A Study on Antibacterial Activity and Chemical Composition of the Petroleum Ether Extract from *Aspergillusniger* Mycelia

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Abstract In order to develop natural antibacterial agents, the antibacterial activity of Aspergillusnigerxj was investigated. After being cultured in potato dextrose liquid medium liquid medium, mycelia was under heating reflux extraction with 90% ethanol. Removal of ethanol under reduced pressure gave a residue, to which water was added and then extracted with petroleum ether and ethyl acetate. In vacuo evaporation of the solvents yielded three crude extracts. Then the disc diffusion method was used to measure the antibacterial activity of these extracts. The petroleum ether extract with antibacterial activity was separated by silica gel column chromatography method, then separated and identified by GC - MS after been methyl esterified. At the concentration of 50 mg/mL, the petroleum ether extract of mycelia exhibited inhibitory effect against Staphylococcus aureus. The petroleum ether extract from Aspergillusnigerxj mycelia contained natural substances with antibacterial activity and fatty acids are the main constituents in it.

Key words *Aspergillusniger*, Mycelia, Antibacterial activity, Petroleum ether fraction, GC - MS

1 Introduction

Since penicillin was discovered, the effective method to control and cure the Staphylococcus aureus infection had been taking antibiotics[1]. However, with the abundant use of antibiotics, drug resistant strains had emerged and a kind of Staphylococcus aureus was completely resistant to all antibiotics being in use, namely superbacteria was sure to emerge, it's just a problem of time[2]. As a result, it's time to investigate and develop new drugs curing Staphylococcus aureus infection. Nowadays, many pharmaceutical companies and research institutions all over the world are attaching great importance to the development of new anti-infective drugs by means of screening and discovering new lead compounds from natural products, and then modifying their chemical structures[3]. Among these natural products, an important source to develop new drugs for every country is microbial agent[4]. Aspergillusniger is a kind of microorganism, which is recognized as safe and consists of various enzymatic system with strong activity[5-6]. Food and industry enzyme production strains approved by Food and Drug Administration (FDA) of America are only about twenty species, such as Aspergillusniger, yeast, Bacillus subtilis. Of these strains, Aspergillusniger has produced the most enzymes[7]. Because of industrial enzymes produced by Aspergillusniger, it has raised more concern, but there are few reports about application of Aspergillusniger in investigation and development of antibacterial materials. Y. C Song *et al.*[8] isolated four naphtho - c - pyrones from the culture of endophyte Aspergillusniger IF B - E003 and investigated the cytotoxic antimicrobial and XO inhibitory actions of these compounds. Li Zhu *et al.*[9-10] isolated a strain Aspergillusnigerxj, which exhibited inhibitory effects against Agrobacterium tumefacines and Staphylococcus aureus. Yoko HASEGAWA *et al.*[11] isolated six new alkylitaconic acids from the culture broth of Aspergillusniger FKI - 2342 by solvent extraction, silica gel column chromatography and HPLC and only testuic acid exhibited moderate grow inhibition against Bacillus subtilis. In this paper, with Aspergillusnigerxj as the material, mycelia collected from fermenter were extracted by petroleum ether and ethyl acetate in turn, the antibacterial activity of these crude extracts were primarily screened and the chemical composition of petroleum ether extract with antibacterial activity was analyzed. We hoped these results could provide a base for the development and application of Aspergillusnigerxj in the aspect of antibacterial drug and lay a certain foundation for the following separation and purification of the antibacterial bioactive metabolites.

2 Materials

2.1 Strains Aspergillusnigerxj, isolated by fungal resources institute of Guizhou University, preserved in CMCC, CCTCC NO: M206021. Staphylococcus aureus, preserved in microorganism laboratory of College of Life Science, Guizhou University.

2.2 Medium Potato dextrose agar medium (containing per liter): 200g potatoes cut into masses and then boiled in water for 30 min, of which the filtrate are collected, 20g dextrose, 20g agar, natural pH. Sterilization condition: 121℃, 30 min (sterilization condition, the same as follows).

Potato dextrose liquid medium: the formula is same to the above except for without any agar.

Nutrient agar medium (containing per liter): 3.0g beef, 10.0 g peptone, 15.0g NaCl, 15 - 20g agar, pH 7.2 - 7.4.

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2.3 The main reagents and equipments Anhydrous alcohol, ethyl acetate, petroleum ether are all of the analytical grade (Shanghai Chemical Reagent General Factory and Chongqing East Sichuan Chemical Group), column chromatography silica gel (Branch of Qingdao Haiyang Co, Ltd., China), mass spectrometer (HPMS 5973, HP Co, Ltd., America); Rotary Evaporator (R-201-B-II, Shanghai Yarong Biochemical Equipment Company, China); sterile workbench (SW-CJ-IBV, Suzhou Antai Air Tech Co, Ltd., China).

3 Methods

3.1 Fermentation of Aspergillusniger The mycelia of Aspergillusnigerxj was subcultured on PDA slant and incubated at 27 ℃ for 3-4 d, activated for 2-3 generation. The inoculum was prepared through inoculating the fresh fungal mycelia into 500ml flasks containing 100mL of potato dextrose broth medium and then followed by incubation in a shaking incubator at 150 rpm for 3 d at 27 ℃. With the proportion of 10% (v/v), the inoculum was inoculated into 19L fermenter containing 13L of potato dextrose broth medium at (28 ± 1)℃ for 5d, stirrer rotation speed, 200-300 rpm, aeration rate, 1-2L/min. The culture was filtered with 4 layers of sterile gauze to collect mycelia which was dried under vacuum at 50 ℃ till constant weight. The dried mycelia were ground into power and stored for use.

3.2 The preparation of test samples The power (234 g) was extracted by heating circumfluence 3 times with 700 ml, 300 ml, and 300 ml of 90% ethanol for 3 h, 2 h, and 1h, respectively. The combination of ethanol extracts were filtered and dried to obtain 39g crude material. This crude material was dissolved in minimal volume of water and then extracted with petroleum ether and ethyl acetate in turn. In vacuo evaporation of the solvents yielded three crude extracts, petroleum ether extract, ethyl acetate extract and aqueous phase.

3.3 Antibacterial bioassay

3.3.1 The preparation of petri plate with test strain. A strain slant of Staphylococcus aureus growing on nutrient agar medium well was injected with 5mL sterile saline and beaten gently and stirred with pipette many times. The bacterial suspension was adjusted to 1×10^8 cfu ml-1 by comparing the test and a McFaland Standard 0.5. An amount of 200uL bacterial suspension was added into the sterilized petri plates which were then added with 45-50 10 mL molten agar medium, mixed well(avoid bubbles).

3.3.2 Antibacterial bioassay. Bioassays of crude extracts from Aspergillusnigerxj mycelia using the disc diffusion method[12] were performed against Staphylocuccus areus and the zone of inhibition (mm) surrounding the disc was recorded as the antibacterial activity of these samples. Suitable amount of samples was dissolved in minimum volume of DMSO and then adjusted to the concentration of 50mg/mL with sterile water, corresponding equivalent solvent as blank control. All of the dissolved samples were under ultraviolet light for 30min. An amount of 20uL of samples was placed onto a sterile disc and the solvent allowed to evaporate by drying through an alcohol lamp a little while. The inhibition zone surrounding the disc on a bacterial plate was measured in three replicates by incubating the plates at 37℃ for 36h. Crossing method was performed to determine the antibacterial activity.

3.4 Chemical composition of petroleum ether extract

3.4.1 Isolation. The petroleum ether extract (7g) was applied on a silica gel column chromatography and eluted stepwise with 1:0-8:1 (v/v) of PE-EtOH solvents. Two fractions were collected and dried to yield yellow oily materials (Fr-1 112.3 mg and Fr-2 85.5mg). The thin-layer chromatograms of the two fractions are different, so each was analyzed by GC-MS respectively.

3.4.2 Methyl esterification of samples. Firstly, 100mg of Fraction 1 and fraction 2 were respectively put in flasks and dissolved in 40 mL benzene-petroleum ester(1:1, *v/v*). Then 20 ml 0.4 mol/L KOH-MeOH were added to each flask which was mixed well meanwhile and kept in water bath at 40 ℃ for 30 min, cooled and added with 40mL distilled water. At last, the supernatant fluid was under reduced pressure to remove of solvent after stratification was clearly formed and yield methyl esterification-products.

3.4.3 Gas chromatography-mass spectrometry analysis. The GC operating condition were as follows: A HP-5MS column (0.25 mm × 30 m, 0.25 μm) was used with helium as a carrier gas at a flow rate of 1.0mL/min and injection volume was 1 μl. The GC oven temperature was kept at 50 ℃ for 2 min and programmed to 280 ℃ at a rate of 5 ℃/min and kept constant at 280 ℃ for 3 min. Splitless injections were one with liquid injection method. The MS operating condition were as follows: carrier gas was He at a rate of 1.0 mL/min; mass spectrometer ionization energy was 70 eV, and electron multiple tube voltage was 1785 V; the ion source temperature was set to 230 ℃, and the quadrupole temperature was set to 150 ℃; the mass scan range was 10-550 amu.

4 Results and analysis

4.1 Antibacterial activity bioassay 4 kinds of crude extracts were collected by treating on mycelia power of Aspergillusnigerxj with the 2.1 method, they were extraction of ethanol, petroleum ester extract, ethyl acetate extract, aqueous phase, respectively. The antibacterial activity of these different crude extracts was shown in table 1, which was measured by means of the 2.3 method.

Table 1 The antibacterial activity of extracts from Aspergillusnigerxj mycelia

	Crude extracts	The inhibition zone(mm)
		Test strain Staphylococcus aureus
Mycelia	Extraction of ethanol	-
	Petroleum ester extract	9.50 ± 0.49
	Ethyl acetate extract	-
	Aqueous phase	-
Control	Solvent	-

The results showed that petroleum ether extract from Aspergillusnigerxj mycelia had certain antibacterial activity against Staphylococcus aureus at the concentration of 50mg/mL, other extracts had no obvious effects.

4.2 The chemical composition of petroleum ether extract Under the experimental condition of 2.4.3, methyl ester of fraction 1 and fraction 2 were isolated by GC into eight peaks and five peaks respectively. The components of the samples were identified by their mass spectra in comparison with those in the NIST 98 and WILEYMS libraries and combination with relevant essays[13]. The results are as follows (Table 2, 3).

Table 2 Constituents and relative contents of fraction 1 from A. nigerxj mycelia

No	tR//min	Compound	Molecular formule	Relative molecular mass	Relative content//%
1	27.084	Octadecanoic acid, methyl ester	$C_{19}H_{38}O_2$	298	0.482
2	26.651	6-octadecenoic acid, methyl ester, (z)-	$C_{19}H_{36}O_2$	296	0.754
3	26.538	9,12-octadecadienoic acid, (z, z)-, methyl ester	$C_{19}H_{34}O_2$	294	8.944
4	24.672	Eicosane,9-octyl-	$C_{28}H_{58}$	394	0.528
5	24.097	Phthalic acid, hexyl octadecyl ester	$C_{32}H_{54}O_4$	502	0.734
6	23.249	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	50.298
7	22.693	Dotriacontane	$C_{32}H_{66}$	451	34.397
8	20.629	Octadecane	$C_{18}H_{38}$	254	3.863

Table 3 Constituents and relative contents of fraction 2 from A. nigerxj mycelia

No	tR//min	Compound	Molecular formule	Relative molecular mass	Relative content//%
1	34.011	Tetracosanoic acid, methyl ester	$C_{25}H50O_2$	382	7.810
2	27.094	Octadecanoic acid, methyl ester	$C_{19}H_{38}O_2$	298	53.420
3	26.660	10-octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296	32.946
4	26.547	9,12-octadecadienoic acid, (z, z)-, methyl este	$C_{19}H_{34}O_2$	294	3.847
5	23.258	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	1.977

The results of GC-MS analysis showed that the main components of the petroleum ester extract from Aspergillusnigerxj mycelia were fatty acids and alkanes. 8 compounds were identified from the fraction 1 and characterized by the presence of saturated fatty acidhexadecanoic acid (50.298), dotriacontane (34.397%), polyunsatuated fatty acid 9, 12 – octadecadienoic acid, (z, z) – (8.944%); 5 compounds were identified from the fraction 2 and characterized by the presence of saturated fatty acid (53.420%), monounsaturated fatty acid 10 – octadecenoic acid(32.946%). It can be concluded from the result that petrolium ester extract from Aspergillus niger xj myceliaconsist of various kinds of fatty acids which varid enomously in the aspect of contents and is found to be rich in saturated fatty acids.

5 Discussions

According to the results of antibacterial activity bioassay, petroleum ester extract from Aspergillusniger mycelia possesses moderate inhibitory effect against Gram negative bacterial Saphylococcusaureus and it can be concluded that Aspergillusniger mycelia consist of natural antibacterial bioactive materials. However, antibacterial activity was not detected in other extracts from Aspergillusniger, which maybe resulted from the monotonousness of the test strain only for screening of antibacterial activity bioassay. Palmitic acid, which is predominant component of petroleum ether extract from Aspergillusnigerxj mycelia, can deduce the amount of serum cholesterol; stearic acid can partly reduce the dissolution of cholesterol well as reduce the cholesterol content in serum and liver through adjusting the formation of cholicacid[14]. Few investigation of 10 – octadecenoic acid was reported, so its potential function and application need to be further studied. Linoleic acid is an essential fatty acid for human body, conjugated linoleic acid (Conjugated Linoleic Acid, CLA) which is an isomer of linoleic acid having some effects on killing melanin tumor, colorectal cancer cells and breast cancer cell culture of human beings[15]. Furthermore, we need to determine the antibacterial activity of the chemical constituents of petroleum ester extract from Aspergillusnigerxj mycelia and interactional effects among them. In summary, this research determines that antibacterial bioactive material of Aspergillusniger mycelia is from the petroleum ester extract. We may suggest that Aspergillusniger has the potential value for producing natural antibacterial bioactive compounds and can be used as a new resource for screening of novel anti-infective drug.

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