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理学博士学位论文

福建沙溪污染地真菌及其次级 代谢产物的研究

**The Screening for Bioactive Strains from the Shaxi Fungi in
Polluted Environment and the Study on the Secondary Metabolites
from Several Fungi**

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*The Screening for Bioactive Strains from the Shaxi Fungi in
Polluted Environment and the Study on the Secondary Metabolites
from Several Fungi*

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福建沙溪污染地真菌及其次级代谢产物的研究

摘 要

天然药物一直是人类获得药物的主要途径。微生物具有广泛的适应性,其代谢产物丰富多样,是天然药物的重要来源之一。但是由于传统微生物药物的长期使用,出现耐药性的情况也越来越多,急需新药来缓解临床用药的压力。另外,扩大微生物的来源也是一条寻找新微生物药物的有效途径。极端环境微生物具有特殊的遗传背景和代谢途径,能够产生各种具有特殊功能的酶类及其它活性物质,在医药、食品、化工、环保等领域有着重大的应用潜力。沙溪是闽江上游三大支流之一。近年来,随着三明市经济的发展,局部污染事件时有发生。环境污染严重地区的土壤或水域与一般的陆地生境不同,微生物在对环境污染物的分解及对抗过程中,有可能造就结构新颖独特具有药理活性的化合物。

本论文已探讨“福建沙溪微生物资源在药物开发中的应用”为切入点,对从福建省三明沙溪河沿岸高污染处或沿岸工厂处理池8个样地采集得到9个不同的土样进行真菌和放线菌的分离,以分离得到的污染地真菌为研究对象,分离筛选具有抗菌、抗肿瘤活性的菌株;对A4-Z-1、SHZK-1、SNSHI-5、HLN-A2和SHZK-2具有一定活性或形态较为特殊的几株真菌菌株的脂溶性次级代谢产物及生理活性进行初步研究,并对SHZK-15和FSNSHII-A1-1具有高活性的两株真菌的主要活性成分进行了分离分析,整个课题取得了一些有意义的结果。

从福建三明沙溪河附近的8处污染点采集到的9个样品中总共分离得到121株真菌菌株。对121株土壤真菌发酵液的乙酸乙酯抽提物进行抗菌活性筛选,发现有55株的菌株对一种或多种指示菌具有抑制作用。另外采用细胞毒筛选模型(MTT法)对这些真菌发酵液的乙酸乙酯抽提物的抗肿瘤活性进行了检测。结果表明共有65株真菌提取物对KB或Raji细胞显示了不同程度的细胞毒作用($IC_{50} \leq 100 \text{ ug/mL}$),其中抗KB细胞的有47株,抗Raji细胞的有53株,抗KB和Raji二种细胞的有35株。这表明环境污染严重地区的土壤或水域中蕴藏着十

分丰富的抗菌、抗肿瘤活性物质产生菌，是研究开发抗菌、抗肿瘤药物的重要资源来源。采用传统的方法对 121 株菌株进行鉴定，结果显示，抗肿瘤活性的真菌主要分布在青霉属、拟青霉属、无孢目、曲霉属和枝孢霉属，分别占总检测菌株数的 9.9%、5.8%、6.6%、6.6%和 5.8%。抗菌活性菌株主要分布在枝孢霉为主的 13 个属中，其中以枝孢霉、青霉属、拟青霉属、曲霉和无孢目为主，各占活性菌株的 20.0%、14.5%、9.1%、12.7%和 12.7%。其它真菌如茎点霉、子囊菌和半知菌球壳孢目的一些分离株也表现出一定的抗菌活性。

A4-Z-1、SHZK-1、SNSH1-5、HLN-3、SHZK-16 等几株真菌具有较强生物活性或形态较为特殊。本文结合形态学和分子生物学方法，对这些菌株进行了分类鉴定。同时，我们将所有测定的各菌株序列及其从基因库中搜索到的最高相似度(>95%)的序列，以 *Apiospora* sp 为外类群，用 DNAMAN 软件进行序列比对和系统学分析，用邻接法构建系统树。

对 A4-Z-1、SHZK-1、HLN-A2、SNSHI-5 和 SHZK-2 具有一定活性的四株真菌菌株的脂溶性次级代谢产物进行了较系统的研究，并对 SHZK-15 和 FSNSHII-A1-1 具有高活性的两株菌株的主要活性成分进行了分离分析。我们总共分离鉴定了 43 个次级代谢产物，主要为油脂类、单苯环衍生物、苯丙素类、苯醚类、不饱和酸酐、不饱和内酯、甾醇类、萜醌类、哌嗪类化合物。通过 Beilstein, CA, SDBS 和 Cambridge 等数据库搜索及图书馆查新，初步确认新结构数为 10 个，它们分别是 A1 (Mycoediketopiperazine), A2 (methyl 2-(4-hexyl-2,5-dioxo-2,5-dihydrofuran-3-yl)acetate), A3 ((4-Octyl-2,5-dioxo-2,5-dihydro-furan-3-yl)-acetic acid), S1-1 (4,6-Dimethyl-tetradeca-2,4-dienoic acid amide), 不饱和内酰胺化合物 S1-2, 不饱和内酯化合物 S1-3, SN-1 (7-Butoxy-3,3,5-trihydroxy-4-methyl-3H-isobenzofuran-1-one), SN-2 (3,3,5-Trihydroxy-7-methoxy-4-methyl-3H-isobenzofuran-1-one)。分离得到的新化合物 A1 具有新颖的骨架结构，为哌嗪类化合物，值得深入研究。另外，S15-1(BFA)具有多种生物活性及潜在的药用价值，是目前国际上抗癌药物研究的一个热点。本文采用 HPLC-MS-MS 联用技术，建立了快速分析和鉴定真菌发酵液中是否含 BFA 的测试方法。

生物活性测试发现，新化合物 A2, A3, S1-2, S1-3, SN-1 和 SN-2 显示了一定的细胞毒活性；新化合物 A3, S1-1 和 SN-2 对其中一种或几种指示菌显示了较强

的抗菌活性；已知化合物中大部分化合物显示了细胞毒活性，且已知化合物 A5, A6, S1-8, S1-10, S1-12, S15-1, H1 和 F1 对其中一种或几种指示菌也显示了强的抗菌活性；H1 除了已经报道的杀线虫活性、酶抑制活性以及对人宫茎癌细胞的细胞毒活性外，我们还初次发现化合物 H1 对 Raji, KB 和 HEPGII 肿瘤细胞都具有一定的细胞毒活性， IC_{50} 分别为 $2.4\mu\text{g/mL}$ ， $10\mu\text{g/mL}$ ， $20\mu\text{g/mL}$ ，对细菌也有很好的抗菌活性（和阳性药物的抑制能力相近）；发现化合物 F1 对肿瘤细胞、细菌、真菌具有广谱活性，并初步发现化合物 F1 具有强抗氧化等新活性；化合物 S15-1 具有强的广谱抗肿瘤活性，化合物 F1 对肿瘤细胞、细菌、真菌具有广谱活性，这提示它们是具有潜在应用前景的活性化合物。

本文结果表明，污染环境中的真菌中蕴藏着较丰富的抗菌和抗肿瘤活性物质产生菌，是一类尚待开发的宝贵的真菌资源，它不仅含有大量已知的生物活性物质，同时也是新颖结构和新的生理活性物质的重要来源。

关键词： 真菌 污染环境 次级代谢产物

The Screening for Bioactive Strains from the Shaxi Fungi in Polluted Environment and the Study on the Secondary Metabolites from Several Fungi

Abstract

Natural product as the main source for the medicine, has made great contribution for human health. Microorganisms for potential pharmaceutical resources have formed a hot spot for searching novel drugs. Many novel secondary metabolites also have been isolated from ordinary surroundings such as: Endophytic fungi, terrestrial microorganisms, and marine organism etc. Furthermore some secondary metabolites of novel structure and high bioactivity may also be isolated from extreme environment such as high temperature, high pressure, extreme acidic or basic conditions etc. But less attention is focused on fungi isolated from polluted environment, which might provide an alternative approach to search for useful natural products. Shaxi is one of the three major upper reaches of Minjiang River which traverses the Sanming city where has many plants for more than 50 years. However, with the economic development of Sanming city, the polluting affairs are a big issue. Therefore, we have studied the antitumor activities and antimicrobe activities of the soil fungi of several samples collected from Shaxi River, a highly polluted river in Fujian, China.

The soil fungal isolated from Shaxi River, in Fujian Province were screened for antimicrobial activity and antitumor activity. And the metabolites of fungus A4-Z-1、SHZK-1、SNSHI-5 and SHZK-2 were investigated in detail. The primary components of several fungus having strong bioactivities, such as HLN-A2, SHZK-15 etc, were studied. Some interesting results were obtained during the progress of investigating the microorganisms from polluted environment, Shaxi River.

Eight soil samples investigated in this study originated from different sites of

Shaxi River. From those soil samples, 121 strains of soil fungi and 45 strains of actinomycetes were isolated by using selective media. Those fungi were cultured on potato dextrose medium. Organic extracts were screened by MTT assay and the filter paper assay. The results showed that 65 strains (53.7% of the total isolates) could inhibit the growth of Raji and/or KB tumor cell lines, while 55 strains (about 45.5% of the total isolates) showed antimicrobial activities against one or more sensitive microbes (*Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans*, *Aspergillus niger*). All the strains were identified by traditional method. The active strains distribute in 13 genera and sporeless class. Most of them belong to *Cladosporium* sp., *Penicillium* sp., *Paecilomyces* sp. and *Aspergillus* sp. Other strains, such as *Phomopsis* sp. and *Ascomyceta* sp. also showed certain antimicrobial or antitumor activity.

Five fungi's internal transcribed spaces (ITS) region was amplified and sequenced by using the general primers ITS1 and ITS4. The ITS regions of the eight fungi (A4-Z-1, SHZK-1, SNSH1-5, HLN-3, SHZK-16, SHZK-2, SHZK-15 and HLKS-9) are 580 bp, 540 bp, 576 bp, 547 bp, 546 bp, 547 bp, 616 bp and 565 bp DNA sequences respectively, and were identified as *Arthrinium* sp., *Arthrinium* sp., *chaetomium globosum*, *Phoma* sp., *Phoma* sp., *Phoma* sp., *Penicillium* sp. and *Penicillium* sp. respectively.

Among the 121 fungi, five strains A4-Z-1 (*Arthrinium* sp.), SHZK-1 (*Arthrinium* sp.), SNSHI-5 (*phialophora* sp.), SHZK-2 (*Phoma* sp.) and HLN-A2 (*Paecilomyces* sp.) with moderate antitumor or antimicrobial activities were selected to study their secondary metabolites. And two strains (SHZK-15 and FSNSHII-A1-1) with high antitumor or antimicrobial activities were selected to study their primary component. Employing different isolation and purification methods, 40 compounds with ten new compounds were isolated from the fermentation broth of fungi. In the later investigation of antitumor, antimicrobial and acetylcholinesterase inhibitory activities, seven compounds with some bioactivity were found.

Among all the new compounds, the A4-Z-1-17 compound is a novel symmetrical metabolite with epoxy group and two -SCH₃. Analyzing its structure character, it

maybe originates from the ramification of tyramine acid. The A1 compound did not show antitumor and antimicrobial activity, but show some antioxygenic property. Some compounds (A2 and A3, SN-1 and SN-2) have the same framework structure with only one different substitute group, but their bioactivity arises to great changes, A3 and SN-2 have preferable activity. Unfortunately, the other new compounds have not exhibited obvious antitumor or antimicrobial activities. Further study on other bioactivities of new compounds will be carried on in the future.

The known compound, F1 (clavatol), demonstrates remarkable antitumor as well as antimicrobial activity in *vitro*. Against human tumor cells line: Raji, KB, Hep-G2, their IC₅₀ were 6.1, 9.1, 14.5, 15.4 µg/mL respectively. And it inhibits the growth of *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans*, *Aspergillus niger* with MIC 200, 200, 100, 25, 50 µg/mL respectively. Other known compound, H1, also exhibits high antitumor activity in *vitro*, against human tumor cells line: Raji, KB, HEPGII, with IC₅₀, 2.4, 10, 20 µg/mL respectively. Brefeldin A, a naturally occurring macrolide, exhibits a diversity of biologic activities, such as antibiotic, antiviral, cytostatic, antimitotic and antitumor effects. In this paper, a rapid method for the identification of BFA in crude microbial extracts is reported, using LC-MS-MS and HPLC-ESI-MS.

In a word, our study indicated that marine lignicolous fungi were reliable resources for metabolites with antitumor and antimicrobial bioactivity.

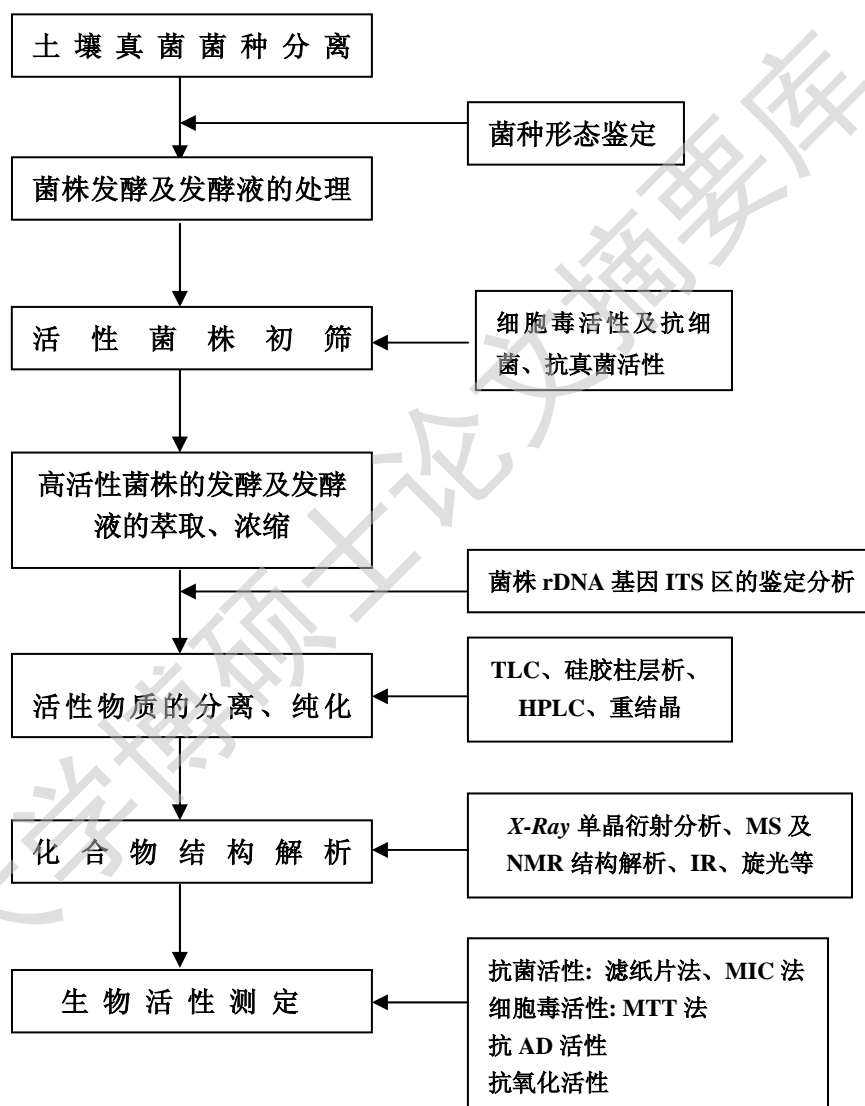
Key words: fungi, polluted environment, secondary metabolites, bioactivity

常用英文缩写词

缩写式	全 称
[α]	Optical rotation 旋光
BFA	Brefeldin A 布雷菲德菌素 A
ACTI	Acetylthiochoine iodide 碘化乙酰胆碱
CC	Column chromatography 柱层析
CDKS	Cyclin-dependent kinases 细胞周期依赖性激酶
CML	Chronic myeloid leukemia 慢性髓样白血病
CID	Collision-induced dissociation 碰撞诱导解离
COSY	Correlated spectroscopy 相关谱
δ	Chemical shift 化学位移
d	Doublet 二重峰
DACH	Diamincyclohexane 环己二胺
dd	doublet of doublet 双二重峰
dt	doublet of triplet 双三重峰
DEPT	Distortionless enhancement by polarization transfer 无畸变极化转移增益实验
DHFR	Dihydrofolate reductase 二氢叶酸还原酶
DPPH	2,2-diphenyl-1,4-phthalazinedione 二苯代苦味酰自由基
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid) 5,5'-二硫代双-(2-硝基苯甲酸)
EC ₅₀	median (or half-maximal) effective concentration
ECM	Extra-cellular matrix 细胞外基质
ECMA	VEGF stimulated endothelial cell mitogenesis assay 内皮细胞生长因子介导的内皮细胞有丝分裂检验
EF	Elongation factor 延长因子
EGF	Epidermal growth factor 表皮生长因子
ESI-MS	Electrospray ionization mass spectrometry 电喷雾质谱
FAB-MS	Fast atom bombardment mass spectrometry 快原子轰击质谱
FTase	Farnesyltransferase 法尼基转移酶
GPAT	Genetic prodrug activation therapy 基因前药活化治疗
Hep-G2	人肝癌 HEp-G2 细胞
HMBC	(¹ H-detected) heteronuclear multiple-bond correlation (检出 ¹ H 的)异核多键相关
HPLC	High Performance Liquid Chromatography 高效液相色谱
HSQC	(¹ H-detected) heteronuclear sigle quantum correlation (检出 ¹ H 的) 异核单量子相关
IAPs	Inhibitor-of-apoptosis proteins 凋亡抑制蛋白
IC ₅₀	concentration giving 50% of maximal inhibition 半数抑制浓度

IL	Interleukin 白细胞介素
IR	Infra-red 红外
ITS	Internal transcribed spaces (核糖体)基因转录间隔区
KB	人口腔皮样癌 KB 细胞
m	Multiplet 多重峰
MAPK	Mitogen activated protein kinase 促分裂源活化蛋白激酶
MDR	Multi-drug resistance 多药耐药性
MIC	Minimum inhibitory concentrations 最小抑制浓度
MMP	Matrix metalloproteases 基质金属蛋白酶
MS	Mass spectrometry 质谱
MTT	Methyl Thiazolytetrazolium 甲基四唑蓝(噻唑蓝)
NCEs	New chemical entities 新化学实体
NCI	National cancer institute 国家癌症研究所
NMR	Nuclear Magnetic Resonance 核磁共振
NMT	N-myristoyltransferase N-豆蔻酰基转移酶
NOESY	Nuclear Overhauser Effect Spectroscopy 核空间效应谱
PBS	phosphate-buffered saline 磷酸缓冲液
PCD	Programmed cell death 程序性细胞死亡
PDGF	Platelet-derived growth factor 血小板生长因子
PTK	Protein tyrosine kinase 蛋白质酪氨酸激酶
q	Quartet 四重峰
Raji	人 B 淋巴瘤 Raji 细胞
RAPD	Randomly amplified polymorphic DNA 随机扩增多肽性
RTK	Receptor tyrosine kinase 受体酪氨酸激酶
RFLP	Restriction fragment length polymorphism 限制性片段长度多肽性
s	Singlet 单重峰
SCF	Stem cell factor 干细胞因子
SDS	Sodium dodecyl sulfonate 十二烷基磺酸钠
SE	Squalene epoxidase 角鲨烯环氧化酶
t	Triplet 三重峰
TA	Tumor angiogenesis 肿瘤新生血管抑制剂
TLC	Thin layer chromatography 薄层色谱
TOF-MS	Time of flight mass spectrometry 飞行时间质谱
TS	Thymidylate synthase 胸苷合成酶
UV	Ultraviolet 紫外
VEGF	Vascular endothelial growth factor 血管内皮生长因子
WHO	World health organization 世界卫生组织

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