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Molluscicidal activity against *Oncomelania hupensis* of endophyte JJ18 from *Pseudolarix kaempferi* Gord

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ABSTRACT

To screen a kind of biological controlling measure against the Oncomelania hupensis snails, This study is to research the molluscicidal effect of JJ18 and identify its species. The alcoholic extract of JJ18 broth effectively killed O. hupensis snails, and the n-butanol fraction of the extract had the best molluscicidal activity compared with other fractions. Ochratoxin A was detected from JJ18 broth with ELISA analysis, and it may be a molluscicidal active substance in the broth. JJ18 strain belongs to Aspergillus niger was identified by morphology characteristics of the slide culture and rDNA ITS sequence analyzing.

Key word: *Oncomelania hupensis;* Endophyte; molluscicidal activity*; Pseudolarix kaempferi* Gord; ITS sequence.

INTRODUCTION

Schistosomiasis is one of the most widespread human parasite diseases in the world. In China, as well as in several African, the Caribbean, the Middle East, and South American countries, the disease continues to be a major public health problem. Integrated schistosomiasis control programs were recommended by WHO(1), and the intermediate host snail control is an important preventive strategy associated with treatment of infected people, together with environmental and socio-economic improvements and health education with community participation. Although chemical molluscicides are the most useful approach for the snail control, the majority of studies have demonstrated rapid re-colonization of the transmission sites by the intermediate hosts. *Oncomelannia hupensis* is the only intermediate host of *Schistosoma japonicun* Katsurade(2), and is the most fragile during its life cycle, and one way to resolve the problem of *Schistosoma* is to destroy the carrier snails and break the life cycle of the parasite, thus it is an essential practice to destroy snails of *O. hupensis*. The treatment with molluscicides of water masses serving as transmission sites is considered an important element in an integrated strategy for the control of the disease. At present, only one synthetic molluscicide (niclosamide) is recommended for schistosomiasis control, but it is expensive and the cost involved in the large-scale use of this compound is prohibitive for most of the developing countries concerned. However, together with concerns about their toxicity to non-target organisms and the possible development of snail resistance, it has lead to a demand for cheaper alternatives(3–4). To find a safe and low-cost alternative, miscellaneous resources should be screened for molluscicidal activity.

Since the 1930s, the molluscicidal properties of numerous plant extracts have been studied, especially

the novel plant species that exhibit molluscicidal activity are greatly facilitated by consideration of traditional folk medicine or the ethnopharmacological literature for use by local communities in the control of the snail vector(5). Endophytic microorganisms exist within the living tissues of most plant species. They are most abundant in medicinal plants. Novel endophytes usually have associated with them novel secondary natural products and/or processes(6–7). On the other side, the molluscicidal effect of plant allelopathy is recognized and established(8), many researchers focus on chemical constituents of plants to explain the allelopathy effect(9), but its mechanism is not clear till now. Plant allelopathy connects with endophyte has been suggested(10–11).

To screen microorganism for molluscicidal activity is one of the most promising categories in biomolluscicides(12). Bacteria and actinomycete are reported microorganisms capable of molluscicidal activity in China(13–14). *Pseudolarix kaempferi* Gord is a Chinese endemic species, which is one of the common and representative plants in China, and used as Traditional Chinese Medicines(15). 106 strains of endophytes were isolated from branches and leaves of *P. kaempferi* Gord(16). JJ18 is one strain of the endophytes isolated. Studies have confirmed that JJ18's fermentation broth has molluscicidal activity(17). On the basis, the present study focused on molluscicidal activity of extracts of JJ18 broth, and on the classification of the species of JJ18 by their morphology characteristics cultured in potato dextrose agar (PDA) medium and by molecular biology.

MATERIAL AND METHODS

Endophyte

JJ18 is one strain of endophytes isolated by Jia He(16) from *P. kaempferi* Gord, and a voucher specimen is kept in the Pharmacognosy Laboratory, College of Pharmacy, Jiangsu University.

Animals

Snails of *O. hupensis* with relatively uniform in size (1.1±0.15 cm in shell height) were collected from the beach of the Yangtze River near Zhenjiang. They were acclimatized in the laboratory at room temperature (25±2°C) for 24h.

Preparation of samples

JJ18 broth (medium: potato20%, glucose2%, the other is water) was treated by centrifugation in 5000r/min and the supernatant, that is to say, the extracellular moiety of broth with pH3, was obtained. The supernatant was salified to pH7 with NaOH, then 6 volumes (v/v) of 95% ethanol were added to the salified supernatant and kept the solution at 4 °C for 24h, and then the deposition contained in the ethanol mixture was removed by centrifugation. Thus obtained supernatant was concentrated by vacuum evaporator to get alcoholic extract.

Then the alcoholic extract was further extracted by four different solvents one after one: petroleum ether, diethyl ether, ethyl acetate and n-butanol, and the obtained solutions were concentrated by vacuum evaporator to get every a fraction, respectively.

Molluscicidal activity assay of the alcoholic extract.

Molluscicidal activity against *O. hupensis* was performed according to WHO guiding(18). 3600 snails were randomly and equally divided into 120 groups with every group containing 30 snails. Dechlorinated tap water was used as diluent. Eight concentrations of 50, 100, 150, 200, 250, 300, 350, and 400mg/L of the alcoholic extract were used to treat test snails, the positive control and negative control groups were performed with 1ppm of niclosamide aqueous and with the dechlorinated tap water, respectively. Every thirty adult snails, as one group, were chosen randomly for the molluscicidal activity test and submerged in every a beaker, containing 300ml of test solutions mentioned above, and kept under normal diurnal lighting condition for 24, 48, 72 and 96h test, respectively. Each test was set in triplicate. At the set time, the suspension in beakers was decanted; the snails were washed with dechlorinated tap water. The test snails were then left in dechlorinated tap water and observed for 72 h, the snails climbing upwards the wall of beakers were judged to be living, and the snails remained in the bottom of beakers were further examined to check mortality by mechanical prodding. The ratio of dead snails to total tested snails was expressed as mortality (%).

Screening of molluscicidal active fractions.

Total 450 snails were randomly and equally divided into 15 groups. Dechlorinated tap water containing 0.03% tween-80 was used as diluent. 200mg/L of the five fractions was used to treat the snails, and the negative control group was performed with the diluent only. The every 30 snails, as one group, were submerged in beakers containing the test solutions for 72h, respectively. Each test was set in triplicate.

Stability of the extracellular broth.

10 ml of extracellular moiety of the broth and 10 ml of its salified one were handled in three ways: (1) The samples heated at different temperature (40°C, 60°C,

80°C and 100°C) for 30 minutes. (2) heated for different periods (30 min 60min 90min 120min 300min) under same temperature (80°C). (3) The samples were placed in an artificial climate box with constant light intensity (˜1200 lux) and temperature (25±1°C), but for different periods (1d, 3d, 6d, 9d and 15d). These treated samples were then diluted to 100ml by dechlorinated tap water as solutions submitted for molluscicidal activity test for 72h to investigate the stability of the molluscicidal active substances. Total 840 snails were randomly and equally divided into 84 groups. Every 10 snails, as one group, were submerged in beakers containing the test solutions for 72h, respectively. Each test was set in triplicate. The other conditions and procedures of the currently mentioned test were the same as mentioned above.

Glycogen (Gn) and Total protein (TPr) assay.

Dechlorinated tap water was used as diluent. Five concentrations of 50, 100, 150, 200 and 250mg/L of the alcoholic extract, which were less than the LC_{50} value in the case of submerged period 48h, were used to treat *O. hupensis* snails. The negative group was performed with the diluent only. Total 1200 snails were randomly and equally divided into 15 groups. Every 80 snails, as one group, were submerged in beakers containing the test solutions for 48h, respectively. Each test was set in triplicate. After exposure for 48 h, the living snails were taken out from the beaker and its soft tissues were weighed before and after drying in oven for 24 h at 40ºC, respectively, the weight ratio of dry/wet were calculated and then the dried soft tissue was grounded into fine powder. Then 2ml aqueous of 30% KOH was added to the test tube with 10 mg of the powder and incubated in boiling water for 20 min, and then cooled to room temperature. Finally 10 ml ethanol was added to the tube after cooling. Glycogen content was analyzed by anthrone colorimetric method.

30mg of the powder were used and the TPr content was detected with KND-04 Kjeldahl nitrogen detection device, and calculated as: (TPr) %=6.25×N%.

Statistical analysis.

The molluscicidal activity test data were statistically analyzed by SPSS13.0, and the result was expressed as mean ±standard error. The LC_{50} and LC_{90} values were calculated by the probit analysis.

JJ18's species classification by Morphology.

The genus of strain JJ18 was classified by slide culture in PDA medium (Glucose 20%, peptone 20%, agar powder 1.8%, w/v). After 3 days culture, the morphological characteristics were observed with a microscope (using a ×40 and the ×100 magnification Olympus BX41, Japan). The genus, which JJ18 belongings to, was then determined according to the observed morphology.

JJ18's species classification by ITS sequence analyzing.

For total DNA extraction from fungus comb (mycelium) or carpophore, we used a simplified Double - Sedimentation Method(19). The entire region ITS1 – 5.8S – ITS2 was amplified by PCR. The total reaction mixture of 50μL composed of 5μL of 10×PCR buffer, 2μL of 5mmol/L dNTP, 2μL of 25mmol/L $MgCl_2$, 1μl of each primer (ITS1 5' TCCGTAGGTGAACCTGCGG 3' and ITS4 5' TCCTCCGCTTATTGATATGC 3' 1μmol/L) 2.5 u of *Taq* polymerase, 2μL of DNA solution (DNA 20ng), the other is water. The tubes were placed in a thermal cycler (My cycler 1.065, bio-rad USA) for amplification under the following conditions: 36 cycles of (1) denaturation at 94°C for 1 min, (2) annealing at 55°C for 1 min, (3) extension at 72°C for 80s. PCR products were electrophoresed in 1% low-melting temperature agarose gel, visualized with ethidium bromide, and excised separately. The separated PCR products were then further purified with Quick PCR Purification Kit (Shanghai Generay Biotech Co. Ltd) according to the manufacturer's protocol. The sequence of the purified DNA was analyzed by the Generay Co. Ltd (Shanghai, China).

The ITS regions of *Aspergillus* from GenBank were referred. The alignments were performed with the Clustal W package(20), and the aligned sequences were corrected manually, focusing on gap positions. DNA sequence data were analyzed to provide pairwise percentage sequence divergence. A phylogenetic tree was created according to the Nucleotide Kimura, 2-parameter method with software of MEGA version3.1. Bootstrap confidence intervals(21) on each branching pattern were calculated for 1000 replications of resampling.

Detection of Ochratoxin A in the alcoholic extract by ELISA.

Ochratoxin A (OTA) calibration and quantitative detection from JJ18 broth's alcoholic extract were carried out according to OTA Kit protocol (Bohom Bio-product Co. Ltd, Nangchang, China).

RESULTS AND DISCUSSION

Molluscicidal activity of alcoholic extract of JJ18's broth.

The alcoholic extract of JJ18's broth showed a moderate Molluscicidal activity (Table 1). The LC_{50} and LC_{90} values

Table 1. The mortality of snails treated by alcoholic extracts (n=3)

Submerged periods (h)	mortality (%) with different dose (mg/L)									
	50	100	150	200	250	300	350	400	LC50*	LC90*
24	10.0±1.1	16.7±1.5	26.7±2.7	36.7±3.3	36.7±3.9	40.0±3.8	33.3±3.5	46.7±5.1	NA	NA
48	26.7±.5	30.0±3.2	33.3±3.1	43.3±4.6	43.3±4.7	53.3±4.9	53.3±5.0	60.0±6.7	284.3±25.8	NA
72	30.0±2.9	33.3±3.2	53.3±6.0	60.0±6.4	60.0±5.7	86.7±8.9	96.7±9.2	100.0±10.2	144.9±15.6	311.9±33.3
96	33.3±3.7	43.3±4.2	60.0±6.3	66.7±6.8	66.7±6.4	93.3±9.2	100.0±9.5	100.0±10.5	117.7±10.3	265.1±27.4

*:Mortalities for dechlorinated tap water and for the water containing 0.03% tween-80 were 0 %, and the mortality for 1mg/L of niclosamide was 100%, for all test time of 24h, 48h, 72h and 96h, respectively. NA: Not Available

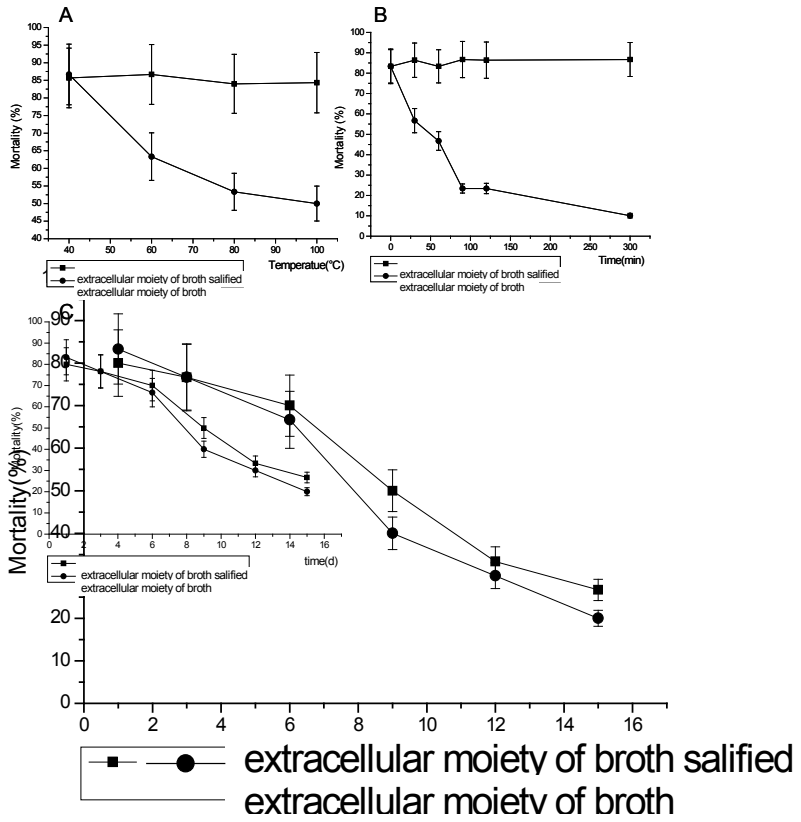

Figure 1. Stability of the extracellular broth against temperature, time and illumination

for different concentrations are shown. There was a significant negative correlation between LC_{50} values and exposure periods. When increasing the exposure time, the LC_{50} of the alcoholic extract was decreased from 419.8 mg/L (24 h) to 117.7 mg/L (96 h).

Stability of the extracellular broth.

Extracellular moiety of JJ18 broth was acetose (pH=3), and was thermostabile obviously after salified (Figure 1). The molluscicidal activity after been salified had no change staying in 80°C for 300min and 100°C for 30min, respectively. The molluscicidal activity was stable within 7 days and then degraded rapidly under the light intensity of 1200 lux. It suggested that the active substance in extracellular moiety of JJ18 broth was thermo-labile under acidic conditions and thermostabile under neutral conditions. That is to say, the active substance could play its role and be little residual under natural conditions.

Glycogen and total protein content of snails' soft tissues tested.

There were no obvious changes of averaged dry and wet weights of soft tissues before and after treatments respectively (Table 2). The Glycogen content decreased greatly after treatments, ranging from 10.42 % to 28.03 % (Table 3). The decreasing rate was parallel to the molluscicidal activity. The stronger the molluscicidal activity, the more decrease the glycogen content. The total

Table 2 Weight of soft tissues of snails treated by alcoholic extracts(n=3)

Item	Concentration (mg/L)					
	50	100	150	200	250	control
Average wet weight(mg/snail)	12.35±1.15	12.13±1.22	12.67±1.18	12.91±1.36	12.60±1.24	12.50±1.39
Average dry weight(mg/snail)	3.06±0.32	3.09±0.29	3.08±0.31	3.05±0.32	2.99±0.30	3.01±0.33
(Average dry weight)/(wet weight)(%)	24.78±2.58	25.47±2.67	24.31±2.45	23.63±2.56	23.73±2.74	24.08±2.21

Table 3 Glycogen Content of soft tissues of snails treated by alcoholic extracts(n=3)

Item	Concentration(mg/L)					
	50	100	150	200	250	control
Content of dry powder(%)	9.97±1.02	9.58±1.11	9.11±1.05	8.94±0.91	8.01±0.98	11.13±1.22
Decreasing ratio (%)	10.42±1.15	13.93±1.48	18.15±1.93	19.68±1.89	28.03±2.94	

Table 4 Total protein content of soft tissues of snails treated by alcoholic extracts(n=3)

Item	Concentration (mg/L)					
	50	100	150	200	250	control
Content of dry powder (%)	27.34±2.69	28.23±2.94	27.44±2.86	26.13±2.57	26.46±2.76	33.81±3.45
Decreasing ratio (%)	19.14±2.04	16.50±1.75	18.84±1.63	22.72±2.06	21.74±2.23	

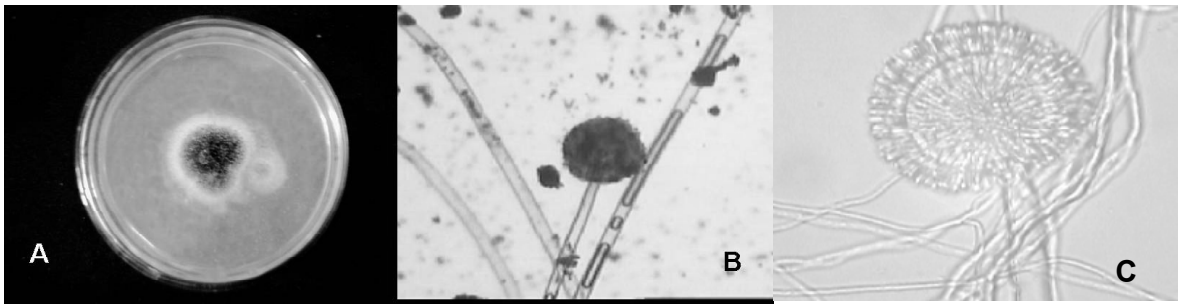

Figure 2. Morphology and hyphal structure of JJ18 on PDA medium

protein content (Table 4) also changed after treatments, ranging from 16.50% to 22.72%, but there was no linear relationship between molluscicidal activity and protein content decreasing rate.

Classfication of JJ18's species.

JJ18 was considered belonging to an *Aspergillus* according to its morphology characteristics cultured in PDA medium (Figure 2)

The rDNA ITS sequence described herein has been assigned GenBank Accession No. EF660198. The rDNA ITS sequence analysis showed that the sequence of JJ18 had a higher similarity to those of *Aspergillus niger* and the differences were located in ITS1 and ITS2 areas, which both belong to variable region. Accordingly, JJ18 strain should been classified into *Aspergillus niger* (Figure 3).

Screening of the active fraction and possible active substance of the alcoholic extract.

The result of molluscicidal activity with concentration 200mg/L of five fractions showed that the n-butanol extract had the best effect (Figure 4).

The above investigation showed that the fermentation broth of JJ18 possesses strong acidic property, its molluscicidal active substances are thermostabile under neutral salified conditions and the unknown substance can be degraded under natural illumination condition, and it had been known that JJ18 belongings to *Aspergillus niger*. Considering that the property of OTA, which is the most representative toxic metabolic product from *Aspergillus niger*, is rather similar to the property of the unknown mulluscicidal active substance contained in the JJ18's broth, we presume reasonably the molluscicidal active substance may be OTA (Appendix A). With ELISA

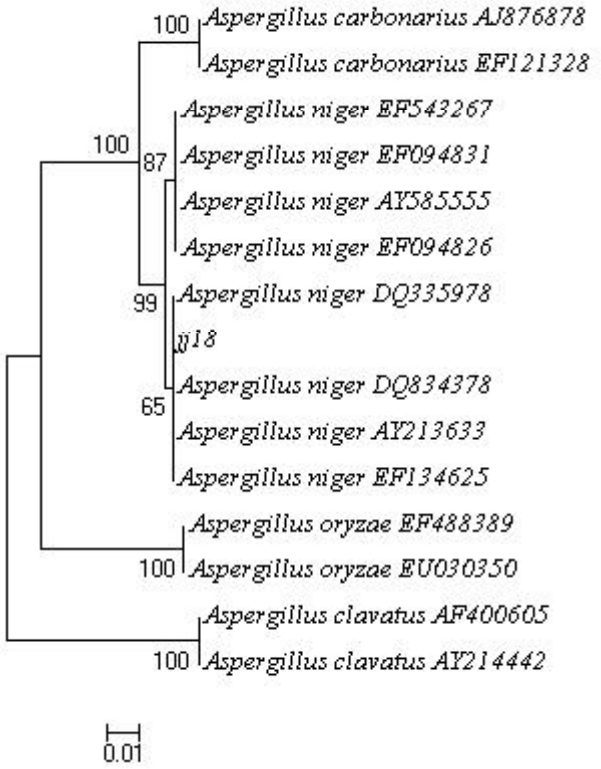

Figure 3. ITS DNA phylogenetic trees of JJ18

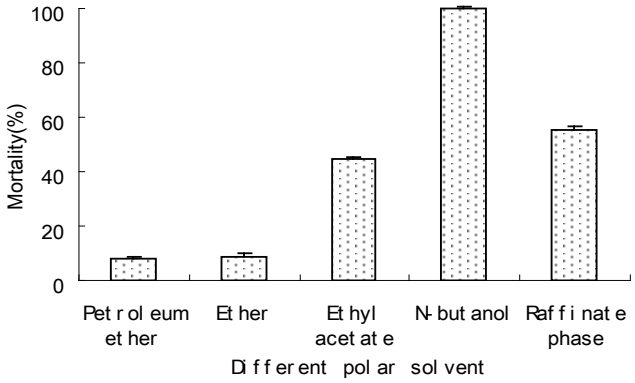

Figure 4. Molluscicidal effect of different solvent extracts

analyzing, OTA was actually detected from the alcoholic extract, and its concentration in the extract, although in a very low level, was 5.072ng/g.

CONCLUSIONS

The alcoholic extract of extracellular moiety of JJ18 broth has molluscicidal activity which was both time and dose dependent. The molluscicidal active substance was stable in natural conditions within 7 days, therefore it showed that the active substance of extracellular moiety of JJ18 broth can be degraded in natural conditions.

The alcoholic extract of JJ18 can decrease the glycogen content in snail's soft tissues suggests that the abnormity of energy metabolism might be the major factor for the molluscicidal activity(22).

Further, OTA was detected in the metabolic production, whether it was the molluscicidal active substance is remained for further research. It should be pointed out that OTA is a mycotoxin produced by several species (fungi and moulds) of *Aspergillus* and *Penicillium*(23), and has been widely studied so far since it is considered as a potential human health hazard.

Classification of endophyte on morphologic features and molecular biology was simple, rapid and accurate. It had been proved that ITS sequence could be used in classification and taxonomy of endophyte(24).

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