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Isolation, Purification, and Structural Identification of an Antifungal Compound from a *Trichoderma* Strain S

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Trichoderma strain T-33 has been demonstrated to have inhibitory effect on the fungus species *Cytospora chrysosperma*. Here, an active antifungal compound was obtained from *Trichoderma* strain T-33 extract *via* combined separation technologies, including organic solvent extraction, liquid chromatography, and thin-layer chromatography. The purified compound was further characterized by advanced analytical technologies to elucidate its chemical structure. Results indicated that the active antifungal compound in *Trichoderma* strain T-33 extract is 2,5-cyclohexadiene-1,4-dione-2,6-*bis* (1,1-dimethylethyl).

Keywords: *Trichoderma*, antifungal, biocontrol, bioactive component, structural identification, *Cytospora chrysosperma*

Introduction

Since the discovery of plant pathogenic fungi in lignin, studies using *Trichoderma* to control plant diseases have gained broad interest worldwide. The early report by Dennis and Webster [9] found that a type of volatile acetaldehyde compound produced by *Trichoderma* had inhibitory effect on pathogenic fungi. One of the main mechanisms of *Trichoderma harzianum* to control the growth of the fungus *Rhizoctonia solani* was to produce a volatile six pentane-pyran and pentenyl pyrano antibiotics [7]. Later, Bruckner *et al*. [2] separated and purified two special antimicrobial peptides from *Trichoderma longibrachiatum* Rifai and *Trichoderma viride*, which were then named as trichobrachin and trichovirin, respectively. Moreover, the amino acid sequence of the antimicrobial peptides was also determined later. Then Sun *et al*. [19] confirmed that the solid fermentation of *Trichoderma* strain SMF2 had an antibiotic compound. This antibiotic compound showed great inhibitory effect on *Pseudomonas solanacearum*. However, they did not identify the structure of this antibiotic. Thereafter, Stoppacher *et al*. [18] analyzed the composition of the volatile organic compounds produced by *Trichoderma* using HS-SPME-GC-MS. Three new acorane sesquiterpenes (1a-isopropyl-4a,8-dimethylspiro [4.5]dec-8-ene-2b,7a-diol, 1a-a,8-dimethylspiro[4.5]dec-8-ene-3b,7a-diol, and 2b-hydroxy-1a-isopropyl-4a,8-isopropyl-4 dimethylspiro[4.5] dec-8-en-7-one) were isolated from the culturing broth of *Trichoderma* sp. YMF1.02647. The structures were elucidated from 1D, 2D NMR and HR-ESI-MS. Thereafter, Li *et al*. [13] isolated three sesquiterpene substances with antifungal activity from *Trichoderma* strain YMF1.02647. Moreover, these three substances also showed growth inhibition effect on HL-60, A549, and MCF-7 cancer cells [13]. Later, Chen *et al*. [5] extracted an antifungal active ingredient from the spores of *T. viride* LTR22 and utilized GC-MS to analyze the chemical composition of the active antimicrobial compound. El-Hasan *et al*. [10] studied *T. harzianum* and discovered that

its metabolite 6-pentyl-α-pyrone suppressed fusaric acid production by *Fusarium moniliforme* with great potency. In other words, there is increasing concern around the world over the widespread use of toxic agricultural chemicals. People have shown an increasing recognition of potential biological pesticides that are much safer. To find fungi as potentially efficient biopesticides, people have tried many antifungal active substances from various sources. Currently, studies on the antifungal active substances of *Trichoderma* are gaining even more attention because there is an increasing requirement that antifungal substances produced by *Trichoderma* be extracted from safer and biodegradable antifungal products, which may be the next generation of biological pesticides. In northwestern and northwestern China, many poplar trees were planted as shelter forests. However, there are increasing diseases for poplar trees, which are also spreading. Among these, *Cytospora chrysosperma* is one of the major diseases that destroy shelter forests. This disease also harms willows, elm trees, and locust trees, *etc*., and is caused by the disease fungus in seedlings and diseased trees. Thus far, the treatment for the disease is mainly to give chemical drugs such as Sanmate. Here, *Trichoderma* strain T-33 was selected from 29 *Trichoderma* strains through preliminary screening for its inhibitory ability on *Cytospora chrysosperma*.

We report the active antifungal compound that was collected from *Trichoderma* strain T-33 extract and further purified *via* combined separation technologies, including organic solvent extraction, liquid chromatography, and thin-layer chromatography (TLC). This compound was studied systematically and its chemical structure was confirmed. The work shown here may bring great benefit in the future development of biopesticides for plant disease control and highlights the potential use of the antifungal compound from *Trichoderma*.

Materials and Methods

Materials

(1) *Trichoderma* strains T-33 (registration number: JF823649) and pathogenic strains (*C. chrysosperma*; code: CHH001) were provided by the College of Forestry, Northeast Forestry University of China. This strain was further identified as *Trichoderma viride* from DNA sequencing.

(2) Culture medium: solid culture using potato dextrose agar (PDA) and liquid culture using potato dextrose (PD).

(3) Reagents: Methanol (HPLC grade and analytical grade), n-butanol, ethyl acetate, *n*-hexane, ether, acetone, chloroform, silica gel (80 mesh, 200–300 mesh), and reagents were purchased from Kermel Reagent Co., Ltd., Tianjin, China.

(4) Ultraviolet spectrophotometer UV-2550PC (Daojin Instrument Co., Ltd., Suzhou, China), biochemical incubator HPG-400H (Donglian Electronic Technology Development Co., Ltd., Harbin, China), rotary evaporator SHB-3 (Dufu Instrument Factory, Zhengzhou, China), vacuum desiccator (WHEATON Company, USA), aluminum thin-layer chromatography (TLC) plates (Beijie Science and Technology Co., Ltd., Kunming, China), full temperature oscillation incubator HZQ-the F100 (Beijing Chengmeng Weiye Science and Technology Co., Ltd., Beijing, China), semipreparative high-performance liquid chromatography (HPLC; Waters HPLC 1525-2489, The WFC, analytical column adopts 4.6 × 150 mm, C18, and particle size of 5 μm; preparative column adopts BEH300, C18, and particle of size 5 μm; USA), GC-MS 6890N-5973 insert (Agilent, USA), FT-IR spectrometer (PE Company, USA), and VLTRASHIELD 400 Fourier transform nuclear magnetic resonance spectrometer (BRUKER Company, Switzerland).

Preparation of *Trichoderma* Strain T-33 Crude Extract and Antifungal Activity Test

Five blocks of *Trichoderma* strain T-33 (10 mm in diameter) were inoculated in flasks with 300 ml of PD medium. The culture flask was incubated in a shaker (26°C; rotational speed: 160 rpm) for 6 days. After that, the culture medium was filtered to separate the cells. The filtrated fermentation broth (250 ml) was collected and precipitated with 750 ml of butanol (v/v) and left for 48 h. Then 750 ml butanol was collected and dried to obtain a crude extract of the fermentation broth (2.34 g). The antifungal activity of the crude extract was then tested by growth rate method [11]. Briefly, the following steps were carried out: (i) 0.2 g crude extract was taken out, to which 10% Tween-80 solution was added to make a volume of 25 ml; (ii) the extract was sterilized, filtered, and further mixed with four volumes of PDA medium into a flat plate. After that, the 10 mm diameter *C. chrysosperma* tablets were directly placed in the center of the flat plate after cooling and were cultured at 26°C. The cross method was used to measure the colony diameter after 7 days. *C. chrysosperma* inoculated in PDA medium without adding crude extract was set as a blank control.

Growth inhibitory rate = [(control plate net growth – sample plate net growth) / control plate net growth] × 100%.

Antifungal Activity Test of *Trichoderma* Strain T-33 Crude Extract *In Vivo*

The antifungal activity test of T-33 crude extract at a concentration of 1.156 mg/ml was subdivided into two groups; the prevention group ($n = 10$) and the treatment group ($n = 10$). Thermal burn was adopted for inoculation of the poplar tree. Briefly, poplar cuttings developed 120 days indoor were burned by spirit lamp, until the tissue fluid dripped from its cutting site, which becomes yellow. A wound with 3 cm in length and half of the diameter of the branches in width was made.

Prevention group. First, the extract from strain T-33 (2 ml at a

concentration of 1.156 mg/ml) was daubed over the wound. After natural drying of the wound (0, 1, 2, 3, and 4 days), the spore suspension (0.2 ml, 2×10^6 CFU/ml) of the pathogen was sprayed on the wound, which was then wrapped with wet cotton wool and tin foil wrap. The wound that was daubed with TW-80 (0.2 ml, 10% (v/v) in 2 ml of poplar bark extract) was used a control. Poplar bark extract (2 ml) was used to keep the cotton wool wet. After 7 days, the wrapped cotton wool and foil wraps were removed. The pathogenetic condition was observed and the grade standard of canker was listed below.

Treatment group. First the spore suspension (0.2 ml, 2×10^6 CFU/ml) of the pathogen was sprayed over the wound, and then the wound site was wrapped with wet cotton wool and tin foil. Poplar bark extract (2 ml) was used to keep them wet three times a day. After inoculation of 0, 1, 2, 3, and 4 days, the wound was daubed with strain T-33 extract (0.2 ml, 1.156 mg/ml), and TW-80 (0.2 ml, 10% (v/v)) as a control. The pathogenetic condition was observed. The grade standard of poplar canker is shown in Table 1.

Disease index = ∑ (number of infected strains × numeric classes)/ [(strains gross × numeric classes of the worst of condition)] × 100

Control effect = (disease index of control – disease index of prevent or treatment)/ disease index of control × 100

Separation and Purification of the Active Antifungal Compound from Crude Extract

Methanol (5 ml) was used to dissolve the strain T-33 crude extract (1 g). The crude extract was subjected to filtration and the insoluble substance on the filter was washed once with methanol (0.5 ml). The filtrate was collected and then concentrated by rotary evaporation. Thereafter, methanol was further volatilized at room temperature in a water bath to obtain a dark brown oily substance. The insoluble substance on the filter was dried and then dissolved in sterile water (2 ml) for antifungal activity test. The antifungal activities of both the methanol soluble substance and insoluble substance were then evaluated using the growth rate method as mentioned above. The methanol-soluble part of the extract with antifungal activity served as the starting material for further separation in the next step.

A wet packed silica gel column (200–300 mesh) was used for further separation of the active substance with gradient elution. The mobile phase composition used in the step elution was *n*-hexane:ethyl acetate = 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, and 1:9 (v/v). Elution volume was 60 ml for each elution step, and the eluate was collected at 15 ml per tube. A spectral scanning analyzer was used for monitoring the eluate fraction in the collection tubes. The eluate fractions in tubes with similar spectra characteristics were combined, concentrated, and tested for antifungal activity. The combined fraction with the strongest antifungal activity was selected for further purification.

Structural Identification of Antifungal Substances in Extract

UV-vis spectroscopy was recorded on UV-2550PC (Suzhou Daojin Instrument Co., Ltd.). Briefly, the purified samples were appropriately reconstituted in ethanol and UV-vis spectra were recorded in the wavelength ranging from 190 to 800 nm [15]. An IR spectrum was made *via* dry sample (1 mg), which was taken and ground with 100 to 200 mg of dry KBr powder in the agate mortar, and then pressed into plates and scanned in the range from 4,000 to 400 cm^{-1} [4]. The UV detector wavelength for liquid chromatography was set at 205 nm. The chromatographic column was a Hypersil ODS column (200 mm × 4.6 mm) and the column temperature was set at 25°C. The mobile phase was methanol:water (8:2 (v/v)) with flow rate at 1.0 ml/min [17]. For gas chromatography, the initial temperature (100°C) was held for 1 min and then it was increased to 250°C at a rate of 10°C/min. The sample inlet temperature was 250°C and carrier gas (helium) flow was 1 ml/min. For mass spectrometry, the ionization mode was electron impact (EI) and electron energy was 70 eV with a ion source temperature at 260°C. The scan mass range was 20–600 amu. The database of mass spectrometry was NIST 08 [20]. Sample was dissolved in d_4-methanol with TMS as internal standard for ^{1}H-NMR, and spectrum acquisition was performed at 22.1°C with a relaxation delay time at 3.958 sec, and 161 scans were performed per sample.

For ^{13}C-NMR, the observation frequency was set at 100.623 MHz, the relaxation delay time was 1.366 sec, and 16,348 scans were performed on each sample [6, 11] with other parameters the same.

Data Processing

The SPSS 11.5 software was used for statistical analysis. The results were presented as mean ± standard error, and the significant difference was analyzed using the Student's *t* test.

Table 1. The five standard grades of poplar canker.

Grade of pathogenetic condition[a]	Numeric classes	Grade standard
I	0	Anosis
II	1	Speckle length is less than1/3 of branches
III	2	Speckle length is 1/3–1/2 of branches
IV	3	Speckle length is over 3/5 of branches
V	4	Dying trees and dead trees

[a]The grades were determined by the ratio of horizontal width of the scab and the length of the tree trunk.

Results

Inhibitory Effect of the T-33 Crude Extract

Typically, 4.695 g of T-33 crude extract could be obtained from 1.5 L of liquid culture after butanol precipitation. T-33 methanol soluble crude extract (2.55 g) was obtained after further extraction with methanol, leaving the methanol-insoluble part, which was also collected thereafter. Hence, the yield for the methanol-soluble extract was 0.17% (g/l). To find whether the methanol-soluble or -insoluble part shows the antifungal activity, we tested both of them. Results are shown in Fig. S1. Compared with the control groups (Fig. S1B), the methanol-soluble part of the extract showed antifungal activity (Figs. S1A and S1C), indicating the active antifungal ingredient is soluble in methanol. Therefore, the active antifungal component is likely to be an organic compound, providing the possibility of purifying and characterizing the active compound.

Antifungal Activity Test of *Trichoderma* Strain T-33 Crude Extract *In Vivo*

The inhibiting effect of T33 extract at different concentrations (from 0.3 to 1.2 mg/ml) on mycelium growth and spore germination of pathogen was tested *in vivo* and the results obtained are explained as follows:

Prevention group. As shown in Table 2, in the disease preventing experiment, after *Trichoderma* extract daubing treatment and inoculation of pathogen, the disease index was the lowest (3.98), and the preventive effect the highest (90.82%) at the beginning. Four days after daubing treatment, the disease index was 21.36 with a preventive effect at 56.38%, which indicates that the disease incidence of seedling increased and the preventive effect declined with the prolonging of inoculation time.

Treatment group. As shown in Table 3, in the disease control experiment (treatment group), after inoculation of pathogen and then *Trichoderma* liquid extract daubing treatment, the disease index was lowest (4.26), and the preventive effect highest (89.99%), at the beginning. Similarly, 4 days after daubing treatment, the disease index was 37.75 with a preventive effect at 45.73%. These results also indicate that the disease incidence of seedling increased and the preventive effect declined with the prolonging of inoculation time.

Taking together, the antifungal test of prevention group and treatment group showed that the extract from strain T-33 has obvious inhibition on the expansion of scab and spore germination of pathogen. At the same time, the results here showed that the control effect of the treatment group is greater than that of the prevention group.

Separation and Purification of Active Antifungal Compound in Crude Extracts

As shown in the previous section, the active antifungal ingredient was soluble in methanol. Strain T-33 methanol extract was further fractionated by column chromatography. Fractions in 60 tubes were collected, which were then combined into six fractions. Here, the combination of fractions was based on UV-vis spectroscopy of the samples in each tube. The six fractions were respectively named as M_1, M_2, M_3, M_4, M_5, and M_6. Thereafter, the six components were evaluated for their antifungal activity respectively. As shown in Table 4, M1 to M6 showed antifungal inhibiting rates ranging from almost 0% (M_3, M_4, and M_5) to 100% (M_2). M_1 and M_2 displayed some inhibiting effect, which was nearly 9-fold less than M_2. Taken together, M_2 was the best as far as the antifungal effect was concerned.

As M_2 was the best one, 10% Tween 80 was used to dilute M_2 by different dilution rates (2 to 16 fold) to further study its antifungal activity [1]. The results are shown in Fig. S2.

Table 2. Effects of indoor pre-biocontrol on mycelium growth and spore germination of pathogen by *Trichoderma* strain T-33 crude extract.

Prevention group	0 day	1 day	2 days	3 days	4 days	Control
Disease index	3.98	8.65	11.58	18.97	21.36	68.62
Preventive effect(%)	90.82	79.26	75.36	60.04	56.38	

Table 3. Effects of indoor biocontrol on mycelium growth and spore germination of pathogen by *Trichoderma* strain T-33 crude extract.

Treatment group	0 day	1 day	2 days	3 days	4 days	Control
Disease index	4.26	5.84	7.79	12.34	37.57	59.58
Preventive effect (%)	89.99	85.46	76.71	62.53	45.73	

Table 4. Inhibition effects of different components in strain T-33 methanol extract.

Different components[a]	Colony diameter (cm)	Inhibiting rate (%)
M_1	7.92^b	11.73^b
M_2	1.11^a	100^a
M_3	8.92^c	0.56^c
M_4	8.98^c	0^c
M_5	9.06^c	0^c
M_6	7.84^b	12.59^b
Control	8.97	

Data in the table are averages. It is such that : a,b and c The same letter indicates no significant difference ($p < 0.05$); [a]Strain T-33 methanol extract was fractionated by column chromatography. Fractions in 60 tubes were collected, which were then combined into six fractions. The six fractions were respectively named as M_1, M_2, M_3, M_4, M_5, and M_6. Thereafter, the six components were evaluated for their antifungal activity respectively.

As the dilution factor increased from 2 to 16 fold, the antifungal potency of M_2 at different dilution folds showed a downward trend (Fig. S2). The inhibition rates of the four different dilutions of M_2 samples were 95.12% (2×), 90.31%(4×), 74.98%(8×), and 45.06%(16×), respectively.

To further identify the antifungal compound, TLC was used to r separate M_2 components. The best developing agent was *n*-hexane:ethyl acetate = 4:6 (v/v) based on the analytical TLC experiment. The iodine color developing method and UV scanning method were also combined for detection. A total of 10 components were therefore collected and named as M_{21}, M_{22}, M_{23}, M_{24}, M_{25}, M_{26}, M_{27}, M_{28}, M_{29}, and M_{210}.Thereafter, the inhibiting activity of the ten components was further studied to unveil which compound was responsible for the antifungal activity. Results in Fig. 1 showed that the components M_{24}, M_{26}, and M_{27} had better antifungal activity than others. As these three components demonstrated higher inhibiting effect, their yield was further calculated. Starting at 2.25 g crude product (methanol-soluble extracts), these three components obtained were 0.642, 0.015, and 0.0196 g, with a yield 25.64% (m/v), 0.60% (m/v), and 0.783% (m/v), respectively. For a potential commercial application of the antifungal substance, the component M_{24} with the highest antifungal activity was selected for further study.

We further separated M_{24}, and the HPLC fraction corresponding to the peak eluent (Fig. S3) was collected. The solvent methanol in this fraction was evaporated to obtain dry sample, which was named as M_{24b}.

Structure Identification of Isolated M_{24b}

The sample M_{24b} was a pale yellow liquid, soluble in methanol and ethanol, and slightly soluble in chloroform and petroleum ether. The color developed with iodine was yellow after TLC. When the modified potassium iodide was sprayed, the color development was positive. The chloroform-concentrated sulfuric acid experiments were positive and the compound was preliminarily identified as terpenes or benzoquinones.

To further find out the structure of the compound, the following characterization was done and the data are shown below:

1) The maximum absorbance was at 254 nm as shown in the UV-vis spectrum (Fig. S4).

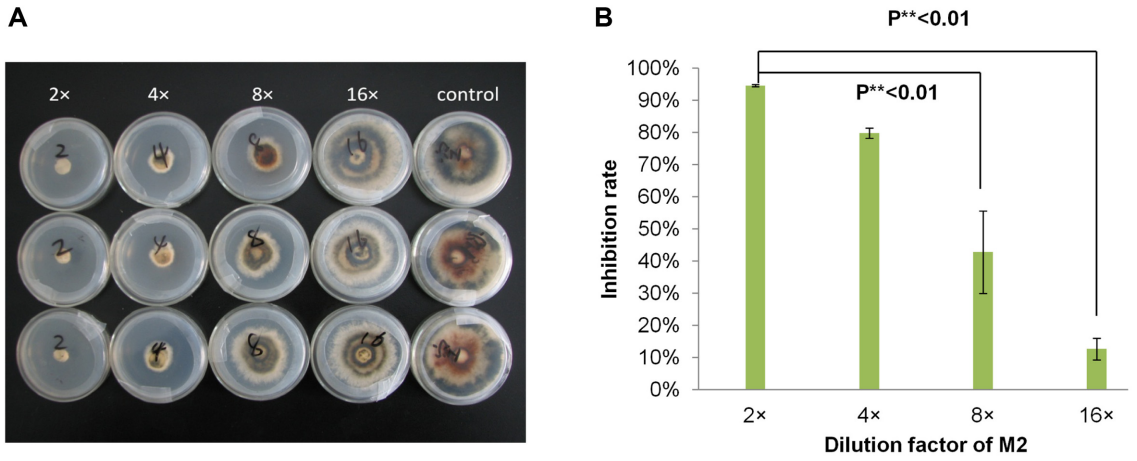

Fig. 1. Inhibiting effects of the different dilutions of M_2 on *C. chrysosperma* YL strain after 5 days of incubation.
Dilution factors (2, 4, 6, 8, 16) are labeled on the plates (**A**). (**B**) Quantification of the inhibiting effect of the different dilutions of M_2. All experiments were conducted in triplicate ($P^{**} < 0.01$).

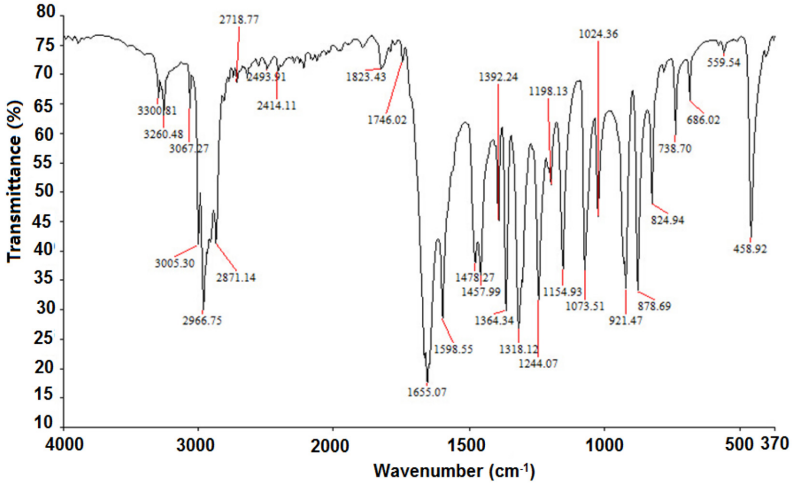

Fig. 2. The IR spectrum of sample M_{24}.

2) IR spectra show that there are carbonyl (-C=O) signals visible at 1,655.07 cm^{-1}; stretching vibration of C-H bond in -CH_3 visible at 2,966.75 cm^{-1}, 2,871.14 cm^{-1}, and 3,005.30 cm^{-1}; bending vibration of C-H bond in -CH_3 at 1,364.34 cm^{-1}, 1,318.12cm^{-1}, and 1,244.07 cm^{-1}; and bending vibration of C-H bond of olefins hydrocarbon at 921.47 cm^{-1} and 878.69 cm^{-1}.

3) The GC sepectrum of M_{24b} in Fig. S5 shows M_{24b} had a purity of 96.57 w% (elution time: 8.01 min).

4) GS-MS (Fig. 3) revealed the main pieces fragmented at different *m/z* as follows: 205.0 (M-CH_3), 177.0 (M-$COCH_3$), 163.0 (177.0-CH_2), 149.0 (163.0-CH_2), 135.0 (149.0-CH_2), 121.0 (135.0-CH_2), 107.0 (121.0-CH_2), 91 (107.0-O), 67.0 (91-C=C), and 53.0 (67.0-CH_2). According to the NIST 2008 gas-mass spectrometry database, the matching rate between spectra of the sample M_{24b} and 2,6-di-*tert*-butyl-1,4-benzoquinone (systematic name: 2,5-cyclohexadiene-1,4-dione-2,6-*bis* (1,1-dimethylethyl)) was 98.6%, indicating M_{24b} could be 2,5-cyclohexadiene-1,4-dione. Moreover, the molecular weight of 2,5-cyclohexadiene-1,4-dione is 220.15, showing a high probability that the sample M_{24b} was 2,5-cyclohexadiene-1,4-dione.

5) The ^{13}C-NMR spectrum (Fig. 4) showed that the compound had a total of six different carbons. Two C connected with oxygen on the benzene ring were found in the C spectrum, at 188.65 and 187.74 ppm, respectively, wherein 188.65 ppm should be attributed to C in the alternative position with *tert*-butyl, and 187.74 ppm should be attributed to the C in the adjacent position with *tert*-butyl. Moreover, the chemical shift of carbon at 157.73 ppm

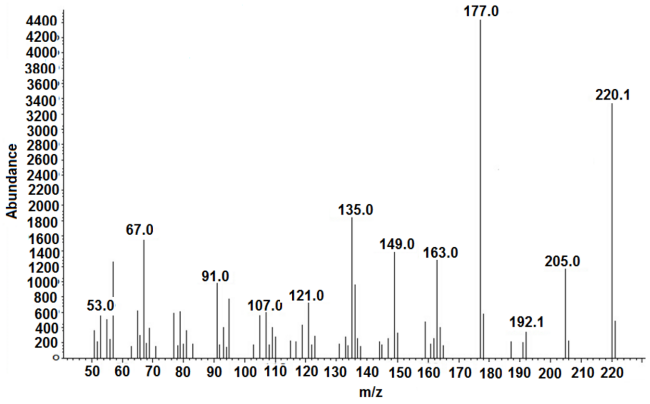

Fig. 3. GC-MS spectrum of sample M_{24}.
Samples were prepared in methanol.

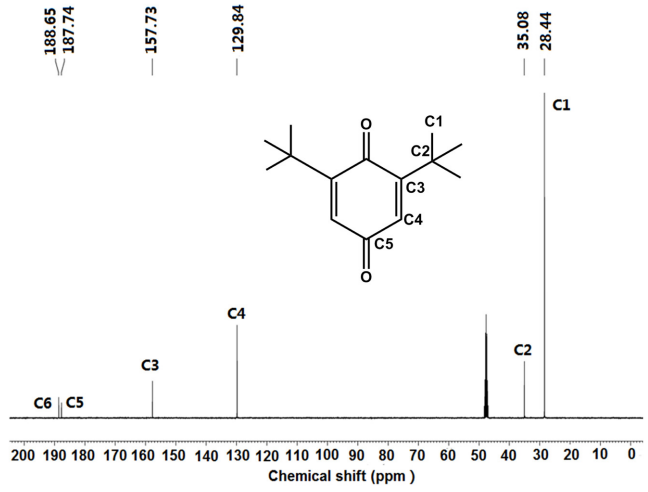

Fig. 4. ^{13}C-NMR of sample M_{24} in CD_3OD at 2 mg/ml (400 MHz, room temperature).

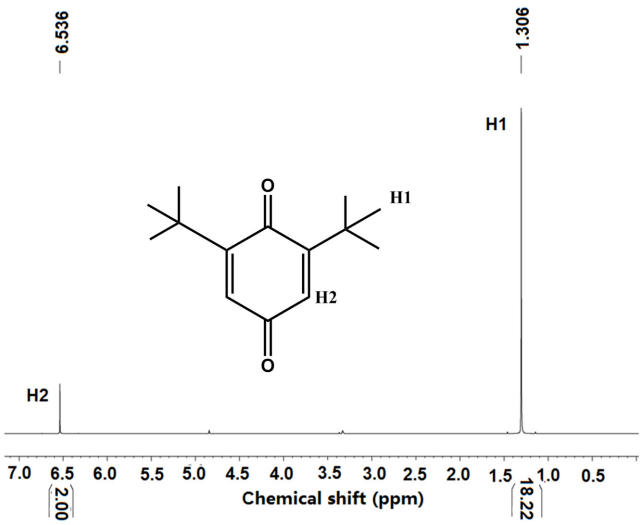

Fig. 5. ^{1}H-NMR of sample M_{24} in CD_3OD at 0.05 mg/ml (400 MHz, room temperature).

could be assigned to two C connected with *tert*-butyl, 129.84 ppm could be assigned to two C connected with H on the benzene ring, 35.08 ppm could be assigned to two C connected with three methyls, and 28.44 ppm could be assigned to 6 methyl-C.

To better elucidate this, the ^{1}H-NMR spectrum was obtained (Fig. 5). The compound had two kinds of hydrogen atoms. The number of hydrogen atoms corresponding to each peak in the ^{1}H-NMR spectrum was quite different judging from the peak heights. The peak at 6.536 ppm could be assigned to two H on the benzene ring. The peak at the position of 1.306 ppm was higher and could be assigned to 18 methyl H.

Taken together, the IR and NMR results further validated the conclusion from the GC-MS. The systematic name of sample M_{24b} could be 2,5-cyclohexadiene-1,4-dione-2,6-*bis* (1,1-dimethylethyl). The molecular formula is $C_{14}H_{20}O_2$, and the molecular weight is 220.15.

Discussion

Trichoderma strain T-33 was previously demonstrated by our research group to have effective inhibitory ability on the plant pathogen fungus *C. chrysosperma* after screening several strains. Preliminary research on the antifungal mechanism of T-33 extract indicates that strain T-33 extract could change the permeability of the pathogen membrane, thereby resulting in an abnormal operation of the glycolytic pathway, TCA cycle, electron transport, and oxidative phosphorylation, and the disruption of the metabolic balance among sugar, lipids, and proteins. The present study adopts solvent extraction, silica gel column chromatography, TLC separation, and GC-MS chromatography to purify and identify the active antifungal compound produced by stain T-33. The main antifungal compound in the T-33 extract is 2,5-cyclohexadiene-1,4-dione-2,6-*bis* (1,1-dimethylethyl), which is used by the pharmaceutical industry as a kind of oxidant. To the best of our knowledge, this is the first time 2,6-di*tert*-butyl 1,4-benzoquinone was discovered and extracted from a live organism. Reports on the effect of benzoquinone materials are rare. Netzly and Butler [16] studied 2-hydroxy-5-methoxy-3-*p*-hydroquinone isolated from the host plant sorghum root exudates, which can stimulate germination of the host plant such as witchweed and Orobanche seed. Chang and Lynn [3] isolated 2,6-dimethoxy-benzoquinone from sorghum root exudates and tested whether the substance has a role of inducing the formation of abstractor in witchweed and Orobanche seed. Deng *et al.* [8] isolated benzoquinones from Changyuan Houpu, a Chinese herbal medicine, and also proved that the benzoquinone materials have antifungal effects. The antifungal mechanism of 2,5-cyclohexadiene-1,4-dione-2,6-*bis* (1,1-dimethylethyl) is not entirely clear and still needs further investigation. The present study provides the best example of obtaining and purifying antifungal substances from *Trichoderma* from safer and biodegradable antifungal products and will possibly find potential use in the near future.

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