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Postprint: Biological Activity of Endophytic Fungi from the Ethnomedicinal Plant Asclepias curassavica

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Abstract

Endophytic fungi from medicinal plants can produce bioactive substances identical or similar to those of their host plants. The ethnic medicine Asclepias curassavica exhibits extensive biological activities. To obtain active endophytic fungal resources from A. curassavica, this study investigated the biological activities of metabolites from 168 endophytic fungal strains based on the research concept of "ethnic medicine-endophytic fungi-bioactive components". The ethyl acetate extracts of fermentation broths from endophytic fungi were evaluated for antitumor, anti-inflammatory, α-glucosidase inhibitory, and antioxidant activities using the SRB method, Griess method, PNPG method, and DPPH method, respectively. Active strains were identified by ITS sequencing. The results showed that among the 168 screened endophytic fungal strains, 22 exhibited varying degrees of biological activity. Specifically, 9 strains demonstrated significant antitumor activity with IC50 values ranging from 0.1 to 40 g·mL-1. Strain MJF-53 at 2.5 g·mL-1 showed significant inhibitory effects on both NO and IL-1β release from LPS-induced Raw264.7 cells. Seven strains exhibited varying degrees of α-glucosidase inhibitory activity with IC50 values between 1.0 and 4.0 mg·mL-1, among which MYF-16 and MYF-55 showed α-glucosidase inhibitory activity comparable to acarbose. Nineteen strains possessed varying degrees of DPPH free radical scavenging activity, with strains MYF-9, MYF-19, and MJF-84 demonstrating moderate antioxidant activity with IC50 values of 13.562, 17.776, and 12.395 g·mL-1, respectively. ITS identification revealed that the 22 active strains belonged to different genera including Alternaria, Colletotrichum, Fusarium, Diaporthe, Talaromyces, and Neofusicoccum parvum. This study demonstrates the diversity of biological activities among endophytic fungi from A. curassavica and lays a foundation for mining potential novel natural compounds with antitumor, anti-inflammatory, antidiabetic, and antioxidant activities from these fungi.

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Full Text

Biological Activities of Endophytic Fungi from Asclepias curassavica

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Abstract: Endophytic fungi in medicinal plants can produce bioactive compounds identical or similar to those of their host plants. Asclepias curassavica, a traditional ethnic medicine, exhibits extensive biological activities. To obtain active endophytic fungal resources from this plant, we investigated the bioactivities of metabolites from 168 endophytic fungal strains isolated from A. curassavica following the research framework of "ethnic medicine-endophytic fungi-bioactive constituents." The ethyl acetate extracts of fungal fermentation broths were evaluated for antitumor, anti-inflammatory, α-glucosidase inhibitory, and antioxidant activities using SRB, Griess, PNPG, and DPPH assays, respectively. Bioactive strains were identified by ITS sequencing. Our results revealed that 22 of the 168 isolated strains exhibited varying degrees of biological activity. Among them, nine strains showed significant antitumor activity with IC_{50} values ranging from 0.1 to 40 $g \cdot mL^{-1}$. Strain MJF-53 at 2.5 $g \cdot mL^{-1}$ significantly inhibited LPS-induced NO and IL-1β release in Raw264.7 cells. Seven strains demonstrated α-glucosidase inhibitory activity with IC_{50} values between 1.0 and 4.0 $mg \cdot mL^{-1}$, with strains MYF-16 and MYF-55 showing inhibitory potency comparable to acarbose. Nineteen strains exhibited DPPH radical scavenging activity, among which MYF-9, MYF-19, and MJF-84 showed moderate antioxidant activity with IC_{50} values of 13.562, 17.776, and 12.395 $g \cdot mL^{-1}$, respectively. ITS identification revealed that the 22 active strains belonged to various genera including *Alternaria*, *Colletotrichum*, *Fusarium*, *Diaporthe*, *Talaromyces*, and *Neofusicoccum parvum*. These findings demonstrate the diverse biological activities of endophytic fungi from A. curassavica and establish a foundation for discovering novel natural antitumor, anti-inflammatory, hypoglycemic, and antioxidant compounds.

Keywords: *Asclepias curassavica*, endophytic fungi, antitumor, anti-inflammatory, α-glucosidase inhibitor, DPPH radical, ITS

Introduction

Microorganisms represent a vital source of natural products, with microbial-derived compounds making significant contributions to nutrition, agriculture, and healthcare (Singh et al., 2017). Endophytic fungi are microorganisms that reside within healthy plant tissues and organs during certain or all stages of their life cycle without causing disease or eliciting host defense responses (Hardoim et al., 2015). Numerous studies have demonstrated that endophytic fungi from medicinal plants possess promising bioactivities, including antitumor, antimicrobial, anti-inflammatory, antioxidant, hypoglycemic, and insecticidal properties. For instance, Wei et al. (2020) isolated endophytic fungus pr10 from *Brassica rapa* that significantly inhibited A549 cell growth, while Ran et al. (2017) obtained endophytic fungi from *Camptotheca acuminata* capable of producing the antitumor compound camptothecin. Sailesh et al. (2020) reported that endophytic fungus QF001 from *Scutellaria baicalensis* roots effectively suppressed LPS-induced expression of pro-inflammatory cytokines NO, TNF-α, and IL-6 in Raw264.7 cells. Geethanjali et al. (2019) found that ethyl acetate extracts of *Chaetomium* sp. from *Catharanthus roseus* exhibited strong DPPH radical scavenging activity, and Afra et al. (2015) documented potent antioxidant capacity in metabolites from endophytic fungi of gourd seeds and bitter apricot. Additionally, Zheng et al. (2015; 2014) isolated an α-glucosidase inhibiting *Alternaria* strain ThF-63 and an antimicrobial and insecticidal *Penicillium* strain ThF-11 from *Tripterygium hypoglaucum*. Consequently, endophytic fungi from medicinal plants are recognized as important sources of bioactive natural products (Sanjana et al., 2012), and exploring novel bioactive compounds from these microorganisms has become a prominent research focus, with their metabolic diversity offering an effective pathway for discovering new natural products.

Asclepias curassavica is commonly used in Dai traditional medicine in Yunnan. With a cool nature and sweet taste, it is traditionally employed to regulate menstruation, activate blood circulation, relieve pain, reduce fever, and reduce inflammation and swelling (Jiang and Feng, 2006). The plant has been used to treat various conditions including inflammation, diarrhea, gonorrhea, rheumatism, and cardiovascular diseases (Jiang and Song, 2017; Yang et al., 2016; Li, 2017). Current research on A. curassavica has primarily focused on pharmacodynamics, revealing antimicrobial, anticancer, cardiovascular, analgesic, and antipyretic activities in its extracts (Ali, 2015; Raja and Ravindranadh, 2014). Regarding mechanisms, Zheng et al. (2019) demonstrated that the ethyl acetate extract of A. curassavica significantly inhibited proliferation of multiple tumor cell lines including A549, Hela, SK-OV-3, MGC-803, and NIC-H1975, and induced apoptosis in NIC-H1975 cells through activation of p38 and JNK MAPK signaling pathways. However, studies on endophytic fungi from A. curassavica and their biological activities remain scarce.

Research has confirmed that medicinal plants harbor diverse endophytic fungi that, through long-term coevolution with their hosts, may acquire the genetic material or information to produce compounds identical or similar to those

of the host (Wang et al., 2020). Stierle et al. (1993) famously isolated a taxol-producing endophytic fungus from Pacific yew. Therefore, exploring endophytic fungi represents an effective strategy for discovering natural bioactive products. Based on this rationale, we hypothesized that endophytic fungi from A. curassavica might possess antitumor, anti-inflammatory, and antioxidant activities. Following the research paradigm of "medicinal plant-endophyte-bioactivity," we conducted the first comprehensive study evaluating 168 endophytic fungal strains from A. curassavica collected in Xishuangbanna for antitumor, anti-inflammatory, α-glucosidase inhibitory, and DPPH radical scavenging activities. This work establishes a foundation for discovering novel antitumor, anti-inflammatory, hypoglycemic, and antioxidant compounds while contributing to the conservation of wild A. curassavica resources.

Materials and Methods

1.1 Materials A total of 168 endophytic fungal strains were isolated from A. curassavica, comprising 106 strains from stems (MJF-1 to MJF-106) and 62 strains from leaves (MYF-1 to MYF-62). These strains were maintained on potato dextrose agar (PDA) and potato dextrose broth (PDB) media. The cell lines used in this study included human non-small cell lung cancer NIC-H1975, human breast cancer MCF-7, human colon cancer HCT-116, human cervical cancer Hela, human hepatocellular carcinoma HepG2, and mouse mononuclear macrophage Raw264.7. NIC-H1975 and MCF-7 cells were cultured in RPMI1640 complete medium, while the other cell lines were maintained in high-glucose DMEM complete medium. All cell lines were purchased from the Cell Bank of Kunming Institute of Zoology, Chinese Academy of Sciences.

1.2 Culture Media Potato dextrose agar (PDA) consisted of potato 200 g, glucose 20 g, agar 15 g, and distilled water 1000 mL, with natural pH. Potato dextrose broth (PDB) contained potato 200 g, glucose 20 g, and distilled water 1000 mL, with natural pH. RPMI1640 complete medium was prepared by mixing 900 mL RPMI1640 basal medium, 100 mL fetal bovine serum, and 10 mL penicillin-streptomycin solution. High-glucose DMEM complete medium was prepared by mixing 900 mL high-glucose DMEM, 100 mL fetal bovine serum, and 10 mL penicillin-streptomycin solution. All media were stored at 4 °C.

1.3 Instruments and Equipment The main instruments included a DHZ-052D constant temperature shaker (Shanghai Bo Cai Biological Technology Co., Ltd.), DHP-9052 incubator (Shanghai Yi Heng Technology Co., Ltd.), Heidolph rotary evaporator (Dexiang Technology Co., Ltd.), SpectraMax i3x multi-mode microplate reader (Molecular Devices Shanghai Co., Ltd.), and CO_2 incubator (Thermo Fisher Scientific).

1.4 Culture of Endophytic Fungi and Preparation of Extracts Isolated endophytic fungi were activated on fresh PDA medium and subsequently inoculated into PDB medium. The cultures were incubated at 28 °C with shaking at 150 rpm for 8 days. Mycelia were removed by filtration, and the fermentation broth was extracted three times with ethyl acetate. The combined extracts were concentrated using a rotary evaporator to obtain crude extract. The extracts were dissolved in DMSO to prepare stock solutions at an initial concentration of 200 $mg \cdot mL^{-1}$ and stored at 4 °C.

1.5 Determination of Antitumor Activity Antitumor activity was evaluated using the SRB method (Skehan et al., 1990). Exponentially growing tumor cells (NIC-H1975, MCF-7, HCT-116, Hela, and HepG2) were seeded in 96-well plates at 4$×10^{4}$ $cells \cdot mL^{-1}$ (100 µL per well) and incubated at 37 °C with 5% CO_2 for 24 h. Various concentrations of extracts (40, 20, 10, 5, 2.5, and 1.25 $\mu g \cdot mL^{-1}$) were added (100 µL per well) with three replicates per concentration, alongside three control wells. After 48 h incubation, the supernatant was removed, and cells were fixed with 100 µL of ice-cold 10% trichloroacetic acid per well at 4 °C for 1 h. The plates were washed five times with distilled water and air-dried. Cells were stained with 100 µL of 4 $mg \cdot mL^{-1}$ SRB solution per well at room temperature for 15 min. After removing the stain, plates were washed five times with 1% acetic acid, air-dried, and 100 µL of 10 $mmol \cdot L^{-1}$ Tris solution was added per well. After 5 min at room temperature, optical density was measured at 560 nm. The inhibition rate was calculated using the formula:

$$I = \frac{A_c - A_y}{A_c} \times 100\%$$

where I represents inhibition rate, A_y is the OD value of the treatment group, and A_c is the OD value of the control group. IC_{50} values were calculated using Origin software.

1.6 Anti-inflammatory Activity Assays 1.6.1 Cell Viability Assessment Cell viability was determined using the SRB method (Skehan et al., 1990). Exponentially growing Raw264.7 cells were seeded in 96-well plates at 3$×10^{5}$ cells per well (100 µL) and treated with various concentrations of extracts (10, 5, 2.5, 1.25, 0.625, 0.3125, and 0.15625 $\mu g \cdot mL^{-1}$). The treatment group received 50 µL of extract plus 50 µL of high-glucose DMEM complete medium, while the control group received 100 µL of medium. After 24 h incubation at 37 °C with 5% CO_2, cell viability was assessed by SRB assay.

1.6.2 NO Production in LPS-Induced Raw264.7 Cells NO levels were measured using the Griess method (Kim et al., 2020). Raw264.7 cells in exponential growth were seeded in 96-well plates at 3$×10^{6}$ cells per well (100 µL) and divided into three groups: blank (DMEM medium only), model (1.5 $\mu g \cdot mL^{-1}$ LPS + DMEM), and treatment (1.5 $\mu g \cdot mL^{-1}$ LPS + various extract

concentrations [2.5, 1.25, 0.625, 0.3125, and 0.15625 μg · mL^{-1}]). Each group had three replicates. After 24 h incubation, culture supernatants were collected for NO determination.

1.6.3 Cytokine Measurement in LPS-Induced Raw264.7 Cells Cells were treated as described in section 1.6.2. Levels of TNF-α, IL-6, and IL-1β in culture supernatants were measured using ELISA kits according to the manufacturer' s instructions. Cytokine concentrations were calculated from standard curves.

1.7 α-Glucosidase Inhibitory Activity Assay Following the method of Zheng et al. (2015), extracts and acarbose were diluted with phosphate buffer (pH 6.8) to appropriate concentrations. α-Glucosidase lyophilized powder was dissolved at 0.8 U · mL^{-1}, and the substrate PNPG was prepared at 2.5 mmol · L^{-1}. The assay included control (buffer + enzyme + substrate), blank control (buffer only), sample (buffer + extract + enzyme + substrate), sample control (buffer + extract), and positive control (buffer + acarbose + enzyme + substrate) groups. After sequentially adding 20 μL of extract or acarbose (5, 2.5, 1.25, and 0.625 mg · mL^{-1}), 20 μL of phosphate buffer, and 20 μL of α-glucosidase, the mixture was incubated at 37 °C for 15 min. Then 20 μL of 2.5 mmol · L^{-1} PNPG was added and incubated at 37 °C for another 15 min. The reaction was terminated by adding 80 μL of 0.1 mol · L^{-1} Na_2CO_3 solution, and absorbance was measured at 405 nm. Each sample was tested in triplicate, and the experiment was repeated three times. The inhibition rate was calculated as:

$$I = \frac{(A_c - A_b) - (A_{st} - A_{sc})}{A_c - A_b} \times 100\%$$

where I is inhibition rate, $A\_\{st\}$ is the OD value of the sample test group, $A\_\{sc\}$ is the OD value of the sample control group, A_c is the OD value of the control group, and A_b is the OD value of the blank control group. IC_{50} values were calculated using Origin software.

1.8 DPPH Radical Scavenging Activity Assay Following the method of Guo et al. (2020), extracts and vitamin C were diluted with anhydrous ethanol to appropriate concentrations, and DPPH was prepared as a 0.2 mmol · L^{-1} solution in anhydrous ethanol. In 96-well plates, 100 μL of each extract concentration (800, 400, 200, 100, and 50 μg · mL^{-1}) was mixed with 100 μL of DPPH solution, gently mixed, and incubated in the dark at room temperature for 30 min. Absorbance (A_s) was measured at 517 nm. The absorbance of 100 μL DPPH mixed with 100 μL ethanol (A_b) and 200 μL ethanol ($A\_\{ref\}$) were also measured. The scavenging rate was calculated as:

$$I = \frac{A_b - (A_s - A_{ref})}{A_b} \times 100\%$$

where I is the DPPH radical scavenging rate, A_s is the OD value of the sample-DPPH mixture, A_b is the OD value of the DPPH-ethanol mixture, and $A\_\{ref\}$ is the OD value of ethanol. IC_{50} values were calculated using Origin software.

1.9 Identification of Active Strains Following the method of Tan et al. (2014), mycelia (0.5–1 g) of purified active strains were rapidly ground in liquid nitrogen, and fungal DNA was extracted using a Tiangen fungal DNA extraction kit according to the manufacturer' s protocol. PCR amplification was performed using universal primers ITS4 (TCCTCCGCTTATTGATATGC) and ITS5 (GGAAGTAAAAGTCGTAACAAGG) under the following conditions: initial denaturation at 95 °C for 4 min; 30 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min; final extension at 72 °C for 10 min. PCR products (5 µL) were analyzed by agarose gel electrophoresis and sequenced by Tsingke Biotechnology Co., Ltd. The resulting sequences were compared against the NCBI database (http://blast.ncbi.nlm.nih.gov/) to determine the taxonomic identity of active strains.

Results

2.1 Antitumor Activity of Endophytic Fungal Metabolites The antitumor activity of 168 endophytic fungal strains was evaluated using the SRB assay. As shown in Table 1 , nine strains exhibited broad-spectrum and significant cytotoxic effects, with MYF-16 showing the strongest activity, surpassing that of cisplatin. Most active strains were more potent against NIC-H1975, HCT-116, and Hela cells than against MCF-7 and HepG2 cells. Strain MJF-33 was particularly sensitive to HepG2 cells ($IC_{50} = 0.147\ \mu g \cdot mL^{-1}$) but showed $IC_{50} > 10\ \mu g \cdot mL^{-1}$ against the other four tumor cell lines. Geographically, active strains were predominantly isolated from stems (8 strains), with only one active strain (MYF-16) obtained from leaves. However, leaf-derived endophytes generally exhibited stronger antitumor activity than stem-derived ones, possibly reflecting that antitumor constituents in the host plant are primarily concentrated in leaves.

2.2 Effects of MJF-53 on IL-6, IL-1β, and TNF-α in LPS-Induced Raw264.7 Cells Using an inflammatory cell model, we examined the effects of endophytic fungal metabolites on NO production in Raw264.7 cells. Strain MJF-53 at concentrations below $2.5\ \mu g \cdot mL^{-1}$ showed no cytotoxicity and concentration-dependently inhibited NO secretion (Figure 1 [Figure 1: see original paper]a, b). Further investigation revealed that MJF-53 extract at $2.5\ \mu g \cdot mL^{-1}$ selectively suppressed IL-1β production without affecting TNF-α or IL-6 secretion (Figure 1c, d), suggesting that MJF-53 may produce anti-inflammatory constituents that inhibit upstream signaling pathways related to IL-1β secretion, thereby suppressing inflammation.

2.3 Effects of Endophytic Fungal Metabolites on α-Glucosidase Activity Seven endophytic fungal strains with promising α-glucosidase inhibitory activity were identified through screening. These strains showed concentration-dependent inhibition, with >70% inhibition at 5 $mg \cdot mL^{-1}$ and IC_{50} values < 4 $mg \cdot mL^{-1}$. MYF-16 and MYF-55 exhibited the strongest inhibitory activity with IC_{50} values of 1.996 and 1.823 $mg \cdot mL^{-1}$, respectively, approaching the potency of acarbose. Results are summarized in Tables 2 and 3 .

2.4 DPPH Radical Scavenging Activity of Endophytic Fungal Metabolites DPPH assay of 168 endophytic fungal extracts revealed that 19 strains showed >50% DPPH radical scavenging activity at 800 $\mu g \cdot mL^{-1}$, with 11 strains achieving >80% scavenging. Strains MYF-9, MYF-19, and MJF-84 demonstrated strong antioxidant activity with IC_{50} values of 13.562, 17.776, and 12.395 $\mu g \cdot mL^{-1}$, respectively. Thirteen active strains were isolated from stems and six from leaves. Results are presented in Tables 4 and 5 .

2.5 ITS Identification of Active Strains NCBI BLAST analysis revealed that all active strains showed approximately 100% sequence similarity and were distributed across six genera: *Alternaria*, *Colletotrichum*, *Fusarium*, *Diaporthe*, *Talaromyces*, and *Neofusicoccum parvum* (Table 6). Notably, nine strains belonged to *Alternaria* and five to *Diaporthe*, while the remaining four genera comprised only eight strains total. The broad-spectrum active strains MYF-16, MJF-53, and MJF-64 were also affiliated with *Alternaria* and *Diaporthe*, suggesting these two genera may represent dominant active endophytic fungi in A. curassavica.

Discussion and Conclusion

Endophytic fungi from medicinal plants produce structurally novel, diverse secondary metabolites with broad biological activities, representing an important source of bioactive natural products (Sunil et al., 2018; Geethanjali et al., 2019). Through long-term symbiotic relationships, host plants may transfer genetic material or information to their endophytic fungi, enabling similar metabolic pathways and resulting in the production of compounds with identical or comparable bioactivities to those of the host. *Asclepias curassavica* is widely used in Dai medicine to treat cancer, asthma, cardiotonic conditions, inflammation, rheumatism, fever, diarrhea, gonorrhea, and cardiovascular diseases (Jiang and Song, 2017; Yang et al., 2016; Li, 2017), indicating diverse bioactivities, particularly antitumor and anti-inflammatory effects. Our previous studies demonstrated that A. curassavica exhibits significant broad-spectrum antitumor activity by inducing apoptosis in NIC-H197 cells through p38 and JNK MAPK pathway activation (Zheng et al., 2019). In the current study, MYF-16 showed remarkable broad-spectrum bioactivity, particularly potent antitumor effects against non-small cell lung cancer NIC-H1975 cells. We hypothesize that MYF-16 may

produce compounds with bioactivities similar to those of the host plant, consistent with previous reports of taxol-producing endophytic fungi from yew (Stierle et al., 1993; Somjaipeng et al., 2015) and camptothecin-producing endophytic fungi from *Camptotheca acuminata* (Ran et al., 2017). Therefore, MYF-16 represents a promising target strain for further investigation of its bioactive metabolites and antitumor mechanisms.

NLRP3 inflammasome activation plays a crucial role in regulating inflammatory responses (Patel et al., 2017; Mao et al., 2017). Signal molecules such as ATP promote intracellular potassium efflux, mediating the assembly of NLRP3, ASC, and caspase-1 into protein complexes that activate caspase-1 autolysis into p20 and p10 subunits. This process drives the maturation and release of pro-inflammatory cytokines IL-1β and IL-18, triggering inflammatory reactions (Ding and Hu, 2018). Our study found that MJF-53 at 2.5 $\mu g \cdot mL^{-1}$ effectively inhibited LPS-induced secretion of NO and IL-1β in Raw264.7 cells without apparent cytotoxicity. We speculate that this strain may produce anti-inflammatory constituents that suppress IL-1β secretion by inhibiting NLRP3 inflammasome activation, thereby blocking IL-1β maturation and release. The specific anti-inflammatory mechanisms and in vivo effects require further validation.

Medicinal plants harbor diverse endophytic fungi that serve as important sources of natural bioactive compounds. ITS identification revealed that the 22 active strains belonged to six genera: *Alternaria*, *Colletotrichum*, *Fusarium*, *Diaporthe*, *Talaromyces*, and *Neofusicoccum*, with nine strains in *Alternaria* and five in *Diaporthe*. The broad-spectrum active strains MYF-16, MJF-53, and MJF-64 were affiliated with these two dominant genera, suggesting that *Alternaria* and *Diaporthe* may represent predominant active endophytic fungi in A. curassavica. This aligns with reports by Tan et al. (2015) identifying *Alternaria*, *Fusarium*, and *Colletotrichum* as dominant fungal communities in medicinal plants. Furthermore, endophytic fungal diversity is closely related to the host plant' s environment. Liu et al. (2010) demonstrated that the species and abundance of endophytic fungi in *Polygonum cuspidatum* correlate with seasonal variations, while Jia et al. (2014) found significant differences in endophytic fungal communities from *Ginkgo biloba* across different geographic regions. Therefore, to obtain more diverse active endophytic fungal resources from A. curassavica, future studies should consider sampling from different seasons, multiple locations, and various plant organs.

In summary, this study identified multiple strains with antitumor, anti-inflammatory, α-glucosidase inhibitory, and DPPH radical scavenging activities, establishing a foundation for discovering novel bioactive compounds from A. curassavica endophytic fungi. These findings provide valuable guidance for developing and expanding the applications of this important Dai medicinal plant.

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