

Griseococcin(1) from *Bovistella radicata* (Mont.) Pat and Antimicrobial Activity

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Abstract

Bovistella radicata (Mont.) Pat was a natural plant with multiple medicinal values. The purified compound purified from *Bovistella radicata* (Mont.) Pat by DEAE-cellulose and sephadex LC-20 column which could inhibit main pathogens leading to tinea pedis. Based on the spectral (HPLC, FT-IR, 1D and 2D NMR etc.) studies, purified compounds were identified as the Griseococcin(1) which were naphthoquinone derivatives, the Chemical formula and MW of Griseococcin(1) was determined as $C_{37}O_{10}H_{43}N$ and 661 Da. Minimum inhibitory concentration(MIC) and zone of inhibition(ZOI) of Griseococcin(1) were 31.2, 31.2 $\mu\text{g/ml}$ and 18.06 ± 0.85 mm, 15.01 ± 1.02 mm respectively against main pathogenic fungus *Trichophyton rubrum* and *Trichophyton mentagrophytes*.

Introduction

Tinea pedis is a chronic fungal infection of the feet (Alteras, et al 1981). Patients that have tinea pedis may be affected by several pathogens, including filamentous fungi named *Trichophyton rubrum* and *Trichophyton mentagrophytes*(Koltin and Hitchcock 1997), as well as a yeast named *Candida albicans* (Erbagci, et al. 2005). *T. rubrum* is the main pathogenic fungi for tinea pedis, having a prevalence as high as 80% among all tinea-pedis-associated pathogenic microbes(Miyajima et al. 2013). Traditionally, to treat tinea pedis, synthetic fungicides such as fluconazole, itraconazole, echinocandins(Daneshmend and Warnock. 1983), and miconazole nitrate, either by oral medication or external use(Patel et al. 2017), have been used to treat this disease. Vermes et al (2000) found that flucytosine and AMB (amphotericin B) were moderately effective in fighting against invasive fungal infections (Francis P and Walsh T J. 1992; Stamm et al.1987; Vermes et al. 2000). Similar studies on Itraconazole have demonstrated that it is effective against fungal infections(Denning et al.1997). However, due to side effects or the continuous drug resistance, some oral medications are unsafe for patients (Subissi et al. 2010), and these chemicals also cause potential deleterious effects on the environment due to their residues(Lushchak. 2016; Rajendra et al.2013). In general, plant natural products have been for decades one of the most successful sources of drugs to treat infectious diseases(Genilloud. 2014) and natural products extracted represent a rich resource for screening bioactive compounds (Ribeiro et al. 2018).

Puffballs are widely distributed in many provinces of China, and are various by more than 100 species(Bates et al. 2009). *Calvatia gigantean*(Batsch ex Pers)Lloyd, *Calvatia lilacina* (Mont.et Berk.) Lloyd, *Lasiochaeta fenzlii* Reich, *Lycoperdon pyriforme* Schaeff.:pers, *Bovistella radicata* (Mont.) Pat, *Handkea utriformis* (HU), *H. excipuliformis* (HE), and *Vascellum pratense* (VP) are all common medicinal puffballs. Although no longer edible in their mature state (because of their powdery consistency), these puffballs have been shown to be a source of active compounds of various biological activities. Puffballs are believed to have several therapeutic properties when used medicinally: hemostasis(LIANG. 2016), cough relief(Jiang. 2017), suppression of cell division, and antitumor(Lam et al. 2001) and antimicrobial(Ye et al. 2017) properties. Petrović P, et al reported noticeable antimicrobial activity diversity for the methanol extracts obtained from *Handkea utriformis* (HU), *H. excipuliformis* (HE), and *Vascellum pratense* (VP) (Petrović et al. 2016). Specimen (*Bovistella radicata* (Mont.) Pat) was dried and deposited

in the Institute of Biology, School of Food and Biological Engineering, Hefei University of Technology (HFUT), China.

The aim of the present study was to evaluate the antifungal activity of Griseococcin(1) extracted from *B. radicata* fermentation broth. The antifungal activities were evaluated in terms of their minimum inhibitory concentration (MIC) values and zone of inhibition (ZOI) values (Negi et al. 2003), the physico-chemical characterization (HPLC, UV, FT-IR) of Griseococcin(1) and the chemical constituents responsible for this activity were also studied (1D and 2D NMR).

Material And Methods

Sample *Bovistella radicata* (Mont.) Pat collection and strain isolation

Bovistella radicata (Mont.) Pat was purchased and collected from Bozhou traditional Chinese medicine trading market, China. The four tested pathogenic fungi included *Trichophyton rubrum* (ATCC 28188), *Trichophyton mentagrophytes* (ATCC 9533), *Epidermophyton floccosum* (ATCC 52066), and *Candida albicans* (ATCC 10231).

Fermentation, extraction and purification of *Bovistella radicata* (Mont.) Pat

The spore powder of *B. radicata* inoculated into 100 mL of potato dextrose broth (PDB). The flask was kept in rotary shaker at 25°C with 115 rpm for 72 h. The pH and moisture content of PDB was also determined according to Maguireboyle (2014) and McAuliffe (2016) (Maguireboyle et al. 2014; McAuliffe L N et al. 2016). For every 12 hours, the fermentation was taken to perform antimicrobial activity against main pathogens *T. rubrum* and *T. mentagrophytes* by Zone of inhibition (ZOI) method. Then the fermentation were centrifuged at 6000 rpm and the final clear supernatant was preserved. 50 ml clear supernatant was applied to 100 ml DEAE-cellulose and sephadex LH-20 column. Different purified fractions (named Griseococcin(s)) were obtained from SPAF, only Griseococcin(1) (250 µg/ml) has antifungal activity and its biochemical characteristics and spectral (HPLC, FT-IR, 1D and 2D NMR etc.) studies were assessed.

Antimicrobial activity

The antimicrobial activity was tested against selected fungi (*T. rubrum*, *T. mentagrophytes*, *E. floccosum*, and *C. albicans*) and bacteria (*S. aureus*, *E. coli* and *P. aeruginosa* from tinea pedis). The examined methods were the minimum inhibitory concentrations (MICs) (Negi et al. 2003) and zone of inhibitions (ZOIs). The MIC value of Griseococcin(1) was determined in the 96-well plates by the double micro dilution method (7.8 ~ 250 µg/mL) against pathogens. The zones of inhibition (ZOI) of Griseococcin(1) (100 µg/ml) was also evaluated (Geetha et al. 2015), Terbinafine and Gentamicin sulfate as the positive control.

General experimental procedures

The UV_{max} absorption spectrum of SPAF was analyzed at full-wave spectra (200–900 nm) by UV/vis 2802 spectrophotometer. The FT-IR spectrum of Griseococcin(s) were recorded on a Thermo Nicolet Spectrum FT-IR in a range of 4000 – 400 cm⁻¹ with KBr pellets. HR-ESI-MS data were obtained on an Agilent 1260 Infinity LC coupled to a 6230 TOF. 20 mg of the dried sample was dissolved in 0.55 mL of deuterioxide (99.99% D) in a NMR tube. 1D and 2D NMR spectra were acquired on an AVANCE-600 NMR spectrometer (Bruker Inc, Rheinstetten, Germany) at 50°C. The chemical shifts were given in δ (ppm) and referenced to the solvent signal (D₂O-d₆, δ H 2.50, δ C 39.5). Column chromatography (CC) was conducted on DEAE-cellulose and Sephadex LH-20. The fractions Griseococcin(s) were also monitored by HPLC(Agilent 1260 chromatography system, USA) which was equipped with a diode array detector (DAD). The DAD detector was set at 215 nm to acquire chromatograms. The separation of the compound was performed on a Hypersil RP-C18 column (5 μm, 250 × 10.0 mm, Thermo Fisher Scientific, USA) at a temperature of 25 °C. Injection volume: 20 μL.

Griseococcin(1)

Griseococcin(1): IR (neat) ν max 3417, 2926, 2356, 1637, 1618, 1456, 1414, 866, 624 cm⁻¹; UV (D₂O) λ max 215 nm; ¹H and ¹³CNMR data see Table 1; HR-ESI-MS m/z 661.1970 [M + H]⁺ (calcd for C₃₇H₄₃NO₁₀, 661.1968).

Table 1. ¹H(700 MHz) and ¹³CNMR (175 MHz) spectroscopic data for streptoxamine(1) in DMSO

Position δ H (mult, J in Hz) δ C Position δ H (mult, J in Hz) δ C

Table 1
¹H(700MHz) and ¹³CNMR (175MHz)
 spectroscopic data for streptoxamine(1) in
 DMSO

Position	δ H (mult, J inHz)	δ C
1	7.81	166
2		128.56
3		175.11
4		138.6
5	7.93	135.7
6	7.72	137.21
7		136.28
8	7.8	135.45
9		138.56
10		175.07
11		128.57
12	7.66	166.07
13	1.07	30.18
14	3.62	166.01
15	3.49	166.02
16	3.55	166.03
17	3.51	166.01
18	1.25	23.15
19	1.94	23.16
20	2.09	184.15
1'	1.94	28.4
2'		215.7
3'	2.14	23.4
4'	4.47	73.6
4'-OH	8.37	
5'	2.12	29.05

6'		215.7
7'	1.08	25.52
8'	1.94	26.81
9'	3.62	71.25
9'-OH	7.81	
10'	1.29	23.45
11'	3.49	71.15
11'-OH	7.8	
12'	1.73	23.47
13'	3.51	71.18
13'-OH	7.93	
14'	1.85	20.27 CH3
15'	2.11	20.27 CH3
16'	1.06	20.27 CH3
17'	1.07	20.27 CH3

Results

Fermentation, extraction and purification of active compound from *B. radicata*

Fermentation broth of *B. radicata* was filtered and centrifuging at 6000 rpm, on 3rd day, the fermentation of *B. radicata* showed the antimicrobial activity against main pathogenic fungi of tinea pedis, *Trichophyton rubrum* and *Trichophyton mentagrophytes*. The fermentation was purified firstly using DEAE-cellulose column and eluted by different concentration NaCl(10%-30%) and get different fractions, 20% NaCl elution fraction showed best antifungal activity against pathogens. Furthermore, 20% NaCl elution fraction was purified by sephadex LC-20 column, different fractions(Griseococcin(s)) were obtain and antifungal activity of Griseococcin (1) was strongest. The UV_{max} of all these fractions were 215 nm, the HPLC chromatograms of SPAF and Griseococcin (1) were shown in Fig. 1(A ~ B). The chromatogram of B showed a single and symmetrical peak for Griseococcin (1)(Fig. 1.B)

1D and 2D NMR of Griseococcin (1)

Griseococcin (1) was isolated as a white amorphous solid powder with the molecular formula of $C_{37}H_{43}NO_{10}$ derived from the high-resolution electrospray ionization mass spectrum (HR-ESI-MS). The complete assignments for all protons and carbons was shown in Table 1. The ^{13}C NMR spectra of

Griseococcin (1) displayed signals of thirty seven carbons, including five carbonyl carbons (δC 215.7–175.1), five aromatic/olefinic methine carbons (δC 128.86, δC 215.7–175.1), seven non-protonated aromatic/olefinic carbons (δC 161.06–109.99), four methyl carbons (δC 20.27), and four olefin carbons (δC 166.01). The 1H NMR spectrum of 1 in D₂O exhibited signals of four methyls at δH 2.14 (3H, s, H-14'), δH 2.12 (3H, s, H-15'), δH 1.06 (3H, s, H-16') and 1.07 (3H, s, H-17'), five aromatic protons [δH 7.80 (1H, s, H-1), δH 7.93 (1H, s, H-5), δH 7.72 (1H, s, H-6), δH 7.81 (1H, s, H-8) and 7.66 (1H, s, H-12)], four hydroxyl groups at δH 8.37 (1H, br s, 4'-OH), δH 7.81 (1H, br s, 9'-OH) and δH 7.80 (1H, br s, 11'-OH) and 9.63 (1H, br s, 13'-OH).

The structure of Griseococcin (1) was deduced by comprehensive analysis of 1H - 1H COSY, HMBC, and HSQC spectra (Fig. 2A). In Griseococcin (1), the naphthoquinone substructure could be identified by the observation of HMBC correlations from H-8 (δH 7.80) to C-6 (δC 137.21), C-4 (δC 138.60) and C-13 (δC 30.18), from H-1 (δH 7.81) to C-3 (δC 175.11), C-12 (δC 166.07) and C-1' (δC 28.40), from H-5 (δH 7.93) to C-3 (δC 175.11) and C-9 (δC 138.56), from H₂-13 (δH 1.07) to C-8 (δC 135.45) and C-6 (δC 137.21), from H₃-14' (δH 1.85) to C-2' (δC 215.7) and C-4'(OH) (δC 73.60), from H₃-15' (δH 2.11) to C-6' (δC 215.70) and C-4'(OH) (δC 73.60), from H₂-7' (δH 1.08) to C-9' (δC 71.25) and C-13' (δC 71.18). The 1H , 1H three-bond couplings observed in the COSY spectrum from H-8' (δH 1.94) to H-9' (δH 3.62), from H-10' (δH 1.29) to H-11' (δH 3.49), from H-12' (δH 1.73) to H-13' (δH 3.51), together with the chemical shifts of the ^{13}C resonances (C-8'-13') observed at alternating higher and lower fields, revealed the presence of cyclohexane with alternating hydroxyl and methyl groups. 1H - 1H COSY correlations from H₂-13 (δH 1.07, m) to H₂-14 (δH 3.62, m), from H₂-14 (δH 3.62, m) to H₂-15 (δH 3.49, m) and from H₂-16 (δH 3.55, m) to H₂-17 (δH 3.51, m) and HMBC correlations from H₂-13 (δH 1.07, m) to C-15 (δC 166.02), from H₂-14 (δH 3.62, m) to C-16 (δC 166), from H₂-15 (δH 3.49, m) to C-17 (δC 166.01) and from H₂-16 (δH 3.55, m) to C-18 (δC 23.15) identified coupled olefins. The key HMBC correlations from H₂-1' (δH 1.94, m) to C-3' (δC 23.4), from H-3' (δH 2.14, m) to C-5' (δC 29.05), from H₃-14' (δH 1.85, m) to C-2' (δC 215.7) and C-4'-OH (δC 73.6), from H₃-15' (δH 2.11, m) to C-6' (δC 215.7) and C-4'-OH identified two meta position carbonyl group and one ortho position hydroxyl group (Fig

2B).

This connectivity was also secured by the observation of the HSQC correlations from H₃-14' to C-3' and from H₃-15' to C-6'. Therefore, the complete structure of naphthoquinone was determined as shown in Fig. 2C.

Physico-chemical characterization of Griseococcin (1)

Griseococcin (1) was white powder and its solubility was 0.063 g/ml in water. It could be slight soluble in methanol and DMSO, but insoluble in n-hexane, dichloromethane, chloroform, ethyl acetate and acetone.

The FT-IR spectrum of Griseococcin (1) showed (Fig. 3) a intense and broad characteristic absorption peaks at 3417.2 cm^{-1} represented the stretching vibration of O–H. The weak absorption peaks at 2356 and 2925.5 cm^{-1} were resulted from the stretching vibration of C–H. The absorption bands at 1637.4 and 1618.1 cm^{-1} are due to the vibration of C = C and C = O in the ester group. The absorptions peaks at 1456.1, 1414 and 624 cm^{-1} were attributed to the presence of an internal C–H deformation. The strong absorption peak at 866 was resulted from aromatics. In conclusion, the typical absorption peak indicated that Griseococcin (1) was naphthoquinone with group O–H, C–H, C = C, C = O and so on (Xie et al. 2018).

In vitro antagonistic assay

Griseococcin (1) was assessed for antifungal activity against selected *T. rubrum*, *T. mentagrophytes*, *E. floccosum*, *C. albicans* and antibacterial activity against selected *Staphylococcus aureus*, *Bacillus subtilis* and *Pseudomonas aeruginosa*. The results were shown in table 2, it displayed strong antifungal activity against *T. rubrum*, *T. mentagrophytes* with ZOI values of 18.06 ± 0.85 , 15.01 ± 1.02 mm and MIC values of 31.2, 31.2 mg/ml, as compared to the positive control Terbinafine with ZOI and MIC values of 20.67 ± 1.58 , 28.33 ± 2.15 mm and 15.6, 7.8 $\mu\text{g/mL}$, respectively. But while antibacterial activity was weak.

Table 2 Antimicrobial activity (ZOI and MIC) of Griseococcin (1) from *Bovistella radicata* (Mont.) Pat

Pathogenic microbiology	Zone of inhibition(mm)	Minimum inhibitory concentration($\mu\text{g/ml}$)	positive control	positive control
	Griseococcin (1)	positive control (1)	Griseococcin (1)	positive control
<i>T. rubrum</i>	18.06 ± 0.85	$20.67 \pm 1.58\text{a}$	31.2	15.6a
<i>T. mentagrophytes</i>	15.01 ± 1.02	$28.33 \pm 2.15\text{a}$	31.2	7.8a
<i>E. floccosum</i>	5.03 ± 2.28	$10.02 \pm 1.02\text{a}$	250	62.5a
<i>C. albicans</i>	1.21 ± 2.19	$6.33 \pm 0.58\text{a}$	250	62.5a
<i>S. aureus</i>	10.05 ± 1.9	$31.7 \pm 1.5\text{b}$	62.4	15.6b
<i>E. coli</i>	8.01 ± 1.1	$28.5 \pm 1.4\text{b}$	125	31.2b
<i>P. aeruginosa</i>	3.7 ± 1.4	$33.6 \pm 1.4\text{b}$	250	31.2b

Discussion

In the present study, selected puffball (*Bovistella radicata* (Mont.) Pat) showed remarkable antifungal activities. These data are consistent with previous findings on the minimum inhibitory concentrations

(MICs) and zone of inhibition (ZOI) of *B. radicata* (Ye et al. 2017).

According to the Chinese Pharmacopeia, the main anti-microorganism activity of the puffball is against *S. aureus* and *Paeruginosa*. The antifungal function of puffballs has not been reported previously, hence, the present study about antifungal function of *B. radicata* is the first report. The novel antimicrobial activities of *B. radicata* might be due to different geographic sources of the material used and different strains used (Ali et al. 2003).

In this study, the purification extraction Griseococcin(s) from fermentation broth of *B. radicata* obtained through macroporous resin D-101, celluoues DE-52 and sephadex LH-20 column, purified fractions were used for biological activity analysis. *In vitro* assays demonstrated that fraction Griseococcin(1) showed better suppress activity for main pathogenic fungi (*T. rubrum* and *T. mentagrophytes*), ZOIs were 18.06 ± 0.85 and 15.01 ± 1.02 mm, MICs were 31.2 and 31.2 mg/ml against *T. rubrum* and *T. mentagrophytes*, respectively. ZOI and MIC values of positive control (Terbinafine) were 20.67 ± 1.58 mm 28.33 ± 2.15 mm and 15.6, 7.8 $\mu\text{g}/\text{mL}$, respectively. The antifungal effect of Griseococcin(1) similar with that of positive control.

The FT-IR spectrum of Griseococcin(1) showed the strong absorption band, stretching vibration and bending vibration of O-H, C = O, C = C and C-H which belong to a unsaturated coupled bond and aromatic form of naphthoquinone. According to HR-ESI-MS analysis, MW of Griseococcin(1) was 661 Da. Based on the results of different spectral (HPLC, FT-IR, DSC, 1D and 2D NMR etc.) studies and physicochemical properties, the molecular formula of Griseococcin(1) was $\text{C}_{37}\text{H}_{43}\text{NO}_{10}$ and the molecular structure of Griseococcin(1) was shown in Fig. 1.

Previously, many authors reported the various biological activity of fermentation broth from puffball like anticancer activity (Silva et al. 2019; Zhong et al. 2018) antioxidant activity (Xu et al. 2018) antifatigue effect (Yange et al. 2017), etc. In the present study, the antifungal activity of *B. radicata* was another important biological function. The biological activities of organic compounds are related to their molecular weight, functional groups, the length of chain, the composition of group and the number of branches, hydrophilic and hydrophobic group. It means that the structure-activity relationship should be disclosed.

Future work concentrating on determining the antifungal mechanisms of Griseococcin(1) will be performed, which will be helpful in laying a foundation for overcoming the drug resistance that pathogens quickly develop against tinea pedis.

In this paper, the antifungal secondary metabolite compound Griseococcin(1) from *B. radicata* were studied. The compound from *Bovistella radicata* (Mont.) Pat was purified. The purified compound can restrain main pathogens (*T. rubrum* and *T. mentagrophytes*) leading to tinea pedis. The antifungal activity of Griseococcin(1) was similar to that of the positive control. Molecular weight and molecular formula were 661 Da and $\text{C}_{37}\text{H}_{43}\text{NO}_{10}$.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

competing interests

The authors declare no competing interests.

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Authors' contributions

Authors' Contributions are according to the ranking. Yong Ye performed the experiments and wrote the manuscript, Kun Liu and Qinghua Zeng performed data analysis, and Qingmei Zeng is the corresponding author. The authors would like to thank Kun Liu and Qinghua Zeng for their excellent technical support.

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Figures

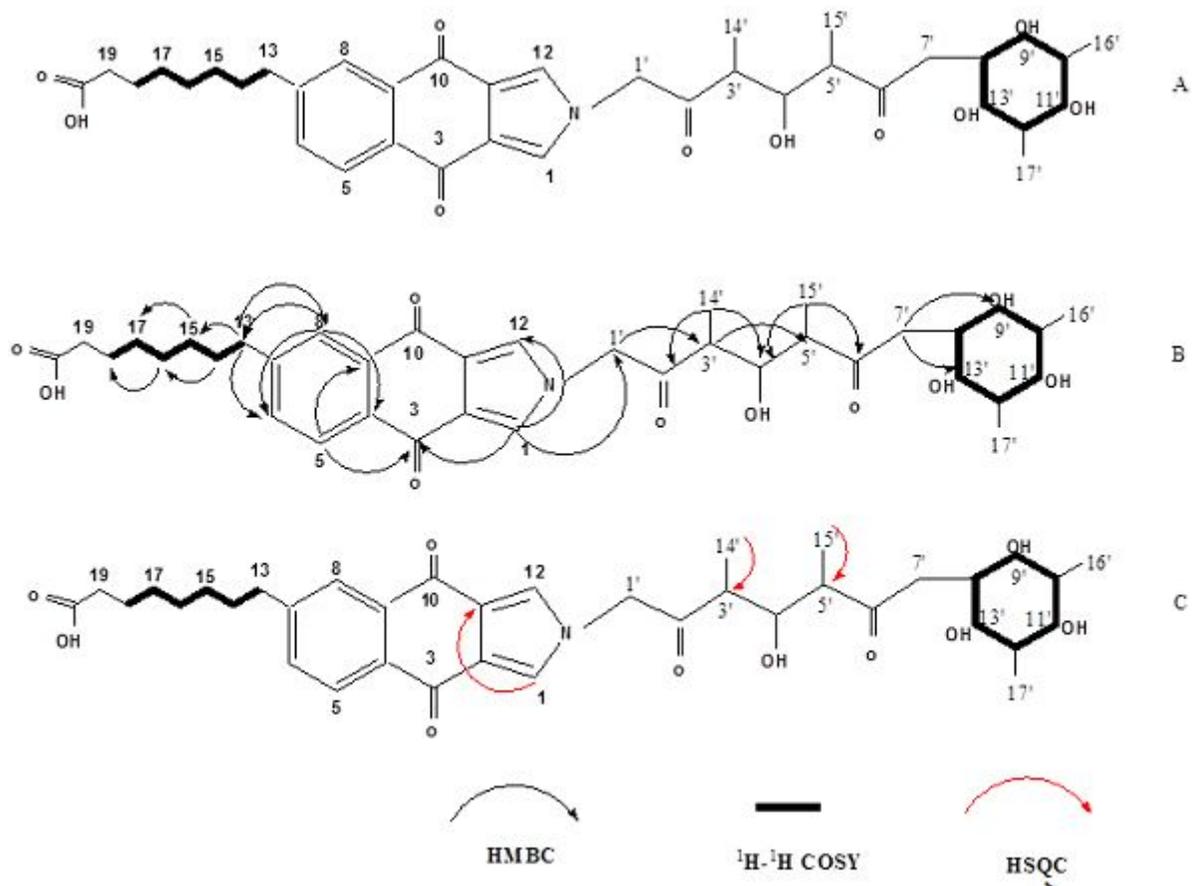


Figure 1

The key $^1\text{H}-^1\text{H}$ COSY, HMBC and HSQC correlations of Griseococcin (1)

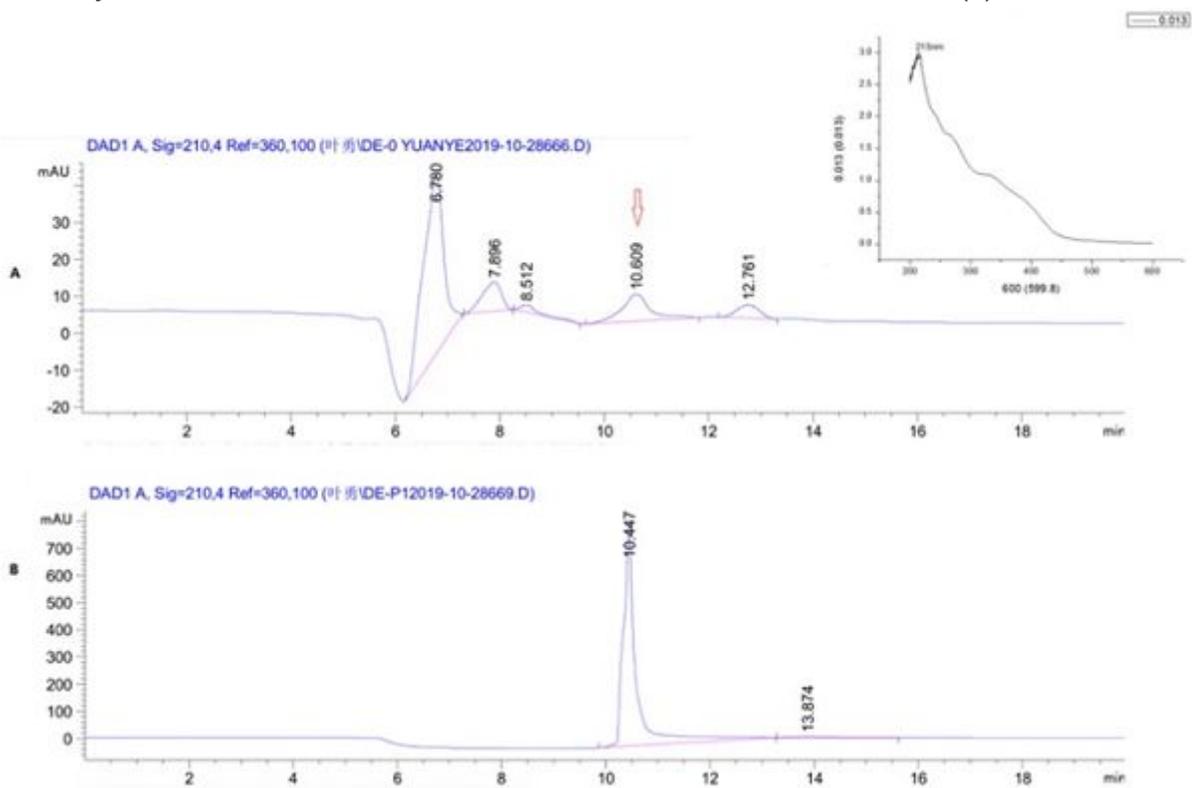


Figure 2

UV spectral and HPLC chromatography of SPAF (A) and purified fraction (Griseococcin (1))(B)

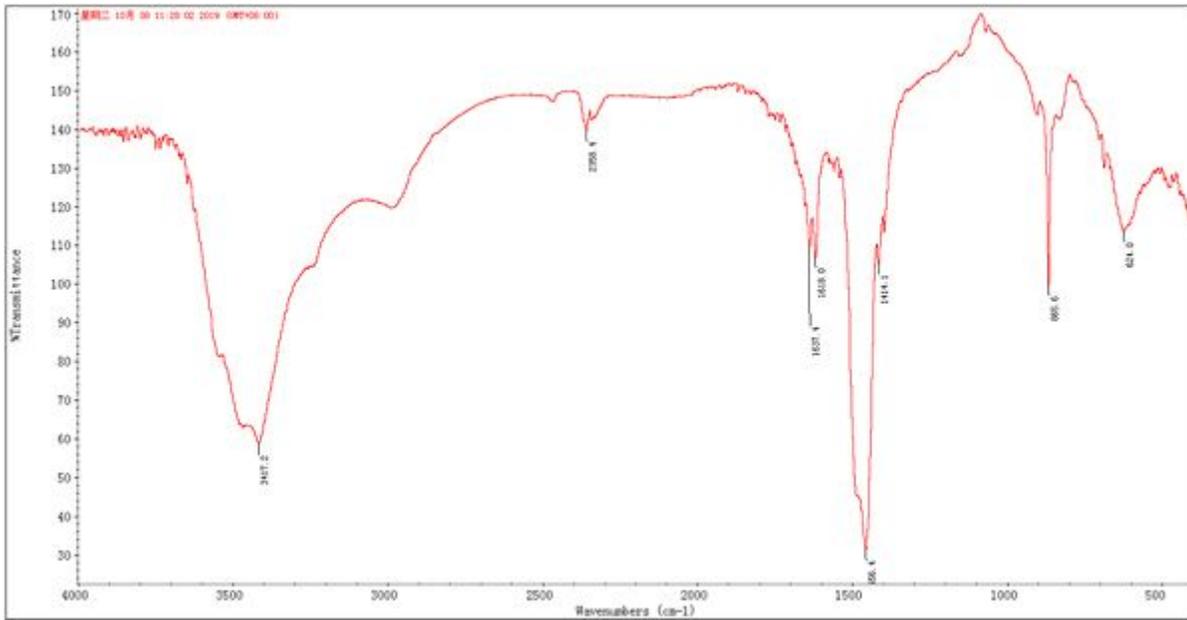


Figure 3

FT-IR of fractions F2 and F3 Note: line A is F2; line B is F3