Griseococcin(1) from *Bovistella radicata (Mont.) Pat* and antimicrobial activity

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Abstract

Background: To evaluate the antifungal activity of *B. radicata* fermentation broth, the broth was purified by DEAE-cellulose and sephadex LC-20 column. The compounds were submitted to spectral analysises(HPLC, FT-IR, 1D and 2D NMR etc.).

Results: The purified compounds were identified as the Griseococcin(s) which were naphthoquinone derivatives, only Griseococcin(1) has antifungal activity among the Griseococcin(s). Minimum inhibitory concentration(MIC) and zone of inhibition(ZOI) of Griseococcin(1) were 31.2, 31.2 μg/ml and 18.06±0.85 mm, 15.01±1.02 mm respectively against main pathogenic fungus *Trichophyton rubrum* and *Trichophyton mentagrophytes,* the Chemical formula and MW of Griseococcin(1) was determined as $C_{37}O_{10}H_{43}N$ and 661Da.

Conclusions: In this paper, the secondary metabolite compound Griseococcin(1) from *B. radicata* 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.

Background

Tinea pedis is a chronic fungal infection of the feet [1]. Patients that have tinea pedis may be affected by several pathogens, including filamentous fungi named *Trichophyton rubrum* and *Trichophyton mentagrophytes*[2], as well as a yeast named *Candida albicans*[3]. *T. rubrum* is the main pathogenic fungi for tinea pedis, having a prevalence as high as 80% among all tinea-pedis-associated pathogenic microbes [4]. Traditionally, to treat tinea pedis, synthetic fungicides such as fluconazole, itraconazole, echinocandins [5], and miconazole nitrate, either by oral medication or external use[6], 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 [7-9]. Similar studies on Itraconazole have demonstrated that it is effective against fungal infections[10]. However, due to side effects or the continuous drug resistance, some oral medications are unsafe for patients [11], and these chemicals also cause potential deleterious effects on the environment due to their residues[12-13]. In general, plant natural products have been for decades one of the most successful sources of drugs to treat infectious diseases[14] and natural products extracted represent a rich resource for screening bioactive compounds [15].

Puffballs are widely distributed in many provinces of China, and are various by more than 100 species[16]. *Calvatia gigantean*(*Batsch ex Pers)Lloyd,Calvatia lilacina (Mont.et Berk.) Lloyd, Lasiosphaera fenzlii Reich, Lycoperdon pyriforme Schaeff.:pers*, *Bovistella radicata (Mont.) Pat, Handkea utriformis (HU), H. excipuliformis (HE)*, and *Vascellum pratense (VP)* are *a*ll 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[17], cough relief[18], suppression of cell division, and antitumor[19] and antimicrobial[20] 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)[21]. Specimen (*Bovistella radicata (Mont.) Pat*) was dried and deposited in the Institute of Biology, School fo 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[22], 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).

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 215nm, the HPLC chromatograms of SPAF and Griseococcin (1) were shown in figure1(A~B). The chromatogram of B showed a single and symmetrical peak for Griseococcin (1)(fig1.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 (δC215.7–175.1), five aromatic/olefinic methine carbons (δC 128.86, δC215.7–175.1), seven non-protonated aromatic/olefinic carbons (δC 161.06-109.99), four methyl carbons (δ C20.27)), and four olefin carbons (δC 166.01). The ^{1}H NMR spectrum of 1in D2O exhibited signals of four methyls at δ H 2.14 (3H, s, H-14’), δ H 2.12 (3H, s, H-15’), δ H 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 δ H 8.37 (1H, br s, 4’-OH), δ H 7.81 (1H, br s, 9’OH) and δ H 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 ^{1}H-^{1}HCOSY, 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 (δ C 137.21), C-4 (δ C 138.60) and C-13 (δ C 30.18), from H-1(δH 7.81) to C-3 (δ C 175.11), C-12(δ C 166.07) and C-1'(δ C 28.40), from H-5(δH 7.93) to C-3(δ C175.11) and C-9(δ C138.56), from H_2-13(δH 1.07) to C-8(δ C 135.45) and C-6 (δC 137.21), from H_3-14' (δH 1.85) to C-2' (δ C 215.7) and C-4'(OH) (δ C 73.60), from H_3-15' (δH 2.11) to C-6' (δ C 215.70) and C-4'(OH) (δ C 73.60), from H_2-7' (δH 1.08) to C-9' (δC 71.25) and C-13' (δC 71.18). The ^{1}H, ^{1}H 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.^{1}H-^{1}H COSY correlations from H_2-13 (δH1.07, m) to H_2-14 (δH3.62, m), from H_2-14 (δH3.62, m) to H_2 -15 (δH 3.49, m) and from H_2-16 (δH 3.55, m) to H_2 -17(δH 3.51, m) and HMBC correlations from H_2-13 (δH 1,07, m) to C-15 (δC 166.02), from H_2-14 (δH3.62, m) to C-16 (δC 166), from H_2-15(δH3.49, m) to C-17 (δC 166.01) and from H_2-16(δH3.55, m) to C-18 (δC 23.15) identified coupled olefins. The key HMBC correlations from H_2-1' (δH1.94, m) to C-3' (δC 23.4), from H -3' (δH2.14, m) to C-5' (δC 29.05), from H_3-14' (δH1.85, m) to C-2' (δC 215.7)and C-4'-OH (δC73.6), from H3-15' (δH2.11, 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 H_3-14' to C-3' and from H_3-15' 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 it's solubility was 0.063g/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 [23].

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±1.02mm 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 ± 2.15mm and 15.6, 7.8 μg/mL, respectively. But while antibacterial activity was weak.

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* [20].

According to the Chinese Pharmacopeia, the main anti-microorganism activity of the puffball is against *S. aureus* and *P.aeruginosa*. 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[24].

In this study, the purification extraction Griseococcin(s) from fermentation broth of *B. radicata* obtained through macroporous resin D-101, celluous DE-52 and sephadex LH-20 column, purified fractions were used for biological activity analysis. I*n 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±1.02mm, 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 ±2.15mm and 15.6, 7.8 μg/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 661Da. 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 $C_{37}H_{43}NO_{10}$ and the molecular structure of Griseococcin(1) was shown in figure1.

Previously, many authors reported the various biological activity of fermentation broth from puffball like anticancer activity[25-26] antioxidant activity [27] antifatigue effect[28], 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.

Conclusions

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 C_{37} $H_{43}NO_{10}$.

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 for72 h. The pH and moisture content of PDB was also determined according Maguireboyle(2014) and Mcauliffe(2016) [29-30]. 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. 50ml clear supernatant was applied to 100ml DEAE-cellulose and sephadex LH-20 column. Different purified fractions(named Griseococcin(s)) were obtained from SPAF, only Griseococcin(1)(250μg/ml) has antifungal activity and it’s 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)[22] 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[31], 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 deuteroxide (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℃. The chemical shifts were given in δ (ppm) and referenced to the solvent signal (D_2O-d_6, 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× 10.0mm, 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, 624cm^{-1} ; UV (D_2O) λ max 215 nm; ^1H and ^{13}CNMR data see Table 1; HR-ESI-MS m/z 661.1970 [M +H] + (calcd for $C_{37}H_{43}NO_{10}$, 661.1968).

Declarations

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Authors' information

Yong ye conceived and designed the experiments. Yong ye performed the experiments and analyzed the data. Qinghua zeng analyzed the data. Yong ye wrote and edited the manuscript. The excellent technical support by Chuanxun Yuan and Qingmei Zeng

Ethics declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

No

Abbreviations

NMR Nuclear Magnetic Resonance

HPLC High Performance Liquid Chromatography

FT-IR Fourier transform infrared spectroscopy

DMSO Dimethyl Sulfoxide

ZOI zone of inhibition

MIC minimum inhibitory concentration

HR-ESI-MS high-resolution electrospray ionization mass spectroscopy

DAD diode array detector

MW molecular weight

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Tables

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Figures

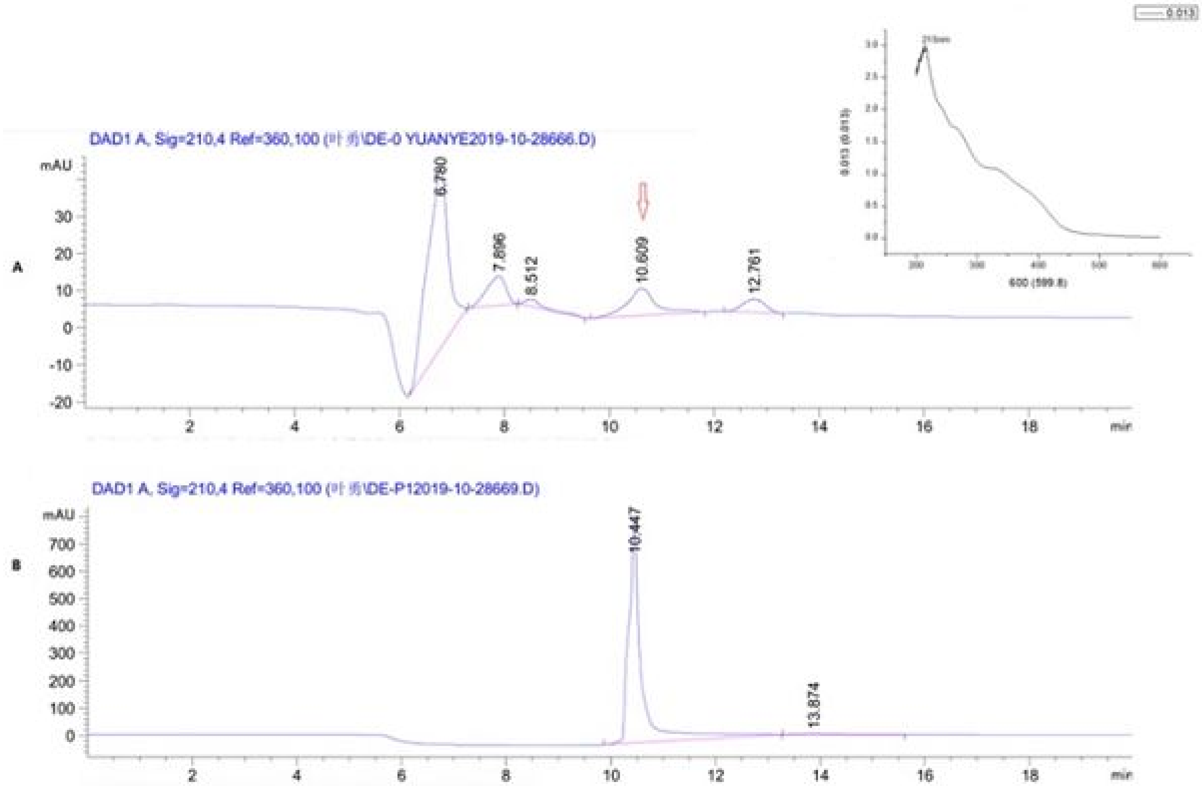

Figure 1

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

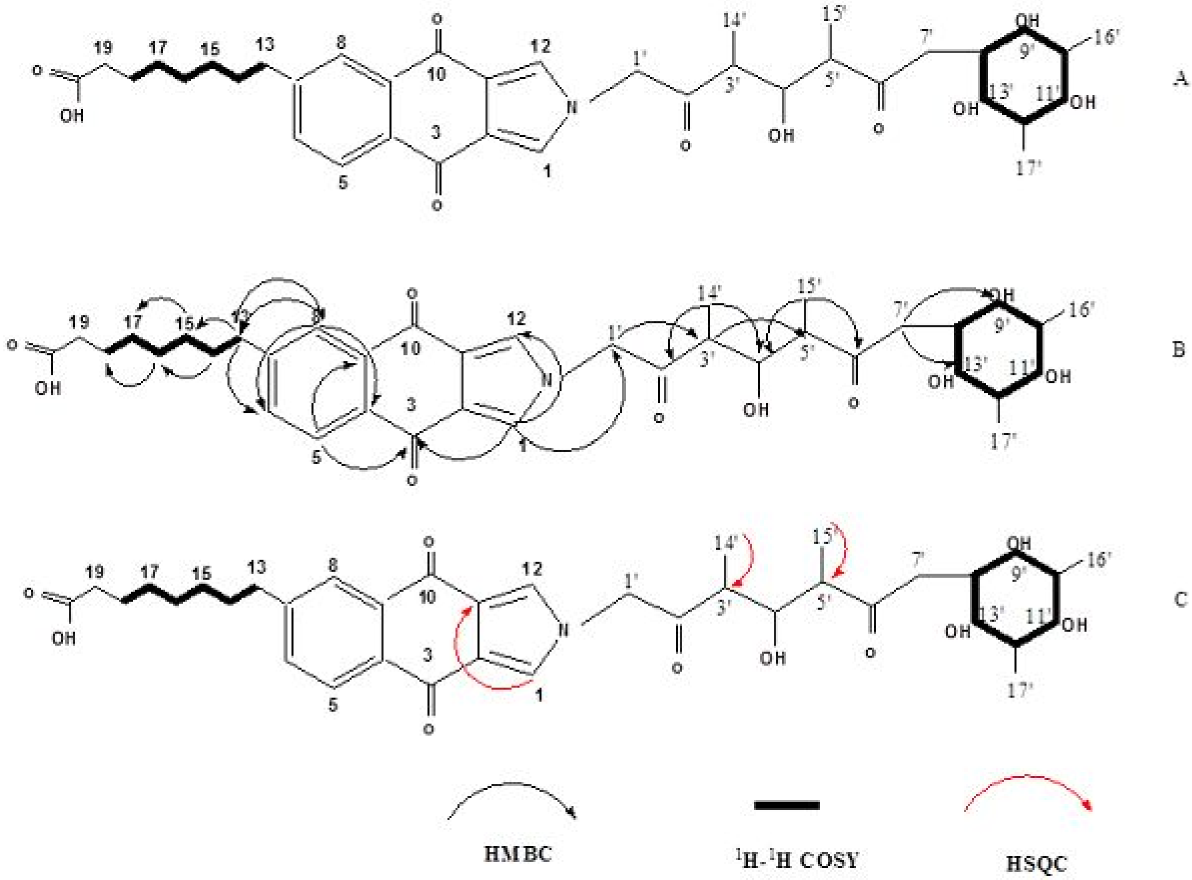

Figure 2

The key 1H-1H COSY, HMBC and HSQC correlations of Griseococcin (1)

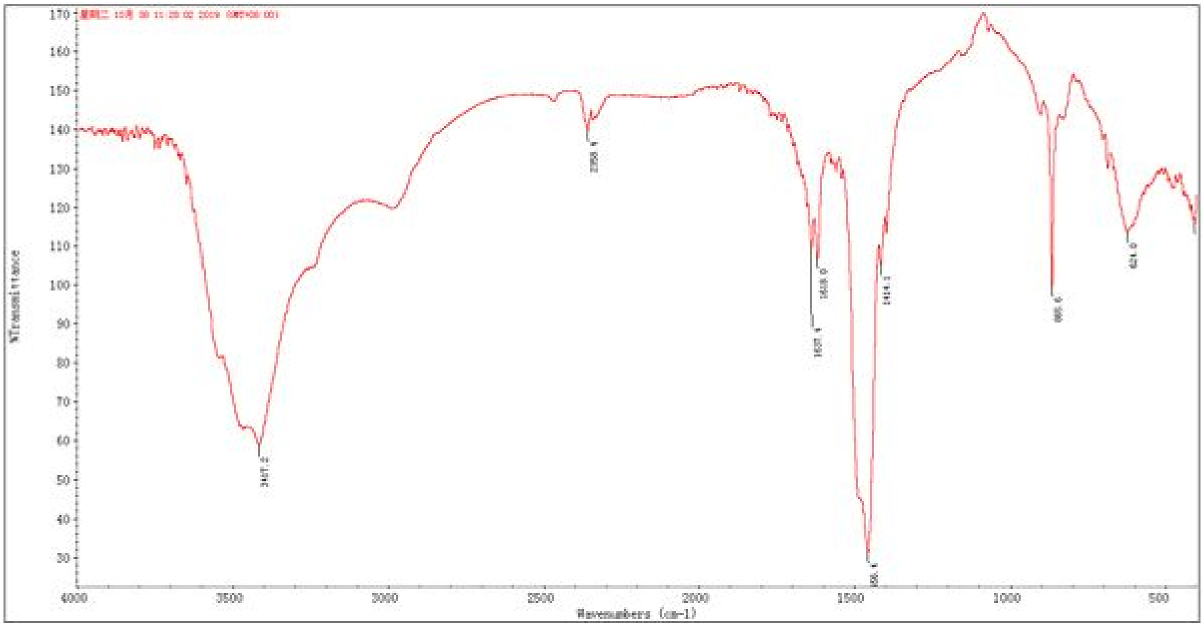

Figure 3

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

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