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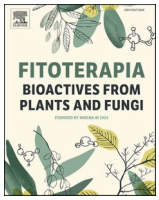

Research Paper

Two new abietane diterpenoids from *Caryopteris trichosphaera*

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ABSTRACT

Two new abietane diterpenoids, cartrisine A (**1**) and cartrisine B (**2**) were isolated from *Caryopteris trichosphaera*. Their structures were elucidated based on HR-ESI-MS and NMR spectral data. Compound **2** exhibited moderate antibacterial bioactivity against MRSA and VRE, and showed a strong synergistic effect with market antibiotics against MRSA and VRE.

1. Introduction

Caryopteris trichosphaera belongs to the family of Lamiaceae containing a wide variety of chemical constituents, including diterpenes, alkaloids, phenylethanoid glycosides and so on, in which abietane diterpenoids were abundant in plants of the genus *Caryopteris* [1–4]. The bioactivities investigation indicated it possesses anticancer, antifibrotic, and neuro-protective effects [3,5,6]. In our continuous search for new antibacterial compounds from plants [7–15], two new abietans (**1**,**2**) were isolated from *C. trichosphaera*, and their antibacterial bioactivities against MRSA and VRE were also evaluated.

2. Experimental

2.1. General experimental procedures

Optical rotation was measured using an Autopol IV polarimeter (Rudolph Research Analytical, Hackettstown, NJ, USA). Ultraviolet (UV) and circular dichroism (CD) spectra were obtained using an Applied Photophysics serial Chirascan V100 CD spectrometer (Applied Photophysics Inc., Charlotte, NC, USA). Infrared (IR) spectra were measured using a NICOLET iS10 infrared spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). High-resolution electrospray mass spectrometry (HRESI-MS) was performed on an Agilent 1290 ultra-high performance liquid chromatography (UHPLC) system (Agilent, Santa Clara, CA, USA) coupled with an Agilent 6545 EST-Q-TOF mass spectrometer. 1D and 2D nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AVANCE NEO-400 MHz spectrometer (Bruker, Billerica, MA, USA), using tetramethylsilane as an internal standard. Coupling constants were expressed in Hz, and chemical shifts (δ) were described in ppm regarding solvent signals. The extracts were concentrated using vacuum rotary evaporators (EYELA OSB-2200, Shanghai, People's Republic of China) and chromatographed on silica gel (200–300 mesh, Qingdao PUKE Co., Ltd., Qingdao, People's Republic of China), Sephadex LH-20 (Cytiva, Uppsala, Sweden), and C-18 silica gel (20–45 μm, Fuji Silysia Co., Kasugai, Japan). The fractions were monitored using thin-layer chromatography (TLC; GF254, Qingdao PUKE Co., Ltd.), and spots were visualized using iodine vapor and a sulfuric acid-ethanol reagent. Semi-preparative HPLC was performed using an Agilent 1260 liquid chromatograph equipped with an Agilent Zorbax SB-C-18 column (150 mm × 9.4 mm, i.d., 5 μm).

2.2. Plant material

Whole plants of *C. trichosphaera* were collected in August 2020 from Changdu City (Tibet, China) and identified by Zhang Jun of the Kunming Zhifen Biotechnology Company. The herb was checked at http://www.theplantlist.org/. A voucher specimen (no. XDL-323) was deposited at the Key Laboratory of Medicinal Chemistry for Natural Resources, Yunnan University, as described previously [16].

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Table 1
^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectroscopic data of compounds **1**–**2**.

No.	1[a]		2[b]	
	δ_H (J,Hz)	δ_C	δ_H (J,Hz)	δ_C
1	Ha: 3.37, m Hb: 1.17, overlap	37.7, t	Ha: 1.38, m Hb: 3.09, m	37.3, t
2	Ha: 1.75, m Hb: 1.48, m	20.4, t	Ha: 1.52, m Hb: 1.70, m	19.0, t
3	Ha: 1.47, m Hb: 1.25, m	42.9, t	Ha: 1.25, m Hb: 1.43, m	43.0, t
4		34.8, s		33.6, s
5	1.26, d	54.9, d	1.71, d, 11.2	53.2, d
6	Ha: 1.81, m Hb: 1.52, m	20.4, t	5.65, dd, 11.2, 7.6	70.4, d
7	2.74, m	34.0, t	4.54, d, 7.6	83.2, d
8		135.1, s		126.7, s
9		134.7, s		133.6, s
10		40.6, s		42.1, s
11		149.4, s		141.9, s
12		143.6, s		140.2, s
13		140.5, s		132.2, s
14	6.42, s	117.8, d	6.78, s	116.8, d
15	3.68, m	26.6, d	3.03, m	27.5, d
16	1.15, d, 6.8	24.2, q	1.26, d, 6.4	22.8, q
17	1.15, d, 6.8	24.2, q	1.24, d, 6.4	22.7, q
18	0.98, s	34.3, q	1.07, s	35.7, q
19	0.96, s	22.6, q	1.01, s	22.8, q
20	1.33, s	20.0, q	2.14, s	22.0, q
21				170.7, s
22			1.49, s	21.6, q
23			3.08, s	53.2, q
1’	4.43, d, 8.0	107.9, d		
2’	3.50, m	75.4, d		
3’	3.43, m	78.1, d		
4’	3.48, m	71.1, d		
5’	3.24, m	78.3, d		
6’	3.78, m	62.3, t		

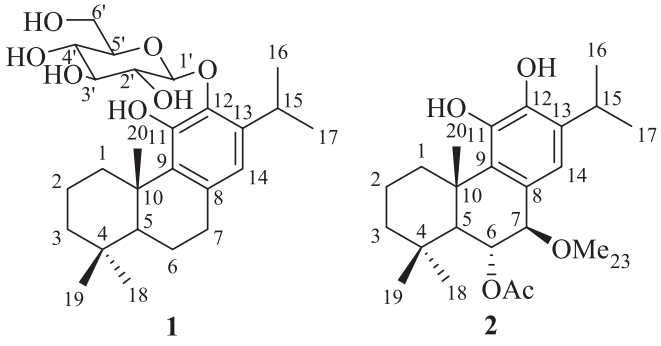

Fig. 1. Compounds **1**, **2**.

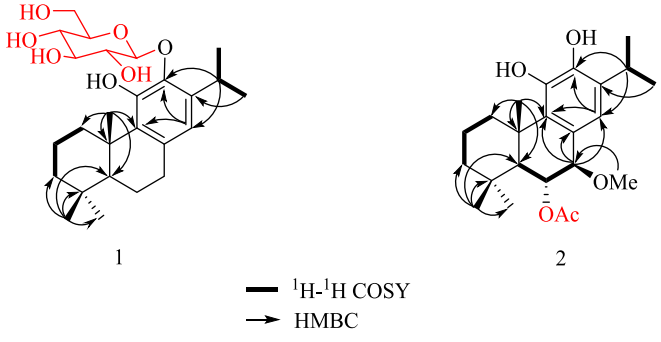

Fig. 2. Key HMBC and ^{1}H – ^{1}H COSY correlations of **1**–**2**.

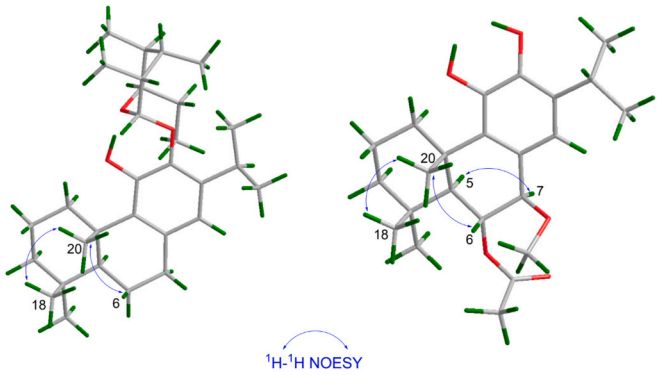

Fig. 3. Key NOESY correlations of **1**–**2**.

2.3. Extraction and isolation

The air-dried and powdered *C. trichosphaera* (700 g) was refluxed and extracted with 85 % ethanol (6 L, 3 × 12 h). The combined extracts underwent evaporation using a rotary vacuum evaporator. The crude extract (Fr. 0; 138 g) was subjected to chromatography on a macroporous resin column and eluted with EtOH-H_2O (0:1, 1:4, 2:3, 3:2, 4:1, and 1:0) to obtain seven fractions (Fr. 1 to Fr. 7). Fr. 5 (5.0 g) was chromatographed on silica gel using a gradient elution of petroleum ether: ethyl acetate (1:0 to 10:1) to obtain six subfractions (Fr. 5. A1 - A6). Fr. 5. A1 was purified using Sephadex LH-20 ($CHCl_3$-CH_3OH) to obtain compound **2** (4.0 mg). Fr. 5.A6 was subjected to silica gel chromatography using gradient elution with $CHCl_3$-CH_3OH (1:0 to 10:1) to afford compound **1** (4.0 mg).

Cartrisine A (1): $C_{26}H_{40}O_7$, white powder; [α]25 D + 4.5 (*c* 0.14, CH_3OH); UV (CH_3OH) λ_{max} (log ε): 204 (5.92), 285 (3.34) nm; IR V_{max} 3321, 2923, 2851, 1456, 1386, 1150, 1008, 858, 590 cm^{-1}; ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectral data, Table 1; HRESIMS *m/z* 463.2689 [M - H]$^-$ (calcd for $C_{26}H_{40}O_7$ 463.2701). (See Fig. 1.)

Cartrisine B (2): $C_{23}H_{34}O_5$; white powder; [α]25 D + 10.4 (*c* 0.20, CH_3OH); UV (CH_3OH) λ_{max} (log ε): 207 (5.94), 285 (3.33), 312 (3.25); IR V_{max} 3212, 2923, 2689, 1723, 1573, 1462, 1376, 1249, 1103 cm^{-1}; ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectral data, Table 1; HRESIMS *m/z* 389.2341 [M - H]$^-$ (calcd for $C_{23}H_{34}O_5$ 389.2333).

2.4. Quantum chemistry calculations

The theoretical calculations of **1** and **2** were performed using Gaussian 09. The possible conformations were initially obtained from the program Spartan’14 and then optimized at b3lyp/TZVP level in the gas phase. Room-temperature equilibrium populations were calculated according to the Boltzmann distribution law. The ECD calculations were performed using Time Dependent Density Functional Theory (TDDFT) at wB97xd/TZVP level in methanol with PCM model. The ECD spectra of **1** and **2** were obtained by weighing the Boltzmann distribution rate of each geometric conformation, and the sigma/gamma value for processing the calculated ECD was 0.3 eV. All calculated curves were shifted +5 nm to better simulate experimental spectra.

2.5. Bacterial strains and cell strains

Clinical MRSA isolates were kindly supplied by Ms. Xiaoqian Li, the chief pharmacist of the Scientific Research Center of Zunyi First People's Hospital. The strains were numbered MRSA3 (170208345) and were isolated from urine. Multi-drug-resistant (MDR) strains of *Enterococcus faecalis* (ATCC 51299) were purchased from Beijing Baiou Bowei Biotechnology Co., Ltd. (Beijing, China). RAW 264.7 murine macrophage cells (Kunming Cell Bank, Kunming, China) were cultured in DMEM (GemCell, USA), as described previously [16].

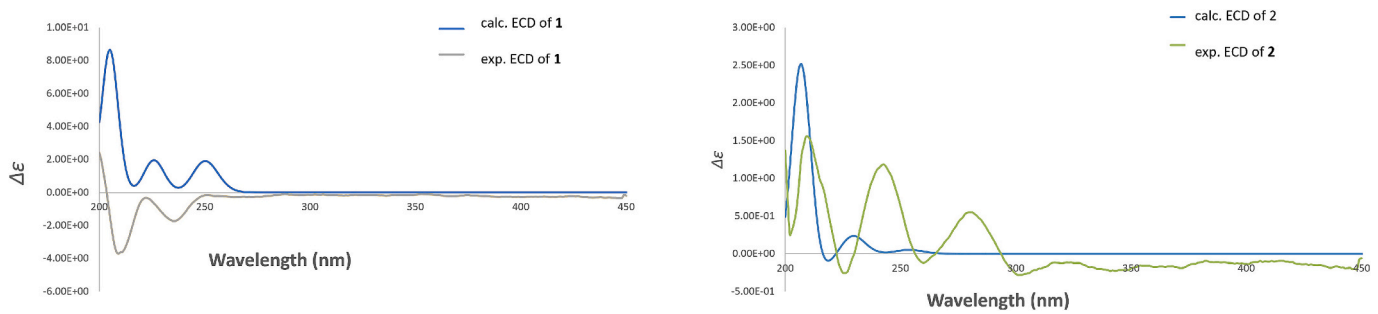

Fig. 4. Circular dichroism spectra of 1–2.

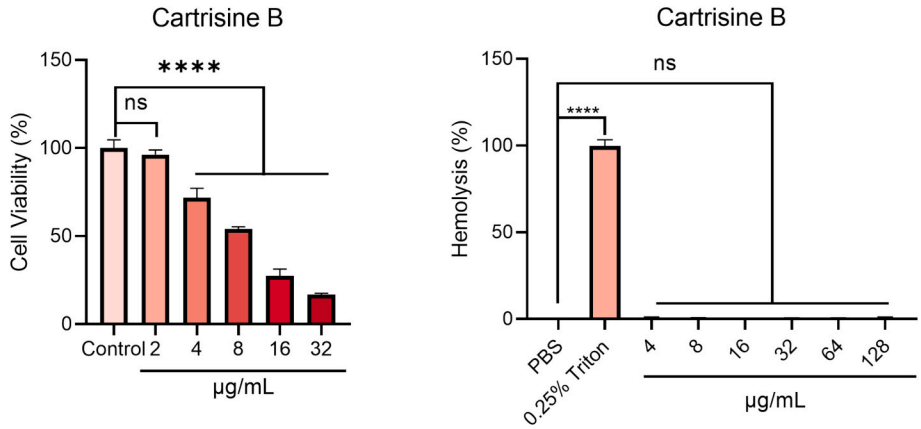

Fig. 5. (A) Cell availability of RAW 264.7 cells after exposure to cartrisine B (**2**) for 24 h. (B) Hemolysis of 4 % sheep red blood cells after exposure to cartrisine B (**2**) for 1 h. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

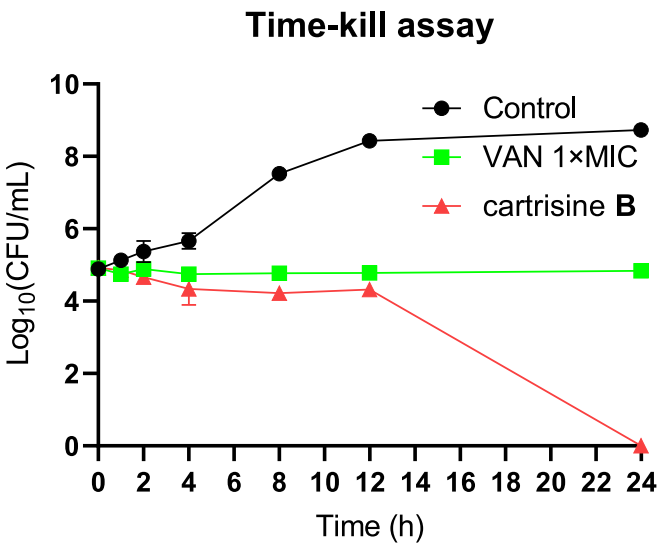

Fig. 6. The time-kill curves of Cartrisine B (**2**).

2.6. Antibacterial test

In accordance with the Clinical and Laboratory Standards Institute (CLSI) 2020 guidelines [15], this study employed the broth dilution method to determine the MIC and MBC of all compounds isolated from *C. trichosphaera* [17]. All isolated compounds were dissolved in DMSO solution. Then, TSB medium was added to the 96-well plate and diluted multiple times by double dilution method to obtain compound solutions with different concentrations. Bacterial solution was added to all wells to give a final concentration of 1×10^5 CFU/mL of bacteria and a final concentration of 1–128 μg/mL of compounds at 37 °C for 12 h, and the minimum concentration corresponding to the wells of clear solution was MIC. MBC was tested after the MIC test, and the MIC solution and above concentrations were transferred to TSA medium; the minimum concentration without colony formation was MBC, as described previously [16].

2.7. Time-kill assay

The time-kill assay was evaluated as in the previous report [16]. MRSA in the logarithmic growth phase was modified to reach a concentration of 1×10^5 CFU/mL. Subsequently, different levels of cartrisine **B** along with vancomycin both at 1 × MIC were applied to the cells for durations ranging from 1 to 48 h. The remaining bacteria were quantified using plating methods.

2.8. Cytotoxicity and hemolysis assay

The viability of RAW 264.7 cells was assessed using the MTT dye reduction assay [18], as described previously [16].

2.9. Checkerboard synergy assay

The checkboard synergy assay was evaluated as in the previous report [16]. Initially, we determined the MIC value for each antibiotic to minimize its usage. Each well contained both the compound and the antibiotic solution. Finally, the absorbance of each well was measured at 600 nm, and the MIC value of each drug was determined when combined [19]. Synergy was assessed using the fractional inhibitory concentration index (FICI) values [20].

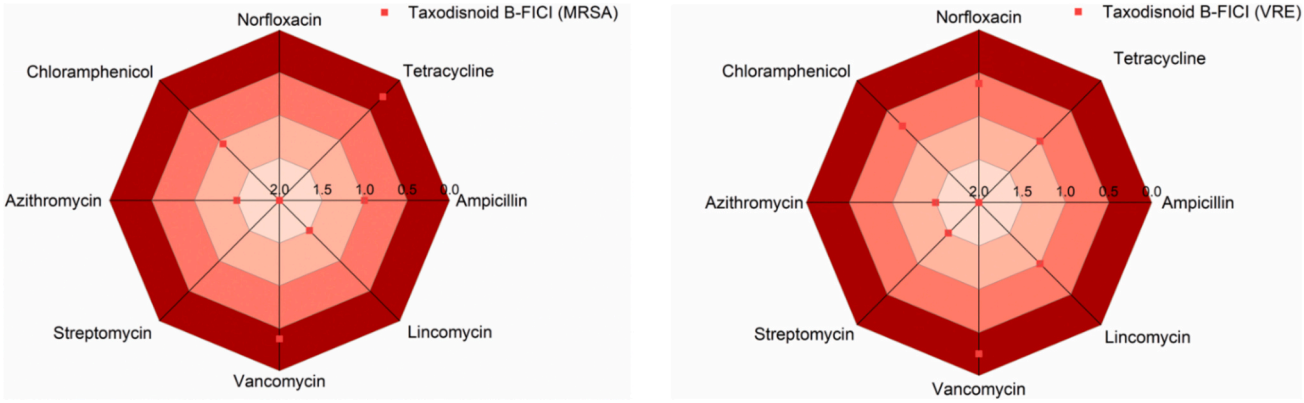

Fig. 7. The spider plot demonstrates the synergistic effect of cartrisine B (**2**) in combination with various antibiotics, where each point on the contour line represents a FICI value accurately drawn to scale.

2.10. Statistical analysis

All results were expressed as the mean ± standard deviation (SD). Statistical analyses were performed using GraphPad Prism 8.0.2 software, and statistical significance was determined using a one-way analysis of variance (ANOVA) and Student's *t*-test. Values of * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ were considered significant.

3. Results and discussion

3.1. Structure elucidation

Compound **1** displayed a quasi-molecular ion $[M - H]^-$ at *m/z* 463.2689 (calculated for m/z 463.2701) in its HRESIMS spectrum, indicating a molecular formula of $C_{26}H_{40}O_7$ with 7 degrees of unsaturation. The IR spectrum showed the presence of an aromatic ring (1608, 1563, 1489, and 1398 cm^{-1}). A total of 26 carbon signals were observed in the ^{13}C NMR spectrum (Table 1, Fig. S1), corresponding to the carbon atoms of an abietane diterpenoid with an additional hexose sugar unit [21]. The abietane-diterpene skeleton was further validated by $^1H - {}^1H$ COSY correlation, from H-1 through H-2 to H-3 and from H-4 through H-5 to H-6, as well as by the HMBC correlations, from H-20 to C-1 and C-5 and from H-18/19 to C-3 and C-5, along with their chemical shift values. The A and B rings, the C-20 methyl group, and the C-5 proton are assumed to be trans-diaxial according to the biogenetically related diterpenoids [22]. Based on the NOESY spectrum, Me-20 and Me-19 were assigned to the β axis, while Me-18 was assigned to the α axis, based on the NOESY data comfirmed from Me-18 to H-3 and from Me-19 to Me-20. The ^1H NMR and ^{13}C NMR spectroscopic data of **1** were very similar to those of 11-hydroxy-8,11,13-abietatriene 12-*O*-β-xylopyranose [21], except for an additional hydroxymethyl signal at the sugar moiety in **1**. The *Rf* value on TLC and optical rotation ([α]25 D + 50) of the hydrolyzed sugar in comparison with an authentic sample of **1** was identical to that of the D-glucose, the assumption was further supported by the correlations of H-6′ (δ_H 3.78) / H-5′ (δ_H 3.24) / H-4′ (δ_H 3.48), H-4′ (δ_H 3.48) / H-3′ (δ_H 3.43), H-3′ / H-2′ (δ_H 3.50) / H-1′ (δ_H 4.43) in its $^1H—^1H$ COSY spectrum (Fig. 2), and the correlations of H-1′ (δ_H 4.43) with C-12 (δ_C 143.6) in the HMBC spectrum (Fig. 2) of **1**, which suggested that the xylopyranose unit of 11-hydroxy-8,11,13-abietatriene 12-*O*-β-xylopyranose was replaced by a D-glucose in **1**. Besides, the calculated ECD approach was utilized to definitively establish the absolute configuration of **1**, the calculated ECD curve of (5S,10S) matched well with the experimental ECD spectrum of **1** (Fig. 4). The coupling constant (J = 8 Hz) at δ_H 4.43 suggested β-configuration for the anomeric proton, so compound **1** was elucidated as 11-hydroxy-8,11,13-abietatriene 12-*O*-β-D-glucose, named cartrisine A.

The molecular formula of compound **2** was determined to be $C_{23}H_{34}O_5$ by its HRESIMS, and it was also supposed to be the same abietane-diterpene skeleton according to the biogenetically related diterpenoids, the same as trans-diaxial of compound **1**. The significant differences between compounds **1** and **2**, were the absence of glucose and the presence of an extra acetyloxy at C-6 and a methoxyl group at C-7 in **2**, which were supported by the correlations of δ_H 3.08 (CH_3–23) with C-6/C-7/C-8, and of δ_H 1.49 (CH_3–22) with C-5/C-6/C-7/C-21 in its HMBC spectrum (Fig. 2). The H-5 and 10-CH_3 were oriented as (5α)- and (10β)- bio-genetically [23–25], and the relative configuration of **2** was established by its NOESY spectrum (Fig. 3), in which NOE correlations of H-6 (δ_H 3.08) with H_3–20 (δ_H 2.14), of H_3–18 (δ_H 1.07) with H_3–20, and of H-5 (δ_H 1.71) with H-7 (δ_H 4.54) positioned 6-OAc at α-orientation and 7-OMe at β-orientation. Additionally, the calculated ECD curve of (5S,10S) matched well with the experimental ECD spectrum of **1** (Fig. 4). Then, compound **2** was elucidated as shown and named cartrisine **B**. All the NMR, IR, UV, CD, and HRESIMS spectra were provided in the Appendix (Fig. S1–22).

3.2. Cytotoxicity and hemolysis assay

Cartrisine B showed cell toxicity to RAW264.7 cells at low concentrations (4 μg/mL), leading to 71 % cell viability (Fig. 4A) and did not induce hemolysis until concentrations reached 128 μg/mL (Fig. 5B).

3.3. Antibacterial assay

Cartrisine B (**2**) inhibited MRSA and VRE with MIC 32 μg/mL and 128 μg/mL, while killing MRSA and VRE with MBC 32 μg/mL and MBC 128 μg/mL, respectively. While Cartrisine A (**1**) exhibited MIC and MBC values exceeding 128 μg/mL against both MRSA and VRE.

3.4. Time-kill assay

Acorrding to the MBC assay, Cartrisine B (**2**) was assumed to be a bactericide. The time-kill assay was used to further investigate the mechanism of Cartrisine B (**2**). The curves showed that Cartrisine B (**2**) reduced the bacterial count below the detectable threshold within 24 h (Fig. 6).

3.5. Cartrisine B (2) exhibited synergistic activity against MRSA and VRE

The broad-spectrum antibacterial potential of cartrisine B (**2**), in combination with multiple antibiotics, against MRSA and VRE was assessed using a checkerboazrd synergy assay. Regarding combating MRSA and cartrisine B (**2**), tetracycline showcased exceptional efficacy by inducing a highly significant synergistic effect, as evidenced by its

low FICI value of 0.28. For VRE and cartrisine B (**2**), the highest level of synergy was achieved with vancomycin, with a FICI of 0.25 (Fig. 7).

4. Conclusion

In summary, two undescribed abietane diterpenoids (**1**,**2**) previously were obtained from *C. trichosphaera* for the first time. In the antibacterial assay, cartrisine B (**2**) showed inhibitory activities against MRSA and VRE. The discovery of these new compounds further enhances the chemical composition profile of the *C. trichosphaera* plant. In future studies, we will clarify the mechanisms by which these compounds act against bacteria.

CRediT authorship contribution statement

Qing-Yu Lu: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Zhao-Jie Wang:** Writing – review & editing, Visualization, Methodology, Data curation. **Li-Yu Bai:** Methodology, Conceptualization. **Wen-Biao Zu:** Methodology. **Zhong-Shun Zhou:** Investigation. **Yan-Yan Zhu:** Writing – review & editing. **Xing-Chao He:** Visualization. **Yun-Li Zhao:** Visualization. **Xiao-Dong Luo:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fitote.2024.106269.

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