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Short communication

Bioactivity-based analysis and chemical characterization of anti-inflammatory compounds from *Curcuma zedoaria* rhizomes using LPS-stimulated RAW264.7 cells

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ABSTRACT

Inflammation is not only a self-defense response of the innate immune system, but also the pathogenesis mechanism of multiple diseases such as arthritis, neurodegeneration, and cancer. *Curcuma zedoaria* Roscoe (Zingiberaceae), an indigenous plant of India, has been used traditionally in Ayurveda and folk medicine. As part of our ongoing efforts to screen traditional medicinal plants exhibiting pharmacological potential and to characterize the compounds involved, we examined the anti-inflammatory effects of the MeOH extract of *C. zedoaria* rhizomes using lipopolysaccharide (LPS)-stimulated RAW264.7 murine macrophage cells and found that MeOH extract inhibited the synthesis of nitric oxide (NO) in a dose-dependent manner (IC_{50}: 23.44 ± 0.77 μg/mL). In our efforts to characterize the compounds responsible for these anti-inflammatory effects, bioactivity-guided fractionation of the MeOH extract and chemical investigation of its active hexane-soluble fraction led to the successful isolation of five sesquiterpenes (**1**–**5**), the structures of which were elucidated by NMR spectroscopic analysis and LC/MS analysis. Among them, curcuzedoalide (**5**) exhibited potent inhibitory effects on NO synthesis (IC_{50}: 12.21 ± 1.67 μM) and also suppressed pre-inflammatory protein expression of iNOS and COX-2. Curcuzedoalide (**5**) was thus determined to be a contributor to the anti-inflammatory effect of *C. zedoaria* rhizomes and could be a potential candidate for therapeutic applications.

1. Introduction

Acute inflammation is a well-known self-defense response of the innate immune system to protect the body from injuries or pathogens. However, any disorder in the regulation of the acute inflammation process can lead to chronic inflammation, which is associated with carcinogenesis [1]. Up to this point, many pathological studies showed that the inflammatory pathway plays a leading role in the pathogenesis mechanisms of cancer [2] and several other diseases such as arthritis, atherosclerosis, Alzheimer's disease, and depression [3–5]. Screening for anti-inflammatory agents is the first step in many studies, which can also target new chemopreventive candidates.

Curcuma zedoaria Roscoe (Zingiberaceae), also known as white turmeric or zedoaria, is a perennial herbaceous plant that has been used in Ayurveda for the treatment of menstrual disorders, indigestion, nausea, and carcinomas [6]. The *C. zedoaria* rhizome is commonly called "Ezhu" in Chinese, and has been used in traditional Chinese medicine to treat various cancers [7]. Previous studies reported that the extracts of this plant demonstrated various pharmacological effects including antimicrobial, anti-cancer, anti-allergen, and analgesic effects [6,8–10]. *C. zedoaria* is a rich natural source of sesquiterpenes, which have exhibited a number of biological activities such as anti-inflammatory [11], antibabesial [12], cytotoxic [9], and anti-fungal properties [13].

In this study, as part of our ongoing efforts to screen traditional medicinal plants exhibiting pharmacological potential and to characterize the responsible compounds [14–20], we found that the MeOH extract of *C. zedoaria* rhizomes exerted a cytotoxic effect on AGS cells [21]. Based on the bioactivity-guided fractionation and chemical investigation of the MeOH extract, cytotoxic sesquiterpenes, which

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contribute to the cytotoxicity of *C. zedoaria*, were identified and the underlying molecular mechanisms for their cytotoxic effect were explored [21]. In this study, in hoping to discover other bioactivities of the *C. zedoaria* rhizome, we examined the anti-inflammatory effects of the MeOH extract of *C. zedoaria* rhizomes using lipopolysaccharide (LPS)-stimulated RAW264.7 murine macrophage cells and found that the MeOH extract inhibited the synthesis of nitric oxide (NO) in a dose-dependent manner. In our efforts to characterize the compounds from the MeOH extract responsible for these anti-inflammatory effects, bioactivity-guided fractionation and chemical investigation of the MeOH extract were performed, which led to the successful isolation of five sesquiterpenes (**1**–**5**). Here, we report the anti-inflammatory effects of *C. zedoaria* rhizomes, the identity of the isolated constituents responsible for the anti-inflammatory effects, and the bioactivity of the isolates with regards to the molecular basis underlying anti-inflammatory activity.

2. Experimental

2.1. General experimental procedures

Optical rotations were measured using a Jasco P-1020 polarimeter (Jasco, Easton, MD, USA). IR and UV spectra were acquired with a Bruker IFS-66/S FT-IR spectrometer (Bruker, Karlsruhe, Germany) and Agilent 8453 UV–Vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA), respectively. LC/MS analysis was performed using an Agilent 1200 HPLC with a photodiode array detector system and an analytical Kinetex C18 100 Å column (100 mm × 2.1 mm i.d., 5 μm) (Phenomenex, Torrance, CA) coupled to an Agilent ESI 6130 mass spectrometer. NMR spectra were obtained using a Bruker AVANCE III 700 NMR spectrometer operating at 700 MHz (^{1}H) and 175 MHz (^{13}C) with chemical shifts given in ppm. A Shimadzu Prominence HPLC system with SPD-20A/20AV UV–Vis detectors (Shimadzu, Tokyo, Japan) was used for semi-preparative HPLC. Column chromatography was performed with silica gel 60 (Merck, Darmstadt, Germany; 70–230 mesh and 230–400 mesh) and RP-C_{18} silica gel (Merck, 230–400 mesh). TLC was performed using precoated silica gel F_{254} plates or reverse-phase (RP)-18 F_{254s} plates (Merck), and spots were detected under UV light or with anisaldehyde-sulfuric acid reagent and heating.

2.2. Plant material

The dried rhizomes of *C. zedoaria* were purchased from Kyoungdong Herbal Market, Seoul, in July 2015. The plant material was identified by one of the authors, Ki Hyun Kim. A voucher specimen (BC-2016) was deposited at the herbarium of the School of Pharmacy, Sungkyunkwan University (Suwon, Korea).

2.3. Extraction and isolation

The dried rhizomes of *C. zedoaria* (1.5 kg) were extracted three times with MeOH at 65 °C and filtered. The resultant filtrate was evaporated under reduced pressure to obtain a crude extract (69.1 g), which was sequentially solvent-partitioned with hexane, $CHCl_3$, EtOAc, and *n*-BuOH to yield hexane (19.4 g), $CHCl_3$ (8.6 g), EtOAc (4.1 g), and *n*-BuOH (1.7 g) fractions. NO assay was conducted after the cells were pre-treated with these fractions at a range of concentrations from 5 to 50 μg/mL. The hexane, $CHCl_3$, and EtOAc fractions notably diminished the level of nitrite in a dose-dependent manner with IC_{50} values of 19.38 ± 1.17, 24.24 ± 0.43, and 30.86 ± 0.41 μg/mL, respectively. The hexane (HX) fraction exhibited the strongest anti-inflammatory effect and the result inspired us to thoroughly investigate the most active fraction, the HX fraction for anti-inflammatory constituents that inhibited NO production.

The hexane fraction (2.0 g) was subjected to silica gel column chromatography using an *n*-hexane:EtOAc gradient (from 15:1 to 1:1, v/v) to yield 15 fractions (BCH1 to BCH15). Fractions BCH3 (19.8 mg), BCH5 (241.1 mg), and BCH7 (33.0 mg) were separately subjected to semi-preparative HPLC (flow rate: 2 mL/min; Phenomenex Luna C18, 250 × 10.0 mm, 10 μm) using an isocratic solvent system of 70% MeOH to furnish compounds **1** (0.9 mg, t_R = 15.0 min), **3** (5.4 mg, t_R = 22.2 min), and **4** (7.4 mg, t_R = 47.3 min), respectively. Compound **2** (6.0 mg, t_R = 43.8 min) was isolated from fraction BCH4 (30.6 mg) by semi-preparative HPLC (flow rate: 2 mL/min; Phenomenex Luna C18, 250 × 10.0 mm, 10 μm) using an isocratic solvent system of 75% MeOH. Fraction BCH8 (347.0 mg) was separated on an RP-C18 silica gel open column with an MeOH gradient (50%–75% MeOH) to yield 11 subfractions (BCH8-1 to BCH8-11). Subfraction BCH8-3 (6.5 mg) was further purified using semi-preparative HPLC (flow rate: 2 mL/min; Phenomenex Luna C18, 250 × 10.0 mm, 10 μm) with an isocratic solvent system of 60% MeOH to afford compound **5** (1.8 mg, t_R = 32.9 min).

2.4. Quantitative analysis of the compounds 1–5 by LC-MS

The detection of each compound was analyzed by an LC-MS, Agilent 1200 Series analytical system equipped with PDA detector combined with a 6130 Series ESI mass spectrometer. Briefly, the MeOH extract (1.0 mg) of *C. zedoaria* rhizomes and its derived fractions (each 1.0 mg) were dissolved in 50% aqueous MeOH (1.0 mL). The solutions were filtered through a 0.45 mm hydrophobic PTFE filter and finally analyzed by injection of 10 μL of each sample using a Kinetex C18 column (2.1 × 100 mm, 5 μm; Phenomenex, Torrance, CA, USA) set at 25 °C. The mobile phase consisting of formic acid in water [0.1% (v/v)] (A) and methanol (B) was delivered at a flow rate of 0.3 mL/min by applying the following programmed gradient elution: 10%-90% (B) for 30 min, 100% (B) for 1 min, 100% (B) isocratic for 10 min, and then 10% (B) isocratic for 10 min, to perform post-run reconditioning of the column. Calibration curves and linear regression equations were generated for the external standard, each compound. Quantification of each compound was based on the peak area obtained from the MS detection and calculated as equivalents of the standard. All contents are expressed as micrograms per 100 g of extract weight.

2.5. Cell culture and MTT cell viability assay

RAW264.7 mouse macrophages were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were grown in DMEM medium (Cellgro, Manassas, VA, USA) supplemented with 10% fetal bovine serum (Gibco BRL, Carlsbad, MD, USA), 100 units/mL penicillin, 100 mg/mL streptomycin (Invitrogen, Grand Island, NY, USA), and 4 mM L-glutamine. The cells were incubated at 37 °C in a humidified atmosphere under 5% CO_2 and sub-cultured every two days.

The cytotoxic effect of the test samples on RAW264.7 cells was determined through a cell viability assay. RAW264.7 cells were seeded on a 96-well plate at a density of 1 × 10^5 cells/well and incubated 24 h for adhesion. Afterwards, the cells were treated with 0.5% DMSO (control) or MeOH extract of *C. zedoaria*, its fractions, and isolated compounds at the indicated concentrations. Twenty-four hours later, cell viability was checked through incubated with the EZ-Cytox reagent for 2 h and absorbance was measured at 450 nm using a PowerWave XS microplate reader (Bio-Tek Instruments, Winooski, VT, USA).

2.6. Measurement of nitric oxide (NO) production

Nitric oxide production was determined through the measurement of accumulated nitrite in the culture medium by the Griess reaction. After seeding on a 96-well plate, the cells were pretreated with phenol red free medium containing 0.5% DMSO (control) or MeOH extract of *C. zedoaria*, its fractions, and isolated compounds at the indicated concentrations. The cells were the exposed to 1 μg/mL *Escherichia coli*

LPS (strain 055:B5) for 24 h. The supernatant was collected and mixed with an equal volume of Griess reagent (supplemented with 1% sulfanilamide, 5% phosphoric acid, and 0.1% N-(1-naphthyl)-ethylenediamine). The absorbance of the mixture was measured at 450 nm using a PowerWave XS microplate reader after being incubated at room temperature for 10 min. Sodium nitrite was used to generate a standard reference curve.

2.7. Western blotting analysis

The RAW 264.7 cells were seeded on a 6-well plate and then treated with a selected compound at the indicated concentrations. After 2 h, 1 μg/mL LPS was added to each well and the cells were incubated for 24 h. The cells were then collected and lysed in RIPA buffer (Cell Signaling Technology, Inc., MA, USA) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) according to the manufacturer's instructions. The protein concentration of each whole-cell extract was determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA). Equal protein amounts of each whole-cell extract (20 μg/lane) were separated by electrophoresis in a 10% sodium dodecyl sulfate-polyacrylamide gel and blotted onto PVDF transfer membranes. Epitope-specific primary antibodies included iNOS, COX-2, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) conjugated with anti-rabbit secondary antibodies (Cell Signaling, Boston, MA, USA) that were used to label the separated proteins. The bound antibodies were detected by ECL Advance Western Blotting Detection Reagents (GE Healthcare, UK) and visualized with the FUSION Solo Chemiluminescence System (PEQLAB Biotechnologie GmbH, Germany).

2.8. Statistical analysis

The data are presented as mean ± standard deviation (SD) from three independent experiments (n = 3). Statistical significance was determined using the paired Student's *t*-test. *P*-value < 0.05 was considered statistically significant.

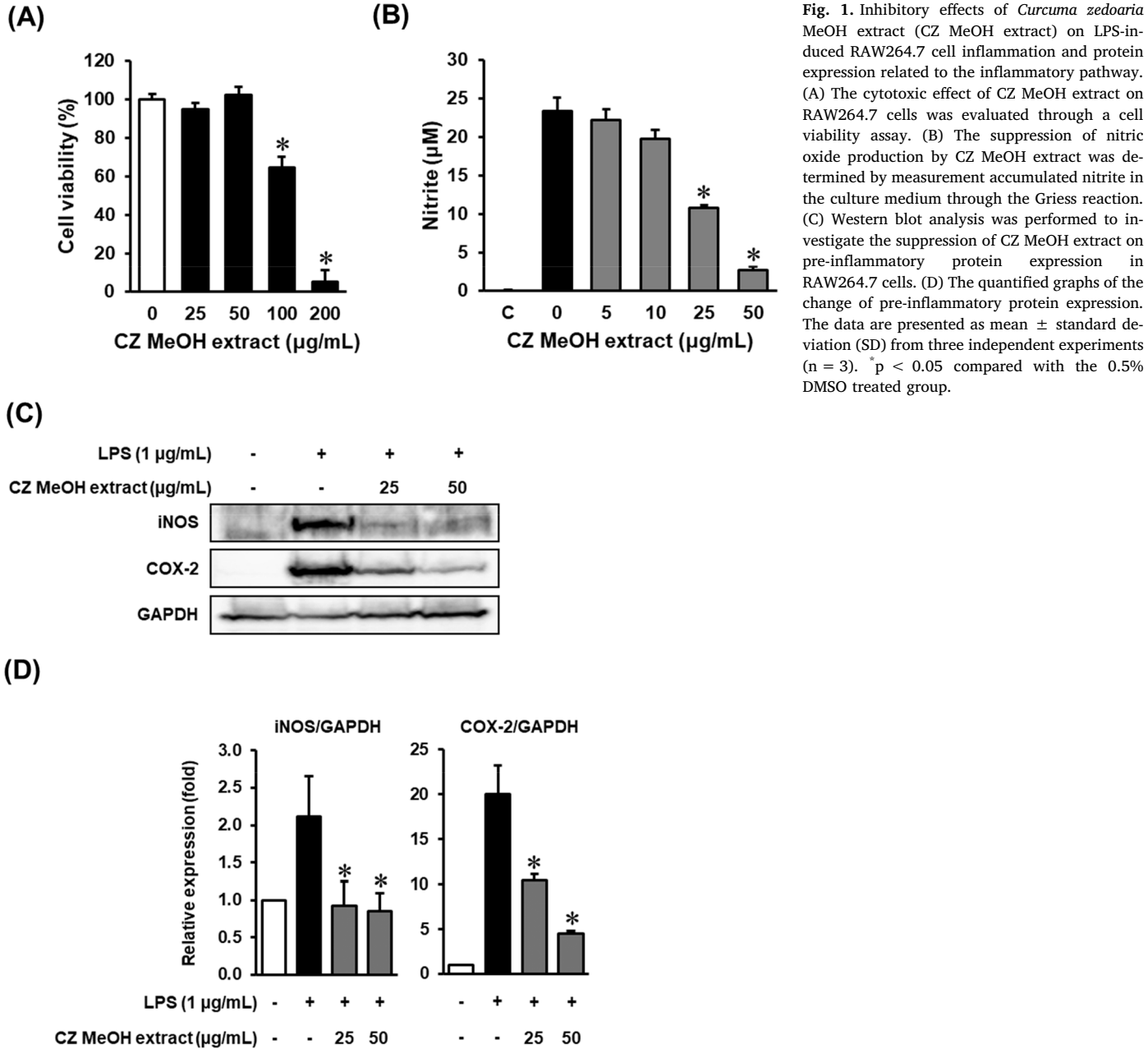

Fig. 1. Inhibitory effects of *Curcuma zedoaria* MeOH extract (CZ MeOH extract) on LPS-induced RAW264.7 cell inflammation and protein expression related to the inflammatory pathway. (A) The cytotoxic effect of CZ MeOH extract on RAW264.7 cells was evaluated through a cell viability assay. (B) The suppression of nitric oxide production by CZ MeOH extract was determined by measurement accumulated nitrite in the culture medium through the Griess reaction. (C) Western blot analysis was performed to investigate the suppression of CZ MeOH extract on pre-inflammatory protein expression in RAW264.7 cells. (D) The quantified graphs of the change of pre-inflammatory protein expression. The data are presented as mean ± standard deviation (SD) from three independent experiments (n = 3). $^{*}p < 0.05$ compared with the 0.5% DMSO treated group.

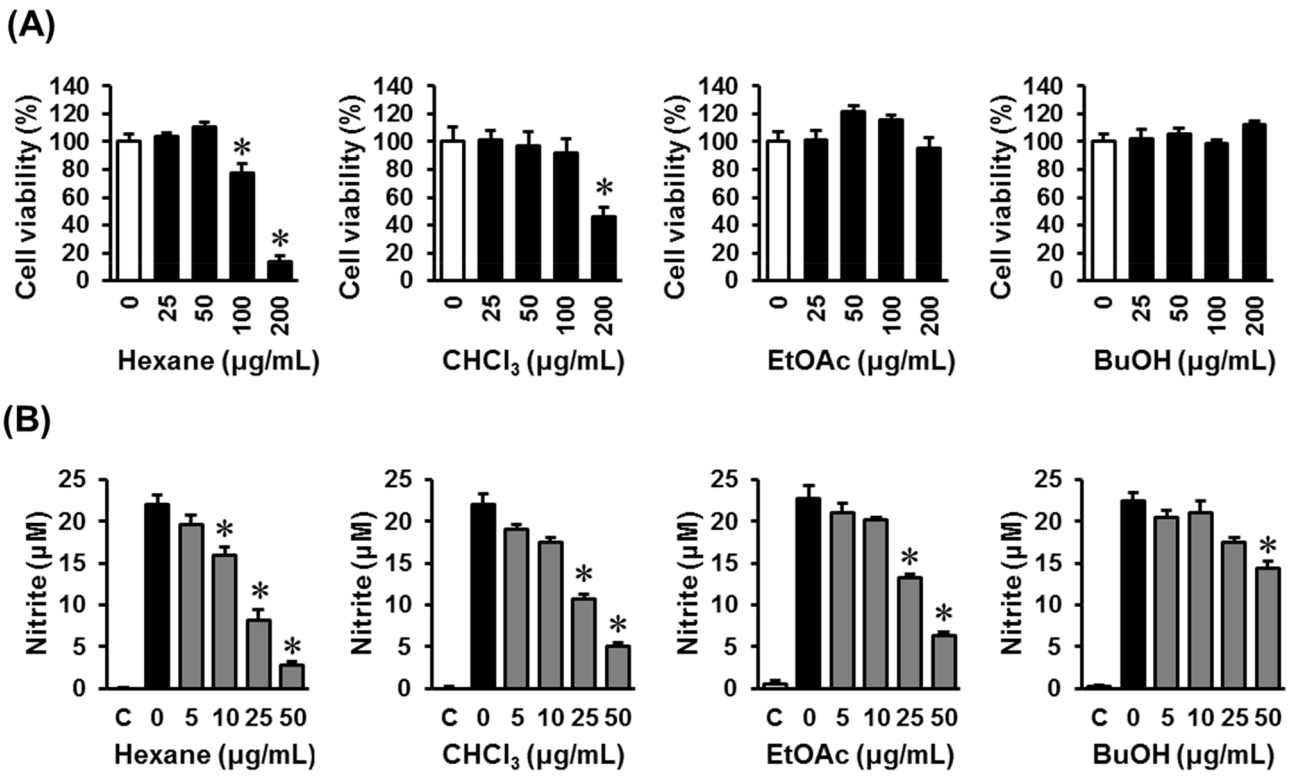

Fig. 2. Inhibitory effects of solvent-fractions from *C. zedoaria* MeOH extract on LPS-stimulated inflammation in RAW264.7 cells. (A) The cytotoxic effect of the solvent-fractions on RAW264.7 cells. (B) The anti-inflammatory effects of the solvent-fractions through the measurement of accumulated nitrite. The data are presented as mean ± standard deviation (SD) from three independent experiments (n = 3). $^{*}p < 0.05$ compared with the 0.5% DMSO treated group.

3. Results and discussion

3.1. Anti-inflammatory effects of MeOH extract of C. Zedoaria rhizomes

The dried rhizomes of *C. zedoaria* were extracted with MeOH at 65 °C and filtered. The filtrate was evaporated under reduced pressure with a rotavapor to obtain a crude MeOH extract, which was examined for anti-inflammatory properties using LPS-stimulated RAW264.7 murine macrophage cells. As shown in Fig. 1, a cell viability assay was conducted to check on the cytotoxic effects of the test samples. The MeOH extract of the *C. zedoaria* rhizomes exhibited cytotoxicity from the concentration of 100 μg/mL (Fig. 1A). Therefore, the range of concentration from 5 to 50 μg/mL of this extract was used for the NO assay. Nitrite, the more stable nitric oxide metabolite, was used for the indirect measurement of nitric oxide in this assay. The level of nitrite was significantly reduced in a dose-dependent manner after treatment with *C. zedoaria* MeOH extract (Fig. 1B, IC_{50}: 23.44 ± 0.77 μg/mL). Western blot analysis was also performed to evaluate the role of *C. zedoaria* MeOH extract on the anti-inflammatory mechanism in RAW264.7 cells. The expression levels of the two pro-inflammatory mediators, iNOS and COX-2, were examined while GAPDH was used as an internal control. RAW264.7 cells were first exposed with LPS to induce inflammation and then treated with *C. zedoaria* MeOH extract at 25 and 50 μg/mL before being subjected to Western blot analysis. As a result, the levels of iNOS and COX-2 significantly decreased after treatment with *C. zedoaria* MeOH extract in a dose-dependent manner (Fig. 1C). All of these results demonstrated that the MeOH extract of *C. zedoaria* exhibited anti-inflammatory effects and could be used for further studies about its chemical components and pharmacology.

3.2. Bioassay guided fractionation of MeOH extract

The MeOH extract was sequentially solvent-partitioned with hexane, $CHCl_3$, EtOAc, and *n*-BuOH to obtain four main fractions. Prior to investigating the anti-inflammatory effects of the four solvent-partitioned fractions, we examined the cellular toxicity of these fractions in RAW264.7 cells with an MTT assay [22–24]. The cytotoxic assay results showed that two of the four fractions significantly suppressed RAW264.7 cell proliferation, hexane (at 100 and 200 μg/mL) and $CHCl_3$ fractions (at 200 μg/mL) (Fig. 2A). An NO assay was conducted after the cells were pre-treated with these fractions at a range of concentrations from 5 to 50 μg/mL. The hexane, $CHCl_3$, and EtOAc fractions notably diminished the level of nitrite in a dose-dependent

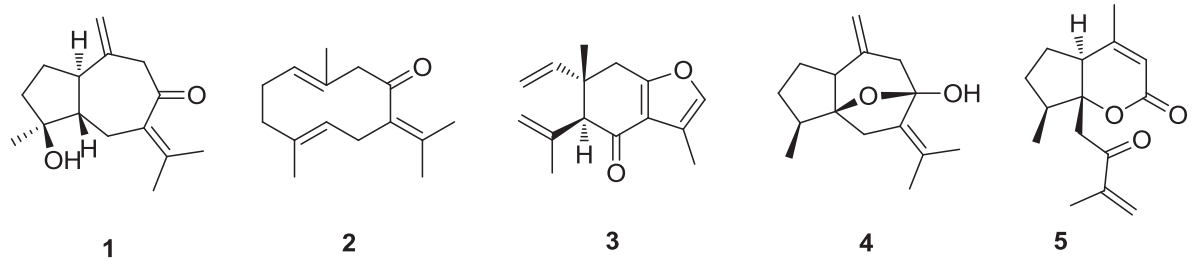

Fig. 3. Chemical structures of compounds 1–5.

Table 1
Contents (μg/100 g of extract weight) of the isolated compounds 1–5 from *C. zedoaria* rhizomes.[a]

Compound	Content in MeOH extract	Compound	Content in hexane fraction
1	3.5 ± 0.8	**1**	53.2 ± 6.7
2	0.9 ± 0.2	**2**	5.1 ± 0.5
3	1.4 ± 0.4	**3**	9.9 ± 1.3
4	11.4 ± 2.1	**4**	982.6 ± 49.1
5	1.3 ± 0.3	**5**	76.9 ± 10.2

[a] Each result shown in the table is the mean of three replicated measurements.

manner when the IC_{50} values were 19.38 ± 1.17, 24.24 ± 0.43, and 30.86 ± 0.41 μg/mL, respectively (Fig. 2B). Among them, the hexane (HX) fraction had the strongest anti-inflammatory effect and thus the most active fraction, the HX fraction, was investigated for anti-inflammatory constituents that could inhibit NO production.

3.3. Chemical identification of anti-inflammatory compounds

In our efforts to characterize the compounds responsible for the anti-inflammatory effects, the HX fraction was subjected to repeated column chromatography and semi-preparative HPLC purification, which resulted in the isolation of five sesquiterpenes (**1**–**5**) (Fig. 3). The isolated compounds were identified as isoprocurcumenol (**1**) [25], germacrone (**2**) [26], curzerenone (**3**) [27], curcumenol (**4**) [28], and curcuzedoalide (**5**) [29] through comparison of NMR spectroscopic data with reported values as well as LC/MS analysis. Quantitative analysis for the isolated compounds was also performed, and their contents in the MeOH extract and hexane fraction are summarized in Table 1. The results demonstrated that curcumenol (**4**) is distinctly predominant

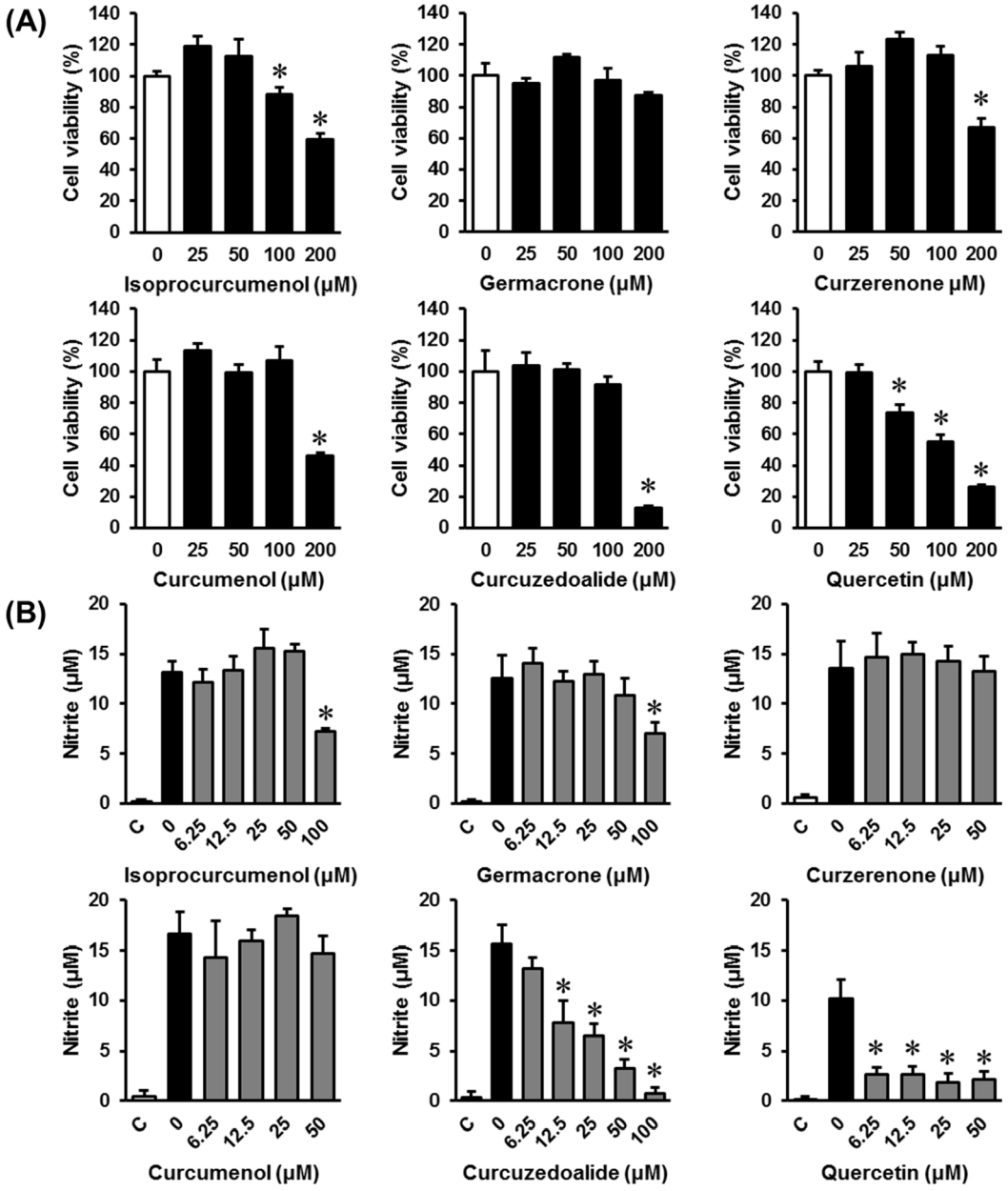

Fig. 4. Inhibitory effects of the isolated sesquiterpenes from the *C. zedoaria* hexane fraction on LPS-induced RAW264.7 cell inflammation. (A) The cytotoxic effect of the isolated sesquiterpenes on RAW264.7 cells. (C) The anti-inflammatory effects of the isolated sesquiterpenes according to measurement of accumulated nitrite. Quercetin was used as a positive control. The data are presented as mean ± standard deviation (SD) from three independent experiments (n = 3). $^{*}p < 0.05$ compared with the 0.5% DMSO treated group.

(982.6 ± 49.1 μg/100 g of the fraction weight) in the active hexane fraction.

3.4. Anti-inflammatory effects of the isolated compounds 1–5

Since all of the isolated sesquiterpenes (**1**–**5**) were purified from active fraction that inhibited NO production, these compounds were then individually tested for NO production inhibition in LPS-activated RAW264.7 macrophages. An NO assay was conducted with the sesquiterpenes (**1**–**5**) at the range of concentrations from 6.25 to 100 μM since four of the five isolated compounds exhibited cytotoxic effects at higher concentrations (Fig. 4A). In Fig. 4B, curcuzedoalide (**5**) exhibited the strongest anti-inflammatory effect, which significantly diminished the level of nitrite in a dose-dependent manner when the IC_{50} value was 12.21 ± 1.67 μM. Quercetin (IC_{50} = 4.15 ± 1.35 μM) was used as a positive control in this assay.

3.5. Mechanism study of the anti-inflammatory compound

To further confirm the anti-inflammatory properties of curcuzedoalide (**5**), we investigated the effects of curcuzedoalide (**5**) on the protein expressions of iNOS and COX-2, which are involved in the pathogenesis of chronic inflammatory disease. The level of iNOS significantly decreased after treatment with curcuzedoalide while the level of COX-2 was also slightly reduced in a dose-dependent manner (Fig. 5). Fig. 5C illustrates the proposed mechanism of the LPS-induced inflammation model in RAW264.7 cells. The inflammatory response of RAW264.7 murine macrophage cells is induced when *Escherichia coli* LPS bind to its pattern recognition receptors, toll-like receptors, or CD14. This conjugate stimulates nuclear factor-κB (NF-κB) as well as activating protein-1 (AP-1), the transcription factors that mediate the expression of iNOS or NOS II and COX-2. iNOS and COX-2 are two inducible enzymes responsible for the production of inflammatory mediators by different mechanisms. iNOS catalyzes the biosynthesis of NO, which converts L-arginine into L-citrulline and NO [30]. As a result,

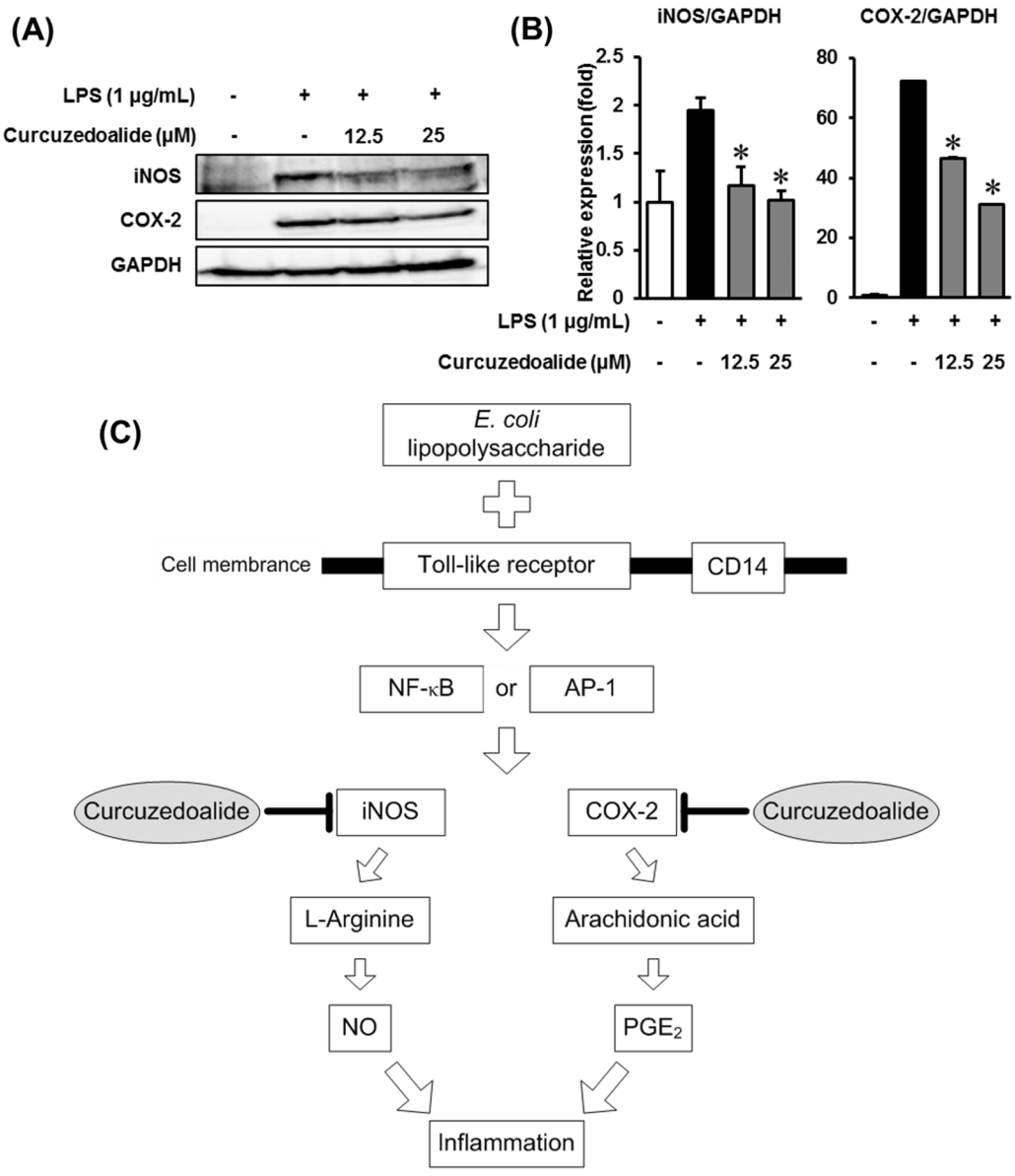

Fig. 5. The effects of curcuzedoalide on the expression of proteins related to the inflammatory pathway. (A) Western blot analysis result showed the inhibitory effect of curcuzedoalide on pre-inflammatory protein expression. (B) Quantified graphs of the change of iNOS and COX-2 levels after treatment with curcuzedoalide. The data are presented as mean ± standard deviation (SD) from three independent experiments (n = 3). $^{*}p < 0.05$ compared with the 0.5% DMSO treated group. (C) The proposed mechanism of LPS-induced inflammation of curcuzedoalide in RAW264.7 cells.

NO induces vascular permeability that leads to the infiltration of leukocytes into inflamed tissues and kills microorganisms as the host defense reaction [31,32]. COX-2 takes part in the formation of prostaglandin E_2 (PGE_2) by stimulating the cyclooxygenase pathway that converts arachidonic acid into prostaglandin endoperoxides (PGG_2 and PGH_2, in that order) and then into PGE_2 [33]. The pro-inflammatory effects of PGE_2 are well known, and it plays a role as a vasodilator to allow for the recruitment of neutrophils and macrophages to the site of injury or infection. PGE_2 also has anti-inflammatory properties through immunosuppressive activities that inhibit IL-2 signaling and T cell activation [34]. As a result, treatment with curcuzedoalide suppressed the expression of iNOS and COX-2 indicating its anti-inflammatory effect.

4. Conclusions

In the current study, we found that the MeOH extract of *C. zedoaria* rhizomes inhibited NO production in LPS-activated RAW264.7 macrophages. In our efforts to characterize the compounds responsible for these anti-inflammatory effects, bioactivity-based analysis and chemical investigation of the MeOH extract led to the successful identification of the anti-inflammatory constituent, curcuzedoalide (**5**), which significantly inhibited NO production in LPS-activated RAW264.7 macrophages with an IC_{50} value of 12.21 ± 1.67 μM. In addition, the treatment of curcuzedoalide (**5**) reduced the LPS-induced increase expression of iNOS and COX-2 proteins. These findings provide experimental evidence that curcuzedoalide (**5**) could contribute to the health benefits of *C. zedoaria* as an anti-inflammatory agent.

Conflicts of interest

The authors declare no competing financial interest.

Acknowledgements

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2018.09.027.

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