

Isolation of endophytic fungi with anti-inflammatory effect *in vitro* from *Zanthoxylum armatum*

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Abstract: Endophytic fungus is a rich source of bioactive metabolites with multiple activities. In the present study, eight endophytic fungal strains (Z-1 to Z-8) from *Zanthoxylum armatum* DC. were isolated and identified, and the anti-inflammatory effect of their extracts was evaluated *in vitro*. The results showed that four endophytic fungal extracts (*Fusarium oxysporum* Schlecht. (Z-1), *Alternaria arborescens* E.G Simmons (Z-3), *Mucor fragilis* Bainier (Z-4) and *Aspergillus terreus* Thom (Z-6)) displayed good activities in inhibition of excessive production of nitric oxide in lipopolysaccharide-activated mouse macrophage RAW264.7 cells. Among them, the extracts of *Fusarium oxysporum* Schlecht showed the maximum inhibition activity with a half maximal inhibitory concentration of 174.0 µg/mL. The underlying mechanism of the anti-inflammatory effect of *Fusarium oxysporum* Schlecht extract was associated with its inhibition on the release of several inflammatory factors. The mRNA expression of interleukin 1β, interleukin 6, inducible nitric oxide synthase in lipopolysaccharide-activated mouse macrophage RAW264.7 cells were all inhibited by Z-1 extract detected by real-time polymerase chain reaction. In summary, several endophytic fungal from *Zanthoxylum armatum* have good anti-inflammatory activity and *Fusarium oxysporum* Schlecht possesses a promising potential to be developed to a novel anti-inflammatory agent.

Keywords: Endophytic fungi; *Zanthoxylum armatum*; Anti-inflammation; Real-time polymerase chain reaction

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1. Introduction

Zanthoxylum armatum DC. (family of Rutaceae), a strongly aromatic shrub, is extensively distributed in the southwest provinces of China and India. It is mainly used for the treatment of stomachache, acute appendicitis and pelvic inflammatory disease in Traditional Chinese

Medicine (TCM)^[1]. Our early studies found that the analgesia mechanism of *Zanthoxylum armatum* mainly involves in the periphery inflammatory analgesia, and its main active components are alkaloids^[2] and lignans^[3,4]. Due to large amount of excavation in recent years, the wild resource of *Zanthoxylum armatum* does not meet the market demand currently. Therefore, searching for new alternative sources of *Zanthoxylum armatum* becomes imminent.

Endophytic fungal from medicinal plants could produce the same or similar compounds as the host produced^[5,6], thus the fermentation of endophytic fungal has been widely used in the mass production of drugs due to its benefits of industrial safety and cost effectiveness^[7,8].

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It will solve the lack of *Zanthoxylum armatum* resource to search for new anti-inflammatory metabolites from endophytic fungi of *Zanthoxylum armatum*^[9,10]. The objective of this study was to screen the endophytic fungi strains from *Zanthoxylum armatum* with potent anti-inflammatory effect. Eight endophytic fungi strains from *Zanthoxylum armatum* were isolated and the anti-inflammatory effect of their extracts on lipopolysaccharide (LPS)-activated mouse macrophage RAW264.7 cells were evaluated with the previous reported method^[11,12]. The results provided an experimental foundation for further study of endophytic fungi strains from *Zanthoxylum armatum*.

2. Materials and methods

2.1. Cell line

This experiment was conducted using mouse mononuclear macrophage RAW264.7 cell line, which was purchased from the Basic Medical Cell Center of the Institute of Basic Medicine (Chinese Academy of Medical Sciences).

2.2. Plant materials

Plant materials, used in this experiment were collected in Longnan, Gansu Province in August 2019. The roots and stems of *Zanthoxylum armatum* were identified by Professor Yang Lin. The specimen was preserved in the specimen room of the School of Life Science and Engineering of Lanzhou University of Technology.

2.3. Drugs and reagents

Dulbecco's Modified Eagle's Medium (DMEM), Lipopolysaccharide (LPS), Trypsin, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Fetal Bovine Serum (FBS), Phosphate Buffer solution (PBS)

and Griess reagent were purchased from Corning Costar Ltd. (Cambridge, MA, USA); Dimethyl Sulfoxide (DMSO), indometacin, TRIZOL, qScript™ cDNA SuperMix. SYBR Green master mix were obtained from Thermo Fisher Scientific (Halethorpe, MD).

2.4. Preparation of extracts from endophytic fungi

Cross sections of the plant tissues were first sterilized with sterile water, sodium hypochlorite, 75% ethanol and sterile water sequentially, then the tissues were inoculated on fresh medium with potato glucose agar and incubated at 37 °C for 5–7 d to magnify endophytic fungi^[13]. The obtained endophytic fungi were isolated and purified, and then cultured in shaking flask for 5 d. The culture medium of the myceliums was filtered and extracted by ethyl acetate and *n*-butanol, respectively. The fungal extracts were finally obtained after concentration by rotary evaporator.

2.5. Molecular identification of endophytic fungi from *Zanthoxylum armatum*

To make an accurate molecular identification, the rDNA ITS regions of the eight endophytic fungi were sequenced and compared with the known species information of the samples in the database and the homology analysis^[14].

2.6. Cell culture and nitrite determination

Mouse macrophage RAW 264.7 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were maintained at 37 °C in a fully humidified environment in incubator with 5% CO₂. For the experiment, cells were cultivated in 96-well plates with 100 μL medium per well (3×10⁵ cell/mL), then incubated overnight under the above conditions for the measurement of cell viability. According to the previous

method of Nitric oxide (NO)^[15], cells were treated with positive control indometacin, DMEM medium, or various concentrations of test samples with or without LPS (1 µg/mL) to stimulate the cells for 24 h. NO concentration were measured using the Griess method. In short, 100 µL of Griess reagent (1% sulfanilamide and 0.1% naphthyl ethylene diaminedihydrochloride in 5% H₃PO₄) were added to aliquots of culture supernatant (100 µL) to form a purple azodye at room temperature for 15 min. Absorbance at 540 nm were measured with Microplate Reader and IC₅₀ values were calculated with origin 7.5 software. Cell viability (> 95%) was evaluated using the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method. Briefly, 0.5 mg/mL MTT was added to the treated cells medium, followed by a further incubation about 4 h under the identical culture condition. Absorbance were recorded at 570 nm wavelength using a microplate reader.

2.7. Real-time polymerase chain reaction

To further evaluated the anti-inflammatory effect of the endophytic fungi, mRNA expression of the cytokines Il-6, iNos, Cox-2 and Il-1β in LPS-induced RAW264.7 cells were analyzed after adding Z-1. The uniformly dispersed RAW264.7 cell suspension was seeded in of 12-well plates with 1 mL per well (3×10⁵ cell/mL) and cultured overnight. 24 h later, the cells were treated with 200 and 400 µg/mL of Z-1 and LPS (1 µg/mL). After cultivation for 6 h, total RNA was extracted from cells using TRIZOL reagent according to the manufacturer's instructions^[16]. The concentration and purity of the total RNA were measured by a NanoDrop spectrophotometer (ND-1000, Thermo Fisher). Under the influence of qScript™ cDNA SuperMix, total RNA was reverse transcribed into cDNA. Real-time PCR assays were performed using SYBR Green PCR master mix (AB Applied Biosystems) in an RT-PCR amplification system (Agilent Mx3000P). Sequences of the primers were listed in Table 1. The mRNA expression level for individual gene

was calculated and normalized to their corresponding β-actin mRNA level.

2.8. Statistical analysis

All statistical analyses were performed on Origin 7.5 software. The results were expressed as the mean ± S.D. *P* < 0.05 was considered as statistically difference.

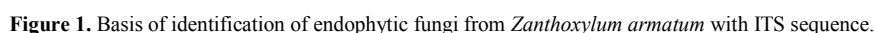
3. Results

3.1. Isolation results of endophytic fungi from *Zanthoxylum armatum*

In this study, eight endophytic fungi were isolated from the stems and roots of healthy *Zanthoxylum armatum*. Using sterile water as a control, no fungus was found grow on the medium. The results showed that the endophytic fungi isolated from *Zanthoxylum armatum* was not contaminated by other impurity fungi and high in purity.

3.2. Identification of endophytic fungi of *Zanthoxylum armatum* with ITS sequence

The endophytic fungi of *Zanthoxylum armatum* were identified with Internal Transcribed Spacer (ITS) sequence. Among the 8 endophytic fungal (laboratory no. Bop214783 to Bop214790) of *Zanthoxylum armatum*, BOP214784 (Z-1) and BOP214787 (Z-2) were identified by ITS sequence as *Fusarium oxysporum* Schlecht. Emend. Snyder & Hansen. BOP214783 (Z-3) is a species of the genus *Alternaria* in the family *Alternaria*, which is most similar to *Alternaria arborescens* E.G. Simmons. BOP214785 (Z-4) and BOP214790 (Z-5) belong to the genus of *Mucor fragilis* Bainier. BOP214789 (Z-6) is *Aspergillus terreus* Thom of *Aspergillus* family. BOP214788 (Z-7) is *Fusarium graminearum* Schwabe. BOP214786 (Z-8) is a species of genus *carnocera* in the family of *carnoceraceae* (*Xylaria* sp.). The basis of identification is showed in Figure 1.



3.3. Anti-inflammatory effect of the endophytic fungi *in vivo*

The endophytic fungi Z-1, Z-3, Z-4, Z-6, Z-7 and Z-8 from *Zanthoxylum armatum* were extracted with ethyl acetate and *n*-butanol and then concentrated into dried powder, respectively. The extracts were separately diluted with DMSO to obtain stock solutions with final concentration of 2 mg/mL. Then the solutions

were diluted to six or eight concentrations, and each concentration was repeated three times. The experiment was carried out according to the procedure established in our lab^[15]. The inhibition effects (IC₅₀) of the endophytic fungi on the NO production were analyzed with Origin 7.5 software. The results (Table 2 and Fig. 2) showed that Z-1 had the best inhibition effect among the six endophytic fungi on the production of NO generated by LPS-induced RAW264.7 cells with IC₅₀ of 174.0 µg/mL (Table 2), though it was weaker than that of the positive drug indomethacin. Z-6 also

Table 1. Sequences of the primers.

Name	Primer	Sequence
IL-1β	Forward	5'-TCAGGCAGGCAGTATCACTC-3'
	Reverse	5'-AGCTCATATGGGTCCGACAG-3'
IL-6	Forward	5'-TACCACTCCCAACAGACCTG-3'
	Reverse	5'-GGTACTCCAGAAGACCAGAGG-3'
Cox-2	Forward	5'-ATAACTGCACCCACTTCCCA-3'
	Reverse	5'-GGGCATCACTTCTACCAGGT-3'
iNos	Forward	5'-ACCCTCACACTCACAAACCA-3'
	Reverse	5'-GGCAGAGAGGAGGTGACTT-3'
β-Actin	Forward	5'-TTCCTTCTGGGTATGGAATC-3'
	Reverse	5'-GAGGTCTAGGAGGTGACTT-3'

Table 2. IC₅₀ of the endophytic fungi on the production of NO in LPS-stimulated RAW264.7 cells.

Named	IC ₅₀ (µg/mL)	SD
Z-1	174.0	4.515
Z-3	1008	33.29
Z-4	784.0	11.20
Z-6	387.0	11.62
Indometacin	55.445	3.714

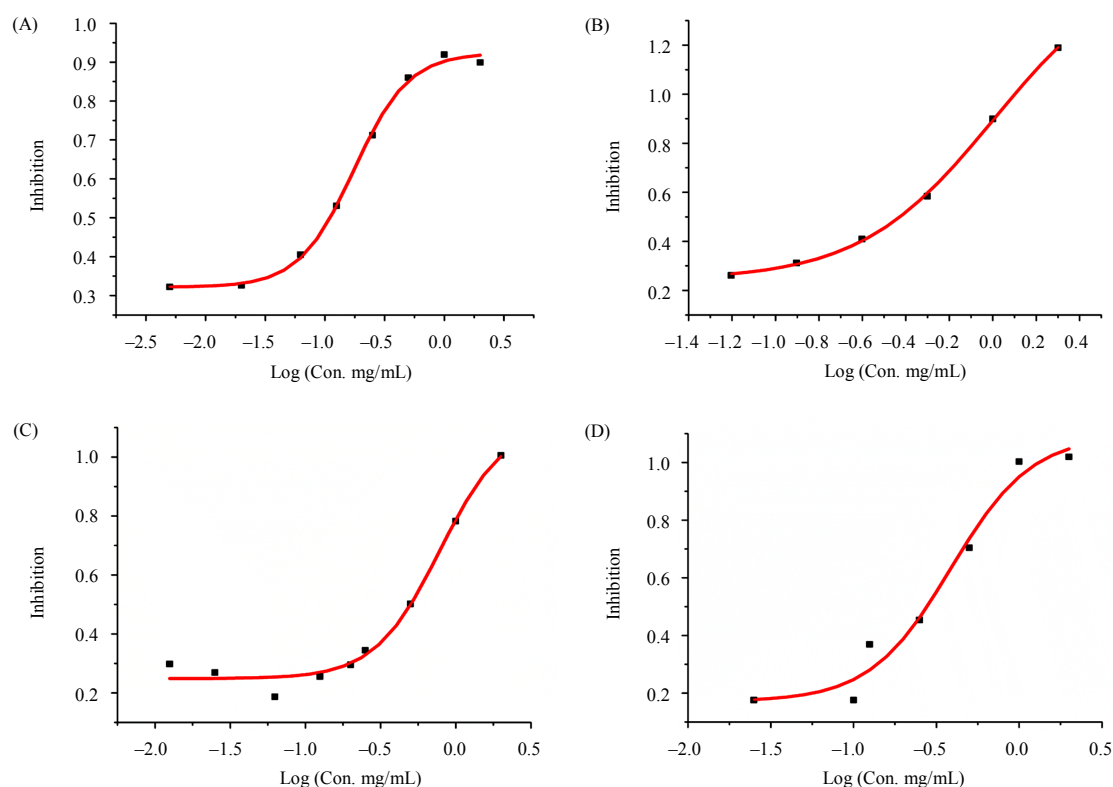


Figure 2. IC₅₀ of the endophytic fungi from *Zanthoxylum armatum* on NO excessive production in RAW264.7 cells. (A) Z-1; (B) Z-3; (C) Z-4; (D) Z-6.

had good activity with IC_{50} at 387.0 $\mu\text{g/mL}$. In addition, the inhibition effect of Z-3, Z-4, Z-7 and Z-8 on the production of NO generated by LPS-induced mouse macrophages RAW264.7 cells was weaker. The IC_{50} values of Z-3 and Z-4 were 1008, and 784.0 $\mu\text{g/mL}$, respectively, and Z-7 and Z-8 did not show inhibition effect.

The results of MTT experiment showed that all the tested endophytic fungi had cell survival rates of > 95% within the concentration ranges, indicating that the endophytic fungi had no toxicity to the cells under the current experimental conditions.

3.4. Effect of Z-1 on the mRNA expression of inflammatory genes

The mRNA expression of cytokines including $IL-1\beta$, $IL-6$, Cox-2 and iNos in the RAW264.7 cells were analyzed (Fig. 3). The results showed that Z-1 had obvious inhibitory effect on the mRNA expression of $IL-1\beta$ and iNos in a concentration dependent manner. It also reduced $IL-6$ mRNA expression at high concentration (400 $\mu\text{g/mL}$), but showed no effect at low concentration of 200 $\mu\text{g/mL}$. Z-1 exhibited no effect for Cox-2 mRNA expression at the two concentrations, suggesting that the anti-inflammatory effect of Z-1 was selective to the pro-inflammatory cytokines.

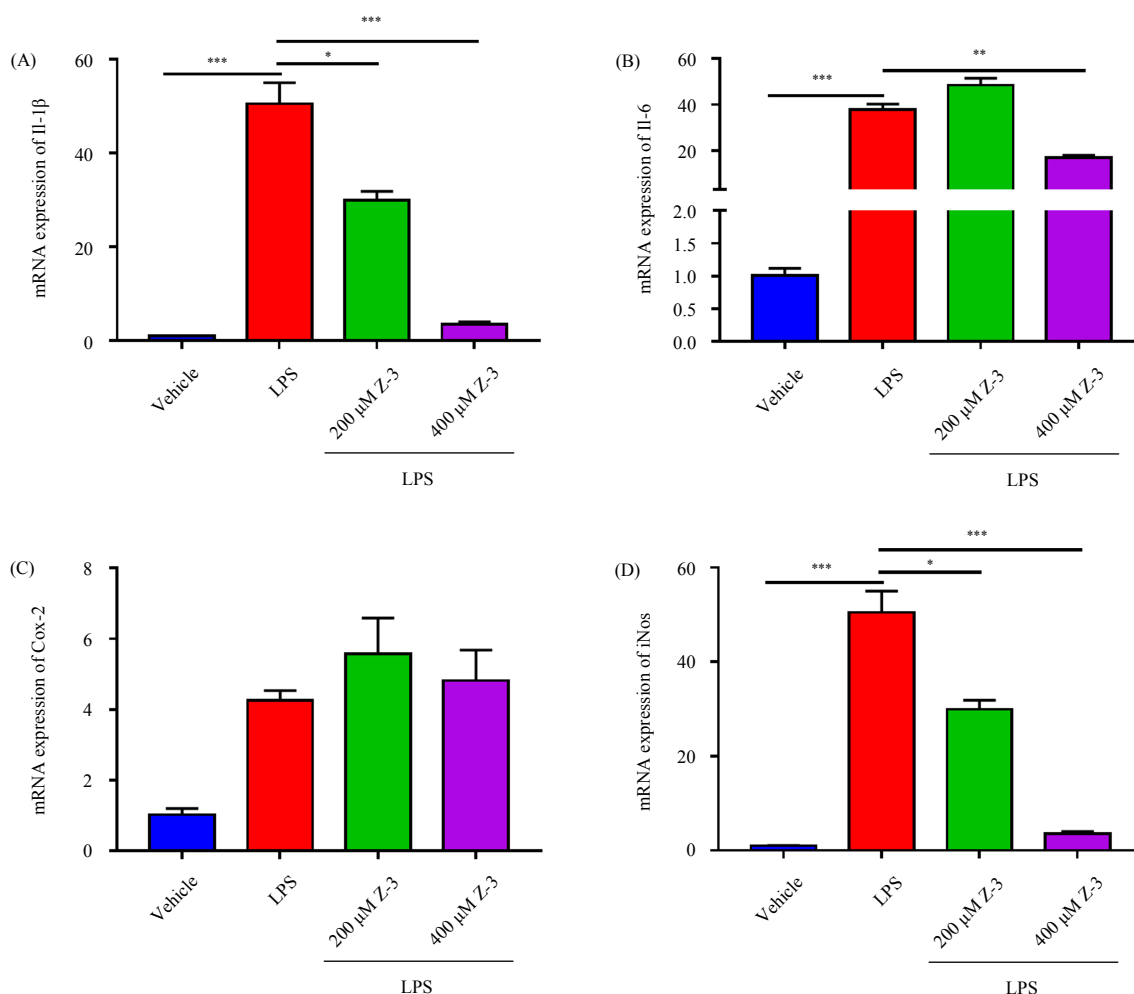


Figure 3. Regulation of Z-1 on the mRNA expression of inflammatory cytokines in RAW264.7 cells. (A) mRNA expression of $IL-1\beta$; (B) mRNA expression of $IL-6$; (C) mRNA expression of Cox-2; (D) mRNA expression of iNos. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Significance was determined by using one-way ANOVA. Data are presented as the mean \pm SEM, $N = 3$ per group.

4. Discussion

Zanthoxylum armatum has a long history of clinical application used as analgesics and anti-inflammatory drug in China. Based on the principle that endophytic fungal from medicinal plants could produce the same or similar compounds as the host produced, the anti-inflammatory activity of endophytic fungi from *Zanthoxylum armatum* was investigated in LPS-activated RAW264.7 cells. Eight endophytic fungal strains from *Zanthoxylum armatum* were isolated and finally identified as six different strains by rDNA ITS sequence. Their anti-inflammatory effect *in vitro* was also evaluated in the present study, respectively. The results showed that four endophytic fungal extracts displayed anti-inflammatory activity in LPS-stimulated RAW264.7 cells. Among them, Z-1 showed the best activity with IC_{50} at 174.0 μ g/mL.

IL-6, iNOS, COX-2 and IL-1 β are important cytokines involved in the inflammatory response in macrophages cell. The mRNA expression results showed that treatment of Z-1 markedly suppressed inflammatory cytokines mRNA expression, including IL-1 β , IL-6 and iNOS, in LPS-stimulated RAW264.7 cells. The present study clearly demonstrated that Z-1 provided effective regulation of the inflammatory reaction by inhibiting the expression of some inflammatory cytokines in macrophages cells.

Due to the potential anti-inflammatory effect, further screening of anti-inflammatory mechanism and active constituents of Z-1 will be performed in the next study. Briefly, this study provides an experimental foundation for further research on potential anti-inflammatory mechanism and compounds from endophytic fungi of *Zanthoxylum armatum*.

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竹叶椒抗炎内生真菌的分离

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摘要: 内生真菌富含具有多种生物活性的次生代谢产物。本研究从竹叶椒中分离鉴定了8种内生真菌, 并通过体外实验对其抗菌活性进行了筛选。结果发现4种内生真菌提取物(*Fusarium oxysporum* Schlecht. (Z-1)、*Alternaria arborescens* E.G Simmons (Z-3)、*Mucor fragilis* Bainier (Z-4)和*Aspergillus terreus* Thom (Z-6))对LPS诱导的RAW264.7细胞一氧化氮过量生成具有显著抑制作用。其中, 真菌 *Fusarium oxysporum* Schlecht提取物具有最好的抗炎活性, 其IC₅₀值为174.0 μg/mL。真菌 *Fusarium oxysporum* Schlecht的抗炎活性与其抑制多种细胞炎症因子的释放有关。实时荧光定量PCR结果显示, 真菌 *Fusarium oxysporum* Schlecht对LPS诱导的RAW264.7细胞中IL-1β、IL-6、iNOS等炎症因子的mRNA表达具有显著抑制作用。总之, 竹叶椒中多种内生真菌具有抗炎活性, 其中*Fusarium oxysporum* Schlecht有望发展成为新的抗炎活性药物。

关键词: 内生真菌; 竹叶椒; 抗炎; 实时荧光定量聚合酶链反应

