Study on Separation and Purification the Secondary Metabolites of \textit{Oudemansiella Mucida}

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Abstract. Oscillation culture was carried out on \textit{Oudemansiella mucida}. The mycelia were extracted with ethyl acetate, separated by silica column chromatography and Sephadex LH-20 dextran gel chromatography. Though determination of chemical and chemical properties of chemical constituents, characterization by organic spectra (MS, 1H NMR, 13C NMR, and DEPT), and comparison with standard compounds, the three secondary metabolites from mycelia were finally identified to be ergosta-4, 6, 8(14), 22-tetraen-3-one; 5α, 8α-epidioxy-(22E, 24R)-ergosta-6, 22-dien-3β-ol, Hexahydropyrrolo [1, 2-α] pyrazine-1,4-dione.

1. Introduction
Medicinal fungi are an important part of natural medicine. Higher fungi contain a variety of secondary metabolites with bacteriostatic activity. Some fungicidal active substances have been developed as pesticide lead compounds into fungicide varieties [1]. In addition, proteins and polysaccharides in higher fungi and their metabolites are the main active ingredients of anti-virus [2].

\textit{Oudemansiella mucida}, commonly known as porcelain fungus, is a basidiomycete fungus of the \textit{Physalacriaceae} family and native to Europe. Recent studies have shown that \textit{Oudemansins contained in Oudemansiella mucida} can significantly inhibit most aerobic saprophytic pathogens [3]. Musilek et al. previously isolated an antifungal active substance mucidin from \textit{Oudemansiella mucida}, then obtained strobilurin and oudemansin [4, 5]. Oudemansins acts to selectively inhibit mitochondrial respiration, and its molecular target is the hydroquinone oxidation center (Qp) that binds to the mitochondrial bc1 complex [6]. In this paper, the mycelia of \textit{Oudemansiella mucida} were extracted with ethyl acetate, separated and identified, to provide a theoretical basis for the further research and development of biological pesticides.
2. Experiment

2.1. Reagents

*Oudemansiella mucida* was purchased from Institute of Botany, Kunming, Yunnan. PDA medium, Ethyl acetate, n-butanol, acetone, Petroleum ether (boiling range 60 ~ 90 °C), chloroform, were purchased from Xi'an Hexin chemical reagent instrument co. LTD, China. All reagents were analytical-reactant grade.

2.2. Instrument

LS-B50L pressure steam sterilizer (Beijing Boya instrument co. LTD), YJ-875SA medical purification device (Thermo Scientific co. LTD), LRH-250A biochemical incubator (Xi'an Keyi instrument co. LTD), SHB-III vacuum pump (Zhengzhou Great Wall Scientific Industry and Trade Co., Ltd.), ZF-6 ultraviolet analyzer (Shanghai Jiapeng Technology Co., Ltd.), X-4 micro melting point instrument (Shanghai Hogon instrument co. LTD), AV-500 nuclear magnetic resonance instrument (Bruker, Switzerland), VG-ZAB-HS mass spectrometer (Thermo Fisher Scientific) were applied in our experiment.

2.3. Sample pretreatment

The plant pathogenic bacteria were activated with a slanted PDA medium, and bacterial suspensions (10^2 to 10^3 cfu/mL) were prepared with sterile physiological saline water solution. The *Oudemansiella mucida* strain was inoculated on a bevel-modified PDA medium, and cultured at 28 °C for 7 days, then inoculated into a 1000 mL Erlenmeyer flask containing 400 mL of liquid culture medium, shake on a rotary shaker at 28 °C for 10 days’ fermentation.

Wash mycelia and dry at 70 °C. The dried mycelium was pulverized and extracted with methanol, and concentrated to the extract. Suspended in water, the suspension was sequentially extracted with ethyl acetate and n-butanol, and then concentrated separately to obtain ethyl acetate and n-butanol Cream. Weigh and record the mass of each extract.

2.4. Isolation and purification of chemical components of mycelium

The mycelium ethyl acetate extract (9.2 g) was dissolved in acetone, and the sample was mixed with crude silica gel (100-200 mesh). 240 g of fine silica gel (200-300 mesh) was packed the column with petroleum ether. It was gradually eluted with petroleum and ether-ethyl acetate (1: 0→50: 1→25: 1→10: 1→5: 1→1: 1→0: 1), followed by TLC to detect 6 fractions (D1, D2, D3, D4, D5, and D6).

2.5. Purity detection and structural identification of single chemical components

Two different elution systems were applied, and the separated compounds were subjected to thin-layer chromatography. The color reaction was performed by ultraviolet lamps (254nm and 365nm), iodine vapor, 5% concentrated sulfuric acid ethanol solution and bismuth potassium iodide solution.

The spots are observed and the melting point is determined to determine the purity. Using modern spectroscopy techniques such as mass spectrometry (FAB-MS) and nuclear magnetic resonance (1H NMR, 13C NMR), refer to the relevant literature spectrum data and compare with standard compounds to determine the structure of the target compound.

2.6. Detection of mixtures

GC-MS was applied to determine the structure of the compounds. The whole isolation process was shown in Fig. 1.

GC-MS conditions:

TRACE DSQ GC-MS (Finnigan Company, USA), DB-5MS (30m × 0.25mm × 0.250μm) flexible quartz capillary column, programmed temperature rise at 40 °C, maintained for 2.5min, and raised to
240 °C at 6 °C / min. Hold for 10min; inlet 250 °C; transfer line 230 °C; carrier gas He gas, flow rate 1.0mL / min; splitless injection, injection volume 1.0μL.

Ionization method EI, 70eV; ion source temperature 250 °C, mass scanning range 35~400amu; emission current 100μA, detection voltage 1.4kV. The random Xcalibur workstation NIST2002 standard library was used to automatically search the mass spectral data of each component, and the content of each component was calculated according to the area normalization method.

**Fig. 1** Isolation and purification of chemical components of mycelium

### 3. Results and discussion

#### 3.1. Separation of chemical constituents from ethyl acetate extract of mycelium

Fragment D2 is a white soap-like solid, which was analyzed by GC-MS. The results were shown in Table 1. The three fragments D3, D4, and D6 were further separated and purified, and three single compounds X-1 were finally obtained. X-2, X-3.

**Tab. 1** The analysis result of fraction D2 from mycelia by GC-MS

| Number | Retention time | Compounds               | Formula               | Relative Content (%) |
|--------|----------------|-------------------------|-----------------------|----------------------|
| 1      | 5.01           | Lauric acid             | C<sub>12</sub>H<sub>24</sub>O<sub>2</sub> | 25.74                |
| 2      | 7.14           | Myristic acid           | C<sub>14</sub>H<sub>28</sub>O<sub>2</sub> | 5.31                 |
| 3      | 9.24           | Palmitic acid           | C<sub>16</sub>H<sub>32</sub>O<sub>2</sub> | 39.48                |
| 4      | 10.87          | Linoleic acid / linolenic acid | C<sub>18</sub>H<sub>32</sub>O<sub>2</sub>/ C<sub>18</sub>H<sub>30</sub>O<sub>2</sub> | 5.33                 |
| 5      | 10.93          | Oleic acid              | C<sub>18</sub>H<sub>34</sub>O<sub>2</sub> | 15.23                |
| 6      | 11.17          | Stearic acid            | C<sub>18</sub>H<sub>36</sub>O<sub>2</sub> | 6.6                  |
Mycelium of *Oudemansiella mucida* contains 7 kinds of fatty acids, with a high content of saturated fatty acids, accounting for 79.44% of the total content. Among them, lauric acid C12: 0 accounts for 25.74%, myristic acid C14: 0 accounts for 5.31%, and palmitic acid C16: 0 accounts for 39.48%. Stearic acid C18: 0 accounts for 6.6%; the content of unsaturated fatty acids is only 20.56%, of which linoleic acid C18: 2 and linolenic acid C18: 3 account for 5.33%, and oleic acid C18: 1 accounts for 15.23%.

**3.2. Purity detection of chemical components**

The purity was determined by TLC analysis, using different elution reagents and different colorimetric methods. The results are shown in Table 3-7.

| Number | Elution reagent               | R_f | colorimetric methods r | Spot color            |
|--------|-------------------------------|-----|------------------------|-----------------------|
| X-1    | Chloroform: Acetone = 9.5: 0.5 | 0.60| ultraviolet (365nm)    | blue-green            |
|        | Petroleum ether: ethyl acetate = 4: 1 | 0.54| 5%H_2SO_4-EtOH         | bright yellow         |
| X-2    | Petroleum ether: acetone = 2: 1 | 0.41| iodine                 | yellow brown          |
|        | Chloroform: methanol = 4: 1    | 0.78| 5%H_2SO_4-EtOH         | purple                |
| X-3    | Chloroform: acetone = 1: 1     | 0.35| iodine                 | yellow brown          |
|        | Chloroform: methanol = 4: 1    | 0.62| Dragendorff            | yellow brown          |

**3.3. Structural identification of chemical components**

**Structural analysis and identification of compound X-1**

Compound X-1 is yellow needle-like crystal (chloroform), melting point (mp): 112 ~ 114 °C, easily soluble in petroleum ether. Applying TLC, the color were yellow-green and pale blue under UV light of 365nm and 254nm, respectively. The color was turned bright yellow in 5% H_2SO_4-EtOH solution, and the Salkowski reaction was positive, which indicated that the molecule may contain a larger conjugate system.

The physical and chemical properties, ¹H NMR, and ¹³C NMR (DEPT) spectrum data of compound X-1 were consistent with the literature values of 4,6,8 (14), 22 (23) -tetraen-3-one-ergostane. The single compound X-1 was finally identified to be ergosta-4, 6, 8(14), 22-tetraen-3-one. The formula is C_{28}H_{40}O, molecular weight 392, and its structure was shown in the Fig2.

**Structural analysis and identification of compound X-2**

X-2 is colorless needle-like crystal (ethyl acetate), melting point (mp): 181 ~ 183 °C, soluble in acetone and chloroform. X-2 has no fluorescence under ultraviolet light, but yellow-brown in iodine. The color was turned purple in 5% H_2SO_4-EtOH solution, with a single spot. Libermann-Burchard reaction was positive, which suggested that compound X-2 is a sterol compound.

The physical and chemical properties, ¹H NMR, and ¹³C NMR (DEPT) spectrum data of compound X-2 were consistent with the literature values. The single compound X-2 was finally identified to be 5α, 8α-epidioxy-(22E, 24R)-ergosta-6, 22-dien-3β-ol The formula is C_{28}H_{40}O, molecular weight 392, and its structure was shown in the Fig2. Compound X-1 and compound X-2 were quite similar, they were homologues.
Structural analysis and identification of compound X-3

X-3 is colorless needle-like crystal (methanol), melting point (mp): 211-213°C soluble in methanol. X-3 has no fluorescence under ultraviolet light, but yellow-brown in iodine with a single spot. The color was turned yellow-brown in Dragendorff reaction, which illustrated that compound X-3 is an alkaloid compound.

The physical and chemical properties, $^1$H NMR, and $^{13}$C NMR (DEPT) spectrum data of compound X-3 were consistent with the literature values. The single compound X-3 was finally identified to be Hexahydropyrrolo [1, 2-α] pyrazine-1, 4-dione. The formula is C$_7$H$_{10}$N$_2$O$_2$, molecular weight 154, and its structure was shown in the Fig3.

4. Conclusion

In this present study, silica gel column chromatography was mainly performed to purify the single compounds in mycelium ethyl acetate extract. With TLC thin layer detection, it is important to accurately collect the product fractions and select the polarity of the eluent. Sephadex LH-20 was applied to separate target compounds due to molecular size differences. Sephadex LH-20 has high separation efficiency and low sample loss, which is very important for the purification of the secondary metabolites of Oudemansiella medical.

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