Research Article

Identification and quantification of oleanane triterpenoid saponins and potential analgesic and anti-inflammatory activities from the roots and rhizomes of Panax stipuleanatus

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abstract

Background: Panax stipuleanatus represents a folk medicine for treatment of inflammation. However, lack of experimental data does not confirm its function. This article aims to investigate the analgesic and anti-inflammatory activities of triterpenoid saponins isolated from P. stipuleanatus.

Methods: The chemical characterization of P. stipuleanatus allowed the identification and quantitation of two major compounds. Analgesic effects of triterpenoid saponins were evaluated in two models of thermal- and chemical-stimulated acute pain. Anti-inflammatory effects of triterpenoid saponins were also evaluated using four models of acetic acid-induced vascular permeability, xylene-induced ear edema, carrageenan-induced paw edema, and cotton pellet-induced granuloma in mice.

Results: Two triterpenoid saponins of stipuleanosides R1 (SP-R1) and R2 (SP-R2) were isolated and identified from P. stipuleanatus. The results showed that SP-R1 and SP-R2 significantly increased the latency time to thermal pain in the hot plate test and reduced the writhing response in the acetic acid-induced writhing test. SP-R1 and SP-R2 caused a significant decrease in vascular permeability, ear edema, paw edema, and granuloma formation in inflammatory models. Further studies showed that the levels of inflammatory mediators, nitric oxide, malondialdehyde, tumor necrosis factor-α, and interleukin 6 in paw tissues were downregulated by SP-R1 and SP-R2. In addition, the rational harvest of three- to five-year-old P. stipuleanatus was preferable to obtain a higher level of triterpenoid saponins. SP-R2 showed the highest content in P. stipuleanatus, which had potential as a chemical marker for quality control of P. stipuleanatus.

Conclusion: This study provides important basic information about utilization of P. stipuleanatus resources for production of active triterpenoid saponins.

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1. Introduction

Panax stipuleanatus H. T. Tsai et K. M. Feng belongs to an evolutionary group of Panax species. It is mainly found in the tropical rain forest, at 1100–1700 m above sea level, in Pingbian, Maguan, and Malipo County, Yunnan Province, China, and its roots and rhizomes can be used as medicinal materials. In traditional Chinese medicine, P. stipuleanatus is known to act as an analgesic, being able to remove blood stasis and provide essential nourishment, promoting wound healing and hemostasis. P. stipuleanatus remains an underutilized natural resource.

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At present, there are only a few studies on *P. stipuleanatus*, limited to plant classification [1], biological characteristics [2,3], and preliminary chemical composition and biological activity [4]. Identified saponins from *P. stipuleanatus* are mainly oleanane-type triterpenoid saponins, e.g., stipuleanosides R1 (SP-R1) and R2 (SP-R2) [4]. Liang et al [5] reported that the oleanane-type triterpenoids isolated from *P. stipuleanatus* had cytotoxic activity against HL-60 cells (leukemia) and HCT-116 cell lines (colov cancer). Liang et al [6] also found that the extract had an inhibitory effect on proinflammatory mediators of nuclear factor kappa B (NF-kB). In HepG2 cells (hepatocellular carcinoma), NF-kB activity was inhibited by reducing the concentration of inflammatory factors, using oleanane-type triterpenoids isolated from *P. stipuleanatus*. However, all currently available studies on the anticancer activity of *P. stipuleanatus* were conducted on cell lines, and its traditionally anti-inflammatory and analgesic effects had not been reported.

At present, the clinical use of both steroids and nonsteroidal drugs in the treatment of inflammatory diseases has been associated with numerous adverse reactions. For example, nonsteroidal anti-inflammatory drugs stimulate the gastrointestinal tract and damage the kidneys, selective cyclooxygenase inhibitors increase the risk of cardiovascular diseases, and glucocorticoid drugs may activate or aggravate infections [7,8]. Recent studies have shown that a variety of natural active ingredients such as most terpene compounds have good anti-inflammatory activity [1,8] (Yang et al, 2006; Yang et al, 2012). Anti-inflammatory terpenes can inhibit production of cytokines, growth factors, adhesion molecules, NF-kB, and nitric oxide (NO) and prostaglandin expression [9]. Drugs derived from natural compounds and plant extracts have the advantage of being less toxic than chemical drugs. The development of such new drugs can represent a potential alternative to the treatment of acute and chronic inflammation with chemical drugs [10]. To develop natural active compounds into treatment of inflammatory diseases, more research is urgently needed for the isolation and identification of the compounds and its functional evaluation from *P. stipuleanatus*.

Therefore, in view of the traditional application of *P. stipuleanatus*, chemical composition and anti-inflammatory and analgesic activities were established in this study. The first objective of this study was to isolate oleanane triterpenoid saponins from *P. stipuleanatus* and determine the contents of saponins in *P. stipuleanatus* of different growth years and from different producing areas. The analgesic and anti-inflammatory activities of oleanane triterpenoid saponins in vivo were evaluated by two analgesic and four acute inflammation animal models. This study provides data support for the development of anti-inflammatory drugs from natural products or plant extracts.

### 2. Materials and methods

#### 2.1. Materials and reagents

Xylene, carrageenan, hematoxylin and eosin, acetic acid, phosphoric acid, and acetonitrile [high-performance liquid chromatography (HPLC)–grade] were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dexamethasone was purchased from Zhejiang Xianchen Pharmaceutical Co., Ltd (Xianju, China). Aspirin was purchased from Shenyang Ojina Pharmaceutical Co., Ltd (Shenyang, China). All the other reagents and solvents were of analytical grade.

#### 2.2. Plant material

The roots and rhizomes of *P. stipuleanatus* H.T. Tsai et K.M. Feng were collected in 2017 in Pingbian, Yanshan, and Wenshan County of Yunnan Province, China (detailed in Table 1). This plant was identified by Professor Xiiming Cui (Faculty of Life Science and Technology, Kunming University of Science and Technology) and given the voucher number KMUST017 at Seed Resource Center of Kunming University of Science and Technology.

#### 2.3. Purification and isolation of SP-R1 and SP-R2

The medicinal parts of *P. stipuleanatus* are shown in Fig. 1. The root and rhizome powder of *P. stipuleanatus* (500 g) was extracted using 70% methanol three times. After filtration, the filtrate was evaporated to get methanol extract (121.36 g). The methanol extract was dissolved in water and partitioned with n-butanol. After obtaining the n-butanol fraction (16.28 g), it was chromatographed on silica gel and then on octadecylysilica gel (ODS) to yield compounds SP-R1 (0.64 g) and SP-R2 (3.67 g). The structures of compounds SP-R1 and SP-R2 were determined by spectroscopic analyses. The NMR spectra were recorded on a Bruker AV III-500 spectrometer (Bruker, Berlin, Germany). HRESIMS data were collected on an Agilent 6530 quadrupole time-of-flight mass spectrometer (Agilent Technologies, California, America).

SP-R1: white powder; \( ^{1}H\)-NMR (CD3OD, 500 MHz) \( \delta \): 0.80 (3H, s), 0.93 (3H, s), 0.93 (3H, s), 0.94 (3H, s), 1.04 (3H, s), 1.15 (3H, s); for \( ^{13}C\)-NMR (CD3OD, 125 MHz) data, see Table 2. The structure of compound SP-R1 is shown in Fig. 2. HRESIMS at m/z 949.4540 [M + Na]+ (calcd for C47H74NaO18, 949.4773). SP-R2: white powder; \( ^{1}H\)-NMR (CD3OD, 500 MHz) \( \delta \): 0.77 (3H, s), 0.78 (3H, s), 0.90 (3H, s), 0.92 (3H, s), 0.93 (3H, s), 1.03 (3H, s), 1.14 (3H, s); for \( ^{13}C\)-NMR (CD3OD, 125 MHz) data, see Table 2. The structure of compound SP-R2 is shown in Fig. 2. HRESIMS at m/z 1111.5023 [M + Na]+ (calcd for C53H84NaO23, 1111.5301).

#### 2.4. HPLC analysis of SP-R1 and SP-R2

The dried and powdered sample (0.25 g) from roots and rhizomes of *P. stipuleanatus* (PS-1 to PS-6 and PS-13 to PS-24) was placed in a tube containing 70% methanol (10 mL). The sample was sonicated for 30 min at room temperature and centrifuged at 3500 rpm for 15 min, and the supernatant was added with the solvent

| Part | Period of growth | Producing area | Code |
|------|----------------|----------------|------|
| Root | One year        | Wenshan, Yunnan province | PS-1 |
| Root | Three years     | Wenshan, Yunnan province | PS-2 |
| Rhizome | Three years      | Wenshan, Yunnan province | PS-3 |
| Rhizome | Five years      | Wenshan, Yunnan province | PS-4 |
| Rhizome | Ten years       | Wenshan, Yunnan province | PS-5 |
| Rhizome | Fifteen years   | Wenshan, Yunnan province | PS-6 |
| Root | One year        | Wenshan, Yunnan province | PS-7 |
| Root | Three years     | Wenshan, Yunnan province | PS-8 |
| Rhizome | Three years     | Wenshan, Yunnan province | PS-9 |
| Rhizome | Five years      | Wenshan, Yunnan province | PS-10 |
| Rhizome | Ten years       | Wenshan, Yunnan province | PS-11 |
| Rhizome | Fifteen years   | Wenshan, Yunnan province | PS-12 |
| Root | One year        | Yanshan, Yunnan province | PS-13 |
| Root | Three years     | Yanshan, Yunnan province | PS-14 |
| Rhizome | Three years    | Yanshan, Yunnan province | PS-15 |
| Rhizome | Five years      | Yanshan, Yunnan province | PS-16 |
| Rhizome | Ten years       | Yanshan, Yunnan province | PS-17 |
| Rhizome | Fifteen years   | Yanshan, Yunnan province | PS-18 |
| Root and rhizome | Three to five years | Yanshan, Yunnan province | PS-19 |
| Root and rhizome | Three to five years | Yanshan, Yunnan province | PS-20 |
| Root and rhizome | Three to five years | Pinbian, Yunnan province | PS-21 |
| Root and rhizome | Three to five years | Wenshan, Yunnan province | PS-22 |
| Root and rhizome | Three to five years | Wenshan, Yunnan province | PS-23 |
| Root and rhizome | Three to five years | Wenshan, Yunnan province | PS-24 |

PS-7 to PS-12 are fresh roots or rhizomes of *P. stipuleanatus*, and others are dried samples.
and made up to 10 mL. The fresh sample (1 g) from roots and rhizomes of *P. stipuleanatus* (PS-7 to PS-12) was chopped and ultrasonically extracted using 70% methanol (20 mL) for 30 min. The sample was centrifuged, and the supernatant was added with the solvent and made up to 20 mL. Each sample solution of the dried and fresh products was filtered through a 0.45-μm filter and prepared for HPLC analysis.

Quantitative analysis of SP-R1 and SP-R2 in *P. stipuleanatus* from different growing areas and harvesting dates was determined by HPLC (Fig. 3). The HPLC data were recorded on a Shimadzu Analytical Instrument (Shimadzu, Kyoto, Japan), equipped with a solvent degasser (DGU-20A3R(C)), a binary pump (LC-20AB), an auto sampler (SIL-20A), and a UV detector (SPD-20A). The separation was performed on a YMC-Pack ODS-A column (250 mm × 4.6 mm, 5μm; YMC Co., Ltd, Kyoto, Japan) at the flow rate of 1.0 mL/min. The mobile phase consisted of 0.05% (v/v) phosphoric acid water (Solvent A) and acetonitrile (Solvent B), and the following gradient program was used: 0–15 min, 30–85% B; 15–30 min, 35–80% B; 30–35 min, 80–85% B. The detection wavelength and the column temperature were at 203 nm and 35 °C, respectively. Reference compounds of SP-R1 and SP-R2 were isolated from roots and rhizomes of *P. stipuleanatus* and identified by NMR and high resolution mass spectrometry (HRMS). The calibration curves for SP-R1 and SP-R2 were y = 7×10^5x − 12851 (R^2 = 0.9996) and y = 4×10^6x + 22414 (R^2 = 0.9997) in the range of 0.2–2 mg/mL, respectively. The samples were analyzed by HPLC three times.

### 2.5. Animals

Kunming mice of clean-grade mice (18–22 g) of both sexes, aged 6–8 weeks, were obtained from Changsha Tianqin Biotechnology Co., China. The qualified number is SCXK 2014-0011. The mice were kept at 25 ± 2 °C and under 12-h dark–light cycle conditions. The animals were fed with normal diet and water. All experimental protocols were approved by the Animal Ethical Committee of Laboratory Animals of Kunming University of Science and Technology.

### 2.6. Hot plate test in mice

The hot plate test was used according to the method described by Sun et al [11]. The latency period of pain response was counted when the mice were placed on a hot plate at 55 °C until the mice licked their hind paw or jumped. Mice with latency longer than 30 s or less than 5 s were removed. The other mice were randomly divided into the negative control group (0.9% saline, same volume), positive group (aspirin, 400 mg/kg), SP-R1 group (1 and 10 mg/kg), and SP-R2 group (1 and 10 mg/kg), with ten mice in each group. The mice were administered orally for 7 days. The hot plate reaction time of 30, 60, and 90 min after administration was determined.

### 2.7. Acetic acid writhing test in mice

The acetic acid writhing test was carried out in mice as described by Meymandi and Keyhanfar [12]. Sixty mice were

### Table 2

C-NMR data of compounds SP-R1 and SP-R2 in CD3OD.

| Carbon no. | Aglycone | Carbon no. | Sugar |
|-----------|----------|------------|-------|
|           | SP-R2<sup>1</sup> | SP-R1<sup>1</sup> | SP-R2<sup>2</sup> | SP-R1<sup>2</sup> |
| 1         | 39.8      | 39.7       | 3-O-GlcUA<sup>3</sup> |       |
| 2         | 26.9      | 26.9       | 1"                    | 106.4  |
| 3         | 91.0      | 91.2       | 2"                    | 76.4   |
| 4         | 40.2      | 40.2       | 3"                    | 79.5   |
| 5         | 57.0      | 57.0       | 4"                    | 79.5   |
| 6         | 19.3      | 19.3       | 5"                    | 78.1   |
| 7         | 33.9      | 34.0       | 6"                    | 176.5  |
| 8         | 40.6      | 40.6       | Glc<sup>2</sup> (1→3)GlcUA |       |
| 9         | 49.1      | 49.1       | 1"                    | 104.4  |
| 10        | 37.9      | 37.9       | 2"                    | 75.6   |
| 11        | 24.5      | 24.5       | 3"                    | 78.2   |
| 12        | 123.8     | 123.67     | 4"                    | 71.0   |
| 13        | 144.6     | 145.2      | 5"                    | 78.3   |
| 14        | 42.9      | 42.9       | 6"                    | 78.2   |
| 15        | 28.9      | 28.8       | Ara(f)<sup>4</sup> (1→4)GlcUA |       |
| 16        | 24.0      | 24.0       | 1"                    | 107.9  |
| 17        | 48.6      | 47.6       | 2"                    | 82.0   |
| 18        | 42.6      | 42.7       | 3"                    | 74.8   |
| 19        | 47.2      | 47.2       | 4"                    | 87.1   |
| 20        | 31.5      | 31.6       | 5"                    | 62.4   |
| 21        | 34.9      | 34.9       | 28-O-Glc-ll<sup>5</sup> |       |
| 22        | 33.1      | 33.7       | 1"                    | 95.7   |
| 23        | 28.5      | 28.4       | 2"                    | 73.9   |
| 24        | 17.0      | 17.0       | 3"                    | 78.0   |
| 25        | 16.0      | 16.0       | 4"                    | 71.0   |
| 26        | 17.7      | 17.7       | 5"                    | 78.7   |
| 27        | 26.3      | 26.4       | 6"                    | 62.2   |
| 28        | 178.0     | 181.82     | 8"                    | 62.2   |
| 29        | 33.5      | 33.8       | 9"                    |       |
| 30        | 24.0      | 24.0       | 10"                   |       |

SP-R1, stipuleanoside R1; SP-R2, stipuleanoside R2.

<sup>1</sup> Recorded at 125 MHz.
<sup>2</sup> GlcUA: β-D-glucuronopyranosyl.
<sup>3</sup> Glc: β-D-glucopyranosyl.
<sup>4</sup> Ara(f): α-L-arabinofuranosyl.
<sup>5</sup> 28-O-Glc-ll: 28-O-D-glucopyranosyl.
randomly divided into six groups, with ten mice in each group. The negative control group (0.9% saline, same volume), positive group (aspirin, 400 mg/kg), SP-R1 group (1 and 10 mg/kg), and SP-R2 group (1 and 10 mg/kg) were orally administered, once a day for seven days. After 1 hour of administration, 0.6% acetic acid (v/v) was injected intraperitoneally (10 ml/kg of body weight) into each mouse. The numbers of writhing in mice were counted for 20 min.

2.8. Acetic acid–induced vascular permeability test

The acetic acid–induced vascular permeability test was carried out as described by Yesilada et al [13]. Sixty mice were divided into six groups, with ten mice in each group. The model group (0.9% saline, same volume), positive group (dexamethasone, 10 mg/kg), SP-R1 group (1 and 10 mg/kg), and SP-R2 group (1 and 10 mg/kg) were orally administered, once a day for seven days. One hour after

Fig. 2. Two compounds identified from *P. stipuleanatus*. SP-R1, stipuleanoside R₁; SP-R₂, stipuleanoside R₂.

Fig. 3. (A) HPLC chromatograms of standards from *P. stipuleanatus*. (B) HPLC chromatograms of 70% methanol extract from *P. stipuleanatus*. Peaks: SP-R₁, stipuleanoside R₁; SP-R₂, stipuleanoside R₂. HPLC chromatogram of the fresh rhizome of three-year-old *P. stipuleanatus* from Wenshan County of Yunnan Province is shown as the red line (top) and that of the dried rhizome of *P. stipuleanatus* of the same age and from the same place is shown as the black line (bottom). HPLC, high-performance liquid chromatography.
the last administration, each mouse was intravenously injected 2% Evans blue in saline (0.1 mL/10 g) and then immediately injected 0.6% acetic acid (10 mL/kg). After 20 min, the mice were killed by removing the cervical vertebra, and peritoneal exudates were collected after the abdominal cavity was washed several times with saline (8 mL) and centrifuged at 3000 rpm for 10 min. The optical density (OD) value of supernatant was measured at a wavelength of 590 nm using a SHIMADZU UV-2600 UV–VIS spectrophotometer (Shimadzu, Tokyo, Japan). The inhibition rate was presented according to the following formula:

\[
\text{Inhibition rate} (\%) = \left( \frac{\text{mean OD value (model group)} - \text{mean OD value (drug experimental group)}}{\text{mean OD value (model group)}} \right) \times 100.
\]

2.9. Cotton pellet–induced granuloma test

The cotton pellet–induced granuloma model was carried out by the method of Wu et al [14]. Sixty mice were divided into six groups, with ten mice in each group. Under ether anesthesia, sterile cotton pellets (10 ± 0.5 mg) were inserted subcutaneously, one in each axilla. The same volume of sterile 0.9% saline (model group), dexamethasone (positive group, 10 mg/kg), SP-R1 (1 and 10 mg/kg), and SP-R2 (1 and 10 mg/kg) were given orally to mice for seven days. After the last administration, each mouse was given a subcutaneous intra-plantar injection with 1% carrageenan suspended in sterile 0.9% saline (30 μL) into the right hind paw. The thickness of paw edema was measured using a digital micrometer (03000002; Guinlin Guanglu Measuring Instrument Co., Ltd, Guinlin, China) 1 h before any treatment and at intervals of 1, 2, 3, 4, 5, and 6 h after the injection of carrageenan. Paw edema degree was presented according to the following formula: Paw edema degree (%) = (T1 − T0)/ T0 × 100%, where T0 and T1 were the thickness of paw edema before and after injection, respectively. The inhibition rate was presented according to the following formula:

\[
\text{Inhibition rate} (\%) = \left( \frac{\text{mean paw edema degree (model group)} - \text{mean paw edema degree (drug experimental group)}}{\text{mean paw edema degree (model group)}} \right) \times 100.
\]

2.12. Histopathological analysis

Ear and paw tissue samples obtained in ear and paw edema assays were preserved in 10% formaldehyde. After decalcification, dehydration, paraffin embedding, and sectioning (5 μm), the tissues were stained with hematoxylin and eosin. The pathological changes were observed using a Nikon Eclipse E100 microscope (Nikon, Tokyo, Japan). Every paw tissue slice was randomly chosen from each group. The images were captured using a Nikon DS-U3 (Nikon, Tokyo, Japan).

2.13. Malondialdehyde and NO assay

The malondialdehyde (MDA) and NO contents of paw tissue samples were determined by thiobarbituric acid reactive substances using the MDA assay kit (Jiancheng Institute of Biotechnology engineering, Nanjing, China) and NO assay kit (Beyotime Biotechnology, Shanghai, China), respectively.

2.14. Tumor necrosis factor-α and interleukin 6 assay

The contents of tumor necrosis factor (TNF)-α and interleukin 6 (IL-6) in paw tissue samples were determined using the enzyme-linked immunosorbent assay kit (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

2.15. Statistical analysis

Single-factor analysis of variance was used to compare between groups. Statistical analyses were performed using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± standard deviation. The values of p < 0.05, p < 0.01, and p < 0.001 were considered to be statistically significant.

3. Results and discussion

3.1. Identification of SP-R1 and SP-R2 from P. stipuleanatus

To confirm the structures, the compounds SP-R1 and SP-R2 have been isolated from P. stipuleanatus and identified using NMR and MS data by comparing with the spectroscopic data reported in the literature [4,5] (Fig. 2).

The molecular formula of the compound SP-R2 was determined to be C_{53}H_{84}O_{23} by positive-mode ESI-MS data at m/z 1111.5023
[M + Na]⁺. The NMR data contained signals for the presence of seven tertiary methyl groups \(\{\delta_{0} 0.77 \text{ (3H, s)}, 0.78 \text{ (3H, s)}, 0.90 \text{ (3H, s)}, 0.92 \text{ (3H, s)}, 0.93 \text{ (3H, s)}, 1.03 \text{ (3H, s)}, 1.14 \text{ (3H, s)}\} \), two olefinic carbons \(\delta c 123.8, 144.6\), one carboxyl group \(\delta c 178.0\), and four anomer carbons \(\delta c 95.7, 104.4, 106.4, 107.9\) (Table 2). The structure of the compound SP-R2 was identified as 3-O-[\(\beta\)-D-glucopyranosyl-(1\(\rightarrow\)3)] [\(\alpha\)-L-arabinofuranosyl-(1\(\rightarrow\)4)]-\(\beta\)-D-glucuronopyranoside-28-O-\(\beta\)-D-glucopyranosyl oleanolic acid and named SP-R2 by comparing the NMR and MS data with the previously reported data [5].

The molecular formula of the compound SP-R1 was shown to be C_77H_120O_45Na_3 based on the (+)-HRESIMS ion peak at m/z 949,4540 [M + Na]⁺. Comparison of the NMR data of SP-R1 (Table 2) with those of SP-R2 suggested that their structures were similar and they differed only in the number of sugar moieties. The sugar moieties was established by the presence of three anomer carbons at \(\delta c 104.4, 106.5, \) and 108.2. The structure of the compound SP-R1 was identified as oleanolic acid 3-O-[\(\beta\)-D-glucopyranosyl-(1\(\rightarrow\)3)] [\(\alpha\)-L-arabinofuranosyl-(1\(\rightarrow\)4)]-\(\beta\)-D-glucuronopyranoside and named SP-R1 by comparing the NMR and MS data with the previously reported data [4].

### 3.2. Analgesic effect of SP-R1 and SP-R2 from P. stipuleanatus

The hot plate and acetic acid–induced writhing tests in mice were two classic models of thermal- and chemical-stimulated acute pain, which were used to evaluate the central and peripheral analgesic effects, respectively [11,12]. To observe the analgesic activity of SP-R1 and SP-R2 on thermal- and chemical-stimulated acute pain, the mice were treated with isotonic saline, aspirin, and two doses of SP-R1 and SP-R2. As shown in Table 3, SP-R1 and SP-R2 (1 and 10 mg/kg) in the hot plate test significantly increased the latency time to thermal pain in the reaction times 30, 60, and 90 min, compared with the saline group. SP-R1 at the dose of 10 mg/kg was found to exhibit stronger analgesic activity, with the latency time of 20.70 ± 4.22 and 20.60 ± 5.36 s in the reaction times 30 and 60 min, than aspirin (400 mg/kg), with the latency time of 18.90 ± 4.01 and 20.10 ± 3.25 s, respectively.

Compared with the isotonic saline group, aspirin, SP-R1, and SP-R2 at the dose of 10 mg/kg significantly reduced the number of acetic acid–induced writhing responses (\(p < 0.05\)), whereas SP-R1 and SP-R2 (1 mg/kg) had no inhibition effect (Table 4). The result indicated that SP-R1 and SP-R2 relieved chemical-stimulated acute pain.

It is reported that triterpenoid saponins have excellent analgesic properties owing to their safety and efficacy. For example, total saponins of *Psammosilene tunicioida* inhibited algesia threshold and effectively decreased the contents of MDA, IL-1β, and TNF-α in the inflammatory tissue soak of rats with adjuvant-induced arthritis.

### Table 3

| Group          | Dose (mg/kg) | Hot plate reaction time (s) |
|---------------|-------------|----------------------------|
|               | 30          | 60                        | 90                        |
| Control       | 0           | 12.50 ± 1.84              | 12.80 ± 4.64              | 12.90 ± 3.06              |
| Aspirin       | 400         | 18.90 ± 4.01***           | 20.10 ± 3.25**            | 19.40 ± 2.95***           |
| SP-R1         | 1           | 17.40 ± 2.84***           | 19.20 ± 2.74**            | 16.10 ± 2.96**            |
|               | 10          | 20.70 ± 4.22***           | 20.60 ± 5.36**            | 18.70 ± 4.37***           |
| SP-R2         | 1           | 17.70 ± 5.06**            | 16.60 ± 3.53              | 16.70 ± 5.36              |
|               | 10          | 18.50 ± 4.93**            | 18.10 ± 3.70*             | 18.10 ± 4.20**            |

\(^{*}p < 0.05, \text{compared with the negative control group.}\)^
\(^{**}p < 0.01, \text{compared with the negative control group.}\)
\(^{***}p < 0.001, \text{compared with the negative control group.}\)

SD, standard deviation; SP-R1: stipuleanoside R1; SP-R2: stipuleanoside R2.

Values are mean ± SD (n = 10).

### Table 4

| Group          | Dose (mg/kg) | Number of writhing |
|---------------|-------------|--------------------|
| Control       | 0           | 56.78 ± 4.78       |
| Aspirin       | 400         | 40.85 ± 5.48       |
| SP-R1         | 1           | 50.47 ± 2.89       |
|               | 10          | 41.14 ± 2.19       |
| SP-R2         | 1           | 54.49 ± 5.26       |
|               | 10          | 40.16 ± 3.28       |

\(^{*}p < 0.05, \text{compared with the negative control group.}\)

### Table 5

| Group          | Dose (mg/kg) | OD value | Inhibition (%) |
|---------------|-------------|----------|---------------|
| Model         | -           | 0.452 ± 0.092 | -              |
| Dexamethasone | 10          | 0.182 ± 0.135 ** | 59.73          |
| SP-R1         | 1           | 0.235 ± 0.048 ** | 48.01          |
|               | 10          | 0.168 ± 0.044 ** | 62.83          |
| SP-R2         | 1           | 0.286 ± 0.084 ** | 36.73          |
|               | 10          | 0.254 ± 0.014 ** | 43.81          |

\(^{*}p < 0.01, \text{compared with the model group.}\)

SD, standard deviation; SP-R1: stipuleanoside R1; SP-R2: stipuleanoside R2; OD: optical density.

Values are mean ± SD (n = 10).

### 3.3. Anti-inflammatory effects of SP-R1 and SP-R2 from P. stipuleanatus

The anti-inflammatory effects of triterpenoid saponins SP-R1 and SP-R2 from *P. stipuleanatus* were evaluated by a series of acute and chronic inflammatory models *in vivo*. In the early stage of inflammation, the main manifestations are telangiectasia, increased permeability, inflammatory exudation, and tissue edema. Acetic acid–induced vascular permeability, xylene-induced ear edema, and carrageenan-induced paw edema in mice are commonly used animal models of acute inflammation, suitable for screening of anti-inflammatory drugs [20,21]. In the acetic acid–induced vascular permeability model, the permeability of abdomen capillaries was significantly decreased by the treatment with SP-R1 and SP-R2 (\(p < 0.01\)) (Table 5). SP-R1 (10 mg/kg) exhibited stronger inhibitory activity with an inhibition rate of 62.83%, than dexamethasone (10 mg/kg). In the xylene-induced ear edema model, the inhibition effect of ear edema was observed in the SP-R1 (10 mg/kg) treatment group, and the inhibition rate was 33.33% (\(p < 0.05\)), while the dexamethasone group (10 mg/kg) showed significant inhibition of ear edema, with an inhibition rate of 45.42% (\(p < 0.001\)) (Fig. 4A). However, SP-R1 had no obvious inhibitory effect on ear edema. Xylene is a commonly used chemical inflammatory agent that can induce the release of proinflammatory mediators (such as histamine and serotonin), thereby promoting vasodilation and leukocyte infiltration [11]. It was suggested that SP-R2 might show inhibitory effect on ear edema by inhibiting vasodilation and leukocyte infiltration. Further histopathological analysis of ear tissue sections showed that SP-R2 at the dose of 10

**[17,18]**. The triterpenoid saponins from *Stauntonia chinensis* enhanced the threshold of thermal- and chemical-stimulated acute pain, which directly increased the inhibitory synaptic response in mouse cortex neurons at the resting state [19]. *P. stipuleanatus* was traditionally used to treat pain-related diseases. In this report, we investigated its antinociceptive properties. Two thermal and chemical nociception models were used to elucidate the analgesic effect of the isolated pure triterpenoid saponins.
mg/kg and dexamethasone significantly reduced xylene-induced edema and inflammatory cell infiltration in the ear tissue (Fig. 5).

As shown in Fig. 4B, at 1 h after injection of carrageenan, acute inflammation of the right hind paw was observed from 1 h to 6 h, and paw edema peaked at 4 h. SP-R1 and SP-R2 at the dose of 1 and 10 mg/kg showed inhibition of paw edema in both early and late phases of the experiment. Compared with the model group, SP-R1 (10 mg/kg) and SP-R2 (10 mg/kg) showed inhibition rates of 25.70% and 29.76% at the 1st h, which increased to 33.76% and 55.40% at the 4th h and decreased to 28.92% and 46.61% at the 6th h in the late phase, respectively. The inhibition rate of SP-R2 (10 mg/kg) at 4 h was 55.40% (p < 0.001) and was better than dexamethasone (51.38%, 10 mg/kg). Pathological analysis of paw tissue sections showed that compared with the negative group, the connective tissue of paw tissue in the model group was significantly damaged. The paw tissue showed swelling, and the connective tissue had obvious inflammatory cell infiltration. SP-R1 (1 and 10 mg/kg), SP-R2 (1 and 10 mg/kg), and dexamethasone significantly reduced carrageenan-induced edema and inflammatory cell infiltration in the connective tissue (Fig. 6).

Fig. 5. Histopathological examinations of xylene-induced mouse ear tissue. (A) Control group: noninflamed ear that received only acetone; (B) model group: inflamed ear that received 30 μL of xylene; (C) positive control group: inflamed ear that was treated with dexamethasone (10 mg/kg); (D) inflamed ear that was treated with SP-R2 (1 mg/kg); (E) inflamed ear that was treated with SP-R2 (10 mg/kg). The tissue sections were observed under a microscope at 200× magnification.

SP-R2, stipuleanoside R2.
Chronic inflammation is caused by the persistent effects of inflammatory factors, characterized by proliferation of small vessels and connective tissue. As a chronic inflammatory model, cotton pellet–induced granuloma in mice was previously used to investigate the effects of drugs on the formation of cellular and inflammatory edema [22,23]. As shown in Table 6, SP-R1 and SP-R2 significantly inhibited granuloma formation. After treatment with SP-R1 and SP-R2 (10 mg/kg), SP-R1 and SP-R2 showed inhibition rates of 25.71% and 24.59%, respectively, and dexamethasone treatment showed an inhibition rate of 48.47%. The two saponins inhibited granuloma formation, and it is possible to inhibit synthesis of collagen and mucopolysaccharide during the formation of granuloma tissue.

Table 6

| Group         | Dose (mg/kg) | Granuloma weight (mg) | Inhibition (%) |
|---------------|--------------|-----------------------|----------------|
| Model         | -            | 9.80 ± 1.61           | -              |
| Dexamethasone | 10           | 5.05 ± 0.93**         | 48.47          |
| SP-R1         | 1            | 8.15 ± 1.26*          | 16.84          |
|               | 10           | 7.28 ± 1.58**         | 25.71          |
| SP-R2         | 1            | 7.95 ± 1.98*          | 18.88          |
|               | 10           | 7.39 ± 1.46**         | 24.59          |

*p < 0.05, compared with the model group.

**p < 0.01, compared with the model group.

SD, standard deviation; SP-R1: stipuleanoside R1; SP-R2: stipuleanoside R2.

Values are mean ± SD (n = 10).

Fig. 6. Histopathological examinations of carrageenan-induced paw tissue swelling and inflammatory cell infiltration. (A) Control group: noninflamed paw that received only sterile 0.9% saline; (B) model group: inflamed paw that received 30 μL of 1% carrageenan; (C) positive control group: inflamed paw that was treated with dexamethasone (10 mg/kg); (D) inflamed paw that was treated with SP-R1 (1 mg/kg); (E) inflamed paw that was treated with SP-R1 (10 mg/kg); (F) inflamed paw that was treated with SP-R2 (1 mg/kg); (G) inflamed paw that was treated with SP-R2 (10 mg/kg). The tissue sections were observed under a microscope at 100× magnification. SP-R1, stipuleanoside R1; SP-R2, stipuleanoside R2.
3.4. SP-R1 and SP-R2 suppressed the production of NO, MDA, TNF-α, and IL-6 in mice with paw edema

To assess the anti-inflammatory effects of SP-R1 and SP-R2, the production of inflammatory mediators (NO, MDA, TNF-α, and IL-6) in the carrageenan-induced paw edema model was determined by NO and MDA assay and TNF-α and IL-6 enzyme-linked immunosorbent assay methods. As shown in Fig. 7, compared with the control group, the levels of NO (Fig. 7A), MDA (Fig. 7B), TNF-α (Fig. 7C), and IL-6 (Fig. 7D) increased significantly in paw tissues after injection of carrageenan \((p < 0.05)\). SP-R1 at the dose of 1 and 10 mg/kg significantly and dose dependently decreased the level of NO, MDA, and IL-6 in paw tissues in comparison with the model group \((p < 0.05, p < 0.01 or p < 0.001)\). The inhibitory effect of SP-R1 on TNF-α was similar at the concentrations of 1 and 10 mg/kg. At the same time, SP-R2 (1 and 10 mg/kg) significantly decreased NO, MDA, and TNF-α levels in paw tissues with edema \((p < 0.05, p < 0.01 or p < 0.001)\). But SP-R2 had no inhibitory effect on IL-6 production.

This proinflammatory cytokines are produced by inflammatory cells after stimulation, as it occurs with injury, infection, and inflammation. TNF-α is an important mediator involved in the inflammatory response. It can activate the cytokine cascade reaction and further induce the release of IL-6 and NO in acute or chronic inflammation [24]. The inhibition of TNF-α, IL-6, and NO production can effectively alleviate the inflammatory response [25]. In our study, the contents of TNF-α, IL-6, and NO in paw tissues were significantly decreased with SP-R1 treatment. SP-R2 significantly reduced the levels of NO and TNF-α in paw tissues with edema, but it had no inhibitory effect on the production of IL-6. IL-6 is a multifunctional cytokine that plays both proinflammatory and anti-inflammatory roles [26]. The anti-inflammatory activity of IL-6 is mediated by classical signaling, and its cell activation is through the combination of IL-6 and the membrane-bound IL-6 receptor. However, the proinflammatory activity of IL-6 is mediated by trans-signaling, and its cell activation expresses the membrane glycoprotein gp130 via the IL-6–soluble IL-6 receptor complex. In our study, SP-R1 and SP-R2 selectively inhibit IL-6 production, possibly owing to the activation of different IL-6 signaling pathways. Accumulation of oxygen metabolites in vivo can damage tissues and lead to inflammatory and pathological changes [27]. The increase of MDA and decrease of superoxide dismutase (SOD) activity indicate the aggravation of inflammation [28]. SP-R1 and SP-R2 can reduce the content of MDA in paw tissues, which alleviates the excessive damage of

![Fig. 7](image-url)
2.2408% to 3.7877%. Comparing the contents of SP-R1 and SP-R2 in three- to fifteen-year-old rhizomes of *P. stipuleanatus* was 20 times higher than that of SP-R1. In fresh products (PS-9), at the content of fresh products (PS-7 to PS-12) was significantly lower than that in dried products (PS-1 to PS-6). As shown in Table 7, the content of SP-R2 in the dried rhizome of three-year-old *P. stipuleanatus* (PS-3) was 20 times higher than that of SP-R1, but only 4 times higher in fresh products (PS-9). At the same time, the change in this ratio was found in other growth years of fresh *P. stipuleanatus*, which may be caused by the drastic decrease of SP-R1 content during the drying process.

### 3.6. Comparison of contents of SP-R1 and SP-R2 in fresh and dried *P. stipuleanatus*

Traditionally, both fresh and dried *P. stipuleanatus* were used for anti-inflammatory treatment. When saponin-containing medicinal materials are processed from fresh products into dried products, saponin components are easily degraded [29]. For the sake of making rational use of fresh and dried *P. stipuleanatus*, the contents of SP-R1 and SP-R2 in fresh and dried *P. stipuleanatus* of different growth years were determined (Fig. 3 and Table 7). The results showed that the content of SP-R1 in fresh products (PS-7 to PS-12) was significantly lower than that in dried products (PS-1 to PS-6). As shown in Table 7, the content of SP-R2 in the dried rhizome of three-year-old *P. stipuleanatus* (PS-3) was 20 times higher than that of SP-R1, but only 4 times higher in fresh products (PS-9). At the same time, the change in this ratio was found in other growth years of fresh *P. stipuleanatus*, which may be caused by the drastic decrease of SP-R1 content during the drying process.

### 4. Conclusions

In summary, the ethanol extract was subjected to chromatography to reveal two oleane triterpenoid saponins, SP-R1 and SP-R2. SP-R1 and SP-R2 possessed potent analgesic and anti-inflammatory activity in vivo, and their main role was suppression of inflammatory mediator production and regulation of antioxidant systems. The contents of triterpenoid saponins from *P. stipuleanatus* collected at different growth years and from different producing areas were also investigated, indicating that the rational harvest of three- to five-year-old *P. stipuleanatus* was preferable to obtain a higher level of triterpenoid saponins. SP-R2 content was the highest in *P. stipuleanatus*, which had potential as a chemical marker for quality control of *P. stipuleanatus*. In this study, the triterpenoid saponins in *P. stipuleanatus* have potential anti-inflammatory effects, which provide a basis for the development and utilization of *P. stipuleanatus*.

### Conflicts of interest

The authors declare that they have no competing interests.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2020.05.002.
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