A high-performance molluscicidal ingredient against *Oncomelania hupensis* produced by a rhizospheric strain from *Phytolacca acinosa* Roxb.

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ABSTRACT

Background: Snail (*Oncomelania hupensis*) control is an important and effective preventive strategy in schistosomiasis control programs, and screening microbial molluscicidal agents is one of the most promising categories in biomolluscicides. **Objective:** To purify and identify the molluscicidal ingredient (MI) obtained from strain SL-30's exocellular broth. **Materials and Methods:** The active extracts extracted from SL-30's exocellular broth was purified on a silica gel column guided by molluscicidal activity assay against *Oncomelania hupensis*, then the MI was obtained. NMR spectroscopy and LC-MS/MS analysis was used to identify the molecular structure of the MI. **Results:** Molluscicidal activity bioassay showed that the MI exhibited significant molluscicidal activity with the LC_{50} values of 0.101, 0.062, and 0.022 mg/L, respectively, in the case of exposure period of 24 h. From ^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY, and ^{1}H-^{13}C HSQC spectra, partial important structure fragment was obtained, and the relative molecular weight of the MI showed 326 according to LC-MS analysis. Then, on these grounds, it was indicated that the molecular structure of the MI had a higher similarity to Gliotoxin with the molecular formula of $C_{13}H_{14}N_2O_4S_2$. The quasi-molecular ion of *m/z* 325.45 was further analyzed by MS^2 as the parent ion, and two daughter ions obtained at *m/z* 295.11 [M-CH_2OH]- and *m/z* 261.08 [M-CH_2OH -2S] – . **Conclusion:** The MI was finally confirmed as Gliotoxin.

Key words: Gliotoxin, Liquid chromatography-mass spectrometry / mass spectrometry analysis, molluscicidal ingredient, nuclear magnetic resonance spectroscopy, *Oncomelania hupensis*, *Phytolacca acinosa* Roxb., strain SL-30

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INTRODUCTION

Schistosomiasis remains a parasitic disease prevailed in many parts of the world, such as Africa, Asia, tropical America, and so on.[1] In China, Schistosomiasis caused by *Schistosoma japonicum* is epidemic in the Southeast, especially in the areas of Yangtze River valley.[2] Snail (*Oncomelania hupensis*) is the only intermediate host of *Schistosoma japonicum*,[3] therefore in schistosomiasis control programs, snail control is an important and effective preventive strategy.[4] Niclosamide, a synthetic compound, is the only commercially available molluscicide recommended by the World Health Organization.[5] However, the high cost of synthetic compound, along with the difficulty of being naturally decomposed, potential resistance of snail to action of the drug, acute and potential toxicity to nontarget organisms,[6] push the scientists to search the alternative biomolluscicides, which are cheap, effective, readily available, and less hazardous to the environment.

Compared with other organisms, microorganisms have diversity of species type, metabolic pathway, and metabolic product,[7-9] furthermore, short growth period and inducible yield ability.[10] Accordingly, screening microbial molluscicidal agents with a rational and effective method is one of the most promising categories in biomolluscicides. In the previous study a strain from *Aspergillus fumigitus* named as SL-30 has been isolated from rhizosphere of medicinal plant *Phytolacca acinosa* Roxb.,

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and studies have confirmed that SL-30's exocellular broth performed excellent molluscicidal activity.[11] Actually many secondary metabolites with bioactivity,[12] such as antitumor activity, antibacterial activity,[13] and antiviral activity,[14] have been obtained from *Aspergillus* sp. However, study on molluscicidal activity of compounds produced by *Aspergillus fumigitus* against *Oncomelania hupensis* has not been reported so far. For this reason, the present study focuses on isolating, purifying, and identifying the molluscicidal ingredient (named as MI) obtained from SL-30's exocellular broth according to molluscicidal activity bioassay, and providing a foundation for further research.

MATERIALS AND METHODS

Microorganism and snails

Strain SL-30 (*Aspergillus fumigitus*) was isolated from rhizosphere of medicinal plant *Phytolacca acinosa* Roxb., and a voucher specimen is kept in the Pharmacognosy Laboratory, College of Pharmacy, Jiangsu University, China.

Snails of *Oncomelannia 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 and acclimatized in the laboratory at room temperature (25°C±2°C) for 2–3 days.

Extracts from SL-30's exocellular broth

Strain SL-30 was cultured at 28°C with 120 rpm of agitation for 4 days, then the SL-30's cultures were collected and filtered. The filtered liquor (namely exocellular broth) was evaporated under a vacuum, and 6 volumes (v/v) of ethanol (95%) were added to the evaporated exocellular broth and the solution was kept at 4°C for 24 h, and then the supernatant was obtained by centrifugation (3000 rpm, 10 min). After evaporation under a vacuum to remove ethanol, the supernatant was further extracted successively with 4 organic solvents of increasing polarity: petroleum ether, diethyl ether, ethyl acetate, and *N*-butanol. Then each obtained extract along with the final raffinate was respectively evaporated to dryness under a vacuum to get totally 5 different polar fractions: petroleum ether polar fraction, diethyl ether polar fraction, ethyl acetate polar fraction, *N*-butanol polar fraction, and the raffinate, which were then followed by molluscicidal activity assay, respectively, to screen molluscicidal active fraction.

Molluscicidal activity assay

Molluscicidal activity against *Oncomelannia hupensis* was performed with the immersion method according to WHO recommended.[15] At room temperature (25°C±2°C), Each 3 bags of live snails as one group (30 snails per bag) was submerged into a beaker containing 900 mL of the test solutions, which was prepared with dechlorinated tap water and kept soaking for 24, 48, and 72 h, respectively. At the same time, niclosamide aqueous (1 mg/L) adopted as the positive control, and dechlorinated tap water as negative control groups. Each test was set in triplicate on 3 different days. Then the test snails were removed from the bags and douched using dechlorinated tap water for 3 times, and finally kept in breeding condition for 3 days. The snails climbing upward the wall of beakers were judged to be alive, 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 (%).

Purification and chemical analysis

The molluscicidal active fraction, after confirmed and obtained according to molluscicidal activity assay as mentioned above, was chromatographed by a silica gel (100–140 mesh) column and eluted stepwise with a petroleum ether:ethyl acetate gradient starting from 45:10 (v/v) to 45:80 (v/v), then every 15 mL volume of elution was obtained and named as collects. For TLC analysis, every collect of elution was then spotted on plates (GF_{254}), with ether:ethyl acetate (2:8, v/v) as developing solvent, then observed under the light of a UV lamp (254 nm). Thus the collects with the same Rf value were gathered together to obtain several candidates, and among which the active candidate was determined by molluscicidal activity assay. Subsequently, the active candidate was further chromatographed to obtain the purified MI.

After evaporated to dryness, the sample was redissolved in methanol and filtered with a membrane filter (0.22 μm) for HPLC analysis. HPLC analysis was performed on an Intersphere ODS-AP RP_{18} 5-μm reverse-phase 4.6- × 250-mm column (Yilite, Dalian, China). Mobile phase of methanol:water (50:50, v/v) was delivered at a constant flow rate of 1.0 mL/min. Detection was performed at 267 nm using an UV detector (SPA-20A). Volumes of 20 μL sample were injected.

Nuclear magnetic resonance spectroscopy

^{1}H nuclear magnetic resonance (NMR), ^{13}C NMR, DQF (Double Quantum Filtered) ^{1}H-^{1}H COSY and ^{1}H-^{13}C HSQC analysis (MeOD, δ ppm/TMS) were run on Bruker AVANCEII 400 spectrometer at room temperature.

Liquid chromatography-mass spectrometry / mass spectrometry analysis

Liquid chromatography-mass spectrometry / mass spectrometry (LC-MS/MS) analysis was carried out using Finnigan LCQ Deca XP MAX system (Thermo, Massachusetts, USA). HPLC analysis was performed on an RP_{18} 5-μm reverse-phase 4.6- × 150-mm column (Agilent,

Palo Alto, USA). Mobile phase was methanol:water (50:50 v/v) with constant flow rate of 0.6 mL/min, and column temperature was at 35°C. Detection was set at 267 nm using a UV detector (SPA-20A). Electrospray ionization source (ESI) was operated in negative mode, and ion trap mass spectrometer was used for mass analysis.

Statistical analysis

The molluscicidal activity test data were statistically analyzed by SPSS13.0. The LC_{50} and LC_{90} values were calculated by the probit analysis with the 95% confident limit (95% CL).

RESULTS

Purification of molluscicidal ingredient

After molluscicidal activity assay, diethyl ether polar fraction from SL-30's exocellular broth showed the highest molluscicidal activity on snails [Table 1]. Two main candidates were obtained from the diethyl ether polar fraction by column chromatography, but molluscicidal activity was only found in the candidate, which was eluted by petroleum ether/ethyl acetate of 45:20. Then the purified MI in the active candidate was obtained by further repeated column chromatography, and finally showed a single absorption peak on HPLC with the peak area content of 97.35% [Figure 1]. After evaporation, it led to a white needle crystal, which exhibited excellent molluscicidal activity with 24 h LC_{50} values of 0.101 mg/L and LC_{90} values of 0.355 mg/L, 48 h LC_{50} values of 0.062 mg/L and LC_{90} values of 0.121 mg/L, and 72h LC_{50} values of 0.022 mg/L and LC_{90} values of 0.066 mg/L, respectively [Table 2].

Liquid chromatography-mass spectrometry analysis

LC-MS analysis was performed to obtain the relative molecular weight. UV chromatogram of the MI by Finnigan LCQ Deca XP MAX system was shown as Figure 2, and the MI showed a single absorption peak with the retention time of 9.69 min.

ESI/IT (electrospray ionization/ion trap) mass spectrum of retention time from 9.79 to 9.92 min showed a quasi-molecular ion at m/z 325.26 ($[M-H]^-$) [Figure 3], and it suggested that the relative molecular weight of the MI was 326. Comparing the information of relative molecular weight and MS spectrum of the MI with the secondary metabolites produced by *Aspergillus fumigitus* reported previously,[16,17] the MI coincided roughly with a known compound Gliotoxin with the molecular formula of $C_{13}H_{14}N_2O_4S_2$, and the molecular structure shown in Figure 4. Furthermore, the previous studies[14,16] showed the biological activities of gliotoxin is entirely dedicated to the oxidized form of the compound with an intact disulfide bridge, and that the reduced dithiol form is inactive.[18]

Nuclear magnetic resonance analysis

^1H NMR(CD_3OD) chemical shifts of the MI were given as follows [Figure 5]: 6.06 (1H, s, H-7), 6.02-5.99 (1H, d, H-8), 5.72-5.70 (1H, d, H-9), 4.86 (1H, s, H-6), 4.73-4.69 (1H, d, H-5a), 4.48-4.45 (1H, d, H-3a), 4.35-4.31 (1H, d, H-3a), 3.79-3.74 (1H, d, H-10), 3.22 (3H, s, 2-CH_3), and 3.08-3.03 (1H, d, H-10). As for ^{13}C NMR(CD_3OD) chemical shift values [Figure 6], 6 carbon signals (110-170) appeared in the downfield region, which included two acidamide-carbonyl (166.50, C-1 and 164.84, C-4), one quaternary carbon atom (132.13, C-9a), and three methenyl (128.76, C-9, 123.54, C-8 and 119.21, C-7). At the same time, seven carbon signals (20-80) appeared in the upfield region, which included two quaternary carbon atom (78.08, C-3 and 75.97, C-10a), two methenyl (73.20, C-5a and 69.24, C-6), two methene (59.06, C-3a and 35.87, C-10), and one methyl (26.78, C-2-Me). The two acidamide-carbonyl (166.50, C-1 and 164.84, C-4) mentioned above suggested that a diketopiperazine-framework existed in the structure of the active ingredient.

DQF ^1H-^1H COSY and ^1H-^{13}C HSQC spectra analysis were applied to clarify proton–proton couplings and proton–carbon correlations. In ^1H-^1H COSY spectrum [Figure 7], H-8 (6.02-5.99) was coupled with H-7 (6.06) and H-9 (5.72-5.70), and the information that H-7 (6.06) was coupled with H-8 (6.02-5.99), and H-9 (5.72-5.70) was coupled with H-8 (6.02-5.99), suggested a diene framework existed in the structure of the active ingredient. In addition, H-6 (4.86) was coupled with H-7 (6.06), H-6 (4.86) was coupled with H-5a (4.73-4.69), H-3a (4.48-4.45) was coupled with H-3a (4.35-4.31), and H-10 (3.79-3.74) was coupled with H-10

Table 1: The molluscicidal activity of different polar fractions (n=3)

Exposure time (h)	Mortality of snails (%)						
	PEF	DEF	EAF	NBF	RAF	DCW	NIC
24	3.33±1	100	0	0	0	0	92.22±1.9
48	6.67±1	100	1.11±0.577	0	0	0	100
72	15.56±1.528	100	3.33±1	2.22±0.577	0	0	100

PEF: Petroleum ether polar fraction (30 mg/L); DEF, diethyl ether polar fraction (30 mg/L), EAF: Ethyl acetate polar fraction (30 mg/L), NBF: *n*-butanol polar fraction (30 mg/L), RAF: The raffinate (30 mg/L), DCW: Dechlorinated tap water as negative control, NIC: Niclosamide aqueous (1 mg/L) as positive control

(3.08-3.03). From ^{1}H-^{13}C HSQC spectrum [Figure 8] the result showed as follows, δ_H 6.06 was correlated with δ_C 119.21, δ_H 6.02-5.99 was correlated with δ_C 123.54, δ_H 5.72-5.70 was correlated with δ_C 128.76, δ_H 4.86 was correlated with δ_C 69.24, δ_H 4.73-4.69 was correlated with δ_C 73.20, δ_H 4.48-4.45 and δ_H 4.35-4.31 were correlated with δ_C 59.06, δ_H 3.79-3.74 and δ_H 3.08-3.03 were correlated with δ_C 35.87, δ_H 3.22 was correlated with δ_C 26.78; furthermore, no hydrogen signal was correlated with δ_C 166.50, δ_C 164.84, δ_C 132.13, δ_C 78.08, and δ_C 75.97. All the information obtained from ^{1}H-^{13}C HSQC spectrum mentioned above confirmed the assignment of H and C, and agreed with the molecular structure of Gliotoxin.

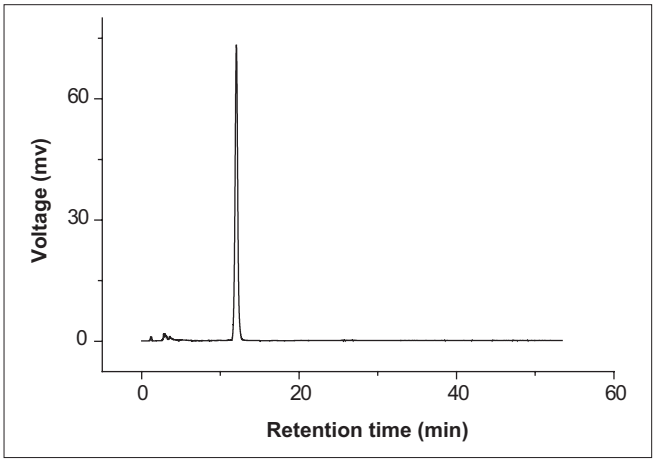

Figure 1: HPLC chromatogram of the molluscicidal ingredient

MS² analysis

MS² analysis was further performed to obtain the information of molecular structure and to confirm the molecular structure of the MI. The quasi-molecular ion of *m/z* 325.45 was analyzed by MS² as the parent ion, and in MS² spectrum [Figure 9], in addition to the parent ion two

Table 2: LC_{50} and LC_{90} (95% CL) of the purified MI against snails (n=3)

Concentration (mg/L)	Mortality of snails (%) in different exposure period (h)		
	24	48	72
0.6	100	100	100
0.4	91.11±1.92	100	100
0.2	63.33±3.33	100	100
0.1	47.78±1.92	82.22±1.92	100
0.08	42.22±1.92	64.44±5.09	100
0.06	36.67±3.33	46.67±3.33	84.44±1.92
0.04		20±3.33	65.56±1.92
0.02		2.22±1.92	51.11±1.92
0.01			17.78±1.92
LC50	0.101 (0.072–0.131)	0.062 (0.058–0.066)	0.022 (0.011–0.033)
LC90	0.355 (0.251–0.655)	0.121 (0.109–0.139)	0.066 (0.041–0.093)
DCW	0	0	0
NIC	95.56±1.92	100	100

DCW: Dechlorinated tap water as negative control, NIC: Niclosamide aqueous (1 mg/L) as positive control

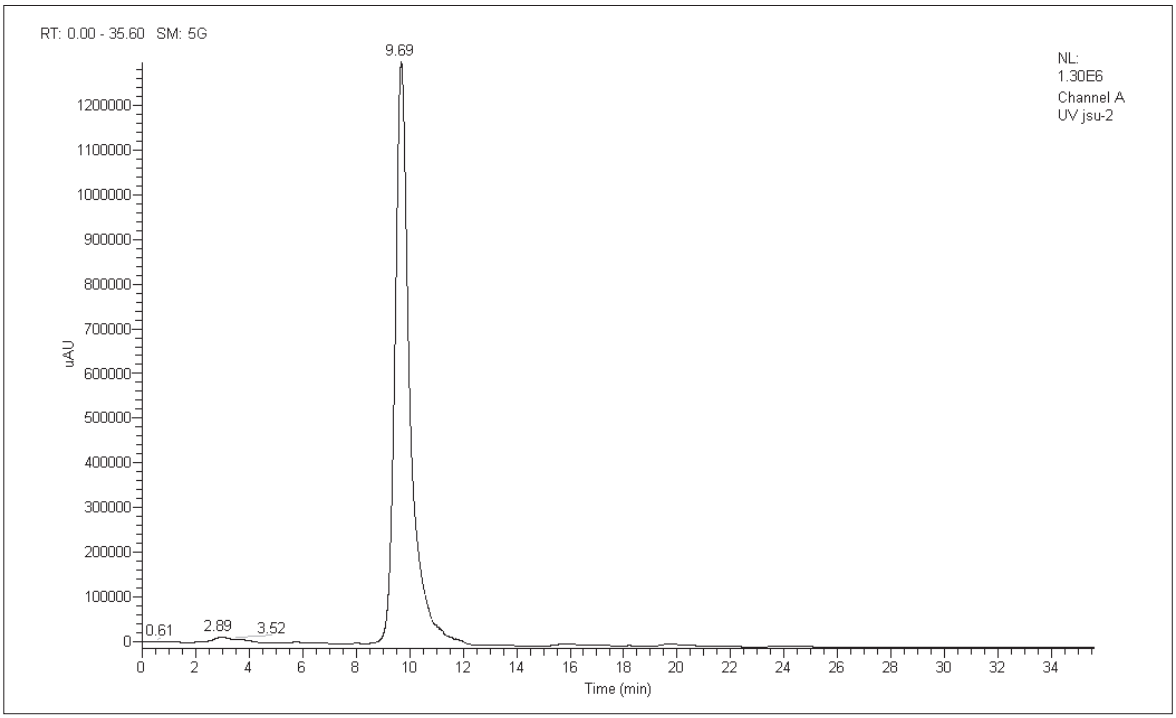

Figure 2: UV chromatogram of the molluscicidal ingredient

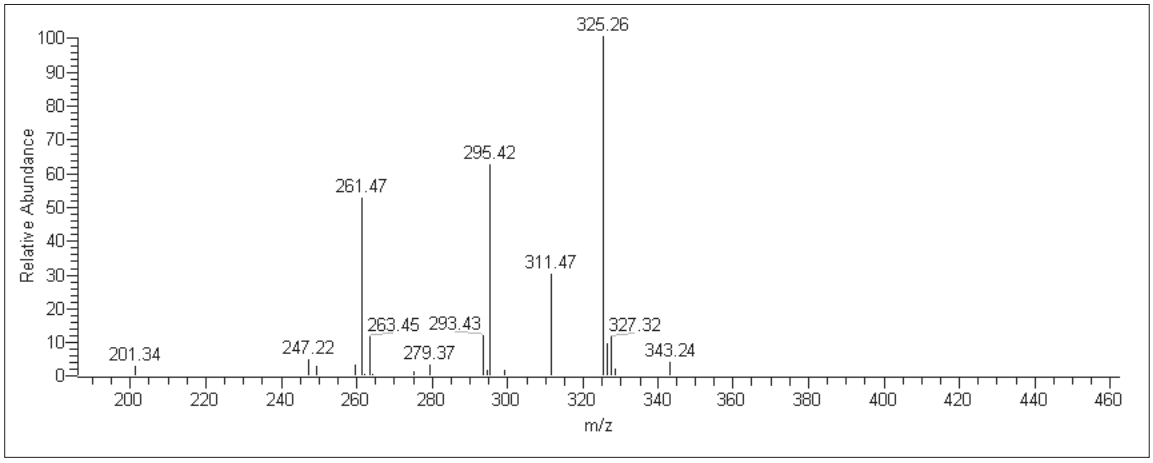

Figure 3: ESI/IT mass spectrum related to the HPLC peak of the molluscicidal ingredient shown in Figure 2

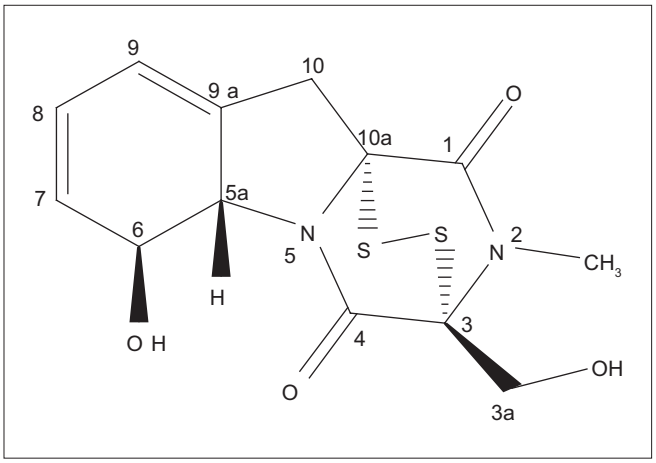

Figure 4: Molecular structure of gliotoxin

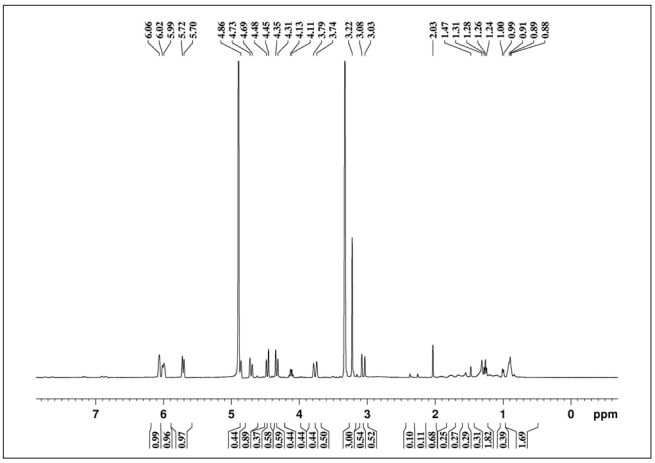

Figure 5: ^{1}H NMR spectrum of the molluscicidal ingredient

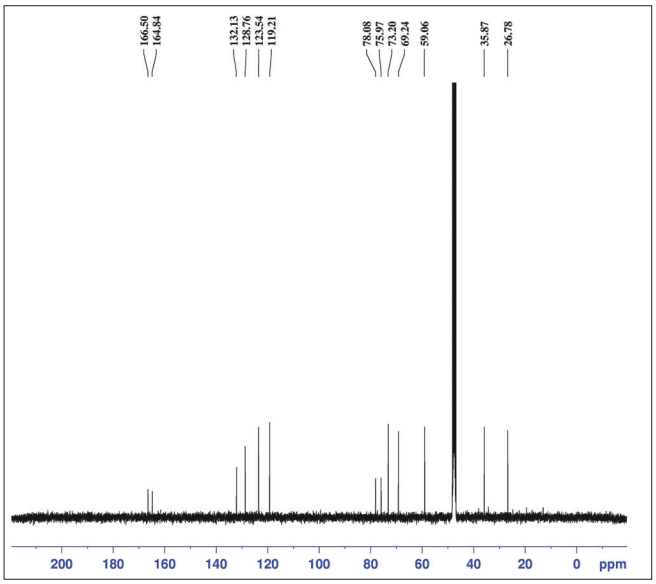

Figure 6: ^{13}C NMR spectrum of the molluscicidal ingredient

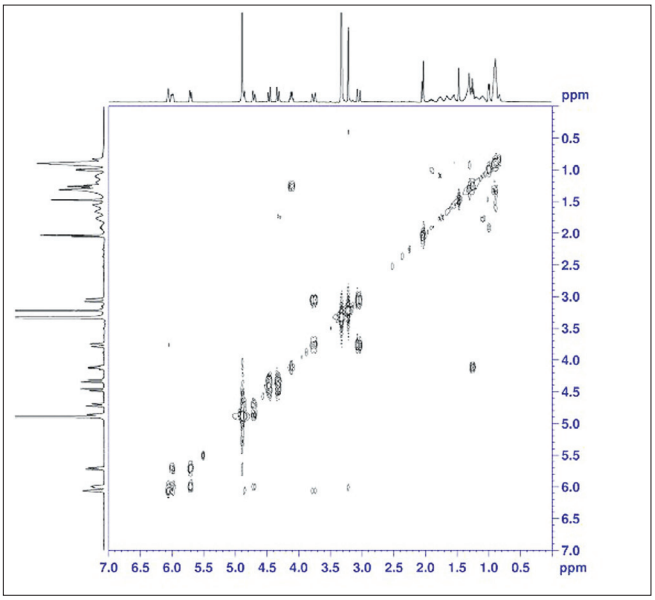

Figure 7: ^{1}H-^{1}H COSY spectrum of the molluscicidal ingredient

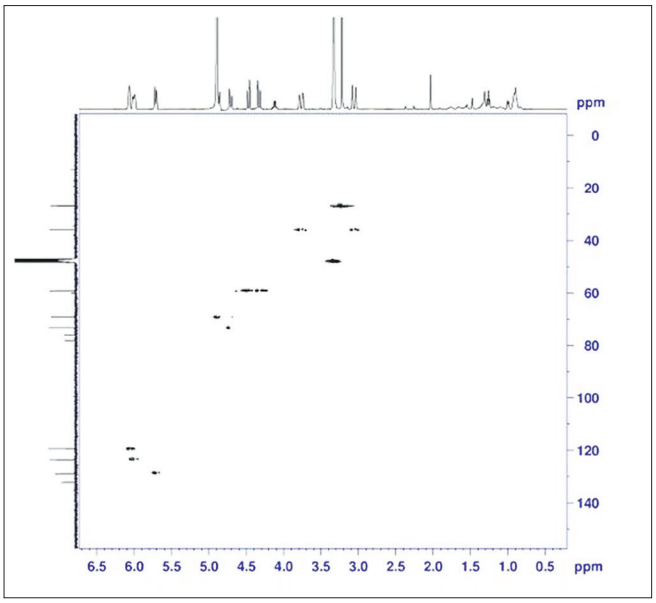

Figure 8: ^{1}H-^{13}C HSQC spectrum of the MI

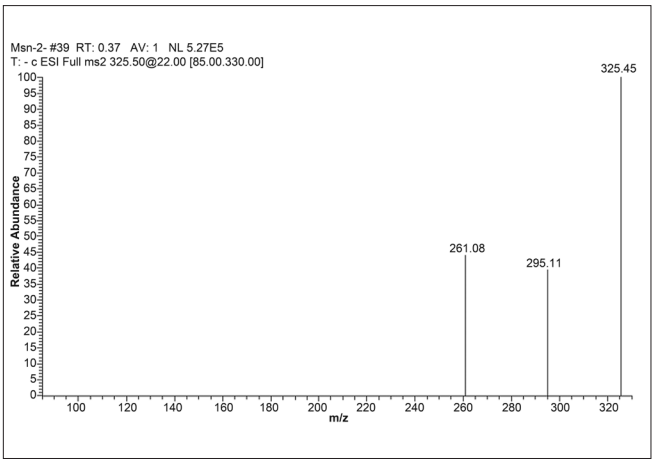

Figure 9: MS^2 of the *m/z* 325.45 ion

daughter ions appeared at *m/z* 295.11 $[M\text{-}CH_2OH]^-$ and *m/z* 261.08 $[M\text{-}CH_2OH\text{-}2S]^-$, which also existed in ESI/IT mass spectrum [Figure 3] with high relative abundance.

The information of MS^2 spectra agreed with the data previously reported,[19] therefore the MI from SL-30's exocellular broth was further confirmed as gliotoxin.

DISCUSSION

Gliotoxin, one of the bioactive agents with antimicrobial,[20] antiviral,[14] and immunosuppressive capabilities,[21] is produced by several genera of fungi, such as *Penicillium obscurum*,[22] *Trichoderma virens*,[23] and *Aspergillus fumigatus*.[16] In 1936, gliotoxin was first isolated from *Trichoderma lignorum* by Weindling and Emerson,[24] and defined as gliotoxin by Weindling in 1941.[25] After this, the chemical structure had not been identified in a long period. Until in 1958, the chemical structure of gliotoxin was finally described by Bell *et al*.[26]

Many data suggested that gliotoxin exhibited potential in transplantation and antitumor medicine due to its immunosuppressive effect on macrophages[27] and inhibitive effect on farnesyltransferase.[28] On the other hand, gliotoxin showed significant antagonism against many phytopathogenic microorganisms.[29] In this regard, gliotoxin provides an important biochemical mechanism in biocontrol effect on plant pathogen.

Here we reported that the MI from molluscicidal strain SL-30's exocellular broth was identified as gliotoxin, and showed significant molluscicidal activity with LC_{50} of 0.101, 0.062, and 0.022 mg/L, respectively, in the case of exposure period of 24, 48, and 72 h. To our knowledge, this is the first report about molluscicidal activity of gliotoxin. First, compared LC_{50} in the case of exposure period of 24 h of the MI and wettable powder of niclosamide, the former (0.101 mg/L) was almost similar to the latter (0.0947 mg/L).[30] The dissolubility in water at 30°C of gliotoxin was about 0.02 mg/mL; therefore, dosage form is promising to be studied for increasing the dissolubility and further decreasing the dosage. In addition, to date, the reported molluscicidal mechanisms of various molluscicidal active substances mainly focused on the following aspects:[31,32] (1) inducing partial liver cell necrosis by affecting hepatic function, then leading to a direct impact on glycogen synthesis; (2) activating or passivating some enzymes relating to glycometabolism, and promoting glycogen decomposition and inhibiting glycogen synthesis, then resulting in a decrease of glycogen content; (3) affecting the digestive tract function, and causing reduction of intake and glucose uptake, then inhibiting glycogen synthesis; and (4) inhibiting activity of acetyl choline esterase, causing the loss of motor function of muscle and accelerating death of snails. Therefore, further experiments are necessary to be carried out to reveal the molluscicidal mechanism of the MI.

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