE on cell viability, RAW264.7 cells were initially seeded in 96-well plates followed by different treatments. Results of the MTT assay after 24 h treatment indicated that none of the treatments with TIE at the different concentrations from 3.125 to 25 μM was toxic as compared to the untreated cells, as well as positive control L-NIL at 50 μM ($P > 0.05$) (Figure 2(a)).

Since TIE has been shown to exhibit inhibition of NO production in our previous screening, increasing concentration of TIE (3.125, 6.25, 12.5, and 25 μM) was tested on RAW264.7 cells with and without stimulation. The result shows that 24 h inflammatory factors treatment triggered 30-fold increase of nitrite concentration, a biomarker of NO production. Application of TIE dose-dependently attenuated NO production while treatments with TIE without LPS stimulation had a mild inhibitory effect on NO production (data not

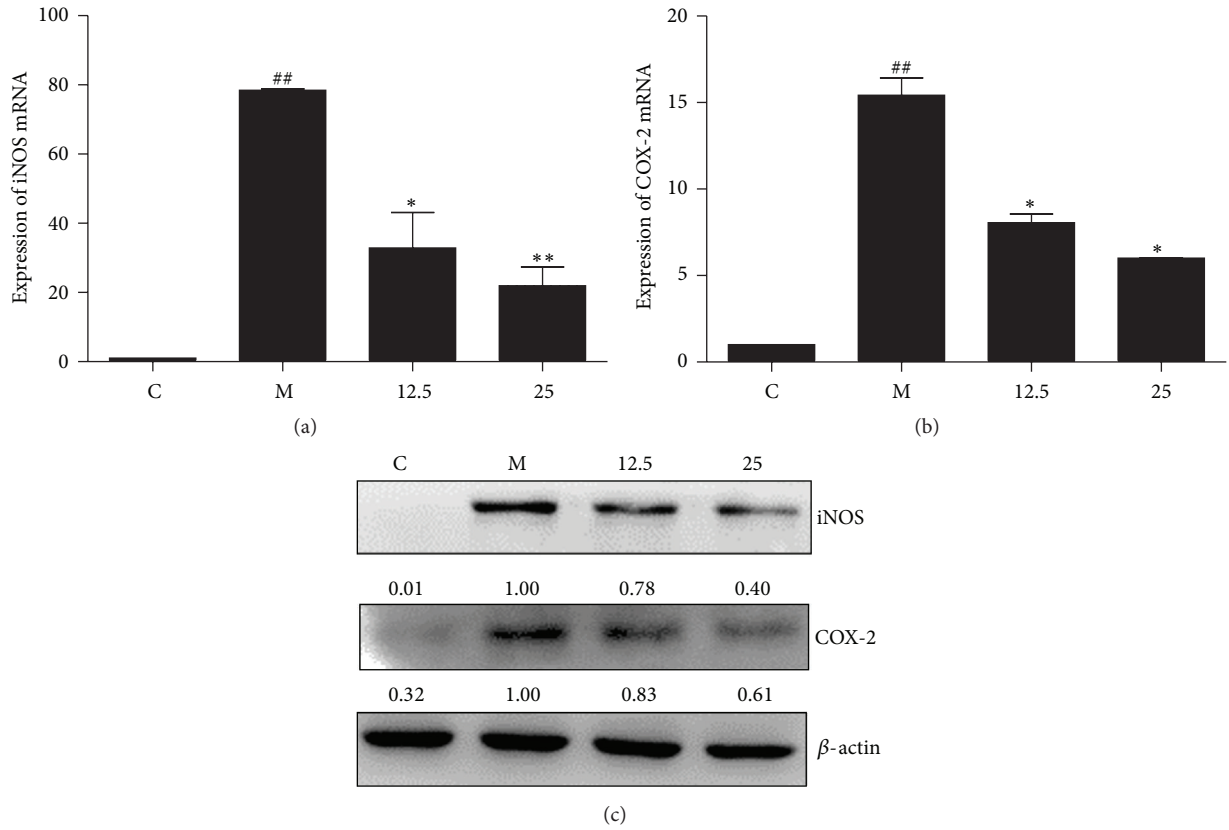

Figure 3: Effect of TIE on iNOS and COX-2 expression in LPS/IFNγ-stimulated RAW264.7 cells. (a, b) RAW264.7 cells were plated at a density of 1×10^6 cells in 30 mm dish overnight and treated with varying concentrations of TIE with treatment of LPS (100 ng/mL) and IFNγ (10 U/mL) for 4 h. Total RNA was isolated and subjected to qRT-PCR. β-actin mRNA was used as an internal control for standardization. (c) RAW264.7 cells were plated at a density of 1×10^6 cells in 30 mm dish overnight. TIE was added with the treatment of IFNγ (10 U/mL) plus LPS (100 ng/mL) for 6 h. Whole cell lysates were prepared and subjected to Western blotting. β-actin protein was used as an internal control for standardization. The data shown are representative of three independent experiments. $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ versus control group; $^{**}P < 0.01$, $^{*}P < 0.05$ versus model group.

shown), with a maximum effect of 62.51% NO reduction with 25 μM TIE (Figure 2(b)). TIE also reduced PGE2 production in a concentration-dependent manner after treatment of 24 h (Figure 2(c)).

These results suggest that TIE selectively inhibits NO and PGE2 production. These results also indicate that the inhibitory effect of TIE on NO and PGE2 production in LPS/IFNγ-stimulated cells is not caused by cellular toxicity.

3.2. TIE Treatment Prevents LPS/IFNγ-Induced iNOS and COX-2 Expression at mRNA and Protein Level. Since TIE can effectively reduce LPS/IFNγ-induced NO production, the fact that iNOS is responsible for LPS/IFNγ-induced NO production indicates that TIE might block NO production by decreasing the amount of iNOS. We hypothesized that TIE might also possess potent inhibition of iNOS expression, since iNOS always regulated by NF-κB at transcription level [14]. We tested this possibility by determining the effect of TIE on iNOS mRNA and protein in LPS/IFNγ-stimulated RAW264.7 cells with the aid of quantitative RT-PCR (qRT-PCR) and Western blot analysis. Results showed that LPS/IFNγ stimulation elevated the mRNA and protein level of iNOS, and TIE pretreatment diminished LPS/IFNγ-induced iNOS gene and protein expression in RAW264.7 cells. TIE was found to decrease the levels of COX-2 mRNA and protein too (Figure 3).

These results suggested that TIE might significantly suppress LPS-induced PGE2 via inhibiting COX-2 expression at the transcriptional level.

3.3. Secretion and Expression of Inflammatory Cytokines Are Suppressed by TIE. The effects of TIE on the secretion and expression of proinflammatory cytokines including IL-6, IL-12, and TNF-α were investigated by ELISA kit and qRT-PCR, respectively.

TNF-α and IL-6 are potent proinflammatory cytokines induced during inflammation progress, accompanied with interleukin-12 (IL-12) playing the essential role in immune defense against infection [15, 16].

Under stimulation of LPS plus IFNγ for 4 h, the mRNA levels from proinflammatory genes IL-6, IL-12p35, and p40 were highly induced and TNF-α enhanced its expression after

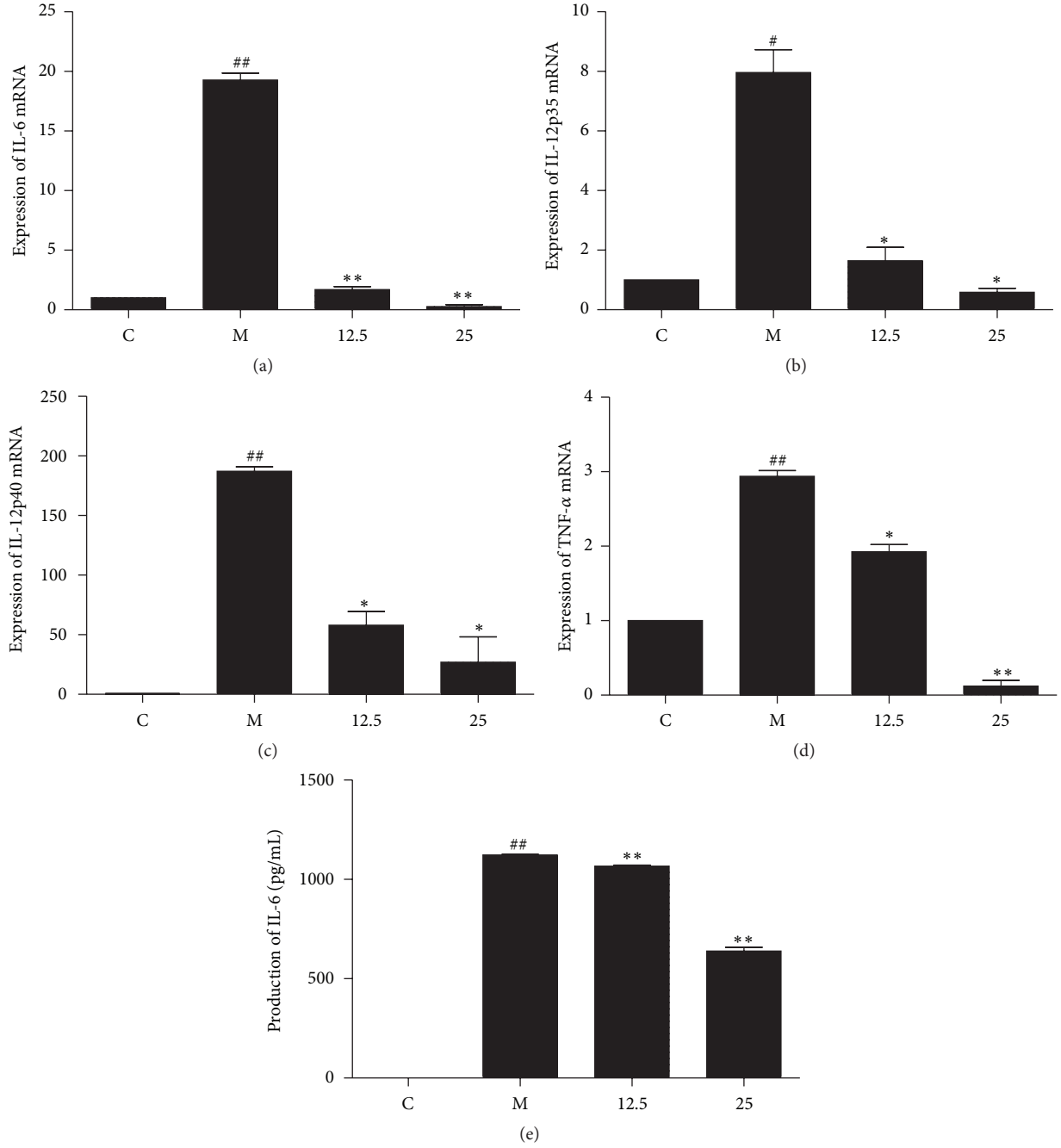

Figure 4: Effect of TIE on proinflammatory cytokines in LPS/IFNγ-stimulated RAW264.7 cells. (a, b, c) RAW264.7 cells (1×10^6 cells/dish) were treated with varying doses of TIE with IFNγ (10 U/mL) plus LPS (100 ng/mL) for 4 h. Total RNA was isolated and subjected to qRT-PCR to determine the level of IL-6 and IL-12p35/p40 mRNA. (d, e) RAW264.7 cells were treated with IFNγ (10 U/mL) plus LPS (100 ng/mL) in the presence of varying concentrations of TIE for 24 h. Total RNA was isolated and subjected to qRT-PCR to determine the level of TNF-α mRNA. Conditioned media were collected and subjected to ELISA to determine the amount of IL-6. The values (means ± SEM) were obtained from three independent experiments. $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ versus control group; $^{**}P < 0.01$, $^{*}P < 0.05$ versus model group.

24 H stimulation. Treatment of cells with TIE significantly decreased the expression of IL-6, IL-12p35/p40, and TNF-α (Figures 4(a), 4(b), 4(c), and 4(d)).

LPS/IFNγ stimulation increased not only IL-6 expression, but also the secretion of IL-6. Coincubation of TIE and proinflammation stimulation for 24 h showed the strong suppression of this proinflammatory cytokine in cell supernatant (Figure 4(e)).

These data showed that TIE maybe interfere in the transcription level and protein secretion progress of IL-6.

Inflammatory cytokines IL-12p35/p40 and TNF-α also can be reduced by TIE.

3.4. TIE Inhibits Induced NF-κB Luciferase Activity via Suppression Nuclear Translocation of the p65 and p50 Subunits. NF-κB plays a pivotal role in regulation of the expression of iNOS, COX-2, and inflammatory cytokines such as IL-6 and IL-12 [5]. To investigate the underlying mechanism of the inhibition of TIE on iNOS and COX-2 expression in stimulated cells, luciferase reporter assay was used to explore the effects of TIE on NF-κB-dependent reporter gene expression following proinflammatory treatment. RAW264.7 cells were transiently cotransfected with a pNF-κB reporter vector.

Cells were incubated with TIE (3.125, 6.25, 12.5, and 25 μM) and LPS plus IFNγ for 18 h.

The results indicated that LPS/IFNγ treatment for 18 h induced NF-κB reporter activity almost 9-fold, and TIE reduced the expression of NF-κB luciferase in a concentration-dependent manner (Figure 5(a)), with a maximum effect of a 63.50% reduction when the cells were treated with 25 μM TIE, which was similar to the results of the positive control L-NIL at the concentration of 50 μM.

Because p65 and p50 are the major subunits of the NF-κB heterodimer, the translocation of p65 and p50 subunits from the cytoplasm to the nucleus after being released from IκBα was well investigated. As shown in Figures 5(b) and 5(c), the concentrations of p65 and p50 subunits were increased in the nucleus after LPS/IFNγ treatment; pretreatment with TIE reversed these trends.

TBK1 belongs to IκB kinase (IKK) family that can active IRF3/IRF7 and NF-κB pathway to regulate inflammatory responses in macrophages [17, 18]. So we also detected the expression of TBK1; the result showed TBK1 was influenced by TIE (Figure 5(d)).

Taken together, these findings confirmed that TIE suppressed the expression of iNOS, COX-2, and cytokines at least in part via NF-κB-dependent mechanism.

3.5. MAPK Signaling Pathways Are the Target of TIE-Mediated Inhibition. LPS induction of cytokine expression occurs via activation of MAPK and key protein phosphorylation following binding to TLR4 [4]. MAPKs play an important role in the transcriptional regulation of LPS-induced expression of iNOS and COX-2 via activation of the transcription factor NF-κB [8]. To determine whether the anti-inflammatory effects of TIE are mediated through the MAPK inactivation, Western blots were performed to analyze the levels of p-ERK, JNK, and p38 in RAW246.7 cells. LPS/IFNγ stimulation (30 min) evocated significant increases in the levels of phosphorylated ERK, JNK, and p38 in RAW246.7 cells. Coincubation of TIE markedly inhibited the extent of ERK and p38 phosphorylation; meanwhile the amounts of nonphosphorylated p38 and ERK1/2 were apparently unaffected by LPS or LPS plus TIE treatment (Figure 5(e) and supplementary data in the Supplementary Material available online at http://dx.doi.org/10.1155/2015/350564). These results suggest that TIE blocks inflammatory responses by the combination of blocking NF-κB, ERK, and p38 activation and slightly enhanced phosphorylation of JNK (p-JNK) ($P > 0.05$). These results suggested that the anti-inflammatory activity of TIE was mediated by inhibition of the LPS-induced phosphorylation of p38 and ERK1/2. We found that TIE attenuated the activation of p38, rather than that of ERK by stimulation.

These results indicated that the inhibitory effects of TIE on NO and PGE2 were mediated partly via the downstream MAPKs pathway.

3.6. miR155 Repression Was Involved in TIE Anti-Inflammatory Effects. MAPKs and NF-κB, two downstream pathways of TLR4 signalling, were shown to positively regulate the expression of miR155 [14]. As miR155 play important roles in the innate immune response and inflammation, we evaluated the effect of TIE on the expression of miR155 by TaqMan MicroRNA assay. As shown in Figure 5(f), miR155 also dramatically increased after 24 h stimulation, and TIE reduced LPS plus IFNγ-induced miR155 expression by 85.87%–90.77% from the dosage from 12.5 to 25 μM.

This result showed that miR155 repression was involved in TIE anti-inflammatory effects of NO, PGE2, and IL-6 production, as a complementary regulation at an epigenetics level.

4. Discussion

Natural production has been used as medicine for treating a wide variety of disorders including acute and chronic inflammation. *Garcinia esculenta* Y. H. Li (Clusiaceae) is a well-known herb in treating inflammation and tumor for hundreds of years according to Chinese traditional medicine though material basis and its mechanism of anti-inflammation are still unclear.

With the help of the activity guiding extraction and separation, we caught some compounds isolated from *Garcinia esculenta* Y. H. Li (Clusiaceae) [13]. Among these compounds, TIE had the strongest capability of inhibition of NO production on stimulated macrophage RAW264.7 during the screening procedure, which indicated the potential of its anti-inflammatory effect *in vitro*.

However the mechanisms of anti-inflammatory effects of TIE have not been delineated yet. Thus, our study aimed to elucidate the mechanisms underlying the anti-inflammatory effects of TIE.

It is well known that the overproduction of NO and PGE2 by iNOS and COX-2 plays a critical role in the regulation of the inflammatory process. Therefore, to study the suppression of NO and PGE2 by iNOS and COX-2 is very important in the development of anti-inflammatory agents [19, 20]. Selective iNOS inhibitor showed the anti-inflammatory and antitumor properties in animal models [21, 22]. Here, we demonstrated that TIE can dose-dependently inhibit LPS plus INF-induced NO production in RAW264.7 macrophages, as well as suppression of PGE2 (Figure 2). Consistent with these findings, TIE also suppressed induced expression of iNOS and COX-2 at the mRNA and protein levels in RAW264.7 macrophages (Figure 3), which suggested that TIE-induced reduction of NO and PGE2 may be due to transcriptional suppression of iNOS and COX-2 genes.

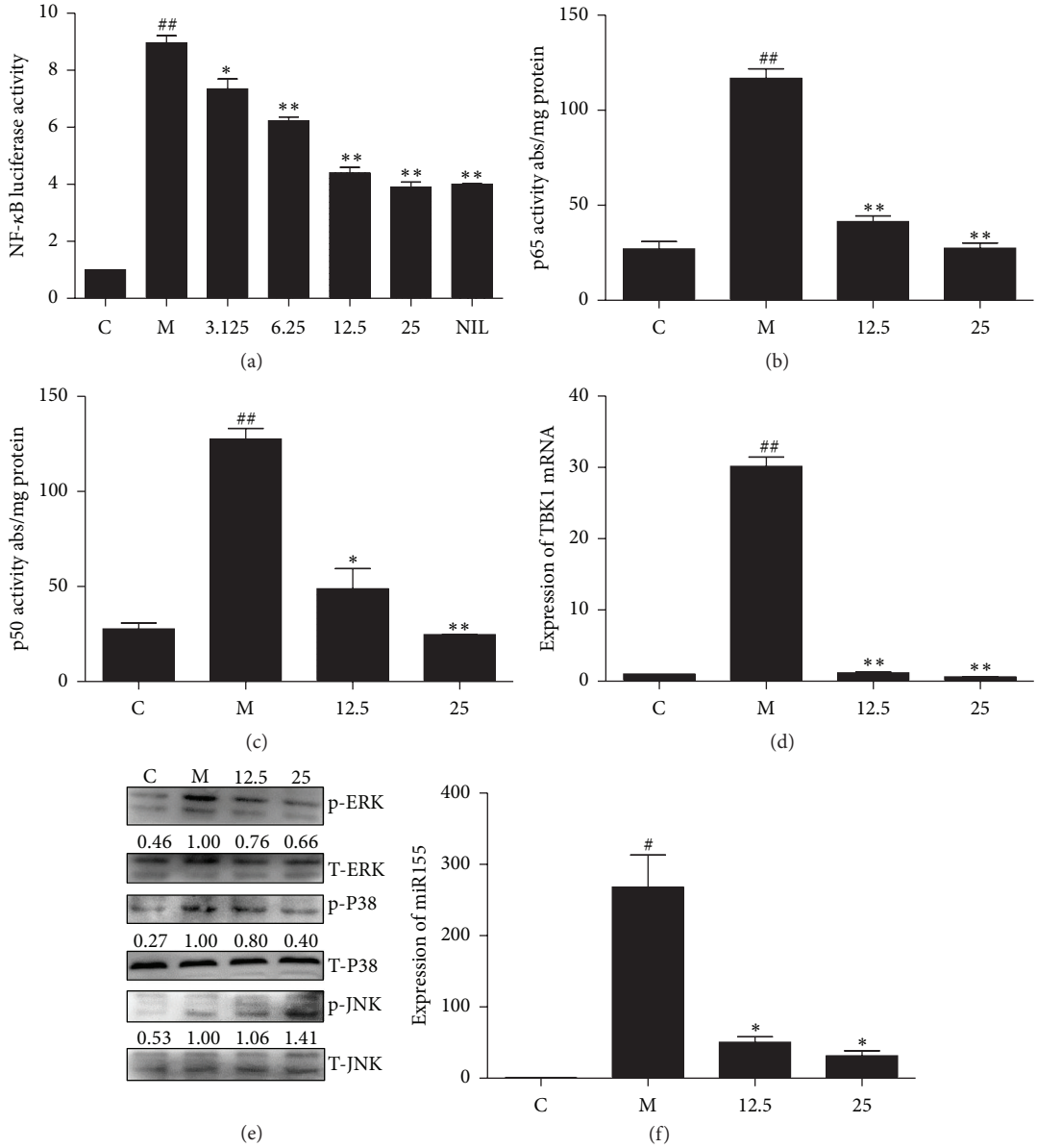

Figure 5: Effect of TIE on NF-κB, MAPK activation, and miR155 expression in LPS/IFNγ-stimulated RAW264.7 cells. (a) RAW264.7 cells were transiently cotransfected with a pNF-κB reporter vector. Cells were incubated with TIE (3.125, 6.25, 12.5, and 25 μM) and L-NIL (50 μM) for 1 h before stimulation with LPS and IFNγ for 18 h. Luciferase activities were measured by Dual Luciferase Reporter reagents following manufacturer's instruction. Luciferase activity was normalized to transfection efficiency as monitored by Renilla luciferase expression. (b, c) DNA-binding activity of p65 and p50 proteins in nuclear extracts was assessed using NF-κBp50/p65 EZ-TFA transcription factor assay. Absorbance was measured at 450 nm in a microplate spectrophotometer. Results were normalized to absorbance/mg protein. (d) RAW264.7 cells (1×10^6 cells/dish) were treated with varying doses of TIE with IFNγ (10 U/mL) plus LPS (100 ng/mL) for 4 h. Total RNA was isolated and subjected to qRT-PCR to determine the level of TBK1 mRNA. (e) RAW264.7 cells were plated at a density of 1×10^6 cells/well in 30 mm dish overnight. TIE was added to cells followed by 30 min stimulation of IFNγ (10 U/mL) plus LPS (100 ng/mL). Whole cell lysates were prepared and subjected to Western blotting. The ratios of immunointensity of p-ERK1/2, p-JNK, and p-p38 were calculated, respectively. Total ERK1/2, JNK, and p38 (T-ERK1/2, T-JNK, and T-p38) were used as a control of the protein amount in the same samples. Data shown are the representative of three independent experiments. (f) The cells were stimulated by IFNγ (10 U/mL) plus LPS (100 ng/mL) with or without 12.5 and 25 μM concentrations of TIE for 24 h. Total RNA was isolated and the expression of miR155 was determined by qRT-PCR. RNU6B was used here as an endogenous control. The data represent the mean ± SD of triplicate experiments. $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ versus control group; $^{**}P < 0.01$, $^{*}P < 0.05$ versus model group.

Induction of iNOS is often accompanied with upregulation proinflammatory cytokines in macrophages [23]. IL-6, IL-12, and TNF-α play a critical role in innate immune responses and it is the principal mediator in response to LPS stimulated tissue injury and shock [24]. Therefore, we also investigated the effect of TIE on LPS plus IFNγ-inducible proinflammatory cytokines expression and secretion. TIE can also diminish the expression and secretion of IL-6 as well as gene expression of IL-12 and TNF-α (Figure 4).

The expression of a number of immunity and inflammatory related genes such as iNOS, COX-2, and IL-6 was modulated by activated NF-κB [25]. Activation of NF-κB involves the phosphorylation and subsequent proteolytic degradation of the inhibitory protein IκB by specific IκB kinases. The free NF-κB (a heterodimer of p50 and p65) then passes into the nucleus, where it binds to NF-κB site in the promoter regions of genes for inflammatory proteins such as cytokines, enzymes, and adhesion molecules [26]. Under inflammatory conditions, inhibitory protein IκB is promptly phosphorylated and degraded from p50 and p65 subunits binding site of NF-κB, and the activated NF-κB subunits migrate to the nucleus. To investigate the possible preventive capability of TIE on NF-κB activation, we studied NF-κB activity, p50/p65 nuclear translocation, and TBK1 expression.

LPS/IFNγ stimulation caused the increase of TBK1 expression, activation of NF-κB and induced p50/p65 movement to nucleus, and TIE repressed luciferase intensity of NF-κB promoter in a dose dependent manner (Figure 5(a)). The present study also showed that TIE inhibited LPS/IFNγ-induced NF-κB activation through the suppression of TBK1 expression (Figure 5(d)) and on the nuclear translocation of the P50/P65 subunit of NF-κB in RAW264.7 macrophages (Figures 5(b) and 5(c)). So TIE displayed the interference in progress of NF-κB active heterology dimmer heading to the nucleus and the binding capability.

The MAPKs pathway is one of the most ancient and evolutionarily conserved signaling pathways and plays essential regulatory roles in both innate and adaptive immune response [23]. LPS/IFNγ stimulation of cytokines production in human monocytes is involved in several intracellular signaling pathways that include three MAPK pathways: ERK1 and ERK2, JNK and p38, and IKK-NF-κB pathway [27]. Thus, we investigated the effect of TIE on activation of ERK1/2, JNK, and p38 in LPS/IFNγ-stimulated cells. Our results showed that the phosphorylation of p38 and ERK in response to LPS/IFNγ was decreased with TIE treatment, whereas JNK phosphorylation was not affected (Figure 5(e)).

MicroRNAs are short noncoding RNAs that are involved in the epigenetic regulation of cellular processes. The macrophage inflammatory response to LPS stimulation involves the upregulation of miRNAs such as miR155 and miR146 and the downregulation of miR125b. Recently, miR155 has been characterized as a component of the primary macrophage response to different types of inflammatory mediators [28]. Given the powerful role of miR155 in inflammation, it may be an ideal candidate target for anti-inflammatory drug development.

Although results above had been made to identify important and classic genes and pathways involved in the anti-inflammatory effect of TIE, knowledge of noncoding genes such as miRNAs and their contributions is less understood. Our results showed that LPS/IFNγ stimulation induced miR155 expression substantially, and TIE reduced LPS plus IFNγ-induced miR155 expression (Figure 5(f)). This study highlights a novel mechanism for TIE effects on the inflammatory response via downregulation of miR155 expression.

miR155 is known to be induced downstream of TLR4 signaling. Inhibition of the NF-κB pathway significantly reduced miR155 expression in LPS treated RAW264.7 cells; meanwhile induction of NF-κB activity rapidly leads to increased levels of mature miR155 transcripts. Current study demonstrated that MiR155HG is a direct NF-κB target gene *in vivo* since NF-κB-responsive site exists in the MiR155HG proximal promoter and miR155 is the diced product of the MiR155HG gene [29, 30]. These evidences reflect the importance of the NF-κB pathway in the induction of miR155.

In addition, TIE reduced the nuclear translocation of p65/p50 by inflammatory stimulation, whereas inhibition of NF-κB activation elicited a downregulation of miR155 in LPS treated macrophages, which indicated the important role of the NF-κB pathway in TIE-regulated miR155 expression.

In conclusion, this is the first investigation of the anti-inflammatory activity of TIE and its functional mechanism in activated macrophages RAW264.7 cells. TIE was found to inhibit the production of NO and PGE2 as well as their upstream enzymes iNOS and COX-2 at protein level as well as secretion and expression of cytokines through LPS/IFNγ induced NF-κB/MAPK/miR155 signaling pathways (Figure 6), indicating that TIE has a potential anti-inflammatory application.

Abbreviations

TIE: 1,3,5,7-Tetrahydroxy-8-isoprenylxanthone
LPS: Lipopolysaccharide
IFNγ: Interferon-γ
NO: Nitric oxide
PGE2: Prostaglandin E2
iNOS: Inducible nitric oxide synthase
COX-2: Cyclooxygenase-2
IL-6: Interleukin-6
IL-12: Interleukin-12
TNF-α: Tumor necrosis factor-alpha
TBK1: TANK {TRAF (TNF (tumor necrosis factor) receptor-associated factor) associated NF-κB activator} binding kinase 1
NF-κB: Nuclear factor-kappa B
ERK: Extracellular signal-regulated kinase
JNK: c-Jun N-terminal kinase
MAPK: Mitogen-activated protein kinase
MiR155: MicroRNA 155
ELISA: Enzyme linked immunosorbent assay
TLR4: Toll-like receptor 4.

Conflict of Interests

The authors declare no competing financial interest.

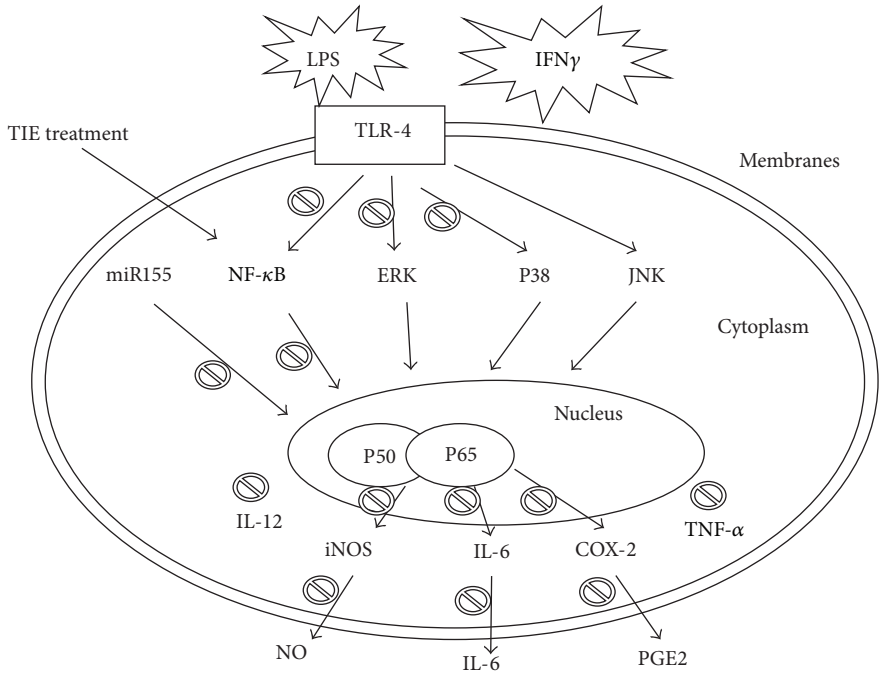

Figure 6: Proposed mechanisms of TIE inhibition of LPS plus IFNγ induced inflammation in RAW264.7 cells. TIE inactivates MAPK and NF-κB signaling pathways in addition to inhibiting expression of miR155, which may result from TIE downregulation of iNOS, COX-2, and IL-6 expression and their production. Arrows indicate the main inflammatory pathway activated by LPS stimulation. The prohibition signs indicate the inhibitory effects of TIE.

Acknowledgments

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Research Article

Downregulation of mPGES-1 Expression *via* EGR1 Plays an Important Role in Inhibition of Caffeine on PGE_2 Synthesis of HBx(+) Hepatocytes

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We investigated the mechanism of caffeine in influencing HBx(+) hepatocytes to synthesize PGE_2. The inhibitory effect of caffeine on hepatocyte proliferation increased with increasing caffeine concentrations (200–800 μM) and treatment times (1–7 days), which was first observed at the second test time point (caffeine treatment for 4 days). The inhibition of caffeine on the growth of HL7702-HBx and HepG2-HBx cells was most obvious at 800 μM caffeine and at caffeine treatment for 7 days. The PGE_2 secretion and the expression of mPGES-1 and EGR1 were downregulated, whereas PPARγ expression was upregulated. The mPGES-1 promoter activity of HBx(+) hepatocytes decreased more significantly than that of HBx(−) hepatocytes. Moreover, the expression of EGR1 and PPARγ changed more significantly in HBx(+) hepatocytes cultured for 12 to 24 hours in the presence of 5 mM caffeine. This limited success may be attributed to caffeine releasing the binding of HBx and PPARγ and furthermore affecting the mPGES-1 expression by EGR1 in HBx(+) hepatocytes. The results indicate that caffeine could effectively reduce PGE_2 synthesis in HBx(+) hepatocytes by specifically blocking the PPARγ-EGR1-mPGES-1 pathway, thereby providing a new evidence of molecular biology for the hypothesis that drinking coffee is beneficial to HBV-infected patients.

1. Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of death worldwide. An estimated 748300 new liver cancer cases and 695900 cancer deaths have been recorded worldwide in 2008 [1]. In China, most HCC patients are infected by hepatitis B virus (HBV), which undergoes a process from hepatitis to liver cirrhosis to HCC. This process is one of the significant differences between HCC and other malignant tumors. HBV, a noncytopathic specific double-stranded DNA virus, could cause acute and chronic hepatitis [2]. Hepatitis B virus x protein (HBx) has various biological functions that could be simultaneously expressed in the nucleus and cytoplasm of hepatocytes. HBx could promote the occurrence and progress of liver cirrhosis and HCC [3].

Prostaglandin E2 (PGE_2) is one of the important products with the most biological activity synthesized by cyclooxygenase. PGE_2 is significantly increased in malignant tumor tissues and plays a critical role in HBV virus infection, as well as in the occurrence and progress of HCC [4, 5]. The synthesis of PGE_2 is higher in hepatocytes with positive HBx [6]. PGE_2 could increase the expression of oncogene in the mRNA and protein levels. The EP_4/GS/AC/CREB/NF-κB molecular signaling pathways promote the growth and invasion of cancer cell [7]. Reduction of PGE_2 could effectively inhibit the invasion of HCC [4].

Relevant epidemiologic studies have shown that the incidence of HCC for the people who drink coffee every day is 30%–80% lower than those who do not. Caffeine has the same protective effect for chronic hepatitis B virus carriers and high-risk populations developing liver cancer [8]. A survey on 63,000 Chinese Singaporeans between the ages of 45 and 74 years conducted by the National University of Singapore shows that consumption of caffeine is negatively related to the incidence of HCC. The incidence of HCC for people who drink three cups of coffee or above every day is significantly

reduced by 44% [9]. A comparison between the 109 HBV carriers and 125 subjects in the control group collected by the Prince of Wales Hospital of Hong Kong shows that drinking coffee in moderation could reduce nearly half of the risk of HCC [8]. Some studies have shown that caffeine could effectively inhibit PGE_2; however, the specific mechanism remains unclear [10]. Moreover, whether caffeine has a particular role in the regulation of PGE_2 synthesis in hepatocytes with HBx expression for the study on HCC associated with HBV infection is the focus of our further investigation. Exploring the effect of caffeine on PGE_2 synthesis pathways may provide a theoretical basis for the study on preventive strategies using caffeine in HBV-infected patients.

2. Materials and Methods

2.1. Material. The recombinant plasmid pcDNA3.0-HBx was constructed by our laboratory [11]. HL7702 cells (a human hepatic cell line, Institute of Biochemistry and Cell Biology, Shanghai, China), previously established HL7702-HBx cells (stable HBx expression by transfection with pcDNA3.0-HBx) [12], HepG2 cells (a human hepatocarcinomal cell line, HB-8065, ATCC, VA), and HepG2-HBx cells (stable HBx expression by transfection with pcDNA3.0-HBx) were grown in DMEM containing 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO_2. The microsomal prostaglandin E synthase-1 (mPGES-1) promoter luciferase reporter plasmid, pGL3B-628 (−628 to +1), and cyclooxygenase-2 (COX-2) promoter luciferase reporter plasmid, pGL3B-COX-2, were constructed by our laboratory [13]. Caffeine (Sigma, St. Louis, MO) was dissolved in culture medium (4×10^3 mM) and a certain volume was drawn to be added to the cell well for reaching the required final concentration.

2.2. Proliferation Assay. Briefly, 5×10^3 of HL7702, HL7702-HBx, HepG2, and HepG2-HBx cells were, respectively, seeded in different wells of 96-well plates and incubated overnight. Caffeine solution was, respectively, added to the different wells with a final concentration (200, 400, or 800 μM); the same amount of medium without caffeine was added to the control wells. 24 hours later, the wells of the first test point (caffeine treatment for 1 day) were washed two times by PBS and incubated with 20 μL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/mL, Sigma, St. Louis, MO) for 4 h. Formazan crystals were subsequently dissolved in 150 μL dimethyl sulfoxide (DMSO). The absorbance of the solution was measured at 490 nm and detected using the Bio-Tek lQuant Universal Microplate Spectrophotometer (Bio-Tek Instruments, Inc., Winooski, VT). For the second (caffeine treatment for 4 days) and third (caffeine treatment for 7 days) test points, the cell wells were, respectively, replaced with new culture medium (containing different concentrations of caffeine) at the third day and the sixth day after cultivation. Each treatment was repeated in triplicate.

2.3. PGE_2 Analysis. Twenty-four hours after 5×10^3 cells/well were plated onto 96-well plates, the caffeine solution was, respectively, added with a final concentration (800 μM); the same amount of medium without caffeine was added to the control wells. One, 4, and 7 days later, the supernatant was, respectively, collected and centrifuged briefly. The amount of PGE_2 in the supernatant was determined using EIA Kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's protocol. Parallel cells were harvested and counted. All assays were performed three times.

2.4. Dual-Luciferase Reporter Assay. 2×10^5 of HL7702, HL7702-HBx, HepG2, and HepG2-HBx cells were, respectively, seeded in different wells of 24-well plates and incubated overnight. The supernatant was removed and replaced with serum-free medium, and DNA transfection solution containing promoter luciferase reporter plasmid and Lipofectamine 2000 (Life Technologies, Carlsbad, CA) was added to the cells, in accordance with the manufacturer's recommendations. Eight hours later, the caffeine solution was, respectively, added with a final concentration (800 μM) and it continued to be cultured for 48 h. The supernatant was removed and the cell lysates were detected for intracellular luciferase activity using Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI) on a luminometer (Orion II Microplate Luminometer, Berthold Detection Systems, Germany) following the manufacturer's recommendations. The relative luciferase units (RLU) were obtained by comparison with control, which was set to 1. Each transfection was performed in triplicate and the data were expressed as the mean ± SD of three separate experiments.

2.5. Western Blotting. 1×10^5 of HL7702, HL7702-HBx, HepG2, and HepG2-HBx cells were, respectively, seeded in different wells of 6-well plates and incubated overnight. Caffeine solution was added with different final concentrations and then the plates were incubated for different time according to the experimental requirement. Cell protein was extracted by conventional method. Each treatment was repeated in triplicate. A total of 40 μg of protein was subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretic transfer to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA). Protein blots were incubated separately with a panel of specific antibodies from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), which included anti-mPGES-1 (1 : 1000, sc-12269), anti-COX-2 (1 : 1000, sc-19999), anti-early growth response 1 (EGR1) (1 : 1000, sc-110), anti-peroxisome proliferator-activated receptor gamma (PPARγ) (1 : 1000, sc-7273), and anti-β-actin (1 : 4000, sc-47778) overnight at 4°C and then incubated with different horseradish peroxidase-(HRP-) conjugated secondary antibody at room temperature for 1 h. Visualization of the immunoreactive proteins was performed by chemiluminescence kit (BeyoECL Plus, Beyotime, Shanghai, China). Intensities of band signals were quantified using the densitometric software Quantity One (Bio-Rad, Hercules, CA) and the relative intensity to internal control (β-actin) was calculated. All measurements were repeated in triplicate.

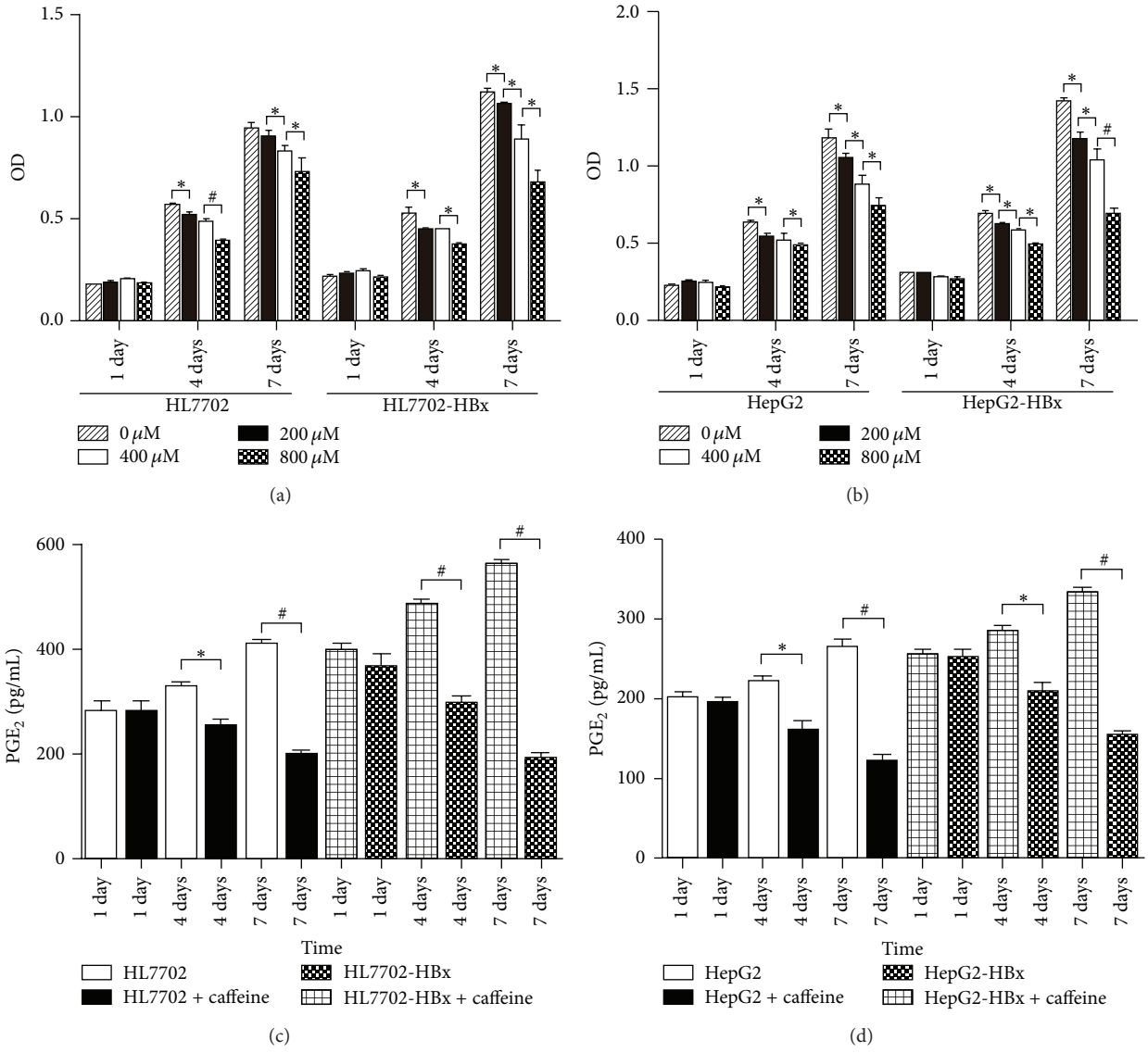

Figure 1: Effect of caffeine on hepatocyte proliferation and synthesis of PGE_2. ((a) and (b)) Cell proliferation at different caffeine concentrations (0, 200, 400, and 800 μM) and different caffeine treatment days (1, 4, and 7 days). The group data represent the mean ± SD ($n = 3$); ∗ denotes a statistically significant difference ($P < 0.05$); # denotes a statistically significant difference ($P < 0.001$). ((c) and (d)) PGE_2 level in the supernatant of different cells in caffeine treatment (800 μM) for 7 days. The group data represent the mean ± SD ($n = 3$); ∗ denotes a statistically significant difference ($P < 0.05$); # denotes a statistically significant difference ($P < 0.001$).

2.6. Statistical Analysis. The data were repeated at least three independent times and expressed with mean ± SD unless otherwise indicated. Assays for characterizing phenotype of cells and expression difference were analyzed by one-way analysis of variance (ANOVA) using SPSS 13.0 software package (SPSS, Inc., Chicago, IL). $P < 0.05$ denoted a statistically significant difference.

3. Results

3.1. Effect of Caffeine on Hepatocyte Proliferation and Secretion of PGE_2. Caffeine with different concentrations was used to treat four strains of hepatocyte (HL7702, HL7702-HBx, HepG2, and HepG2-HBx). Figures 1(a) and 1(b) show that the inhibitory effect of caffeine on hepatocyte proliferation increased with increasing caffeine concentrations (200–800 μM) and treatment times (1–7 days), indicating that caffeine inhibits cell proliferation in a dose- and time-dependent manner. The inhibition activity of caffeine on the four strains of hepatocyte was first to be observed at the second test time point (caffeine treatment for 4 days) ($P < 0.05$). Meanwhile, the inhibition of caffeine on HL7702-HBx and HepG2-HBx cells was most obvious at the concentration of 800 μM and at the third test time point (caffeine treatment for 7 days), which was significantly higher than that on HL7702 and HepG2 cells ($P < 0.05$), respectively. Therefore, we chose 800 μM as

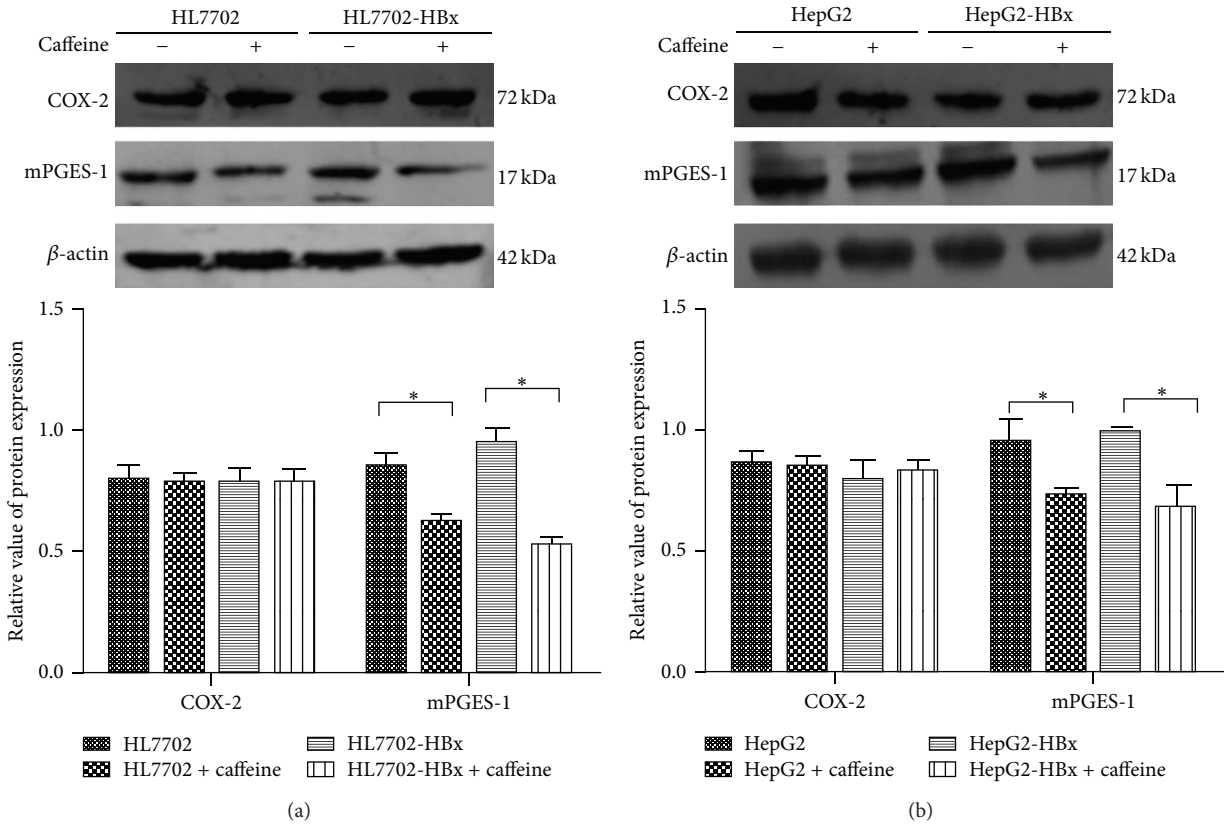

Figure 2: Effect of caffeine on the expression of COX-2 and mPGES-1 in HBx positive hepatocyte. (a) Representative immunoblots for COX-2, mPGES-1, and β-actin in HL7702 and HL7702-HBx cells. (b) Representative immunoblots for COX-2, mPGES-1, and β-actin in HepG2 and HepG2-HBx cells. The group data represents the mean ± SD ($n = 3$). The densitometry data were normalized to β-actin. Note: $^{*}P < 0.05$, statistically significant difference compared to HL7702 cells (a) or HepG2 cells (b).

the optimum concentration of caffeine for subsequent experiments.

Four strains of cells were cultured in a medium containing caffeine (800 μM) for 7 days. The cell supernatant was collected to test the secretion of PGE_2. Figures 1(c) and 1(d) show that the amounts of PGE_2 secreted from the four strains of cells were gradually reduced after culture with caffeine (800 μM) for 1, 4, and 7 days ($P < 0.05$). Compared with the first test time point (caffeine treatment for 1 day), the amounts of PGE_2 secreted from the four strains of cells cultured at the third test time point (caffeine treatment for 7 days) were reduced by 29.4% (HL7702), 47.5% (HL7702-HBx), 38.6% (HepG2), and 43.0% (HepG2-HBx), respectively. This result indicates that the inhibition of caffeine on PGE_2 secreted from HL7702-HBx and HepG2-HBx cells was higher than that secreted from the HL7702 and HepG2 cells.

3.2. Effect of Caffeine on the Expression of COX-2 and mPGES-1 in HBx Positive Hepatocyte. The synthesis of PGE_2 covers the effects of a series of enzymes among which COX-2 and mPGES-1 are the rate-limiting enzymes that play key roles [14]. In the presence of 800 μM of caffeine for 7 days, the cell proteins of HL7702, HL7702-HBx, HepG2, and HepG2-HBx cells were, respectively, extracted for detecting the expressions of COX-2 and mPGES-1. Figure 2 shows that caffeine had no insignificant effect on COX-2 expression of the four strains ($P > 0.05$). The mPGES-1 protein expression with the addition of caffeine was significantly lower than that without caffeine ($P < 0.05$). The mPGES-1 protein expression of HL7702-HBx and HepG2-HBx cells with the addition of caffeine was significantly lower than that of HL7702 and HepG2 cells ($P < 0.05$).

3.3. Effect of Caffeine on the Transcriptional Activity of COX-2 and mPGES-1 Gene Promoter in HBx Positive Hepatocytes. We further investigated whether caffeine caused the change in protein through transcriptional link. Approximately 800 μM of caffeine was used to treat HL7702, HL7702-HBx, HepG2, and HepG2-HBx cells to detect the fluorescent activity of the gene after the mPGES-1 and COX-2 promoter had been transiently transfected for 48 hours. Figure 3 shows that addition of caffeine had no effect on the transcriptional activity of the COX-2 promoter of four strains ($P > 0.05$), but the transcriptional activity of the mPGES-1 promoter was downregulated more significantly with the addition of caffeine than that without caffeine ($P < 0.05$). After the addition of caffeine, the activity of the mPGES-1 promoter in the HL7702-HBx and HepG2-HBx cells decreased more significantly than that in HL7702 and HepG2 cells ($P < 0.05$). This result indicates that caffeine could inhibit the activity of

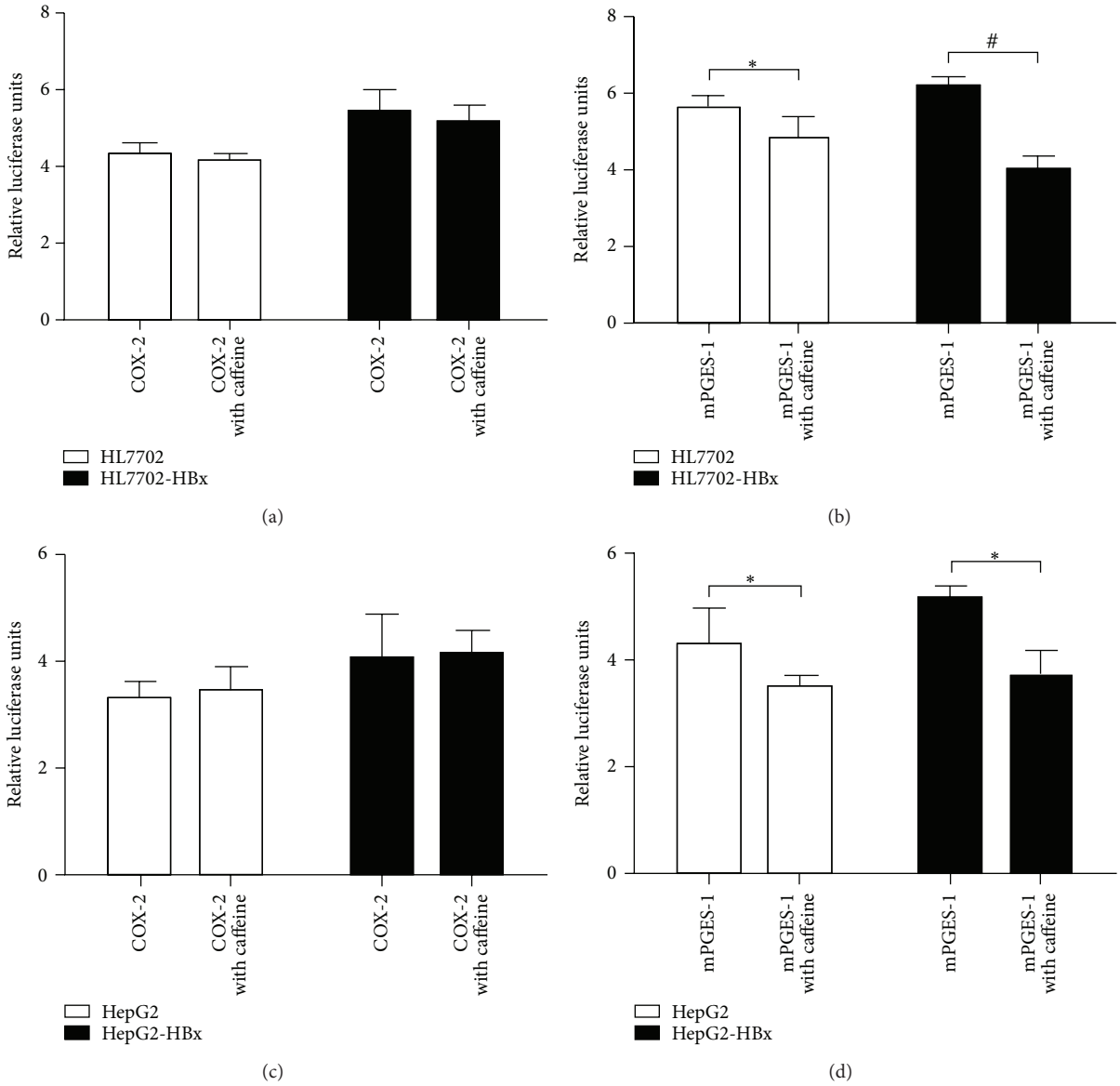

Figure 3: Effect of caffeine on the transcriptional activity of COX-2 and mPGES-1 gene promoter in HBx positive hepatocytes. (a) The COX-2 promoter activity (RLU value) in HL7702 and HL7702-HBx cells. (b) The mPGES-1 promoter activity (RLU value) in HL7702 and HL7702-HBx cells. (c) The COX-2 promoter activity (RLU value) in HepG2 and HepG2-HBx cells. (d) The mPGES-1 promoter activity (RLU value) in HepG2 and HepG2-HBx cells. Cells were cotransfected with 1 μg of pGL3B-COX-2 (COX-2 promoter luciferase reporter plasmid) or pGL3B-628 (mPGES-1 promoter luciferase reporter plasmid) and 100 ng pRL-TK, and pGL3-basic served as the negative control. $^{*}P < 0.05$ or $^{\#}P < 0.001$ versus HL7702 or HepG2 cells (RLU was set to 1, $n = 3$).

the mPGES-1 promoter, thereby affecting the expression of mPGES-1.

3.4. Inhibition of Caffeine on the Expression of mPGES-1 through EGR1 in HBx Positive Hepatocytes. Our previous research showed that HBx could upregulate the transcription of mPGES-1 promoter through EGR1, thereby enhancing the expression of mPGES-1 in promoting the hepatocytes to secrete PGE_2 [13]. The role of EGR1 is very important in this process. Therefore, caffeine may inhibit the expression of mPGES-1 through the EGR1 pathway; that is, caffeine could inhibit the synthesis pathway of HBx-EGR1-mPGES-1-PGE_2. To verify this tentative idea, we detected the EGR1 expression of HL7702, HL7702-HBx, HepG2, and HepG2-HBx cells in the presence of caffeine (800 μM). Figure 4 shows that the EGR1 protein expression of the four strains of cells was downregulated ($P < 0.05$), indicating that caffeine could downregulate mPGES-1 expression by inhibiting the EGR1 expression in the hepatocytes. Meanwhile, we observed that HL7702-HBx and HepG2-HBx cells in response to caffeine stimulation were more significant than HL7702 and HepG2 cells ($P < 0.05$).

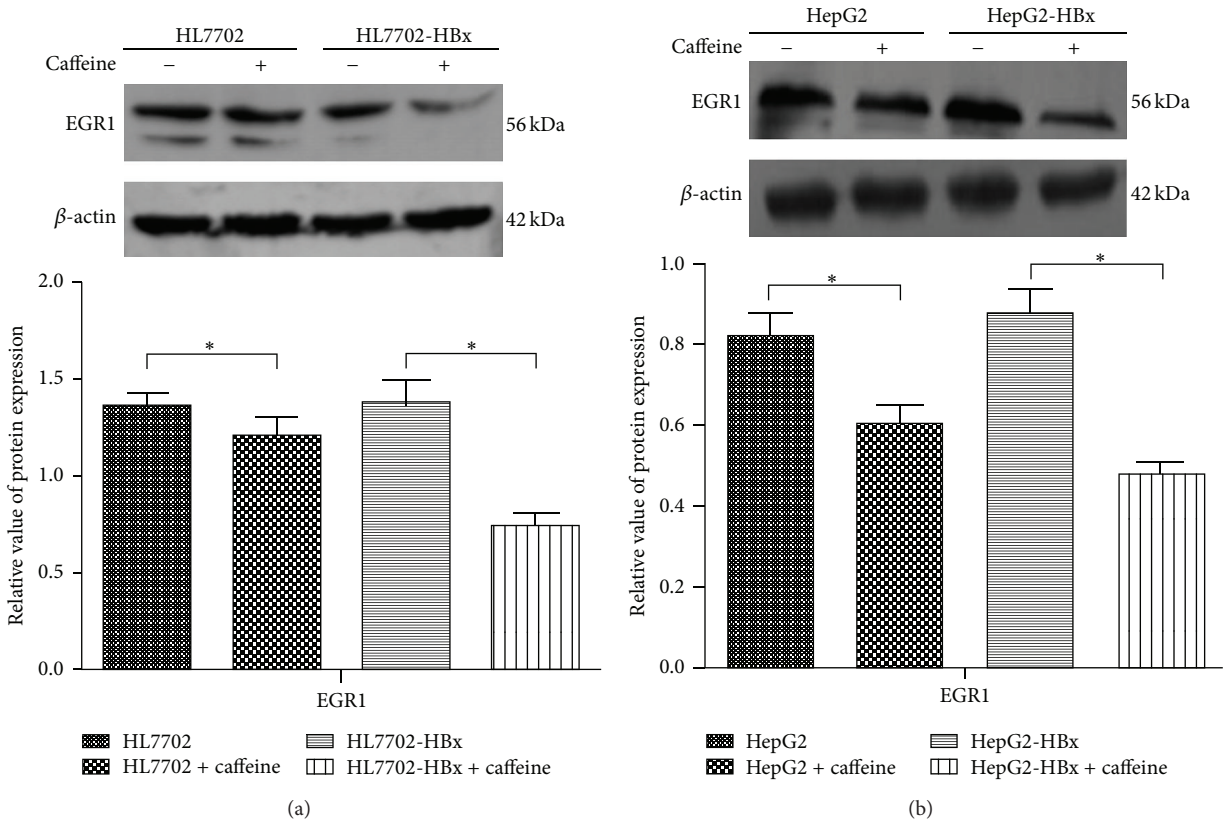

Figure 4: Inhibition of caffeine on the expression of mPGES-1 through EGR1 in HBx positive hepatocytes. (a) Representative immunoblots from three independent studies for EGR1 in HL7702 and HL7702-HBx cells treated with 800 μM caffeine or not for 7 days. The densitometry data were normalized to β-actin. $^*P < 0.05$ ($n = 3$), statistically significant difference compared to HL7702 cells. (b) Representative immunoblots from three independent studies for EGR1 in HepG2 and HepG2-HBx cells treated with 800 μM caffeine or not for 7 days. The densitometry data were normalized to β-actin. $^*P < 0.05$ ($n = 3$), statistically significant difference compared to HepG2 cells.

3.5. Effect of Caffeine on EGR1 Expression through PPARγ in HBx Positive Hepatocytes. Previous studies showed the interaction of proteins between HBx and PPARγ, which inhibited the nuclear orientation of PPARγ and the DNA binding function and affects the expression of the relative growth-inhibited gene regulated by PPARγ [15]. To verify whether caffeine could affect EGR1 expression by PPARγ to block the secretion of PGE2 from hepatocytes caused by HBx, we used 800 μM caffeine (a lower dose) to treat HL7702, HL7702-HBx, HepG2, and HepG2-HBx cells for 7 days. The results showed that the expression of PPARγ was increased, and the expression of PPARγ increased more significantly in the presence of HBx ($P < 0.05$) (Figures 5(a) and 5(b)). Approximately 5 mM caffeine (a higher dose) was further used to treat these cells for 6, 12, and 24 hours. Figures 5(c) and 5(d) show that the expression of EGR1 and PPARγ did not significantly change after 6 hours of cultivation. After 12 and 24 hours, the expression of PPARγ in the case with addition of caffeine was significantly higher than that without caffeine. The increase was more significant over time ($P < 0.05$). Meanwhile, the EGR1 expression with the addition of caffeine was significantly lower than that without caffeine; the decrease was more significant over time ($P < 0.05$). The changing trend of EGR1 and PPARγ expression was more significant in the presence of HBx.

4. Discussion

PGE_2 is one of the important products with the most biological activities synthesized by cyclooxygenase. Studies have shown that PGE_2 is significantly increased in malignant tumor tissue and could promote the growth of tumor cells [16]. Therefore, inhibition of PGE_2 has become one of the valuable research directions against inflammation to cancer. Currently, many drugs or compounds that could inhibit cells in producing PGE_2 have been found. These drugs could destroy or affect various enzymes expression in the generation process of PGE_2 [17–19]. The biosynthesis of PGE_2 is regulated by phospholipase A2, cyclooxygenase (COX), and mPGES-1. Inhibition of the above-mentioned enzymes could prevent the synthesis of PGE_2. Previous studies have shown that the expression level of COX-2 in tumor cells increased, and specific COX-2 inhibitor could inhibit tumor cell proliferation, induce apoptosis, and enhance the sensitivity of tumor cell to chemotherapy drugs. However, improper use

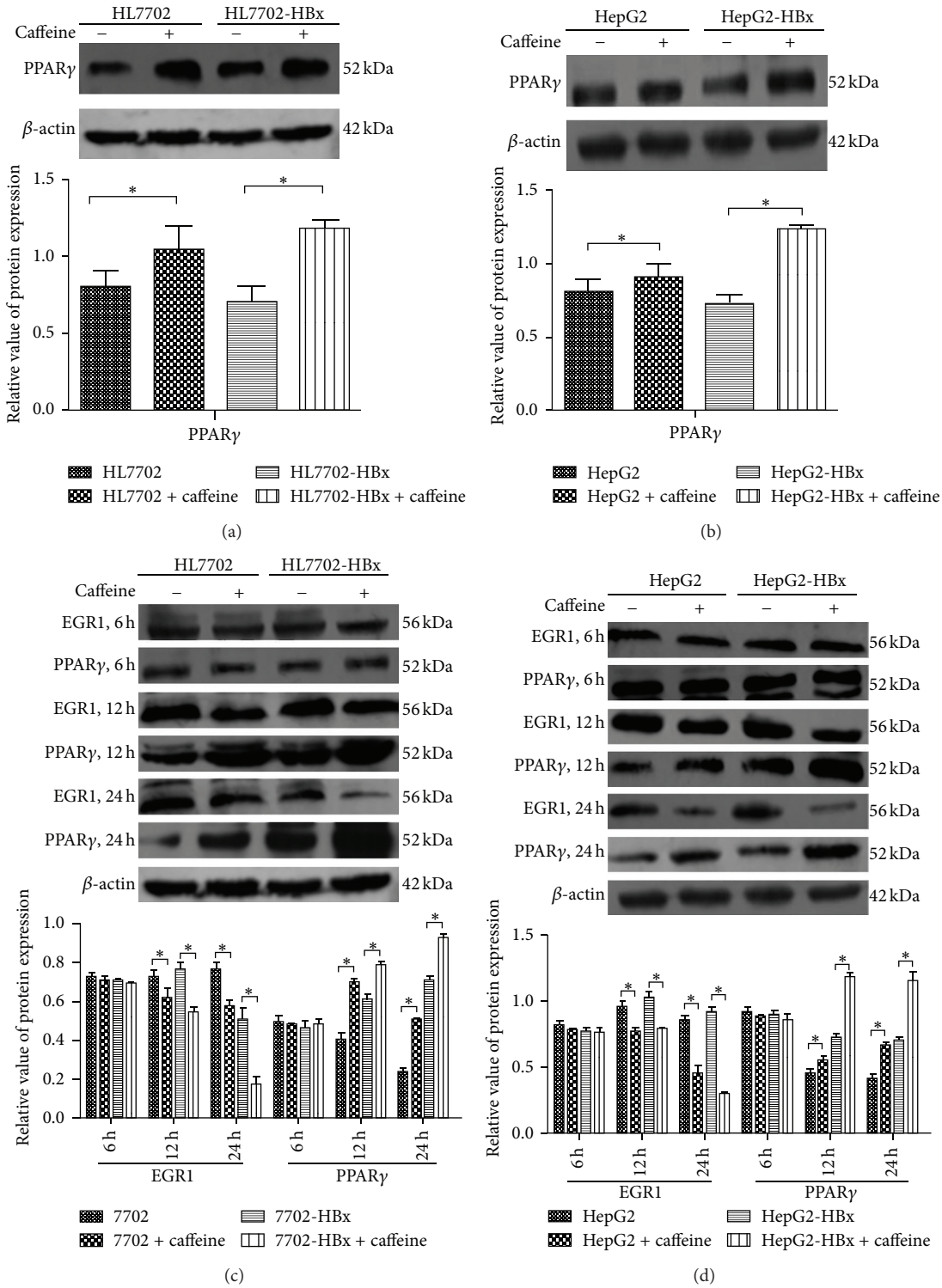

Figure 5: Effect of caffeine on EGR1 expression through PPARγ in HBx positive hepatocytes. (a) Representative immunoblots from three independent studies for PPARγ in HL7702 and HL7702-HBx cells treated with or without 800 μM caffeine for 7 days. The densitometry data were normalized to β-actin. $^{*}P < 0.05$ ($n = 3$), statistically significant difference compared with HL7702 cells. (b) Representative immunoblots from three independent studies for PPARγ in HepG2 and HepG2-HBx cells treated with or without 800 μM caffeine for 7 days. The densitometry data were normalized to β-actin. $^{*}P < 0.05$ ($n = 3$), statistically significant difference compared with HepG2 cells. (c) Representative immunoblots from three independent studies for EGR1 and PPARγ in HL7702 and HL7702-HBx cells treated with or without 5 mM caffeine for 6, 12, and 24 h. The densitometry data were normalized to β-actin. $^{*}P < 0.05$ ($n = 3$), statistically significant difference compared to HL7702 cells. (d) Representative immunoblots from three independent studies for EGR1 and PPARγ in HepG2 and HepG2-HBx cells treated with or without 5 mM caffeine for 6, 12, and 24 h. The densitometry data were normalized to β-actin. $^{*}P < 0.05$ ($n = 3$), statistically significant difference compared to HepG2 cells.

of COX-2 inhibitor could cause kidney damage and increase the incidence of cardiovascular disease and thrombus [20].

In our previous study, we found that HBx protein could regulate the transcriptional activity of mPGES-1 promoter through EGR1. During this process, PPARγ plays an important role in inhibiting the combination of EGR1 and mPGES-1 promoter to prevent the transcription of mPGES-1 [13]. An interaction exists between HBx and PPARγ, which could affect the expression of the relative growth-inhibited gene regulated by PPARγ by inhibiting the nuclear orientation of PPARγ and DNA binding function to release the inhibition on cell growth [15]. 15d-PGJ_2, a ligand of PPARγ, was used to interfere in this process. The result showed that the combined action of 15d-PGJ_2, PPARγ, and EGR1 could regulate the secretion of PGE_2 in the cell through mPGES-1. For hepatocytes expressed by HBx, the presence of 15d-PGJ_2 breaks the relationship between HBx and PPARγ. Activated PPARγ could inhibit the combination of EGR1 and mPGES-1 promoter to prevent the transcriptional activity of mPGES-1, thereby inhibiting the occurrence of PGE_2. This finding indicates that compound intervention in the relationship between PPARγ and EGR1 for affecting HBx in the expression of mPGES-1 is an effective way. Interestingly, although the relationship between proinflammatory mediator PGE_2 and liver disease has been given significant attention, studies on the influence of HBV infection on PGE_2 synthesis and the relevant intervention are rare. Therefore, we use two hepatic cell lines with different backgrounds, as well as their derived cell lines with HBx expression, to observe the characteristics of caffeine interfering in PGE_2 synthesis in HBx(+) hepatocytes.

Coffee is a common drink. Various biological activities of caffeine, the main constituent of coffee, have been widely studied. In the prevention and treatment of diseases, caffeine has a positive effect. Some studies have shown drinking coffee could reduce the risk of liver cancer. A study has shown that caffeine could enhance the sensitivity of hepatocytes to 15d-PGJ_2, PGE_2 specific inhibitor, by upregulating the expression of PPARγ receptor in hepatocytes. Caffeine could also promote the degradation of SMAD2 and inhibit phosphorylation of SMAD1 and SMAD2 [21]. The above-mentioned effects of caffeine downregulate the hepatic fibrosis-related connective tissue growth factor of inflammatory cytokines induced by TGF-β (CCTG), thereby inhibiting the progress of hepatic fibrosis. The interaction between caffeine and mPGES-1 has not been reported yet. In this paper, we found that the mPGES-1 and EGR1 expression and mPGES-1 promoter activity in the hepatocytes treated by caffeine are significantly lower than those of the group without caffeine. The expression of PPARγ was significantly higher than that of the group without caffeine. These changes are more significant in HBx(+) hepatocytes. Therefore, we speculated that caffeine has an effect similar to 15d-PGJ_2, which could release the binding of HBx and PPARγ in DBA hinge regions and activate PPARγ, to play the role of PPARγ in the inhibition of cell growth. Moreover, we also found that caffeine has insignificant effect on COX-2 expression and promoter transcription of PGE_2 biosynthetic enzymes. That is, caffeine does not have side effects similar to COX-2 inhibitor-like drugs in inhibiting HCC growth. In addition, the potential adverse side effects caused by inhibition of normal levels of PGE_2 produced by hepatocytes must be taken into consideration. Based on our current experiments *in vitro* using low concentration of caffeine, we speculated that the side effects of PGE_2 inhibition by caffeine may be fewer; however, the exact effects should be verified in animal experiments in the future.

Apparently, our study reveals that caffeine could effectively reduce PGE_2 synthesis in HBx(+) hepatocytes by specifically blocking the PPARγ-EGR1-mPGES-1 pathway and delay the effect of PGE_2 in promoting HCC growth, which provides a new evidence of molecular biology for the hypothesis that drinking coffee is beneficial to HBV-infected patients.

Conflict of Interests

None of the authors have any conflict of interests to disclose.

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