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Article

Isolation and Purification of an Antibiotic Polyketide JBIR-99 from the Marine Fungus *Meyerozyma guilliermondii* by High-Speed Counter-Current Chromatography

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Abstract: JBIR-99 is a secondary metabolite of marine fungi that has been shown to possess strong antibiotic activity. An efficient approach using a combination of size exclusion chromatography with a Sephadex LH-20 and high-speed counter-current chromatography (HSCCC) has been successfully developed for the isolation and purification of a polyketide from the solid-state fermentation of *Meyerozyma guilliermondii*. The active compound was isolated with purity >95% by HSCCC using an optimized solvent system composed of petroleum ether–ethyl acetate– 95% ethanol–water (5:3:5:3, v/v/v/v) after size exclusion chromatography. This compound was successfully purified in the quantity of 68 mg from 120 mg of the crude extract. The structure of JBIR-99 was elucidated and assigned by 1D, 2D NMR spectroscopic, and positive HRESITOFMS. Moreover, the relative configuration of compound JBIR-99, displaying a quite complex multi-ring structure, is determined by X-ray crystallography for the first time. The purification method developed for JBIR-99 will facilitate the further investigation and development of this antibiotic agent as a lead compound. Furthermore, it is suggested that the combination of size exclusion chromatography and HSCCC could be more widely applied for the isolation and purification of polyketides from marine fungi.

Keywords: JBIR-99; high-speed counter-current chromatography; polyketide; NMR spectroscopy; mass spectroscopy; X-ray crystallography; *Meyerozyma guilliermondii*

1. Introduction

Polyketides are a class of secondary metabolites produced by certain living organisms. Many polyketides, such as geldanamycin, doxycycline, erythromycin A, clarithromycin, and azithromycin *et al* have antimicrobial properties and tacrolimus has immunosuppressive property, and many

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mycotoxins produced by fungi are polyketides too. Structurally, polyketides are complex organic compounds that are often highly active biologically [1]. *Meyerozyma guilliermondii* (formerly known as *Pichia guilliermondii* until its rename in 2010) is a species of yeast of the genus Meyerozyma whose asexual or anamorphic form is known as *Candida guilliermondii. C. guilliermondii* has been isolated from numerous human infections [2]. JBIR-99 (**1**; Figure 1) is a recently reported xanthoquinodin-like compound isolated from marine sponge-derived fungus *Tritirachium sp.* SpB081112MEf2 [3], then Wu et al [4] reported the isolation of this compound from the marine fungus *Engyodontium album* strain LF069. The compound exhibited inhibitory activity against methicillin resistant *Staphylococcus aureus* (MRSA) that was 10-fold stronger than chloramphenicol. It is a promising compound for further investigation.

However, the reported isolation and purification of JBIR-99 has included multiple column chromatography steps and preparative HPLC, which is time-consuming, leads to the loss of target compound due to degradation during the long process, and is not industrially viable because of the cost of solid supports (silica gel or resin) for separation. Accordingly; these techniques are not typically suitable for purification of large quantities of material unless no other methods can complete the task [5-6]. Alternatively, high-speed counter-current chromatography (HSCCC) is a liquid-liquid separation chromatography that can reduce the separation time and cost, and provide effectively total sample recovery due to the lack of a solid support matrix that can degrade or permanently retain target molecules [7]. HSCCC has recently been applied to the isolation of various natural products, most typically coming from marine natural products [8-9]. The purpose of this study was to develop an efficient method for the preparative isolation and purification of JBIR-99 using the combined methods Sephadex LH-20 and HSCCC, which are both nondestructive and nonabsorptive techniques.

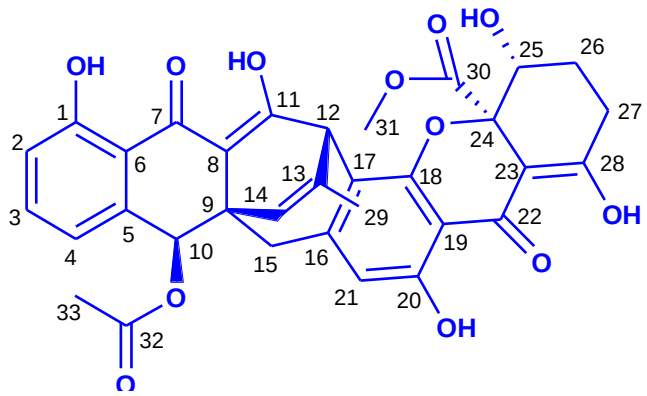

Figure 1. Chemical structure of JBIR-99 (**1**).

2. Results

2.1. Enrichment of JBIR-99 by Sephadex LH-20 Size Exclusion Chromatography

In order to remove the large amounts of pigments and other unknown compounds contained in the crude acetonitrile extract of *M. guilliermondii* was first subjected to size exclusion chromatography on a Sephadex LH-20 gravity column. The LH-20 column was eluted with ethanol (95%). A total of 100 fractions (25 mL each) were successively collected, Fractions 67-71 were combined and determined to contain **1** in high quantity, and this sample was concentrated to dryness and stored in a refrigerator (4 °C) for later HSCCC separation.

2.2. Optimization of HPLC Analysis for JBIR-99

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A high-performance liquid chromatography (HPLC) method was developed to ensure the baseline separation of the target compound and impurities, and evaluate the size exclusion chromatography fractions. Different flow rates, elution modes, detection wavelengths were screened. The result indicated that the target compound was baseline separated with acetonitrile-water (50 %, containing 0.1% formic acid) as the solvent system, when the flow rate, column temperature and detection wavelength were set at 1.0 mL/min, 25 °C and 220 nm. Preliminary assignment of **1** in the chromatogram was made by comparison of peak retention time with reported reference [4]. The HPLC chromatogram of fractions showed the major, but not only peak, as being **1** (Figure 2A).

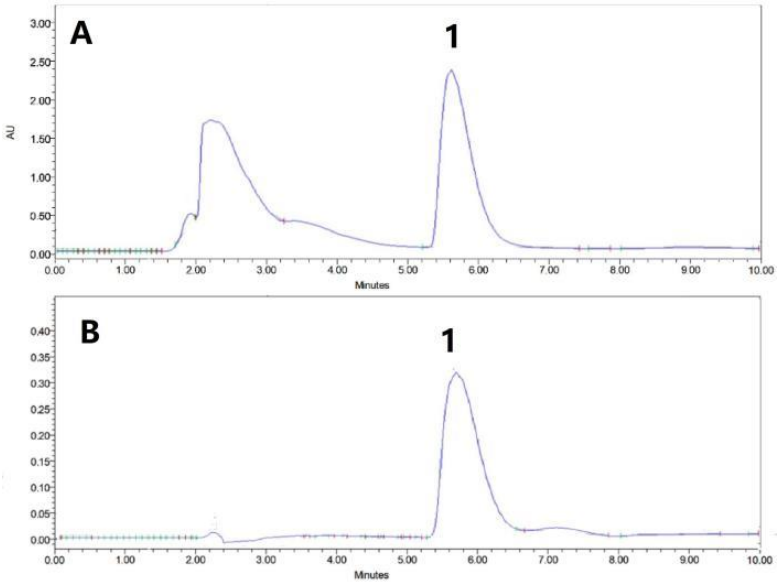

Figure 2. Representative high-performance liquid chromatography (HPLC) chromatograms (220 nm) of samples prepared from *M. guilliermondii* (A). Fractions 67-71 from the size exclusion chromatography of the crude acetonitrile layer; (B) Subfraction that contains **1** after preparative separation by high-speed counter-current chromatography (HSCCC).

2.3. Selection of the HSCCC Two-Phase Solvent System

Satisfactory HSCCC purification relies on two immiscible liquids to serve as stationary and mobile phases, the selection of a suitable biphasic solvent system plays a vital role in successful separations. It has been suggested that the partition coefficient (K) is the most important parameter in solvent system selection, which should be $0.5 \leq K \leq 2$ (close to 1, best) to get a good separation for HSCCC in a suitable run time [10]. As previously reported in the literature [11], the two-phase solvent system "HEMWat", comprising n-hexane–ethyl acetate–methanol–water, has been widely applied in the separation of natural products by HSCCC. In our study, to green the process of isolation, petroleum ether and 95% ethanol were used to replace n-hexane and methanol respectively.

Six sets of different proportional two-phase petroleum ether/ethyl acetate/95% ethanol/water (PEEW) solvent systems were carried out to determine the partition value, K, of the target compound at various volume ratios of petroleum ether/ethyl acetate/95% ethanol/water (4:4:4:4, 3:5:4:4, 2:6:2:6, 1:7:1:7, 5:3:5:3, 6:2:6:2, all v/v/v/v) by HPLC analysis of each partition. The results,

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shown in Table 1, indicated that the two-phase solvent system of 5:3:5:3 PEEW, v/v/v/v, provided a suitable partition value for JBIR-99 of K = 0.58 with good resolution and short elution time.

Table 1. *K* values of target compound **1** in different ratios of the PEEW at solvent system.

Solvent System	Ratios (v/v/v/v)	*K*
petroleum ether/ethyl acetate/95% ethanol/water	4:4:4:4	2.07
petroleum ether/ethyl acetate/95% ethanol/water	3:5:4:4	23.99
petroleum ether/ethyl acetate/95% ethanol/water	2:6:2:6	56.69
petroleum ether/ethyl acetate/95% ethanol/water	1:7:1:7	26.63
petroleum ether/ethyl acetate/95% ethanol/water	5:3:5:3	0.58
petroleum ether/ethyl acetate/95% ethanol/water	6:2:6:2	0.25

2.4. HSCCC Separation

The selected fractions 67-71 from the size exclusion chromatography of the extract from *C. guilliermondii* (120 mg) was applied for HSCCC separation with the chosen two-phase solvent system, petroleum ether/ethyl acetate/95% ethanol/water (5:3:5:3). In order to optimize the resolution and reduce the separation time, different flow rates and rotation speeds were evaluated. It was found that when the flow rate was 8 mL/min and rotation speed was 1100 rpm, a good separation was achieved for elution of **1** with a good stationary phase retention of 70 %. The HSCCC peak fraction corresponding to **1** (68 mg) was collected and determined to have purity of 95.12% by HPLC analysis (Figure 2B). The resulting HSCC chromatogram is shown in Figure 3, demonstrating the good resolution and peak shape of compound **1** at t_R = 31 min (peak 4).

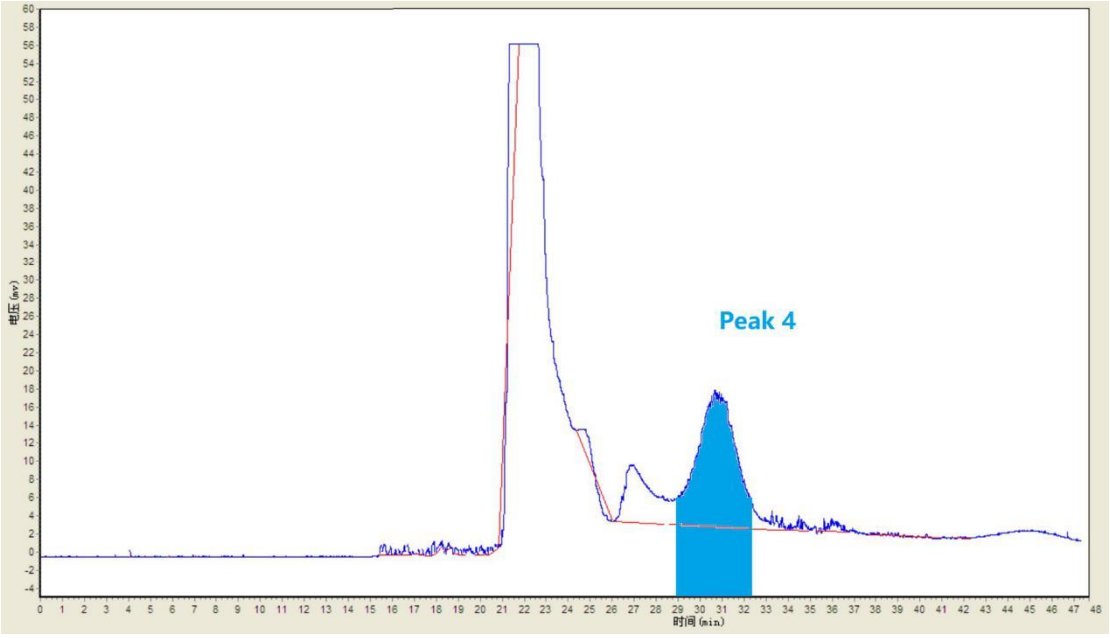

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Figure 3. HSCCC chromatogram of the fraction 67-71 of the ethanol extract from *C. guilliermondii* using the two-phase solvent system composed of petroleum ether/ethyl acetate/95% ethanol/water (5:3:5:3, v/v/v/v); stationary phase: upper phase of solvent system; mobile phase: lower aqueous phase of solvent system; column capacity 500 mL; rotation speed 1100 rpm; column temperature 25 °C; flow rate 8.0 mL/min; detection, 220 nm; sample injected, 120 mg in 20 mL biphasic solution; retention of the stationary phase,70%; peak4 identification: JBIR-99 (**1**).

2.5. Identification of Chemical Structure

Compound **1** was identified by HR-ESI-MS, ^{1}H-NMR, ^{13}C-NMR, DEPT, ^{1}H-^{1}H COSY, HMQC and HMBC after purification by HSCCC, and its detailed data are shown in Table 2. The HMBC correlations are shown in Figure 2. Its molecular formula $C_{33}H_{28}O_{12}$ was deduced by HR-ESI-TOF-MS data at m/z 639.1472 $[M+Na]^{+}$. Compound **1** was identified as a xanthoquinodin-like compound, JBIR-99, with the chemical structure as shown in Figure 1. The structure of JBIR-99 was first disclosed by Ueda J. [3], but the single-crystal X-ray diffraction data were never reported before.

Table 2. ^{1}H (400 MHz) , ^{13}C-NMR (100 MHz) and HMBC spectroscopic data of JBIR-99 (**1**) in CD_3COCD_3.

Pos.	δ_H	δc	HMBC	**Pos.**	δ_H	δc	HMBC
1		162.6		18		156.8	
2	7.00, d(8.4)	119.7	C1,C3	19		105.6	
3	7.59,t(8.4)	137.1	C1,C5	20		160.9	
4	7.13, d(8.4)	122.9	C2,C6,C10	21	6.17, s	114.4	C15,C17, C19,C20
5		137.0		22		188.2	
6		116.3		23		100.8	
7		187.5		24		86.5	
8		107.5		25	4.47, m	72.1	
9		42.1		26	2.27, 2.09, m	25.7	C24,C25, C27,C28
10	6.05, s	73.8	C4,C5,C6,C8, C9,C15,C32	27	2.65, m	28.5	C23,C25, C26,C28
11		187.8		28		180.4	
12	4.95, s	43.4	C8,C11,C13, C14,C16,C17, C18,C29	29	1.94, s	20.6	C12,C13,C14
13		142.9		30		170.4	
14	5.78, s	126.2	C8,C9,C10, C12,C29	31	3.66, s	53.1	C30

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15	2.92, d(17.8), 2.72, d(17.8)	36.2	C8,C91,C10, C14,C16,C17, C21	32		171.5	
16		149.5		33	2.07,s	20.9	C32
17		115.9		1-OH	11.65,s		C1,C2

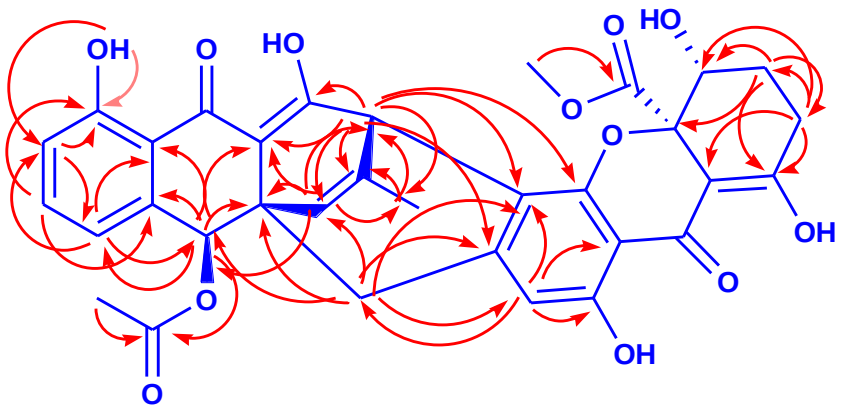

Figure 4. HMBC correlations of JBIR-99 (**1**)

2.6 X-ray crystallography

X-ray crystal data for **1**. Colorless crystals of **1** were obtained in methanol. Crystal data (CCDC 1945666) were collected on a Bruker Smart APEX II diffractometer equipped with graphite-monochromitized Mo-Kα radiation (Wavelength = 0.71073). Orthorhombic, space group $P2_12_12_1$ (no. 19), a = 7.9628(7) Å, b = 15.1359 (16) Å, c = 25.686(2) Å, α = 90, β = 90, γ = 90, V = 3095.8 (5)) Å^3, Z = 4, T = 296 K, μ (Mo-Kα) = 0.108 mm^{-1}, Dcalc = 1.389 g/cm^3, F(000) = 1356, R1 = 0.0510, wR2 = 0.1466, GOF = 1.007. Crystal dimensions 0.12 × 0.10 × 0.10 mm^3. The total number of independent reflections measured was 5443, of which 3907 were observed. The structure was solved by direct method and refined by full-matrix least-squares on F2 using SHELX-2017 [12]. All non-hydrogen atomic positions were located in Fourier maps and refined anisotropically, while all of the hydrogen atoms were refined with isotropic displacement parameters (Figure 5). Moreover, these results are consistent with the relative configuration of **1** that was proposed in [3,4] on the basis of NMR data and X-ray crystal data.

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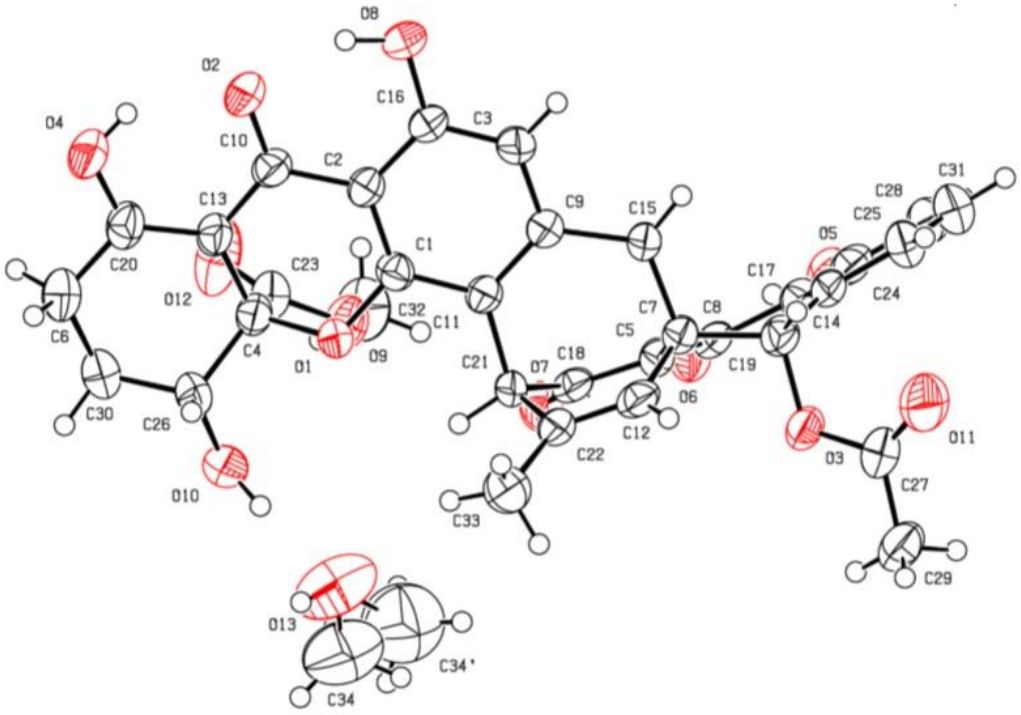

Figure 5. X-ray structure of JBIR-99 (**1**)

3. Materials and Methods

3.1 Reagents and Materials

All solvents used for HSCCC were of analytical grade (Fuyu Chemical Co., Ltd., Tianjin, China). 95% ethanol was bought from Hebei Ruikang Medical Science and Technology Co., Ltd. (Hengshui, China) . Reverse osmosis Milli-Q water (18 M) (Millipore, Bedford, MA, USA) was used for all solutions and dilutions. Acetonitrile used for HPLC analyses was of chromatographic grade and purchased from Shanghai Aladdin Bio-Chem Technology Co.,Ltd (Shanghai, China). The CD3COCD3 used for NMR analyses was purchased from Tenglong Weibo Technology (Qingdao, China). The fungus Y39-1 was was kindly provided by First Institute of Oceanography State Oceanic Administration (SOA) Qingdao, China, which was isolated from sea water samples collected from the Indian Ocean at a depth of about 30 m (88°59′51″ E，2°59′54″ S).

3.2. Apparatus

HSCCC was carried out using an OptiChrome-500 PLUS high-speed counter-current chromatograph (Counter Current Technology Co., Ltd., Jiangyin, China) equipped with three multilayer coil separation columns. The three multilayer coil columns were connected in series and had a combined volume of 500 mL. The inner diameter (i. d.) of the tubing was 2.1 mm, and the sample loop contained 30 mL. The revolution radius was 5 cm, and the β values of the multilayer coils varied from 0.6 at the internal terminal to 0.8 at the external terminal. The rotational speed of the apparatus was regulated with a speed controller in the range of 0 to 1200 rpm. The apparatus was also equipped with one P-3000 metering pumps (Beijing Tong Heng Innovation Technology Co., Ltd, Beijing, China), an HD-3 UV detector (Heqin Analytical Instrument Co., Ltd Shanghai,

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China.), and an N2000 data analysis system (Institute of Automation Engineering, Zhejiang University, Hangzhou, China) was employed for HSCCC data collection and analysis. The HPLC equipment was using a Waters Xbridge™ C18 column (250 mm × 4.6 mm, 5 μm particle size) equipped with a UV 3000 spectrometer (Beijing Tong Heng Innovation Technology Co., Ltd, Beijing, China), a CXTH-3000 workstation (Beijing Tong Heng Innovation Technology Co., Ltd, Beijing, China). NMR experiments including ^{1}H, ^{13}C, DEPT, ^{1}H-^{1}H COSY, HMQC, and HMBC were carried out using a 400 MHz Bruker Avance nuclear magnetic resonance spectrometer (Bruker Biospin, Rheinstetten, Germany) spectrometer. HR-ESI-MS data was measured using a Waters HRESITOF Premier LC/MS spectrometer (Waters Co., Milford, MA, USA). Column chromatography (CC) was carried out with Sephadex LH-20 (Pharmacia, Uppsala, Sweden).

3.3 Fungal Materials

The fungus Y39-1 was stored at 4 °C on PDA slants as provided by one of the co-authors (Dr. X.L.). Fungal identification was carried out by Shandong Lige Technology Co., Ltd. (Jinan, China) using a molecular biological protocol by DNA amplification and sequencing of the ITS region The sequence data of the fungal strain was most similar (100%) to the sequence of *Meyerozyma guilliermondii*. A voucher strain was deposited at the Biology Institute, Qilu University of Technology (Shandong Academy of Sciences) with the access code Y39-1.

3.4. Preparation of Crude Sample from Y39-1 for HSCCC

The fungus Y39-1 on PDA slants was cut into small pieces and grown under static conditions at 25 °C for 35 days in a solid autoclaved rice substrate medium containing 80 g of rice and 120 mL of water in 500 mL flasks (30 flasks in all). After incubation, the mycelia and solid rice were extracted with EtOAc, and the extracts were concentrated to yield 12.8 g of residue under reduced pressure. The residue was partitioned between 200 mL of petroleum ether and 200 mL of acetonitrile, the lower layer was concentrated to yield 2.2 g of residue, which was subjected to a glass column packed with Sephadex LH-20 and eluted with 95% ethanol to obtain 100 fractions. Fractions 67-71 were combined based on TLC monitoring and used for later HSCCC separation.

3.5. Preparation of Two-Phase Solvent System and Sample Solution

The HSCCC experiments were performed using a two-phase solvent system comprising petroleum ether/ethyl acetate/95% ethanol/water (5:3:5:3, v/v/v/v) solvent. The two phases were separated after thoroughly equilibrating the mixture in a separating funnel at room temperature. The upper organic phase was used as the stationary phase, and the lower aqueous phase was employed as the mobile phase.

3.6. HSCCC Separation

The organic stationary phase (upper phase) was pumped into the HSCCC column (flow 30 mL/min), after it was full, the mobile phase was pumped into the column (8 mL/min) while in the normal rotating mode (1100 rpm). When the mobile phase emerged from the column, it indicated that hydrodynamic equilibrium had been achieved. The sample (fractions 67-71, 120 mg) obtained from the Sephadex LH-20 column chromatography was dissolved in 10 mL of a 1:1 (v/v) mixture of the two HSCCC solvent system phases and injected to the sample port. The effluent from the HSCCC was monitored by UV at 220 nm, and peaks were collected.

3.7. Analysis and Identification of the Target Compound

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The fraction generated by preparative HSCCC was evaluated by HPLC (50% acetonitrile, flow 1 mL/min, monitored at 220 nm). Peak 4 (t_R = 31 min) showed only one peak in the HPLC chromatogram, which was concentrated under reduced pressure to yield compound **1** (68 mg). The purity of compound **1** was 95.21%.

4. Conclusions

To draw a conclusion, a rapid method relying on HSCCC after size exclusion chromatography on Sephadex LH-20 was utilized to separate JBIR-99 from the extract of the fungus *C. guilliermondii* in a lossless procedure. It was important to pre-treat the crude extract by partition between two phase solvents and pass through a Sephadex LH-20 column for HSCCC separation to improve the resolution and efficiency. The solvent system of petroleum ether/ethyl acetate/95% ethanol/water (5:3:5:3, v/v/v/v) was optimized to isolate JBIR-99 (**1**). The separation condition was selected as follow: flow rate 8.0 mL/min, rotary speed 1100 rpm, column temperature 25 °C. Under the optimized HSCCC condition, 68 mg of JBIR-99 with the high purity of 95.21% was isolated from 120 mg of fractions 67-71 of *C. guilliermondii*. This is the first report of the isolation of JBIR-99 by integrating size exclusion chromatography and HSCCC , and this method could be used for the effective isolation of different xanthoquinodin-like polyketides from different natural materials. This convenient and economical approach will be applicable for scale-up production of JBIR-99 to increase the yield. The purification procedure optimized for JBIR-99 will also facilitate the further development of this antibiotic agent as a lead compound and further structure-activity relationship studies.

Supplementary Materials: The ^{1}H NMR, ^{13}C NMR, DEPT, COSY, HMQC, HMBC, HRESIMS spectra are available online

Author Contributions: Conceptualization, Hankui Wu; Formal analysis, Yongchun Zhang; Funding acquisition, Xiaobin Li; Investigation, Jianmin Liu, Ninghui Duan, Ru Han, Xinxin Zhang and Xiaoyu Leng; Methodology, Hankui Wu, Liwen Han and Yongchun Zhang; Project administration, Liwen Han, Shu Xing and Mingyang Zhou; Resources, Xiaobin Li; Supervision, Xiaobin Li and Mingyang Zhou; Validation, Wenjie Liu; Writing – original draft, Hankui Wu; Writing – review & editing, Shu Xing.

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Conflicts of Interest: The authors declare no conflict of interest.

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