The fermentation and chemical characters of the endophytic fungus from *Dendrobium huoshanense*

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碩士學位論文

霍山石斛內生真菌發酵培養及化學特徵分析

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ABSTRACT

The fermentation and chemical characters of the endophytic fungus from *Dendrobium huoshanense*

by Hu Dejun

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Major: Chinese Medicinal Sciences

Dendrobium huoshanense Tang et Cheng, (*Huoshanshihu* in Chinese), a well-known traditional Chinese medicine (TCM) recorded as first grade in *Shen Nong Ben Cao Jing*, is used for treatment of asthenia pyrosyndrome, dim vision and retching, etc. Modern pharmacology showed that it also has anti-oxidation, enhance immunity, anti-tumor and other effects. *D. huoshanense* grows in the stones of mist-shrouded cliffs or among the towering trees gaps. The harsh growth environment of *D. huoshanense* leads to the scarcity of natural resources of *D. huoshanense*. It is worse for the over-excavation of wild resources. From the late 50s in the 20th century, in China, many research focused on the sustainable development of *D. huoshanense*. The main ways include: (1) the way of sterile germination on the seed of *D. huoshanense*; (2) Plantlets organogenesis of *D. huoshanense*; (3) PLB (Protocorm-like body) occurring pathway; (4) Artificial cultivation of *D. huoshanense*.[1] Although plantlets of *D. huoshanense* were successfully transplanted in 1985, but so far, the scale is very small, the problem that low survival rate on transplanting into field has not been fundamentally solved, it is hard to achieve large-scale production. *Dendrobium* has important medicinal value, but the amount of the plants is rare, especially *D. huoshanense*. Endophytic fungi have an important relationship with plants, which have been isolated from many plants, including the species of *Dendrobium*, and many of these endophytic fungi had pharmacologically

activity, they have a symbiotic relationship with the host plants and their chemical compositions also have a certain similarity with host plants. With the development of fermentation industry, mycelium and broth can be obtained from the fermentation of the endophytic fungi isolated from *Dendrobium*, which may have fully/partially replace of the effective components in *Dendrobium*.

An endophytic fungus called HS-01 was isolated from living *D. huoshanense* in this subject, and was fermented for the accumulation of mycelium. The mycelium was extracted by ethanol and the extract was used for chemical separation. Meanwhile, the quantitative analysis of *D. huoshanense* and HS-01 were done by different methods including GC and HPLC.

The thesis includes five chapters. **Chapter 1** reviewed the advance detailed on the research progress of *D. huoshanense*, the chemical constituents of *Dendrobium*, the overview on the endophyte of *Dendrobium* and medicinal plants. In **Chapter 2**, the isolation and culture of endophyte isolated from *D. huoshanense* (HS-01) was taken to get the mycelium. A comparison of all components in endophytic fungus and *D. huoshanense* was taken by the way of established multi-column chromatography. The results showed that the response is not enough for the quantative analysis of the polysaccharides by this mass condition, and the column Aq-C18 is not suitable for the analysis of the low polar conponents. From the MS data, it also showed that the *D. huoshanese* contained fructose, glucose, and sucrose, while the HS-01 contains mannitol. **Chapter 3** made an initial analysis on the alcohol extract of *D. huoshanense* and HS-01 by liquid chromatography; four peaks were found with the same retention time and the same spectrum. Indexing to the peaks at the same chromatogram time, the extraction conditions were optimized. After the separation of the target compounds, four fatty acids including palmitic acid, linoleic acid, oleic acid and stearic acid were got to be identified by NMR and GC-MS. Then all the fatty acids in endophytic fungus and *D. huoshanense* were compared by GC-MS through the one step derivatization using BSTFA. The result showed that the palmitoleic acid, palmitic acid, linoleic acid, oleic acid and stearic acid are both in *D. huoshanese* and

its endophyte HS-01. Meanwhile, the *D. huoshanese* contains myristic acid, pentadecanoic acid, docosanoic acid; but HS-01 contains lauric acid, lignoceric acid, and ergosterol. In **Chapter 4** an established method, saccharides mapping, based on enzymatic (carbohydrase) digestion and subsequent chromatographic analysis of enzymatic hydrolysate, was successfully applied to find same glycosidic bond of crude polysaccharides in the fungus and host plant *D. huoshanense*. Both of the two samples have response on cellulose and pectinase, the corresponding glycosidic bond are β -(1→4)-D-glucose glucosidic linkage and α -(1→4)-D-galacturonic acid glucosidic linkage. So the possibility of the same polysaccharides produced by the two samples existed **Chapter 5** was a summary of this study.

Key words: *Dendrobium huoshanense*; Endophyte; multiple components; fatty acids; GC-MS; polysaccharides; saccharides mapping.

摘要

霍山石斛內生真菌發酵培養及化學成分分離分析

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霍山石斛 *Dendrobium huoshanense* Tang et Cheng ，別名爲米斛，屬蘭科石斛屬多年生草本植物,爲常用名貴中藥材，爲石斛中的上品, 主產于大別山安徽霍山等地, 據《增補本草備要》記載: “斛出霍山, 養胃清熱, 生津止渴, 清虛熱, 功勝全面。” 由於其具有上述功能, 用它代茶生津潤喉、嗓音不衰。現代藥理表明其又具有抗氧化、提高免疫力，抗腫瘤等多種功效。該類未進化的氣生蘭科植物，大多生長在雲霧繚繞的懸崖峭壁崖石縫隙間和參天古樹上，由於霍山石斛生長環境苛刻，野生資源被過度採挖使其生長環境遭到嚴重破壞，導致天然霍山石斛資源稀缺。從 20 世紀 50 年代末開始,國內開始霍山石斛資源可持續開發利用的研究。主要途徑如下:①霍山石斛種子的無菌萌發途徑，②霍山石斛試管苗的器官發生途徑，③霍山石斛試管苗的類原球莖發生途徑，④霍山石斛的人工栽培試驗,等四個主要的方面。[250] 雖然霍山石斛試管苗移栽在 1985 年獲得成功,但至今規模很小,大田移栽後成活率低的問題還沒有從根本上得到解決,很難實現大規模化生產。石斛具有重要的藥用價值，但其數量稀少，尤其是霍山石斛菌更是珍稀，內生真菌與植物有重要的關係，已經從很多植物包括石斛中分離到很多內生真菌，而這些內生真菌有很多具有藥理活性，其與宿主植物在化學成分上具有一定的相

似性，並與宿主植物有共生培養的關係。隨著發酵產業的興起，對石斛內生真菌進行發酵培養，得到的菌絲體對石斛有效成分進行取代或部分取代。

本課題從霍山石斛活體中分離出內生真菌 HS-01，並對其進行發酵累積菌絲體，對菌絲體進行醇提，化學分離。同時對霍山石斛及其內生真菌進行色譜分析，比較其中相似成分。

全文共分五章。**第一章**由淺入深的依次對霍山石斛研究進展，石斛屬植物化學成分，石斛屬內生真菌研究概況進行較爲詳細的綜述。**第二章**霍山石斛內生真菌 HS-01 的分離，培養並採用已建立的多柱色譜全成分分析對霍山石斛及其內生真菌進行初步比较，其中多糖部分对 MS 检测器响应较差，而 Aq-C18 柱对小极性小分子分离效果不好，而从其中正相柱对大极性小分子分析来看，霍山石斛药材主要含有果糖，葡萄糖以及蔗糖，而 HS-01 中主要含有甘露醇。**第三章**對霍山石斛及其內生真菌醇提物進行初步液相色譜分析，发现在 32.8 分钟，37.1 分钟，38.1 分钟以及 42.2 分钟处出现具有相同光谱，相同保留时间的峰出现，即出现相同峰的可能性较高，对其进行进一步分析，以色譜圖中相同時間所出的峰爲指標，優化提取條件，得到以 80%乙醇水系统对其进行提取，並以他們爲目標化合物進行化學分離，經核磁以及 GC-MS 的方法鑒定得到 4 個脂肪酸，分別为棕榈酸，亚油酸，油酸，十八烷酸。並採用 BSTFA 衍生化结合 GC-MS 的方法對霍山石斛及其內生真菌中脂肪酸成分進行定性比較，二者都包含棕榈油酸、棕榈酸、亚油酸、油酸、十八烷酸，其中霍山石斛药材中还含有十四烷酸、十五烷酸、二十二烷酸，而其内生真菌中则另包含十二烷酸、二十四烷酸以及麦角甾醇。 **第四章**採用已建立的糖譜方法對霍山石斛及其內生真菌中所含的糖苷鍵進行初步分析，从分析结果可得，二者对纤维素酶以及果胶酶均有响应，其对应的糖苷键分别为 1,4-β-D-葡萄糖苷键，1,4-α-D-半乳糖醛苷键，其多糖结构相似

的可能性存在。**第五章**總結並討論了研究結果。

關鍵字：霍山石斛；內生真菌；多成分；脂肪酸；GC-MS；多糖；糖譜

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LIST of Abbreviations

CE	Capillary electrophoresis
DAD	Diode array detector
EC	Enzyme Commission
ELSD	Evaporative light scattering detection
ESI	Electrospray ionization
GC	Gas chromatography
HPLC	High performance liquid chromatography
HPSEC	High performance size exclusion chromatography
LOD	Limit of detection
LOQ	Limit of quantification
MS	Mass spectrometry
MW	Molecular weight
NMR	Nuclear magnetic resonance
PDA	Photo diode array
PLE	Pressurized liquid extraction
PMP	1-Phenyl-3-methyl-5-pyrazolone
RSD	Relative standard deviation
S/N	Signal-to-noise ratio
TCMs	Traditional Chinese medicines
TIC	Total ion chromatogram
TLC	Thin layer chromatography
UPLC	Ultra performance liquid chromatography
UV	Ultraviolet

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