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Full Length Research Paper

Preliminary characterisation of the phytotoxin of sheath-blight disease of rice caused by *Rhizoctonia solani*

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The culture filtrates of *Rhizoctonia solani* was significantly toxic to rice leaves, being similar to that caused by *R. solani*. The culture filtrate from *R. solani* was extracted using ethyl acetate and isolated by silica gel column chromatography, which showed significantly high toxicity. The toxin was thermally stable even after incubation at 121°C for up to 30 min. A pH of 2.0 was helpful for pathogenesis; after 12 h, yellow lesions were found. The solvent system ethyl acetate/methanol (10:1, v/v) was the optimal solvent system used for preparative thin-layer chromatography. Eight fractions were chosen to be collected by silica gel column chromatography, but only three spots were obtained in the thin-layer chromatography analysis. The second fraction was found to be the one with the highest activity and the toxin appeared to be mainly present in this fraction. The ultraviolet and infrared spectral data indicated that lactone, ketone and benzene groups existed in the culture filtrate. This report is the first describing the preliminary characterisation of phytotoxins from an *R. solani* culture filtrate; it briefly presents the isolation, purification and bioassay method for the phytotoxin produced by *R. solani.*

Key words: Richard's medium, crude extracts, bioassay, thin-layer chromatography, silica gel column chromatography.

INTRODUCTION

Rice sheath blight caused by *Rhizoctonia solani* is a serious disease in all rice-growing countries (Roy, 1993). Symptoms of the disease include greenish gray elliptical or oval-shaped spots with yellow margins mostly found on leaf sheaths; however, at times, leaf blades are also infected (Vidhyasekaran et al., 1997; Huang et al., 2009). Similar symptoms were produced by the culture filtrates of *R. solani* and a strong correlation was found between rice cultivar sensitivity to *R. solani* and *R. solani* culture filtrates, suggesting the possible involvement of phytotoxins in lesion development (Vidhyasekaran et al., 1986, 1992). The phytotoxic metabolites of several types of *R. solani* have been partly isolated or structurally characterised (Akoi et al., 1963; Mandava et al., 1980; Ramalingam, 1986; Lakpale et al., 1996; Vidhyasekaran et al., 1997; Betancourt et al., 2000; Chen et al., 2001). All these structurally characterised toxins are low-molecular-weight secondary compounds with diverse chemical structures. Several workers (Chen, 1958; Akoi et al., 1963; Frank,and Fancis 1976; Kenning and Hanchey, 1980; Iacobellis and Devay 1987; Chen et al., 2001) found that *R. solani* isolated from rice produced non-host-specific toxins which were phenyl acetate and its derivatives; and caused symptoms normally associated with leaf blight on a variety of plants. Other workers (Vidhyasekaran et al., 1997), using hosts and non-hosts of the pathogen, showed that toxins produced by *R. solani* were host-specific; the host-specific toxin from the rice sheath-blight pathogen induced the characteristic symptoms of the disease only on hosts of the pathogen and they were carbohydrates. However, the phytotoxins produced by different types of *R. solani* may be different. The objectives of this study were to isolate, purify and characterise the phytotoxin produced by *R. solani*.

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MATERIALS AND METHODS

Fungi and media

R. solani (Anastomosis Group IA) was obtained from Sichuang Agricultural University; all the other chemicals and reagents used in the experiment were purchased from Kelong Reagents Company (Chengdu, China). The media used in the experiments were as follows: potato sugar agar (PSA; potato, 200 g; sugar, 20 g; agar, 15 g; and distilled water, 1,000 ml; pH 7.0), potato sucrose broth (PSB; potato, 200 g; sucrose, 20 g; distilled water, 1,000 ml; pH 7.0), Richard's medium (potassium nitrate, 10 g; potassium dihydrogen phosphate, 5 g; magnesium sulfate·$7H_2O$, 2.5 g; ferric sulfate, 0.25 g; sugar, 50 g; and distilled water, 1,000 ml; pH 7.0).

Fungal cultures and toxin production

Cultures of a virulent isolate (Anastomosis Group IA) of *R. solani* were grown on PSA plates at 25°C for seven days. Further, 6-cm-diameter mycelial plugs from PSA cultures were transferred into a 150-ml flask containing 50 ml of PSB. The cultures were then incubated at 28°C on a rotary shaker (180 rpm) for two days and then stored at 4°C. For production of toxin, four mycelium groups from PSB cultures were transferred into a 500-ml flask containing 200 ml of Richard's medium; *R. solani* produces toxins in this type of medium (Chen, 1992; Xu et al., 2004; Lu et al., 2005). The cultures were then incubated under stationary conditions at 28°C on artificial vibration once a day for 15 to 20 days. This method was followed by Xu et al. (2004). Culture filtrates were obtained by passing the liquid through four layers of cheesecloth and Whatman No. 1 filter paper. Unless otherwise stated, the Richard-medium-cultured filtrate was used for all subsequent tests.

Leaf-necrosis assay and data analysis

The biological activities of crude and processed extracts were determined by the leaf-necrosis assay on a partial resistant cultivar (9311), and a susceptible cultivar (Lemont). Briefly, for each treatment, 10 leaves at the three-leaf stage were detached, the abaxial surface was lightly wounded by pricking gently with a sterilised needle and a 30-µl solution was applied to the wound site (Song et al., 1993; Zheng et al., 2010). The leaves were then incubated in a moist chamber at 25°C for three days and the lesion area around each wound was assessed. Each bioassay was repeated three times for the five plants; leaves treated with sterile distilled water and un-inoculated media served as controls.

The data of leaf-necrosis assay were subjected to analysis of variance, and when significant treatment differences were found, means were compared by the test of least significant difference (LSD, $P<0.5$).

Bioactivity of soluble macromolecular compounds obtained from culture filtrates

In this test, 50 ml of culture filtrate was first concentrated to half the original volume in vacuo at 40°C. An equal volume of methanol or acetone was added to the culture filtrate and stored overnight at 4°C. Precipitates were collected by centrifugation at 8,500 rpm for 10 min. The precipitates were then dissolved in 50 ml of sterile distilled water and the bioactivity was assessed by the leaf-necrosis assay, as previously described. The supernatant was evaporated to dryness at 50°C, dissolved in 50 ml of sterile distilled water and bioassayed similarly.

Adsorption of toxins from culture filtrates by active carbon

Following the method of Vidhyasekaran et al. (1997), activated carbon (60 g/l) was added into the culture filtrate (50 ml), chilled overnight at 4°C, and then filtered through two Whatman No. 1 filter papers. The filtrate was then evaporated to dryness at 50°C, dissolved in 50 ml of sterile distilled water and bioassayed as previously described. The carbon remaining in the filter paper was washed with 50 ml of hot methanol (50°C), shaken (80 rpm) for 30 min and filtered; this step was repeated once. The methanol was evaporated at 50°C and the residue was dissolved in 50 ml of sterile distilled water and bioassayed as afore stated.

Solvent extraction

The extraction followed the procedure described by Vikrant et al. (2006), with minor modifications. The culture filtrate (50 ml) was extracted three times with half volumes of ether, benzene, ethyl acetate or chloroform using a separatory funnel. Both water and the solvent fractions were evaporated to dryness at 50°C. The residues were dissolved in 50 ml of sterile distilled water and their toxicity was measured using the leaf-necrosis assay. Sterile distilled water and the culture filtrate served as controls.

Effective concentration in the bioassay

Culture filtrates were evaporated to dryness at 50°C; diluted to 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50%; and bioassayed as previously done. Sterile distilled water and culture filtrate served as controls. The minimum concentration of toxins required to induce symptoms was regarded as the dilution endpoint assay and this concentration was used for all further leaf assays.

Effect of heat, strong acid and strong alkali on toxin activity

The culture filtrates were subjected to temperatures of 60, 100 or 121°C for 30 min in a water bath (or autoclaved) and bioassayed as previously done. Sterile distilled water and untreated culture filtrate served as controls.

To test whether the toxin is sensitive to pH, the pH of the culture filtrates was adjusted to 2.0, 7.0 and 12.0 using 12 mol/l hydrochloric acid and 1 mol/l potassium hydroxide; incubated for 30 min and the pH was then adjusted to 7.0. The culture filtrates of pH 2.0 to 7.0 (from 2.0 adjusted to 7.0); 7.0; and 12.0 to 7.0 (from 12.0 adjusted to 7.0) were used for the bioassay; every treatment was repeated three times. Sterile distilled water and the untreated culture filtrate served as controls.

Isolation, purification and characterisation of the phytotoxin from culture filtrate

The culture filtrates were acidified to pH 2.5 with 12 M hydrochloric acid and extracted three times with an equal volume of ethyl acetate. The combined extracts were concentrated to a small volume in vacuo at 40°C and then washed three times with equal volumes of 5% aqueous sodium bicarbonate. The aqueous layers were acidified to pH 2.5 with 12 M hydrochloric acid and extracted three times with ethyl acetate. This method of Iacobellis and Devay (1987) was followed, with little modifications. The combined extracts were evaporated to dryness *in vacuo* at 40°C. The residue was obtained and dissolved in 1 ml methanol for analysis by thin-layer chromatography (TLC). Preparative analytical TLC plates, coated with a GF-254 fluorescent silica gel (5 × 10 cm, Qingdao, China) and spotted with the samples were developed separately in solvent

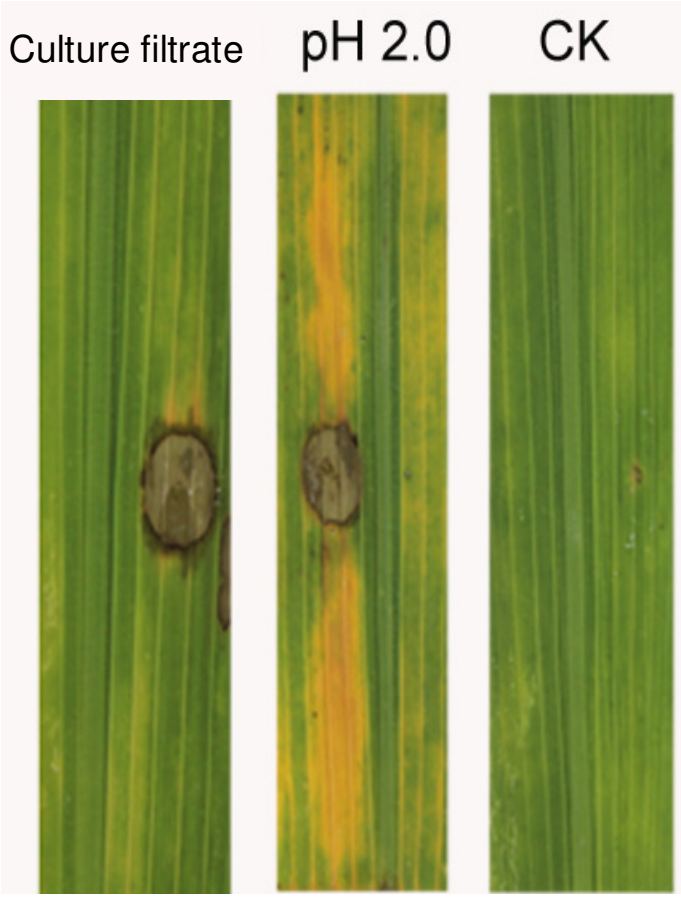

Figure 1. Necrosis lesions caused by *R. solani* culture filtrate on wounded rice leaves of cultivar 9311. All treated leaves were incubated at 25°C for 3 days; when the pH of culture filtrate was adjusted to 2.0, necrosis was also found and the rice leaf was turned to be yellow; sterile distilled water served as control and no necrosis was found.

systems containing ethyl acetate/methanol (1:1 to 20:1, v/v). The spots on the TLC plates obtained using the solvent aforementioned were marked under ultraviolet (UV) light at 254 nm and the Rf value of each spot was recorded. The solvent system that could yield clear and unattached spots on the TLC plates was used to purify the crude toxin by elution in a silica gel column.

Silica gel column chromatography and infrared spectrum

The crude toxin was dissolved in 2 ml of methanol and applied to a 15 × 500 mm column filled with silica gel (200 to 300 mesh, Qingdao, China), which had been activated at 150°C overnight and pre-equilibrated with methanol. The dissolved toxin was added on top of the column and the column was eluted using a linear gradient with the following eluant strength: 100% ethyl acetate, methanol/ethyl acetate (1:10, 1:5, 1:1, 5:1 and 10:1; v/v) and 100% methanol. Elution was carried out at the rate of 0.25 ml min^{-1}. 2 ml fractions were collected, analysed by TLC on GF254 using ethyl acetate/methanol (10:1. v/v) and visualised by UV light (254 nm); the fraction that had spots on the TLC plate was assessed by detaching the leaves of six species of plants: rice, corn, soybean, rape, Chinese radish and Chinese cabbage. Each bioassay was repeated three times for the six plants; the leaves treated with sterile distilled water and un-inoculated media served as controls. Identification of the biologically active compound was conducted using infrared (IR; NEXUS 670, American Electric Company, United States) and UV spectroscopy (Beckman DU-70, Germany). The IR and UV spectra were recorded in KBr and ethanol, respectively.

RESULTS

Toxin production and preliminary *in vitro* characterisation

The culture filtrates of *R. solani* grown on Richard's liquid media can induce leaf necrosis (Figure 1); this result is consistent with that of Robert et al. (1984). Necrosis was found in both resistant and susceptible cultivars; however, the area with necrosis was smaller in resistant cultivars than that in susceptible ones. The symptoms produced in rice leaves by the culture filtrates of *R. solani* were similar to those produced by pathogenic *R. solani* (Figure 2); moreover, a strong correlation was found

Figure 2. Necrotic lesions produced by *R. solani* and *R. solani*-toxins on wounded rice leaves of cultivar 9311 (susceptible). Leaves were inoculated with fungal plugs 4 mm in diameter or treated with 10 drop of *R. Solani* toxins. All treated leaves were incubated at 25°C for 2 days.

between rice-cultivar sensitivity to *R. solani* and sensitivity to *R. solani* culture filtrates, suggesting the possible involvement of phytotoxins in lesion development. However, the filtrate from 20-day-old Richard's medium cultures, which produced an average lesion size of 27 mm^2, was significantly toxic to rice leaves. Filtrates from un-inoculated media caused no lesions on the tested rice leaves. Hence, all subsequent tests were conducted with Richard's medium filtrates.

Preliminary characterisation of the toxic activity was carried out *in vitro* before toxin isolation. No toxicity was detected in the precipitates, which were obtained after the addition of methanol or acetone (Table 1). When the crude toxin was treated with active carbon, toxicity could be detected, both in the solution and the absorbed fraction; however, the absorbed fraction showed higher toxicity, showing that a portion of the toxin could be absorbed by the active carbon, whereas others could not (Table 1). The toxic components were found only in the solvent fraction after the culture filtrates were partitioned with ethyl acetate or chloroform; and in the water fraction, after partitioning with petroleum ether or ether. After partitioning with benzene, the toxins were detected in both water and the solvent fractions (Table 1). Because the ethyl acetate extract showed significantly higher toxicity than the one from chloroform, ethyl acetate was chosen for extraction of the toxins from the culture filtrate.

The toxic activity in the culture filtrates was thermally stable even after incubation at 121°C for up to 30 min, indicating that they were highly thermostable. Moreover, a pH of 2.0 was helpful to the pathogenesis process. After 12 h, yellow lesions were found on the treated leaves (Figure1). The other symptoms were found after 24 h and there was no necrotic area on the controls, which suggested that low pH helped the toxins to infect rice (Iacobellis,and Devay 1987; Jayaraman et al., 2010). The necrotic region was isolated to prove the relationship between phytotoxin concentration and necrotic lesion development. The effective concentration causing serious leaf necrosis was 40%; concentrations less than 40% could cause only slight necrosis or not at all and concentrations more than 40% could not increase the necrotic region.

Isolation and purification of *R. solani* toxin

To obtain a sufficient amount of the bioactive compound, the mycelium of *R. solani* was grown in one litre of Richard's medium for 15 to 20 days. Extraction with ethyl acetate gave a light yellow powder (289.8 mg) with high phytotoxic activity after being dissolved in sterile distilled water and used in the leaf-necrosis assay. Spots were obtained with the solvent system ethyl acetate/methanol (10:1, v/v), wherein the Rf value was 0.42. This optimal solvent system was used for preparative TLC. The Rf value of the isolated toxin was 0.6, which showed that the isolated toxin has a small polarity, but not all the toxins in the isolate. Eight fractions were chosen to be collected in the silica gel column chromatography, whereas in the fractionation by TLC, three spots were obtained with the solvent system ethyl acetate/methanol (10:1, v/v) (Figure 3). The second, fifth, and sixth fractions produced average lesion sizes of 5, 2 and 1 mm^2, respectively, in the rice leaf–necrosis assay (Table 2). The second fraction was found to be the one with the highest activity ($P>0.5$), which showed that the toxin is mainly in this fraction. Leaves of corn, soybean, rape, Chinese radish, and Chinese cabbage showed no necrosis. When the

Table 1. Phytotoxicity of crude toxin, isolated from culture filtrates of *Rhizoctonia solani* after three days, during a leaf-necrosis assay on the leaves of rice cultivar 9311.

Experiment	Treatment	Fraction	Leaf-necrosis assay (mm^2)
1	Methanol	Water fraction	16.1^{a}
		Precipitate	0^{b}
	Acetone	Water fraction	15.7^{a}
		Precipitate	0^{b}
	Culture filtrate(control)		18.1^{a}
2	Active carbon	Non adsorbed fraction	5.4^{bc}
		Adsorbed fraction	12.1^{b}
	Culture filtrate(control)		18.1^{a}
3	Petroleum ether	Water fraction	2.1^{c}
		Solvent fraction	0^{d}
	Ether	Water fraction	2.4^{c}
		Solvent fraction	0^{d}
	Benzene	Water fraction	1.8^{c}
		Solvent fraction	1.4^{cd}
	Ethyl acetate	Water fraction	0^{d}
		Solvent fraction	8.6^{b}
	Chloroform	Water fraction	0^{d}
		Solvent fraction	3.2^{c}
	Culture filtrate(control)		18.1^{a}

Column means followed by the same letter(s) are not significantly different ($P \geq 0.05$) by DMRT. Culture filtrates from *R. solani* cultures grown for 20 days in Richard's medium served as controls.

Figure 3. Eight fractions of Silica gel column chromatography were analyzed by TLC. The solvent system was ethyl acetate/methanol, [10:1, (v/v)]; only three pots were found, and the second fraction had the highest bio-activity.

Table 2. The results of eight fractions of silica gel column chromatography.

Fraction	Rf value	Leaf-necrosis assay (mm^2)
1	0	0^d
2	0.71	5^a
3	0	0^d
4	0	0^d
5	0.57	2^b
6	0.43	1^c
7	0	0^d
8	0	0^d

Column means followed by the same letter(s) are not significantly different (P ≥ 0.05) by DMRT.

concentration of the fraction was increased, there was no necrosis in the leaves, however, necrosis was evidently found in rice leaves, suggesting that the toxins of *R. solani* are host-specific in nature.

UV (EtOH) λ_{max} (nm) (logε) was 227.2 (2.27) and 296.73 (3.575); IR (KBr, cm^{-1}): 3383.67 (–OH); 2933.05 ($–CH_2–$); 2886.46 and 1386.54 ($–CH_3$); 1767.61 and 1724.08 (lactone, ketone or carboxylic acid); 1643.94 (benzene C=C); and 1254.46, 1145.10 and 918.17 (C–O–C) (Figure 4). The absorption bands of the UV spectrum suggest that the toxin that was purified and had a higher absorption at 296.73 nm was the compound we had analysed. The IR spectrum showed bands characteristic of the lactone, ketone (1767.61, 1724.08) and benzene (1643.94) groups. Compared with the information from the database, the presence of lactone, ketone and benzene groups in *R. solani* culture filtrates was confirmed.

DISCUSSION

Phytotoxins produced by fungi are often released into the artificial medium in very low amounts, causing difficulties in their isolation and purification; hence, we prepare larger amounts of the growth medium to cultivate the pathogen so that we may obtain a greater yield of the compounds (Strobel, 1982; Strange, 2007). In this study, *R. solani* cultivates grown in Richard's medium for 20 days yielded more than 298.8 mg/l of crude toxins. When the fungus was incubated for 25 days, there was a reduction in the overall toxic activity, which is probably due to the degradation of the toxin in the culture medium. *R. solani* toxin is heat stable because the culture filtrate still showed high toxicity after exposure to a temperature of 121°C for 30 min (Chen, 1992). Solvent extraction to recover organic compounds with novel activity has been considered one of the most effective methods to isolate phytotoxins from other fungal metabolites (Vikrant, 2006). We used ethyl acetate to extract the *R. solani* toxin from the culture filtrate. Variable phytotoxicity of different batches of culture filtrates was observed in this study and this might be related to the age of the cultures, because toxin production of *R. solani* isolates was reduced after transferring for several generations. However, the major phytotoxin, *R. solani* toxin, which was the most phytotoxic compound observed in the culture filtrate, was purified in this study by a combination of two chromatography steps after extraction with ethyl acetate. Although the chemical structures of the phytotoxins produced by several types of *R. solani* are known, the physical characteristics of the *R. solani* toxin differ from the previously described phytotoxins produced by other types of *R. solani*. Preliminary characterisation has shown that the partial *R. solani* toxin is an acidic compound containing phenolic hydroxyl groups (Chen, 1958; Akoi et al., 1963; Iacobellis and Devay 1987; Chen et al., 2001). Full structural characterisation and identification of *R. solani* toxin is currently in progress. The activity of phytotoxins in terms of symptom production on rice leaves was further substantiated in dosage–response curves, wherein increased concentrations of the purified phytotoxin caused increased severity of symptoms. A high concentration of the pure phytotoxin caused severe leaf-blight symptoms similar to those produced by *R. solani* in the later stages of infection. This implies that the *R. solani* toxin may be involved in the virulence of *R. solani* on rice leaves (Robert, 1984; Jiang, 2008). A more-intensive study of the ability of resistant rice cultivars to withstand the effects of the toxin and of the vulnerability of susceptible rice cultivars may shed light on the mechanism of symptom development in infected plants at the macroscopic, cellular and molecular levels.

Conclusion

The *R. solani* toxin was isolated and showed significantly high toxicity. The toxin was thermally stable even after incubation at 121°C for up to 30 min. A pH of 2.0 was helpful for pathogenesis; after 12 h, yellow lesions were found. The solvent system ethyl acetate/methanol (10:1,

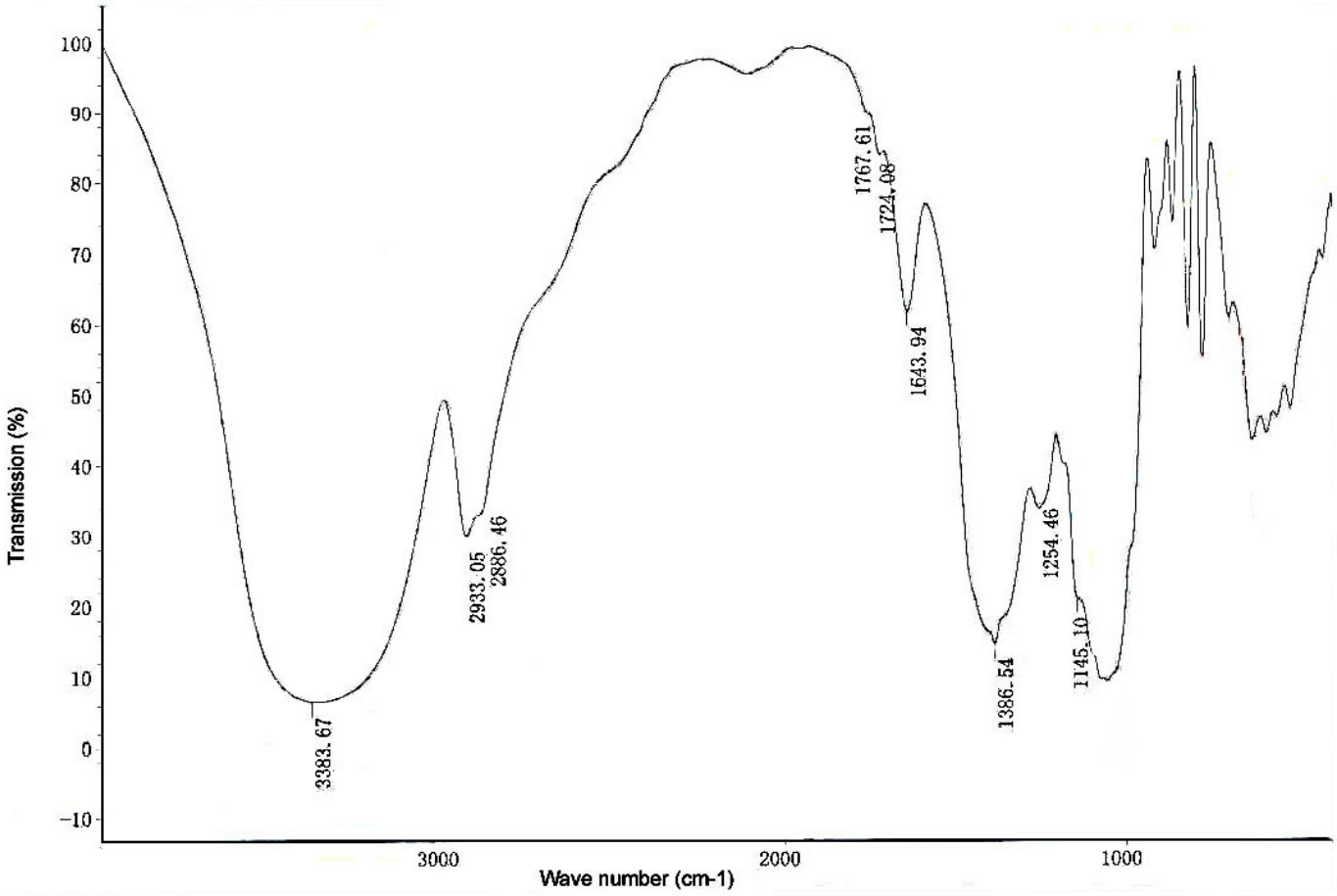

Figure 4. Infrared spectrum (IR spectrum) used to conduct the biologically active compound obtained from silica gel column chromatography. The IR spectra were recorded in KBr.

v/v) was the optimal solvent system used for pre-parative thin-layer chromatography. Eight fractions were collected by silica gel column chromato-graphy, but only three spots were obtained in the thin-layer chromatography analysis. The second fraction was found to be the one with the highest activity and the toxin appeared to be mainly present in this fraction. The ultraviolet and infrared spectral data indicated that lactone, ketone and benzene groups existed in the culture filtrate.

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