INTRODUCTION

Meliaceae plants are well known for their important biological activities and diversified limonoid compounds, and they have aroused widespread interest in organic chemistry and agricultural chemistry. The genus *Chukrasia* (Meliaceae) includes one species, *Chukrasia tabularis*, and a variant *Chukrasia abularis* var. *velutina* (Kaur & Arora, 2009; Liao et al., 2009; Mulholland et al., 2000; Roy & Saraf, 2006). In recent years, a large amount of limonins with diverse structures have been separated from this genus (Fan...
et al., 2007; Zhang, Fan, et al., 2008; Zhang, Yang, et al., 2008), some of them have anti-inflammatory, potassium channel blocking, and antibacterial activity (Zhang, Fan, et al., 2008; Zhang, Yang, et al., 2008; Zhang, Yang, Liao, et al., 2007; Zhang, Yang, Zhu, et al., 2007). Phragmalin limonoids and carbonate are characteristic compositions of the Chukrasia genus (Abdelgaleil et al., 2006; Nakatani et al., 2004; Saad et al., 2003; Wu et al., 2005). Chukrasia tabularis A. Juss., an economically important evergreen tree, is widely cultured in tropical areas like Malaysia, southeastern China, and India (Luo et al., 2012). Its bark is traditionally used in China and India as astringent, antidiarrheal, and anti-influenza agent, and its leaf extract exhibits activity on bacteria and fungi (Luo et al., 2010). Previous chemical research on this plant provided a series of phragmalin limonins. Limonin is a kind of nortriterpene with diversified structure, which has a wide range of bioactivities, like antifeeding insects, antimalarial and anticancer activities (Liao et al., 2009; Tan & Luo, 2011; Fang et al., 2011).

In the physiologic responses to infection or damage, macrophages have a special impact on the progress of inflammatory processes (Alivernini et al., 2020). Both the production of pro-inflammatory mediator and the aggravation of inflammation are impossible to separate from the action of macrophage (Elisa et al., 2018). Many pro-inflammatory cytokines, like tumor necrosis factor alpha (TNF-α), interleukin 1β (IL-1β), and interleukin 6 (IL-6), are originated from macrophages. Nuclear factor kappa B (NF-κB) is an example of signal transduction and gene modulation associated with macrophages’ immmersion and activation (Sae-Tan et al., 2020). When activated, NF-κB causes the production of pro-inflammatory cytokines like TNF-α, IL-6, and IL-1β. Given the potential relevance of inflammation and macrophages, it is important to find a way to modulate the expression of inflammatory cytokines and control the activation of macrophages. Lipopolysaccharide (LPS) has been widely used to stimulate macrophages in inflammatory models in experiments on anti-inflammatory mechanisms. After LPS stimulation, NF-κB signaling cascade was activated, resulting in changes in related protein expression (Ren et al., 2020).

In recent years, the anti-inflammatory, antitumor, and antioxidant activities of Chukrasia tabularis have been widely reported (Kaur et al., 2011). In our studies on the anti-inflammatory constituents of Meliaceae plants, two new phragmalin limonoid orthoesters Chukrasitin D and E (1 and 2) (Figure 1) were isolated and identified from the root barks of C. tabularis, together with 12 known limonoids (3–14). In this study, we report the separation, structure identification, and bioassay results of the extracts and isolated compounds. The in vitro anti-inflammatory assay of compounds 1–14 on LPS-mediated macrophages showed that limonins 1 and 2 displayed a significant inhibitory effect. In addition, the effects of limonin 1 on the production of nitric oxide, NF-κB, and TNF-α in RAW 264.7 cells induced by LPS and their possible anti-inflammatory mechanisms were also evaluated. Therefore, the current study focused on anti-inflammatory evaluation of C. tabularis extracts and isolated limonins.

## 2 MATERIALS AND METHODS

### 2.1 Reagents and materials

The optical rotation was obtained using a JASCO P-1020 polarimeter. Infrared (IR) spectra were measured on a Nicolet 1700 FT-IR spectrometer, ultraviolet (UV) spectra were detected on a 210A UV spectrum. The nuclear magnetic resonance (NMR) spectra were recorded on a 400MHz Bruker spectrometer. Electrospray ionization mass spectrometry (ESIMS) and high-resolution electrospray ionization mass spectrometry (HRSEIMS) were measured on a 2020 LCMS spectrum and Bruker APEX II mass spectrum, respectively. Semipreparative high-performance liquid chromatography (HPLC) was performed on a RP-18 column (250×10mm, Waters). The root bark of Chukrasia tabularis was provided and identified by Dr. Y. H. Zhang, School of Pharmacy, Fujian Medical University, Fuzhou, China. Dulbecco’s modified Eagle’s medium (DMEM) was offered by Gibco Company (USA). The enzyme immunosassay kit of NF-κB, TNF-α, and IL-6 was supplied by the R&D System Company (USA). Lipopolysaccharide (LPS) was provided by Sigma Chemical Corp. (USA). Nitric oxide was supplied by Nanjing Jiancheng Bioengineering Inc. The electronic circular dichroism (ECD) spectrum was measured in methanol (MeOH) on a Jasco J 1500 spectropolarimeter (JASCO Corporation).

### 2.2 Preparation of extracts from C. tabularis and bioassay-guided separation

The anti-inflammatory test of xylene-induced ear edema in mice showed that the dichloromethane extract had significant anti-inflammatory activity (Table 1), so the dichloromethane phase was selected for further separation. Subfractions of dichloromethane extracts Fr.C and Fr.D showed significant anti-inflammatory activity by mouse xylene auricle swelling experiments (Table 1), so isolation and purification focused on these two fractions.

The chopped root bark of C. tabularis (5.6 kg) was extracted three times with MeOH at room temperature for 7 days each (20L). The obtained solution was evaporated in vacuo to gain a brownish extract (890g). The residue was suspended in H2O and divided by petroleum ether (PE), dichloromethane (CH2Cl2), ethyl acetate, and n-butanol. The CH2Cl2 extract (290g) was fractionated by a MCI gel column and eluted by 10% (Fr.A), 30% (Fr.B), 50% (Fr.C), 70% (Fr.D), 90% (Fr.E), and 100%MeOH (Fr.F). Fr.C (98g) was fractionated to silica gel column and eluted by petroleum ether–ethyl acetate (PE-EtOAc) (8:1–0:1, each 5 L) to gain seven fractions (Fr.s C1–C7). Fr.C3 (22.7g) was fractionated to Sephadex LH–20 (CH2Cl2-MeOH) to give six fractions (Fr.s C3–1–C3–6). Fr.C3-2 (12.8g) was applied to semi-HPLC (MeCN:H2O = 7:3) to gain compounds 12 (15.6 mg, tR 13.2 min), 4 (25.9 mg, tR 17.1 min), 3 (21.8 mg, tR 20.4 min), and 9 (15.4 mg, tR 24.9 min). Fr.C3-4 (10.2g) was purified to HPLC (MeCN:H2O = 3:2) to obtain compounds 13 (12.5 mg, tR 19.3 min), 14 (14.6 mg, tR 21.9 min), 8 (37.6 mg, tR 24.1 min),
and 5 (27.4 mg, t<sub>R</sub>32.7 min). Fr.D (78 g) was fractionated with silica gel column and eluted by PE–EtOAc (8:1–0:1, each 5 L) to gain nine fractions (Frs. D1–D9). Fr.D3 (26.7 g) was applied to Sephadex LH-20 (MeOH) to gain four fractions (Frs. D3-1–D3-4). Fr.D3-1 (11.7 g) was subjected to RP-18 column and semi-HPLC (MeCN:H₂O = 13:7) to gain limonins 6 (24.3 mg, t<sub>R</sub>17.2 min) and 11 (23.4 mg, t<sub>R</sub>22.6 min). Fr.D5 (13.8 g) was applied to Sephadex LH-20 (MeOH) and semi-HPLC (MeCN:H₂O = 13:7) for compounds 1 (21.3 mg, t<sub>R</sub>15.1 min), 2 (13.7 mg, t<sub>R</sub>16.5 min), 7 (17.8 mg, t<sub>R</sub>24.5 min), and 10 (19.7 mg, t<sub>R</sub>33.7 min) (Figure S17).

2.3 | Laboratory animals

Male Institute of Cancer Research (ICR) mice (18 ± 2 g), specific pathogen free, were supplied by the experimental animal center of Fujian
Medical University. All animals were acclimatized to environment for 3 days before experiment, and fed and drank ad libitum. The animal experiments complied with the guidelines for the care and use of laboratory animals and were approved by the Laboratory Animal Ethics Committee of Fujian Medical University.

2.4 | Xylene-induced ear edema in mice

The extracts were dissolved in 0.5% CMC-Na (sodium carboxymethyl cellulose) and Aspirin was applied as a positive control. After gavage of the extracts or control for 1 h, the right ear of each mouse was treated with 40 μl of xylene solution, and the left ear served as a control. One hour after xylene treatment, mouse was executed due to cervical dislocation. A circular part with a diameter of 6 mm of each ear was weighed with an electronic analytical balance, and its inhibitory activity on ear edema was calculated (Table 1).

2.5 | In vitro anti-inflammatory activities

RAW 264.7 cells obtained from the China Center for Cultivated Studies (Shanghai, China) were maintained in DMEM contained with 1% penicillin and streptomycin and 10% fetal bovine serum, and under 5% CO2 at 37°C. Cells were stimulated with LPS. In brief, cells were placed on the 96-well plate (1×10^5 cells/well). After 2 h of preincubation, the LPS (2 μg/ml) and compounds were added and the samples incubated for 24 h. The supernatant of cell culture was collected 24 h later and NO was detected by the Griess reagent (Gasparotto et al., 2013).

2.6 | Measurement of NF-κB, IL-6, and TNF-α production

The levels of NF-κB, TNF-α, and IL-6 were determined by enzyme-linked immunosorbent assay (ELISA) based on manufacturer’s protocol. The standard solution and the antibody-bearing sample were placed at 37°C for 60 min, added to the working solution, incubated in 37°C for 30 min, and washed. Tetramethylbenzidine (TMB) was then added and the TMB termination solution was added after 20 min. In the end, the absorbance at 450 nm was recorded by ELISA.

2.7 | Statistical analysis

The data obtained were expressed as mean±SD. All experiments had 3 replicates. The t-test was used to verify differences between groups by IBM SPSS Statistics 24.

3 | RESULTS

3.1 | Bioactivity-guided abstraction and isolation of active components

The anti-inflammatory activities of methanolic, petroleum ether, dichloromethane (CH2Cl2), EtOAc, and n-butanol extracts and fractions from the root barks of C. tabularis were assessed in vivo by xylene-induced ear edema in mice. The result showed that the dichloromethane extract displayed significant anti-inflammatory activities with an inhibition rate of 42.41% (400 mg/kg) (Table 1). The subfractions of dichloromethane extract Fr.C and Fr.D exhibited significant anti-inflammatory activities with inhibitory values of 43.65% and 42.93% (400 mg/kg). Two novel phragmalin limonins, Chukrasitin D (1) and E (2), together with 12 known limonins (3–14) were separated and identified from Fr.C and Fr.D (Figure 1).

3.2 | Structural elucidation of isolated compounds

Chukrasitin D (1) was isolated as white amorphous powder, and its molecular formula was demonstrated as C33H50O14 by the HRESIMS.

### Table 1

| Extracts/Fractions | Dose (mg/kg) | Edema degree (x±SD, mg) | Inhibition rate (%) |
|--------------------|-------------|------------------------|-------------------|
| Control group      |             | 6.71 ± 0.33            |                  |
| Aspirin            | 200         | 4.13 ± 0.96            | 38.45             |
| Methanol extract   | 400         | 5.12 ± 0.77            | 38.50             |
| Petroleum ether fraction | 400        | 5.47 ± 0.81            | 24.22             |
| Dichloromethane fraction | 400       | 4.39 ± 0.52            | 42.41             |
| Ethyl acetate fraction | 400       | 5.31 ± 0.69            | 31.89             |
| n-Butanol fraction | 400         | 5.69 ± 0.46            | 19.21             |
| Fr. B(30%MeOH)     | 400         | 5.98 ± 0.83            | 12.83             |
| Fr. C(50%MeOH)     | 400         | 4.10 ± 0.64            | 43.65             |
| Fr. D(70%MeOH)     | 400         | 4.95 ± 0.85            | 42.93             |
| Fr. E(90%MeOH)     | 400         | 5.83 ± 0.79            | 17.78             |

*p < .01 versus Control group.
ion at m/z 718.3162 [M + Na + H]+ (calcd. for C35H51O14Na, 718.3175) which indicated 11 degrees of unsaturation. The infrared (IR) spectrum analysis indicated that 1 contained hydroxyl (3459 cm⁻¹) and ester groups (1740 cm⁻¹). The 13C-NMR (carbon nuclear magnetic resonance) spectrum indicated 35 carbon resonances, including 10 methyl groups (three methoxys), seven methylene groups, five methine groups (two oxygenated), and 13 quaternary carbons (five oxygenated). In addition, a comprehensive analysis of its 1H-NMR (proton nuclear magnetic resonance) and 13C-NMR (carbon nuclear magnetic resonance) and data (Table 2) showed the presence of three methyl esters, one orthoacetoxy moiety, one propanoyl, and one 3-methylbutyryl group. In molecule 1, there are 11 unsaturates, of which 5 are occupied by 5 ester carbonyls, and the remaining 6 unsaturates require 1 to be hexacyclic in the center. The foregoing data indicated that 1 was a limonoid orthoester of phragmalin type (Lin et al., 2009).

Extensive 2D-NMR (two-dimensional nuclear magnetic resonance) spectral analysis, especially HMBC (heteronuclear multiple bond correlation) data, assigned most of the functional units to limonoid core and identified D-Seco Phragmalin limonoid framework of 1. In HMBC spectra, the main correlation between H-18/ C-13/ C-12/ C-17 and between an OMe to C-17 at δ174.8 and the correlation between H-15/OMe of C-16 δ171.2 indicated that the two OMe units were connected, respectively, to C-17 and C-16 (Figure S1), suggesting that 1 was ring D-opened limonin (Lin et al., 2009). The mutual HMBC correlations of H-3 and C-3′ of 3-methylbutyryloxy moiety showed that it is situated in C-3. The HMBC correlation between H-30 and carbonyl (C-1′) of a propanoyl group δ174.1 exhibited the presence of a propanoyloxy in C-30. The quaternary carbon δ120.4 (C-31) corresponds to methyl δ1.62(s), showing the presence of orthoacetoxy part. In addition, the strong HMBC correlation between C-1/H-29/H-19, C-2/H-29/H-3, C-18/H-30/H-15, and C-9/H-12/H-11 allocated four oxygenated quaternary carbons as C-2, C-1, C-9, and C-8. Phragmalin limonoids, separated from Chukrasia, are usually 8, 9, 14-, 8, 9, 30-, and 1, 8, 9- orthoacetate (Lin et al., 2011; Silva et al., 2008; Zhang, Fan, et al., 2008; Zhang, Yang, et al., 2008). The presence of 1, 8, 9 orthoacetate in 1 was determined temporarily.

The relative structure of compound 1 was deduced by ROESY (Rotating Frame Overhauser Enhancement Spectroscopy) spectra, and there were strong cross peaks between Me-18a/H-14 and H-30/H-15, which showed that H-30 and H-15 were coplanar, and they were β directed. The ROESY correlation of H-3/H-29 and Me-4′/H-5 exhibited that the 3-methylbutyryl moiety was β oriented. The main correlation of H-18/H-14 revealed that H-14 was α directed. H-32 is associated with methyl of propanoyl and H-14, indicating that propanoyl and 1, 8, 9 orthoacetate groups were α directed. The ROESY relationship between H-29a/H-19/Me-28 and between H-29b/Me-28/H-3 can determine the two protons of C-29. According to the above results, the relative configuration of 1 was completed, as shown in Figure 1. By comparing experiments and computational ECD data, the absolute configuration of 1 was finally proved, which is a suitable method for solving the absolute configuration of natural compounds. (Table 2)

**Table 2**: 1H-NMR (proton nuclear magnetic resonance) (400 MHz) and 13C-NMR (carbon nuclear magnetic resonance) (100 MHz) spectroscopic data for 1 and 2

| No.  | δH (J in Hz) | δC      | δH (J in Hz) | δC      |
|------|--------------|---------|--------------|---------|
| 1    | 873          | 88.3    | 2a           |           |
| 2    | 810          | 80.9    |              |          |
| 3    | 4.54(s)      | 85.6    | 4.55(s)      | 85.4    |
| 4    | 46.7         | 46.6    |              |          |
| 5    | 2.94(brd, 11.7) | 37.8 | 2.85(m)      | 38.0    |
| 6    | 2.49(dd, 13.5, 6.0) | 35.7 | 2.51(m)      | 35.6    |
| 7    | 174.7        | 175.3   |              |          |
| 8    | 87.2         | 87.0    |              |          |
| 9    | 88.5         | 87.2    |              |          |
| 10   | 47.3         | 47.1    |              |          |
| 11α  | 1.28(m)      | 29.4    | 1.30(m)      | 30.3    |
| 11β  | 1.32(m)      |         | 1.35(m)      | 30.8    |
| 12α  | 1.33(m)      | 26.1    | 1.19(m)      | 26.1    |
| 12β  | 1.38(m)      | 1.23(m) |              |          |
| 13   | 35.5         | 35.3    |              |          |
| 14   | 2.19(m)      | 44.0    | 2.17(m)      | 44.2    |
| 15   | 2.78(dd, 12.3, 7.0) | 26.3 | 2.28(dd, 12.4, 5.0) | 27.2 |
| 16   |              | 171.2   |              |          |
| 17   | 174.8        | 174.8   |              |          |
| 18   | 1.14(s)      | 20.7    | 1.13(s)      | 20.7    |
| 19   | 1.18(s)      | 16.6    | 1.18(s)      | 16.7    |
| 28   | 0.95(s)      | 15.2    | 0.91(s)      | 15.0    |
| 29a  | 1.98(d, 13.0) | 40.5 | 1.65(d, 10.7) | 40.5 |
| 29b  | 1.66(d, 13.0) | 1.96(d, 10.7) |          |          |
| 30   | 5.90(s)      | 72.6    | 5.81(s)      | 72.6    |
| 31   | 120.4        | 120.5   |              |          |
| 32   | 1.62(s)      | 21.3    | 1.62(s)      | 21.3    |
| 1'   | 179.3        | 179.2   |              |          |
| 2'   | 2.33(m)      | 28.7    | 2.35(m)      | 28.7    |
| 3'   | 2.93(m)      | 35.4    | 2.75(m)      | 34.9    |
| 4'   | 1.24(d, 7.2) | 18.7    | 1.14(d, 6.5) | 18.7    |
| 5'   | 1.17(d, 7.2) | 18.7    | 1.19(d, 6.5) | 18.7    |
| 1''  | 174.1        | 174.3   |              |          |
H-30 and H-15 were coplanar, and they were \( \beta \) directed. The ROESY correlation of H-3/H-29 and Me-3’/H-5 exhibited that the propa

Table 2

| No. | \( \delta_H (\text{in Hz}) \) | \( \delta_C \) | \( \delta_H (\text{in Hz}) \) | \( \delta_C \) |
|-----|----------------------------|------------|----------------------------|------------|
| 2’’ | 2.30 (q, 8.0)              | 28.7       | 2.36 (q, 7.5)              | 28.7       |
| 3’’ | 1.03 (t, 8.0)              | 9.0        | 1.04 (t, 7.5)              | 9.0        |
| 7’-OMe | 3.73 (s)                  | 52.8       | 3.73 (s)                   | 52.8       |
| 16’-OMe | 3.73 (s)                 | 52.8       | 3.73 (s)                   | 52.8       |
| 17’-OMe | 3.73 (s)                  | 52.8       | 3.73 (s)                   | 52.8       |

\*Recorded in \( \text{CD}_3\text{OD} \).

Figure 2 Calculated and experimental electronic circular dichroism (ECD) spectra of 1

Chukrasitin D (1): white powder; \([\alpha]_D^{28} = -0.68^\circ (c \ 0.36, \text{CHCl}_3)\); UV (MeOH)209 nm; IR (KBr): \( \nu_{\text{max}} \) 3459, 2970, 1740, 1406, 1150, 1043 cm\(^{-1}\); \(^1\)H and \(^13\)C NMR data, see Table 2; HRESIMS \( m/z \) 718.3162 [M + Na + H]+ (calcd. For \( \text{C}_{35}\text{H}_{50}\text{O}_{15}\text{Na} \). 718.3175).

Chukrasitin E (2): white powder; \([\alpha]_D^{28} = -0.66^\circ (c \ 0.30, \text{CHCl}_3)\); UV (MeOH)209 nm; IR (KBr): \( \nu_{\text{max}} \) 3450, 2971, 1742, 1460, 1151, 1047 cm\(^{-1}\); \(^1\)H and \(^13\)C NMR data, see Table 2; HRESIMS \( m/z \) 756.2718 [M + Na + K]+ (calcd. 756.2753).

3.3 Anti-inflammatory effects of separated limonins from C. tabularis

Nitric oxide is a major bioinformatics molecule with dual roles of biological messenger and cytotoxic molecule. It is involved in the pathogenicity of inflammation, is overexpressed in LPS-mediated macrophages, and is an indicator of inflammation (Jeon et al., 2016; Keisuke et al., 2021). The in vitro anti-inflammatory effects of limonins (1-14) were determined by LPS-stimulated RAW 264.7 cells by evaluating the production of NO. Cell viability determination showed that limonins (1-14) have no toxicity to RAW 264.7 cells at a concentration of 100 \( \mu \)M. To determine whether limonins (1-14) suppressed NO production by LPS-mediated RAW 264.7 cells, the concentration of NO in medium containing these limonins was evaluated. As shown in Table 3, 14 limonins exhibited anti-inflammatory effects at the tested concentration. The result exhibited that D-ring-opened phragmalin limonoid orthoester (1-2) showed strong NO inhibitory activities, while limonins (3-14) showed potent to moderate activity. Limonins 1-2 displayed significant anti-inflammatory activities.
with IC₅₀ values of 6.24 and 6.13 μM. Limonoids 3–14 showed effective anti-inflammatory effect with the inhibition rate between 12.30 and 50.19 μM. Considering that anti-inflammatory components are found in the root bark of *C. tabularis*, it can be determined that they are a source of natural anti-inflammatory molecules. It is worth noting that limonins 1 and 2 showed the strongest anti-inflammatory activity. Therefore, the potential anti-inflammatory activity and molecular mechanism of compound 1 were further studied.

3.4 | Effects of limonin 1 on LPS-stimulated production of TNF-α, IL-6, and NF-κB

TNF-α, IL-6, and NF-κB are major pro-inflammatory cytokines identified as anti-inflammatory markers that can be released by LPS-mediated macrophages (Dong et al., 2018; Lee et al., 2021). Excessive production of pro-inflammatory cytokines exacerbates a variety of diseases, including allergies, autoimmune disease, and cancer (Benedetto et al., 2019; Guo et al., 2019). We investigated the activity of limonin 1 on LPS-stimulated pro-inflammatory cytokines in RAW 264.7 cells. The results in Figure 3 exhibited that the levels of NF-κB, IL-6, and TNF-α in the LPS group were notably higher than those in the control group. As shown in Figure 3a–c, the addition of limonin 1 notably suppressed production of NF-κB, IL-6, and TNF-α in a dose-dependent manner. The result indicated that limonin 1 could suppress the expression of NF-κB, IL-6, and TNF-α in LPS-stimulated macrophage, and achieved anti-inflammatory activity. The regulation of anti-inflammatory activity on macrophages may be partly involved in the protective activity of *Chukrasia tabularis* on inflammatory diseases.

TABLE 3 Inhibitory activity of compounds 1-14 on lipopolysaccharide-induced (LPS-induced) nitric oxide (NO) production in RAW 264.7 cells

| Compounds | IC₅₀ (μM) | Compounds | IC₅₀ (μM) |
|-----------|-----------|-----------|-----------|
| 1         | 6.24 ± 0.80 | 9         | 31.79 ± 7.11 |
| 2         | 6.13 ± 0.99 | 10        | 36.78 ± 3.66 |
| 3         | 25.56 ± 3.21| 11        | 27.83 ± 1.11 |
| 4         | 21.78 ± 4.2 | 12        | 50.19 ± 11.78|
| 5         | 12.30 ± 4.21| 13        | 48.25 ± 5.38 |
| 6         | 20.46 ± 6.38| 14        | 44.95 ± 7.74 |
| 7         | 25.76 ± 2.12| Indomethacin* | 26.18 ± 1.56 |
| 8         | 19.86 ± 3.90|

*Positive control.

FIGURE 3 Effects of compound 1 on lipopolysaccharide (LPS)-induced macrophages. The macrophages were incubated with LPS (1 μg/ml) and treated with compound 1 (6.25 and 25 μM) for 24 h. (a) The levels of tumor necrosis factor alpha (TNF-α), (b) nuclear factor kappa B (NF-κB), and (c) interleukin 6 (IL-6) in the supernatant were assayed using enzyme-linked immunosorbent assay (ELISA) kits. All values are the mean ± SD. **p < .01 compared to the LPS group.

4 | DISCUSSION

Macrophage is involved in most inflammatory responses, including LPS stimulation, and secretes pro-inflammatory cytokines like NF-κB, TNF-α, and IL-6 (Kim et al., 2020). Modern studies have suggested that natural product may inhibit inflammation by modulating NO or inflammatory factor in macrophage (Fang et al., 2008). Among these natural products, a thorough in-depth research on homologous medicinal and edible plants, it is found that limonin is the main anti-inflammatory active ingredient (Fan et al., 2019), mainly through inhibition of inflammatory mediators’ NF-κB signaling cascade (Jin et al., 2018). *C. tabularis* bark and fruit extract was proven to have anti-inflammatory activities by inhibiting pro-inflammatory cytokines such as NO, TNF-α, and IL-6 (Perianayagam et al., 2004). However, few reports have focused on anti-inflammatory activities and mechanism of limonins in *C. tabularis* extracts (Yang et al., 2020). In this study, a combination of octadecyl silica gel, Sephadex LH-20, and HPLC was used to separate anti-inflammatory limonins from *C. tabularis*, and 14 compounds were identified by NMR, ECD, and mass spectrometry, including two novel limonins Chukrasitin D and E (1 and 2) and 12 known limonins (3–14). Further research revealed that limonins 1–2 exhibited notable anti-inflammatory activities by LPS-mediated RAW 264.7 cells. Furthermore, limonin 1 can suppress the release of TNF-α, NF-κB, and IL-6 stimulated by LPS, thereby contributing to the anti-inflammatory activity of the extract.

Lipopolysaccharide is a common pathogenic endotoxin constituent of the outer membrane of Gram-negative bacteria. LPS is an...
effective trigger for inflammatory responses (Pandher et al., 2021). Inflammation is the main risk element for many diseases, and macrophages are the primary immune cells and the first line of defense against pathogen invasion (Lesageur et al., 2020). In the process of inflammation, macrophages produce excess induced nitric oxide synthase as inflammatory mediators and pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β (Huang et al., 2019). NO is a biological signal and effect or element that modulates the expression of pro-inflammatory cytokine (Zamora et al., 2000). Inflammatory damage is thought to be caused by the excessive production of NO-induced pro-inflammatory cytokine (Kany et al., 2019; Zhang et al., 2017). Excessive production of nitric oxide occurs in inflammation and various diseases, where NO plays a cytotoxic role in the pathological process (Lea et al., 2020; Shao et al., 2013). Therefore, suppression of NO production is important for the prevention of inflammatory disease. Among inflammatory stimulants, LPS induces macrophage activation leading to the release of pro-inflammatory cytokine in the inflammatory response (Bonizzi & Karin, 2004; Ronchetti et al., 2017). Cytokines arouse fever, stun, and various inflammatory diseases. Therefore, it is essential to suppress the overproduction of pro-inflammatory cytokines. The in vitro anti-inflammatory effects of limonins (1-14) were determined by LPS-stimulated RAW 264.7 cells by evaluating the production of NO. The result exhibited that D-ring-opened phragmalin limonoid orthoester (1-2) showed strong NO inhibitory activities while limonins (3-14) showed potent moderate activity. We also investigated the activity of limonin 1 on LPS-stimulated pro-inflammatory cytokines in RAW 264.7 macrophages. The results showed that limonin 1 notably inhibited the expression of NF-κB, IL-6, and TNF-α in LPS-stimulated macrophage, and achieved anti-inflammatory activity. The regulation of anti-inflammatory activity on macrophages may be partly involved in the protective activity of Chukrasia tabularis on inflammatory diseases.

Our results showed that fractions of C. tabularis root barks’ ethanol extract indicated varying degrees of anti-inflammatory effect on ear swelling induced by xylene in mice. Two isolated anti-inflammatory limonins, Chukrasitin D (1) and Chukrasitin E (2), exhibited remarkable inhibitory activity. The result showed that limonin 1 suppressed the production of NO and pro-inflammatory cytokines in LPS-stimulated RAW 264.7 cells in a dose-dependent manner. This is the first time that Chukrasitin D (1) and Chukrasitin E (2) have been identified in C. tabularis root bark, and they have been proved to have strong anti-inflammatory activities, providing a theoretical basis for the application of C. tabularis in anti-inflammatory activity.

5 | CONCLUSIONS

Screening for anti-inflammatory activity of Chukrasia tabularis root bark extracts led to the separation of 14 limonins, including two novel phragmalin limonoids (1-2), 12 known limonoids (3-14). All isolated compounds were determined for NO production by LPS-mediated RAW 264.7 cells. The result exhibited that D-ring-opened phragmalin limonoid orthoester (1-2) showed strong NO inhibitory activities while limonins (3-14) showed potent moderate activity. Limonins 1-2 displayed significant anti-inflammatory activities with IC₅₀ values of 6.24 and 6.13 μM. Compound 1 inhibited the production of NO and TNF-α in stimulated cells and reduced the secretion of NO and TNF-α levels during inflammatory processes. These results provide basic information for further research on utilizing C. tabularis as natural anti-inflammatory resource.

ACKNOWLEDGEMENT

This work was financially supported by the National Natural Science Foundation of China (No. 41806173), Fujian Natural Science Foundation (Nos. 2020J01619 and 2018J01847), Key Laboratory of Marine Biological Genetic Resources of the State Oceanic Administration (HY201807, HY201604), and the Science and Technology Innovation Platform Project of Fujian Province (2018Y2001).

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT.

The data presented in this study are available in the Supplementary Materials.

ETHICAL APPROVAL

All experiments involving the use of animals have been approved by the Institutional Animal Protection and Use Committee of Fujian Medical University (Approval No. 2017-0102).

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