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Postprint: Isolation, Identification, and Microbial Transformation of Endophytic Fungus *Colletotrichum queenslandicum* KJT-1 from *Tinospora sinensis*

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Abstract

To fully exploit the endophytic fungal resources of Tinospora sinensis and obtain strains capable of microbial transformation of the host medicinal material, this study employed tissue isolation method combined with ITS sequence analysis to identify the isolated endophytic fungi; the differences in biological activity and chemical composition of extracts before and after microbial transformation were analyzed and evaluated through antimicrobial experiments, DPPH free radical scavenging assays, and liquid chromatography-mass spectrometry techniques. The results showed that: (1) One strain of endophytic fungus capable of microbial transformation of the host medicinal material was isolated from Tinospora sinensis and identified as Colletotrichum queenslandicum. (2) All extracts from Tinospora sinensis before microbial transformation showed no inhibitory effect against the tested strains, whereas the n-butanol extract from Tinospora sinensis after microbial transformation exhibited inhibitory activity against Staphylococcus aureus, with an MBC value of 31.3 $mg \cdot mL^{-1}$. (3) The DPPH free radical scavenging capacities of the crude extract, n-butanol extract, and ethyl acetate extract from Tinospora sinensis after microbial transformation were decreased compared to those before transformation, while the petroleum ether extract showed enhanced DPPH free radical scavenging capacity compared to before transformation. (4) A total of 33 and 23 compounds were identified and characterized from the n-butanol extracts of Tinospora sinensis before and after microbial transformation, respectively, indicating changes in chemical composition. This study isolated and identified an endophytic fungus with biotransformation capability toward Tinospora sinensis medicinal material, providing theoretical support for the efficient utilization of Tinospora sinensis and biotransformation research.

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Full Text

Isolation, Identification, and Microbial Transformation of Endophytic Fungus *Colletotrichum queenslandicum* KJT-1 from *Tinospora sinensis*

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Abstract

To fully exploit the endophytic fungal resources of *Tinospora sinensis* and obtain strains capable of microbially transforming the host medicinal material, this study employed tissue isolation combined with ITS sequence analysis to identify isolated endophytic fungi. The bioactivity and chemical composition differences of extracts before and after microbial transformation were evaluated through antibacterial assays, DPPH radical scavenging experiments, and liquid chromatography-mass spectrometry (LC-MS). The results demonstrated: (1) One endophytic fungus with microbial transformation capability was isolated from *T. sinensis* and identified as *Colletotrichum queenslandicum*. (2) Pre-transformation extracts of *T. sinensis* showed no inhibitory effects on tested strains, whereas the post-transformation n-butanol extract exhibited inhibitory activity against *Staphylococcus aureus* with a minimum bactericidal concentration (MBC) of 31.3 $mg \cdot mL^{-1}$. (3) The DPPH radical scavenging capacity of crude, n-butanol, and ethyl acetate extracts decreased after microbial transformation, while the petroleum ether extract showed enhanced scavenging ability. (4) Thirty-three compounds were identified in the pre-transformation n-butanol extract, compared to twenty-three compounds post-transformation, indicating altered chemical composition. This study isolated and characterized an endophytic fungus capable of biotransforming *T. sinensis*, providing theoretical support for the efficient utilization and biotransformation research of this medicinal plant.

Keywords: endophytic fungi, *Tinospora sinensis*, microbial transformation, chemical constituents, biological activity

Introduction

Tinospora sinensis (Lour.) Merr., known as "Kuanjinteng" in traditional Chinese medicine, is the dried stem of the Menispermaceae plant *Tinospora sinensis*. Characterized by its cold nature and bitter taste, it is traditionally used to soothe the liver and meridians according to the *Chinese Materia Medica* (State Administration of Traditional Chinese Medicine, 1999) and *Flora of Hunan* (Editorial Board of Flora of Hunan, 2000). Widely distributed in Guangdong, Guangxi, and Yunnan provinces, *T. sinensis* has been traditionally employed for relaxing muscles and tendons, dispelling wind and pain, tonifying deficiency, and regulating menstruation (Jia & Zhang, 2016; Liu et al., 2023). Previous studies have revealed diverse chemical constituents in *T. sinensis*, including alkaloids, terpenoids, flavonoids, phenols, and other compounds exhibiting various bioactivities such as antioxidant, anti-inflammatory, analgesic, and anti-rheumatoid arthritis effects (Jain et al., 2010; Xue et al., 2014; Wu et al., 2014; Jiang, 2017; Gesang et al., 2017; Yang et al., 2019; Sun et al., 2020; Gao et al., 2022; Xu et al., 2024).

With advances in interdisciplinary technologies, microbial transformation has emerged as a promising approach for novel drug discovery and innovative pharmaceutical development. This technology utilizes enzymes produced during microbial growth and metabolism to structurally modify substrates through catalytic reactions including decomposition and conversion, thereby altering active substances in medicinal materials to reduce toxicity and enhance efficacy, generating new compounds or more potent derivatives with bioactive metabolites (Guan et al., 2018; Xie et al., 2018; Ma et al., 2024; Gao, 2024). Yang et al. (2009) reported that microbial transformation by photosynthetic bacteria significantly enhanced the antitumor activity of *Viscum coloratum* extracts. Zhan et al. (2017) utilized *Cunninghamia elegans* to transform artemisinin, yielding a derivative with superior antimalarial activity and solubility. Cao et al. (2015) demonstrated that *Bacillus subtilis* could transform aristolochic acid A to reduce toxicity. These findings illustrate that microbial transformation integrates traditional medicinal values while improving efficacy and reducing potential toxicity, thereby facilitating the modernization of traditional Chinese medicine.

Endophytic fungi reside within plant tissues without causing disease, establishing mutualistic symbiosis with enhanced host adaptation. Research indicates they can synthesize identical or analogous compounds to their hosts, promoting the production of similar active ingredients (Ding et al., 2013). As important microbial resources, endophytes have become a research hotspot for producing bioactive substances through microbial transformation. Xie et al. (2018) found that endophytic fungi from *Tripterygium wilfordii* could transform crude extracts to produce antitumor compounds with enhanced efficacy and reduced toxicity. Liu et al. (2022) reported that endophytic fungus *Fusarium*-C39 significantly increased the content of total saponins and specific paridis saponins in *Paris polyphylla* var. *yunnanensis* after biotransformation.

Despite *T. sinensis* being traditionally used as an ethnic medicine with research focusing on its extracts and chemical constituents, studies on its endophytic fungi and microbial transformation remain unreported. It remains unclear whether endophytes can biotransform the host and whether differences exist in chemical composition and bioactivity before and after transformation. Therefore, this study isolated and identified endophytic fungi from *T. sinensis* and employed them for microbial transformation, investigating material changes and bioactivity differences to provide a theoretical basis for further development and utilization of *T. sinensis* and its microbial resources.

Materials and Methods

Sample Collection and Strains

Fresh *T. sinensis* 药材 was collected in May 2022 from Yizhou District, Hechi City, Guangxi, and identified as the stem of *Tinospora sinensis* by Professor Xie Yanjun from Hechi University. The voucher specimen is stored in Laboratory 201 at Hechi University. Indicator strains including *Staphylococcus aureus*, *Candida albicans*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Streptococcus* were kindly provided by Professor He Haiyan' s laboratory at Hechi University. Potatoes and rice were purchased from local supermarkets, and *T. sinensis* 药材 was obtained from local pharmacies.

Culture Media

PDA medium, PDB medium, LB liquid medium, rice medium (50 g rice in a 500 mL conical flask with approximately 80 mL distilled water), and *T. sinensis* 药材 medium (药材 powder sieved through 80 mesh, 50 g placed in a 500 mL conical flask with approximately 80 mL distilled water) were prepared and sterilized by autoclaving before use.

Reagents and Instruments

Reagents including 1,1-diphenyl-2-picrylhydrazyl (DPPH), ampicillin sodium, amoxicillin, fluconazole, dimethyl sulfoxide (DMSO), sodium chloride, glucose, potassium dihydrogen phosphate, and sodium hydroxide were purchased from Guangxi Yiling Medical Equipment Co., Ltd. Analytical-grade n-butanol, ethyl acetate, methanol, petroleum ether, and anhydrous ethanol were obtained from Xilong Scientific Co., Ltd. Peptone, beef extract, agar powder, and yeast powder were purchased from Beijing Aoboxing Biotechnology Co., Ltd.

Instruments included a rotary evaporator (Shanghai Yarong Technology Co., Ltd.), high-performance liquid chromatography-mass spectrometer (Waters Corporation, USA), biochemical incubator (Shandong Boke Biological Industry Co., Ltd.), pipettes (Eppendorf, Germany), and multifunctional microplate reader (Shandong Boke Biological Industry Co., Ltd.).

Isolation and Purification of Endophytic Fungus KJT-1

Endophytic fungi isolation and purification followed the method described by Xie et al. (2018). Fresh *T. sinensis* stems were cut into segments with sterilized scissors, surface-sterilized by immersion in 75% ethanol for 3 minutes, then in 5% sodium hypochlorite solution for 30 seconds, followed by 4-5 rinses with sterile water and drying. Sterilized dissecting knives were used to cut the tissue into small pieces (5 mm $\times$ 5 mm), which were evenly inoculated onto PDA plates containing ampicillin sodium in a triangular pattern and incubated at 25-28°C until hyphae emerged. The final rinse water was plated onto identically treated PDA plates as a surface sterilization control; absence of fungal growth after 5-7 days confirmed complete surface sterilization, indicating that isolated fungi were endophytic. Upon hyphal growth from the inoculated stem tissue, sterile inoculating loops were used to transfer hyphae to fresh PDA plates for incubation at 25-28°C. Repeated transfer of apical hyphae was performed until pure colonies were obtained. The purified single colony' s apical hyphae were transferred to new PDA plates to obtain the pure strain KJT-1, which was stored at 4°C (Xie et al., 2018).

Identification of Endophytic Fungus KJT-1

Morphological characteristics of KJT-1 were observed and recorded after inoculating onto PDA plates. For molecular identification, KJT-1 hyphae were inoculated into PDB liquid medium and cultured on a shaker at 25-28°C for 3-4 days. Mycelia were collected by centrifugation at 1,500 $r \cdot min^{-1}$, and DNA was extracted using a DNA extraction kit. The ITS region was amplified using primers ITS1 (5 -TCCGTAGGTGAACCTGCGG-3) and ITS4 (5 - TCCTCCGCTTATTGATATGC-3). Product purity testing and sequencing were performed by Qingdao Pengxiang Biotechnology Co., Ltd. The obtained sequences were compared against the NCBI nucleotide database, and a phylogenetic tree was constructed using MEGA 7 software to determine the species (Liu et al., 2018).

Microbial Transformation Fermentation

Microbial transformation medium preparation and fermentation were modified from literature methods (Liu, 2018; Zou, 2019). Activated KJT-1 hyphae were inoculated into PDB medium and cultured on a shaker at 25-28°C for 3 days to obtain seed fermentation broth. Under sterile conditions, 5 mL of the homogenized seed broth was inoculated into two types of sterilized test media (*T. sinensis* 药材 medium and rice medium) and fermented at 25-28°C for 28 days. A blank control consisting of *T. sinensis* 药材 medium with 5 mL sterile water instead of seed broth was prepared with identical processing.

Preparation of Extracts Before and After Microbial Transformation

Preparation of *T. sinensis* extracts followed the method of Gao et al. (2022). The uninoculated *T. sinensis* 药材 medium was extracted three times with 1 L of 70% ethanol for 2 hours each, filtered, and the combined extracts were concentrated under reduced pressure to obtain the crude extract (CE-1). The crude extract was dispersed in water and sequentially extracted with 1 L each of petroleum ether, ethyl acetate, and n-butanol. Each fraction was concentrated under reduced pressure to obtain petroleum ether extract (PE-1), ethyl acetate extract (EA-1), and n-butanol extract (n-BE-1). Post-transformation extracts were prepared identically, yielding CE-2, PE-2, EA-2, and n-BE-2. KJT-1 metabolites from rice medium fermentation were extracted three times with 1 L of 70% ethanol for 2 hours each, filtered, combined, and concentrated under reduced pressure.

Antimicrobial Activity Assay

Antimicrobial activity was evaluated using the agar punch method to measure inhibition zone diameters (Xu et al., 2018). Pre- and post-transformation extracts (CE-1, PE-1, EA-1, n-BE-1, CE-2, PE-2, EA-2, n-BE-2) were dissolved in DMSO to prepare 1 $g \cdot mL^{-1}$ sample solutions. Positive controls fluconazole and amoxicillin were prepared at 20 $mg \cdot mL^{-1}$ and 0.1 $mg \cdot mL^{-1}$, respectively. KJT-1 metabolites were prepared as 1 $g \cdot mL^{-1}$ solution for blank control. Test strains (*S. aureus*, *E. coli*, *C. albicans*, *Streptococcus*, *P. aeruginosa*) were spread on LB agar plates. Six-millimeter filter paper discs soaked in sample, blank control, or positive control solutions were placed equidistantly on plates, with three replicates per sample. Plates were incubated at 37°C for 20 hours. Inhibition zone diameters were measured using the "cross" method and expressed as mean ± standard deviation ($\bar{x} \pm s$) to evaluate inhibitory effects against the five indicator strains.

Minimum Bactericidal Concentration (MBC) Determination

The MBC of post-transformation n-butanol extract was determined using the tube double-dilution method (Li, 2017). Ten sterile tubes were numbered 1-10, each receiving 2 mL sterile broth. Tube 1 received 2 mL of 1 $g \cdot mL^{-1}$ test sample in DMSO, mixed thoroughly. Serial dilution was performed from tube 1 to tube 9, yielding concentrations of 500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.91, and 1.95 $mg \cdot mL^{-1}$. Tube 10 received saline as control. Each tube was inoculated with 0.1 mL bacterial suspension, with three parallels per tube. All tubes were incubated statically at 37°C for 20 hours. After incubation, 0.1 mL from each tube was plated on LB agar and spread evenly. Plates were incubated at 37°C for 20 hours. Growth was recorded as "+" (no bactericidal effect) or "-" (bactericidal effect). The lowest concentration showing no bacterial growth was designated as the MBC.

DPPH Radical Scavenging Assay

The DPPH assay was performed according to Wang (2021). Pre- and post-transformation extracts (CE-1, PE-1, EA-1, n-BE-1, CE-2, PE-2, EA-2, n-BE-2) were diluted 20-fold in anhydrous ethanol and sonicated at 60°C for 10 minutes. Stock solutions (50 $mg \cdot mL^{-1}$) were further diluted to working concentrations of 1, 0.5, 0.1, 0.05, and 0.01 $mg \cdot mL^{-1}$. Vitamin C (VC) served as positive control. Three milliliters of each sample solution was mixed with 2 mL DPPH solution and reacted in darkness for 30 minutes. Absorbance at 517 nm was measured using anhydrous ethanol as blank control. DPPH radical scavenging rate was calculated according to Li et al. (2015) and Liu et al. (2023) using Formula 1:

Formula 1:
DPPH radical scavenging rate = $[1 - (A_2 - A_1)/A_0] \times 100\%$

Where:
A_0 = absorbance of 2 mL DPPH + 3 mL anhydrous ethanol
A_1 = absorbance of 2 mL anhydrous ethanol + 3 mL sample solution
A_2 = absorbance of 3 mL sample solution + 2 mL DPPH solution

Chemical Composition Analysis by LC-MS

LC-MS analysis was performed in negative ion mode to identify compounds in pre- and post-transformation n-butanol extracts (n-BE-1, n-BE-2). Total ion chromatograms were obtained and compounds were identified by comparing mass spectral data with the NIST23.L database.

Chromatographic conditions: ACQUITY UPLC-BEH C18 column (2.1 mm × 100 mm, 1.7 µm); mobile phase: 0.1% formic acid in water (A) and methanol (B) with gradient elution (0–40 min, 10% B to 100% B); flow rate: 1 $mL \cdot min^{-1}$; column temperature: 35°C; injection volume: 1 µL; detection wavelength: 254 nm.

Mass spectrometry conditions: Electrospray ionization (ESI) source in negative ion mode; capillary voltage: 3.0 kV; cone voltage: 30 V; cone gas flow: 50 $L \cdot h^{-1}$; desolvation gas flow: 1,000 $L \cdot h^{-1}$; source temperature: 230°C; desolvation temperature: 300°C; scan time: 0.2 s; mass range: m/z 50–1,000. Data acquisition and analysis were performed using UNIFI™ 1.9.4 software (Guo, 2022).

Results

Identification of Endophytic Fungus KJT-1

During microbial purification, ten endophytic fungal strains were isolated (designated KJT-1 through KJT-10). Transformation studies revealed that only

the n-butanol extract of KJT-1 post-transformation (n-BE-2) exhibited significant antimicrobial activity against *S. aureus*, while KJT-1 metabolites and pre-transformation extracts showed no activity against the five indicator strains. Other strains showed no significant differences in antimicrobial activity before and after transformation, indicating KJT-1' s superior transformation capability worthy of further investigation. Additionally, the absence of antimicrobial activity in KJT-1 metabolites both before and after transformation minimized confounding effects on results. Therefore, KJT-1 was selected for identification.

As shown in [Figure 1: see original paper]A, KJT-1 grew well on PDA plates with white, soft, smooth hyphae that were easily picked. Based on gene sequencing and BLAST comparison, phylogenetic analysis using MEGA 7 software and the neighbor-joining method clustered KJT-1 with *Colletotrichum queenslandicum* OBP22 ([Figure 1: see original paper]B), showing 99.67% sequence similarity. Thus, KJT-1 was identified as *Colletotrichum queenslandicum*.

Antimicrobial Activity of Extracts Before and After Microbial Transformation

As shown in , pre-transformation extracts of *T. sinensis* showed no inhibitory effects on the five test strains, consistent with the lack of reported antimicrobial activity for *T. sinensis* extracts. and demonstrate that post-transformation crude, ethyl acetate, and petroleum ether extracts also showed no inhibition, while the n-butanol extract inhibited *S. aureus* growth with an inhibition zone diameter of (9.82 $\pm$ 0.2) mm. MBC determination revealed the post-transformation n-butanol extract had an MBC value of 31.3 $mg \cdot mL^{-1}$ against *S. aureus* but no activity against the other four test strains.

DPPH Radical Scavenging Activity

As illustrated in [Figure 2: see original paper], all extracts exhibited concentration-dependent DPPH radical scavenging capacity. At 1 $mg \cdot mL^{-1}$, the pre-transformation crude extract showed 37.5% scavenging activity, which decreased to 36.58% post-transformation (0.92% reduction). Petroleum ether extract scavenging efficiency increased from 88.8% to 95.7% (6.88% increase). Ethyl acetate extract decreased from 90.5% to 78.92% (11.62% reduction). n-Butanol extract decreased from 93.85% to 89.23% (4.62% reduction). These changes in DPPH scavenging capacity indicate that microbial transformation altered the chemical composition of *T. sinensis* extracts.

Chemical Composition of n-Butanol Extracts Before and After Transformation

Total ion chromatograms of pre- and post-transformation n-butanol extracts are shown in [Figure 3: see original paper] and [Figure 4: see original paper]. [Figure 3: see original paper] and identify 33 compounds in the pre-transformation extract, including 1 flavonoid, 8 terpenoids, 3 phenylpropanoids, 4 esters, 7

phenols, 2 steroids, 3 alkaloids, 3 fatty acids, and 2 organic acids. [Figure 4: see original paper] and identify 23 compounds post-transformation, comprising 1 terpenoid, 4 phenylpropanoids, 5 phenols, 1 quassinoid, 6 fatty acids, 1 ester, 2 steroids, and 1 alkaloid. The altered compound types and quantities, particularly notable changes in terpenoids, suggest that microbial fermentation significantly transformed the chemical constituents, likely accounting for the newly observed antimicrobial activity.

Discussion and Conclusion

Microorganisms possess powerful abilities to decompose and transform substances through enzymatic catalysis, generating structurally diverse bioactive metabolites. Lai et al. (2024) demonstrated that fungal fermentation can modify flavonoids, alkaloids, terpenoids, and phenylpropanoids through glycosylation, hydroxylation, methylation, oxidation, reduction, and hydrolysis, producing various metabolites. This study employed the endophytic fungus *C. queenslandicum* KJT-1 to biotransform *T. sinensis*. Antimicrobial results showed that KJT-1 metabolites and pre-transformation extracts lacked activity against the five test strains, while post-transformation n-butanol extract exhibited clear inhibition against *S. aureus*. The marked changes in compound types and quantities, particularly terpenoids, before and after transformation confirm that the endophytic fungus indeed transformed host constituents into bioactive compounds.

Colletotrichum queenslandicum belongs to the *Colletotrichum gloeosporioides* species complex and can produce metabolites that directly or indirectly inhibit microbial growth. For instance, *Colletotrichum gloeosporioides* isolated from *Artemisia mongolica* produces collector acid with antibacterial and antifungal activity (Wang, 2016). However, KJT-1 metabolites showed no antimicrobial activity, possibly due to culture medium effects or other factors influencing metabolite production, as different media significantly impact microbial metabolite profiles (Jin et al., 2023).

T. sinensis contains diverse chemical constituents including terpenoids, alkaloids, phenylpropanoids, flavonoids, steroids, and phenols (Gao et al., 2022; Liu et al., 2023), consistent with our findings. Flavonoids exhibit multiple bioactivities and function as antioxidants by scavenging and inhibiting free radical generation (Tu, 2016). Our results show that all extracts possessed concentration-dependent DPPH scavenging capacity. Post-transformation decreases in crude, ethyl acetate, and n-butanol extracts, coupled with a 6.88% increase in petroleum ether extract, likely reflect changes in flavonoid composition and content. The altered antimicrobial and antioxidant activities presumably resulted from significant changes in compound types or quantities, with alkaloids and terpenoids showing notable variation in n-butanol extracts, confirming endophytic transformation of host constituents.

Gingerol components exhibit broad-spectrum antimicrobial activity (Liu et al., 2015; Lee et al., 2018; Lu et al., 2021). For example, 6-gingerol inhibits *E. coli* growth (Li et al., 2022). Chemical analysis revealed 6-gingerol in both pre- and post-transformation n-butanol extracts, yet antimicrobial activity only emerged post-transformation, suggesting that biotransformation may have increased antimicrobial substance content or generated novel compounds. Post-transformation extracts contained newly added alkaloid *N*-cis-feruloyltyramine and terpenoid eucommiol, further confirming endophytic transformation. Zheng (2021) reported that *N*-cis-feruloyltyramine inhibits LPS-induced NO release in BV-2 microglial cells, while Li et al. (2011) identified eucommiol as an iridoid that promotes granulation tissue formation and collagen synthesis, though other activities remain unreported. These findings suggest that activity changes correlate with chemical composition alterations, though further verification is needed.

This study has limitations, including lack of systematic isolation and identification of active compounds from post-transformation extracts, absence of mechanistic investigation into the transformation process, and no genomic analysis of antimicrobial metabolite biosynthetic gene clusters. Future work will focus on systematic chemical isolation, pharmacological validation, microbial transformation mechanisms, and gene cluster regulation studies. In conclusion, we obtained an endophytic fungus (*C. queenslandicum* KJT-1) capable of microbially transforming *T. sinensis*, converting host constituents into antimicrobial and antioxidant substances. This provides a theoretical foundation for microbial transformation and lead compound discovery, offering a basis for resource development of *T. sinensis* and its endophytic fungi.

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Note: Figure translations are in progress. See original paper for figures.

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