Supporting Materials

***Rec. Nat. Prod*. 19:3 (2025) 263-277**

Chemical Composition, Antioxidant, Acetylcholinesterase and β-Lactamase Inhibitory Activities of Essential Oils from *Clerodendrum cyrtophyllum* Turcz. and *Clerodendrum fortunatum* L.

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S1: Materials and Methods

S.1.1. Plant Materials

Herbarium numbers of the *Clerodendrum cyrtophyllum* Turcz. and *Clerodendrum fortunatum* L. were IBK00191733 and 02244446 from CVH (Chinese Virtual Herbarium). Aerial parts of *C. cyrtophyllum* were collected from Pingnan County, Guigang City, Guangxi Province, China (23.555 N, 110.356 E), in October 2022, while aerial parts of *C. fortunatum* were collected from Dianbai County, Maoming City, Guangxi Province, China (21.230 N, 111.196 E) in November 2022. The plant species was confirmed as *Clerodendrum cyrtophyllum* Turcz. and *Clerodendrum fortunatum* L., by Hong Zhao, plant taxonomist of Marine College (Shandong University), where the accession is deposited under EO2222 (*C. cyrtophyllum*) and EO2223 (*C. fortunatum*). Plant samples are kept at -18 degrees Celsius until EO extraction.

S.1.2. Extraction of EOs

Aerial parts of the plants (1.5 kg) were washed with pure water and then pulverized into small pieces. The pieces were transferred to a 5.0 L round-bottom flask and mixed with approximately 3.0 L ultrapure water. The EOs were extracted through hydrodistillation using a Clevenger-type apparatus for about four hours. The EO was then separated from the aqueous layer using diethyl ether. The extracted EO was subsequently dried using the anhydrous sodium sulfate and Termovap Sample Concentrator. The obtained EO was stored at a low temperature (4 °C) for further analysis.

S.1.3. GC-MS and GC-FID Analysis

The EO components and their content were determined using gas chromatography-mass spectrometry (GC-MS) and gas chromatography-flame ionization detector (GC-FID) methods. GC-MS analysis was carried out using an Agilent 7890–5975C gas chromatograph–mass spectrometer equipped with a fused silica capillary column type HP-5MS (30 m × 0.25 mm with film thickness, 0.25 microns, Agilent Technologies, Santa Clara, CA, USA). The injector temperature was set at 260 °C, and the interface temperature was 280 °C. The oven temperature was initially 50 °C. It was maintained for 4 min, programmed from 50 °C to 280 °C at a rate of 6 °C/min, and held steady for 3 min. Helium was used as the carrier gas at a 1.1 mL/min velocity. The mass spectrometer conditions were as follows: electron impact (EI) mode (electron energy = 70 eV), scan range of 25-500 amu, scan rate of 4.0 scans/s, and quadrupole temperature of 150 °C. A 1% w/v sample solution in n-hexane was prepared, and 0.3 µL was injected using a splitless mode. GC-FID analysis was performed using an Agilent 7890 gas chromatograph with a type HP-5 fused silica capillary column (30 m × 0.25 mm with film thickness 0.25 microns, Agilent Technologies, USA). The injector temperature was 260 °C, and the detector temperature was 305 °C. The oven temperature was initially 50 °C, was maintained for 4 min, and then programmed from 50 °C to 280 °C at the rate of 6 °C/min and held steady for 3 min. Helium was used as the carrier gas at a 1.1 mL/min velocity. Identifying those compounds in EO is primarily based on comparing mass spectrometric data and Kovat

retention indices relating to retention time with commercial libraries (NIST/EPA/NIH 2023 Mass Spectral Database) [23,24]. Specifically, the Kovat retention indices were calculated using a series of n-alkanes (C_8-C_{30}) with linear interpolation on the HP-5MS column. The data analysis was carried out by Aglient MassHunter Qualitative Analysis 10.0 Program.

S. 1.4 Antioxidant Activity Evaluation

1.4.1. DPPH Method

The DPPH (2,2-diphenyl-1-picrylhydrazyl) experimental procedure was modified from previous studies [25,26,27]. The DPPH ethanolic solution was prepared at a concentration of 0.17 mM. During DPPH free-radical scavenging activity evaluation, 96-well microplates were prepared. Following the addition of 200 µL of the stock DPPH solution, 50 µL of each dilution of the oil in methanol (50, 25, 10, 5, 2.5, 1, and 0.5 mg mL^{-1}) was added. For comparison, the positive solution was prepared with BHT (butylated hydroxytoluene) or Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) but without the EO sample. The control was prepared with 200 µL of the stock DPPH solution and 50 µL ethanol, and the sample blank was prepared with 50 µL of EO solution and 200 µL ethanol. After 30 min, readings were taken using a microplate reader (Epoch, Biotech company, Minneapolis, MN, USA) at a wavelength of 516 nm. All tests were performed in triplicates, and the results were averaged. The DPPH free-radical scavenging capacity (*RSC*%) was calculated using the following equation:

$$RSC\% = \left(1 - \frac{A_{Sample} - A_{Sample\ Blank}}{A_{Control}}\right) \times 100\%$$

Where *RSC*% assesses the "radical scavenging activity" of the DPPH radical, A_{Sample} is the absorbance of the solution in the microplate with the sample at different concentrations, $A_{Control}$ is the absorbance of the DPPH solution without the EO sample, and $A_{Sample\ Blank}$ is the absorbance of the ethanol sample without DPPH.

S. 1.4.2. ABTS Method

The ABTS (2,2-azinobis(3-ethylbenzothiazoline-6-sulfonate)) scavenging capacity was performed according to the method of the previous paper [26,27]. Equal volumes of ABTS stock solution (7.4 mM) and $K_2S_2O_8$ stock solution (2.6 mM) were mixed and reacted at room temperature for at least 12 hours under dark conditions. The solution was diluted 45 times with ethanol to obtain $ABTS^{\bullet+}$ reagent. The ethanolic EO solution was prepared according to the following gradients: 25, 10, 5, 2.5, 1, and 0.5 mg mL^{-1}. During free-radical scavenging activity evaluation, 96-well microplates were prepared. Following the addition of 200 µL of the stock $ABTS^{\bullet+}$ reagent, 50 µL of each dilution of the oil in methanol (25, 10, 5, 2.5, 1, and 0.5 mg mL^{-1}) was added. For comparison, the positive solution was prepared with BHT or Trolox but without the EO sample. The absorbance of the solution was determined as 734 nm seven minutes into the reaction using a microplate reader (Epoch, Biotech company, USA). Ethanol

was used as a blank and gradient-diluted solvent. All tests were performed in triplicates, and the results were averaged. The scavenging capacity was calculated using the equation below:

$$RSC\% = \frac{A_0 - A}{A_0} \times 100\%$$

Where *RSC*% assesses the "radical scavenging activity" of the ABTS•+ radical, A_0 is the absorbance of 200 µL diluted $ABTS^{\bullet+}$ solution mixed with 50 µL ethanol at 734 nm, and *A* is the absorbance of 200 µL diluted $ABTS^{\bullet+}$ solution mixed with 50 µL sample solution at 734 nm. IC_{50} was then calculated using GraphPad Prism 9.5.

S. 1.4.3. FRAP Method

The FRAP (ferric reducing antioxidant power) assay was conducted using the method described in the previous publication [28]. In this assay, stock solutions included (i) 0.3M pH 3.6 acetate buffer solution, (ii) 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution, and (iii) 20 mM Fe^{3+} solution. The stock solutions were mixed at the proportion of 10: 1: 1, and were diluted 50 times with ethanol. Next, 50 µL of different dilutions of EOs (4000, 2000, 1000, 500, 250, and 100 µg mL^{-1}) and 0.25 mg/mL Trolox solution (2, 5, 10, 15, and 20 µL) were mixed with 200 µL FRAP working reagent in a 96-well microplate. The blank solution was similarly prepared by replacing EOs with ethanol. After 30 min of reaction at 37°C, the absorbance of the resulting solution was measured at 593 nm using a microplate reader (Epoch, Biotech company, USA). The standard curve was created based on the mass of Trolox and its corresponding absorbance. The absorbance of the EO sample at the specified concentration was referenced against the standard curve equation to obtain the equivalent value of 1.0 nM of Trolox as the standard (X nmol). If the concentration is Y µg/mL, the volume of the solution is 50 µL, and the antioxidant capacity of the compound can be expressed as 20X/Y µmol/g. All tests were performed in triplicates, and the results were averaged.

S.1.5. Anti-Acetylcholinesterase Activity test

The acetylcholinesterase inhibitory effect was measured using the previously described method with minor modifications [26,29]. The EO sample was prepared with ethanol at a series of concentrations of 5, 10, 25, 50, 100, 500, 1000 µg/mL. Then, 125 µL of 0.1 mM, pH 8.0 phosphate-buffered saline (PBS), 20 µL of the sample, and 35 µL of acetylcholinesterase solution containing 0.28 U/mL were mixed in a microplate and left to incubate at 4 °C for 20 min. Subsequently, 10 µL 2 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and 10 µL 15 mM s-acetyl choline iodide (ATCI) (15 mM) were added to the cells. The absorbance at 412 nm was read in 60-second intervals for 6 minutes, and the initial reaction rate (*K*) of the enzyme under inhibition was determined through linear regression. Simultaneously, the enzyme's initial reaction rate (K_0) under non-inhibitory conditions was obtained by replacing the 20 µL of sample solution with 20 µL solvent. Galantamine was used as a positive control. All tests were performed in triplicates, and the results were averaged. The acetylcholinesterase inhibition rate was then calculated as follows:

$$\text{Inhibition\%} = \frac{K_0 - K}{K_0} \times 100\%$$

Where K_0 is the initial reaction rate of the enzyme without the inhibition, while K is the initial reaction rate of the inhibited enzyme, the IC_{50} value was calculated using nonlinear regression by GraphPad Prism 9.5.

S. 1.6. Anti-β-lactamase Activity Test

The *β*-lactamase inhibitory test was modified from the previously described method [30]. Ethanolic EO solutions were gradient-diluted to 50, 100, 250, 500, 1000, 2500, and 5000 µg/mL using pH 7.0 PBS. 20 µL EO samples, 30 µL 0.1 M pH 7.0 PBS, and 100 µL *β*-lactamase solution (1000 U/mL) were mixed and then incubated at 30 °C for 10 minutes. Then, 50 µL 0.1 mg/mL substrate nitrocefin was added and incubated at 30 °C for another 10 minutes. The absorbance at 489 nm was measured. Clavulanate Potassium was used as the positive control. The *β*-lactamase inhibition rate was calculated using the formula:

$$\text{Inhibition\%} = (1 - \frac{A_S - A_{Sb}}{A_e - A_b}) \times 100\%$$

where A_S is the absorbance of the samples, A_{sb} is the blank reaction absorbance of the samples with 130 µL PBS replacing 100 µL *β*-lactamase solution, A_e is the enzymatic determination absorbance, and A_b is the blank reaction absorbance with 100 µL PBS replacing 100 µL *β*-lactamase solution. The IC_{50} value was calculated using nonlinear regression by using GraphPad Prism 9.5.

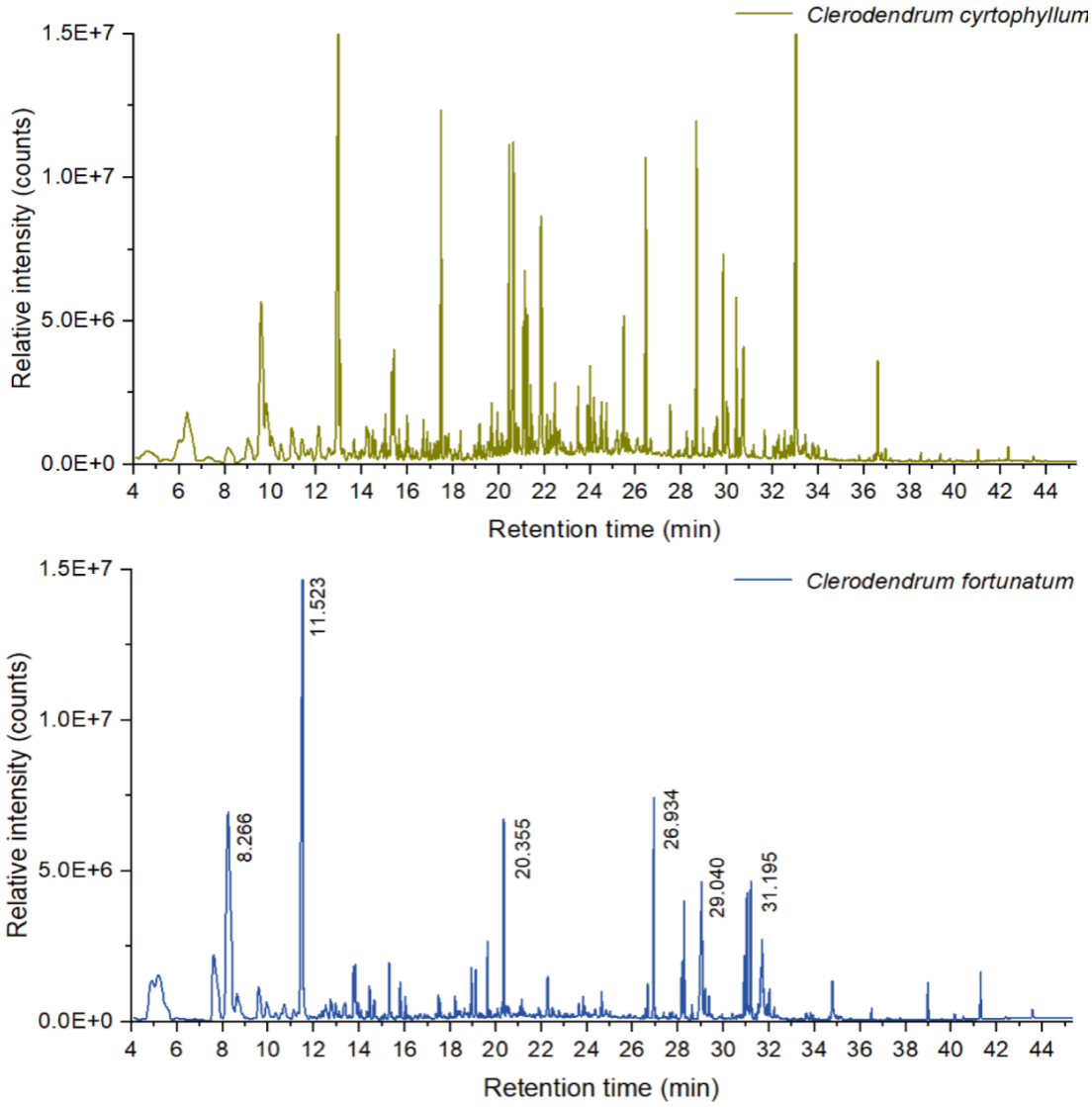

Figure S1: The total ion chromatograms of the EOs distilled from two plants

分析报告

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样品信息

名称	KB	数据文件路径	E:\【SDU科研资料】\研究工作\挥发油\4-数据处理\20230110\【Test data】\【Raw data】\20230202\KB.D
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仪器	GCMS	方法路径 (Acq)	D:\MassHunter\GCMS\1\methods\GCMC测试-1.M
MS 类型	Q	版本 (Acq SW)	MassHunter GC/MS Acquisition B.07.06.2704 18-Jul-2017 Copyright © 1989-2017 Agilent Technologies, Inc.
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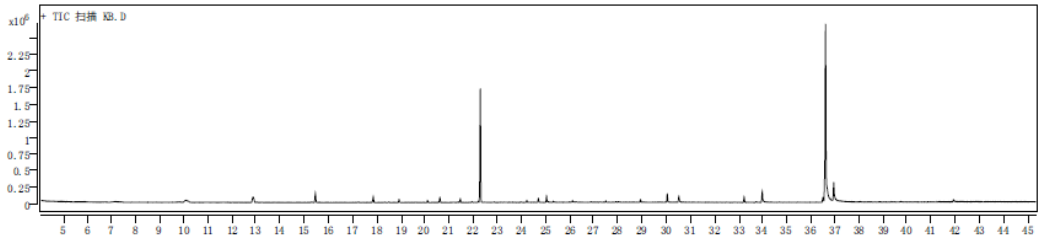
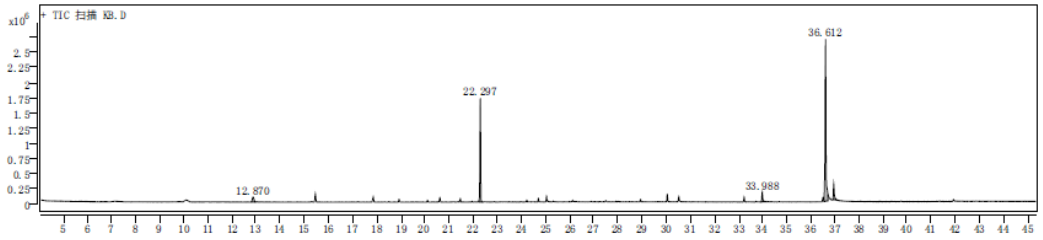
操作者		结果摘要	5 已识别 (5 已找到)

样品色谱图





色谱峰

峰	起始	RT	终止	峰高	面积	面积 %	SNR
1	12.792	12.870	12.963	81730	377043	5.22	
2	22.248	22.297	22.379	1714980	3563319	49.36	
3	33.930	33.988	34.113	159626	419015	5.80	
4	36.541	36.612	36.716	2678454	7219523	100.00	
5	36.915	36.950	37.087	262745	685608	9.50	

化合物摘要

Cpd	名称	分子式	RT	质量	CAS	ID 来源	分数	分数 (Lib)	分数 (DB)	分数 (MFG)	算法
1	Undecane	C11 H24	12.870		1120-21-4	Lib 检索	91.72	91.72			积分
2	Butylated Hydroxytoluene	C15 H24 O	22.297		128-37-0	Lib 检索	96.61	96.61			积分
3	Dodecanamide	C12 H25 N O	33.988		1120-16-7	Lib 检索	84.45	84.45			积分
4	9-Octadecenamide, (Z)-	C18 H35 N O	36.612		301-02-0	Lib 检索	94.86	94.86			积分
5	Octadecanamide	C18 H37 N O	36.950		124-26-5	Lib 检索	87.82	87.82			积分

MassHunter Qual 10.0
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Figure S2: Blank background chromatogram