Chemical Constituents of *Carpesium cernuum* and Their In Vitro Anti-leukemic Activity (Post-print)

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Abstract

To investigate the chemical constituents of Carpesium cernuum and their in vitro inhibitory effects on leukemia cells, this study employed silica gel column chromatography, gel column chromatography, macroporous adsorption resin, and other methods to separate and purify the ethyl acetate fraction of Carpesium cernuum. The structures of the compounds were identified using spectroscopic techniques including 1H NMR, 13C NMR, and MS. The MTT assay was utilized to evaluate the in vitro inhibitory effects of compounds 1-10 against leukemia cells (K562, HEL). The results demonstrated that: (1) A total of 11 compounds were isolated and identified from the ethyl acetate fraction of Carpesium cernuum, namely 2, 9-epoxy-5, 9-dihydroxy-8-angeloyloxy-11-methoxymethyl-4(15)-germacraen-6, 12-olide (1), cardivin D (2), cernuumolide I (3), cernuumolide J (4), 8-hydroxy-9, 10-diisobutyryloxythymol (5), (2E, 6Z, 10E, 12R)-7-[(acetyloxy)methyl]-3, 11, 15-trimethylhexadeca-2, 6, 10, 14-tetraene-1, 12-diol (6), 9, 10-dihydroxyoctadecanoate (7), 1, 6-dihydroxy-8-hydroxymethyl-anthraquinone (8), emodin (9), 4-megastigmen-3, 9-dione (10), and β-sitosterol (11). Among these, compound 1 is a new compound; compounds 5 and 7-10 were isolated from the genus Carpesium for the first time; and compounds 2 and 5-10 were isolated from Carpesium cernuum for the first time. (2) The activity assay results indicated that compounds cardivin D (2), cernuumolide I (3), and cernuumolide J (4) exhibited favorable in vitro inhibitory effects on leukemia cells, with IC50 values against K562 cells of (2.27$\pm$0.46), (5.53$\pm$0.41), $and(3.90\pm0.80)\mu mol\cdot L-1, respectively, andIC50 values against HEL cells of(1.84\pm0.14), (2.36\pm0.90), and(2.31\pm$1.17) mol · L-1, respectively. These findings enrich the chemical constituents of Carpesium cernuum and provide a material basis for the development of anti-leukemia drugs.

Full Text

Chemical Constituents from Carpesium cernuum and Their Anti-Leukemia Activities *In Vitro*

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Abstract

To investigate the chemical constituents of *Carpesium cernuum* and their inhibitory effects on leukemia cells *in vitro*, the ethyl acetate fraction of *C. cernuum* was isolated and purified using silica gel column chromatography, Sephadex LH-20 column chromatography, and macroporous adsorption resin. The structures were identified by spectroscopic techniques including ^{1}H NMR, ^{13}C NMR, and MS. The inhibitory effects of compounds 1–10 on leukemia cells (K562, HEL) were evaluated by MTT assay.

The results showed that: (1) Eleven compounds were isolated and identified as 2,9-epoxy-5,9-dihydroxy-8-angeloyloxy-11-methoxymethyl-4(15)-germacraen-6,12-olide (1), cardivin D (2), cernuumolide I (3), cernuumolide J (4), 8-hydroxy-9,10-diisobutyryloxythymol (5), (2E,6Z,10E,12R)-7-[(acetyloxy)methyl]-3,11,15-trimethylhexadeca-2,6,10,14-tetraene-1,12-diol (6), 9,10-dihydroxyoctadecanoate (7), 1,6-dihydroxy-8-hydroxymethyl-anthraquinone (8), emodin (9), 4-megastigmen-3,9-dione (10), and β-sitosterol (11). Among them, compound 1 was a new compound; compounds 5 and 7–10 were isolated from the genus *Carpesium* for the first time; and compounds 2 and 5–10 were obtained from *C. cernuum* for the first time. (2) The activity tests revealed that cardivin D (2), cernuumolide I (3), and cernuumolide J (4) exhibited potent inhibitory effects against leukemia cells *in vitro*. The IC_{50} values against K562 cells were $(2.27\$\pm0.46), (5.53\pm0.41), and (3.90\pm0.80)\mu mol \cdot L^{\{-1\}, respectively, while those against HEL cells were (1.84\pm0.14), (2.36\pm0.90), and (2.31\pm1.17)\mu mol \cdot L^{\{-1\}}\$$, respectively. These findings enrich the chemical composition of *C. cernuum* and provide a material basis for the development of anti-leukemia drugs.

Keywords: *Carpesium cernuum*; chemical constituents; isolation and purification; structure identification; leukemia cells; inhibitory effects

Introduction

Carpesium cernuum, also known as "shaower vegetable" or "pipewort," is the type species of the genus *Carpesium* (Asteraceae). It is widely distributed in Sichuan, Yunnan, Guizhou, Hunan, and Shaanxi provinces of China, typically growing in roadside wastelands, slopes, and ditch sides. In folk medicine, it is often used interchangeably with *Carpesium divaricatum* (Editorial Committee of China Flora of Chinese Academy of Sciences, 1979). The whole herb is used medicinally with warm and slightly bitter properties and mild toxicity. It has been traditionally used to treat malaria, laryngitis, sores (fresh leaves applied externally), dysentery, toothache, uterine prolapse, and rectal prolapse (Zhang et al., 2005). Modern pharmacological studies have demonstrated its antitumor (Kim et al., 2007; Liu et al., 2016; Dang et al., 2019), anti-inflammatory (Liu et al., 2010), and antiparasitic activities (Chung & Moon, 2009; Kim et al., 2009). Chemical investigations have revealed that *C. cernuum* contains sesquiterpenoids, monoterpenoids, diterpenoids, triterpenoids, coumarins, lignans, volatile oils, flavonoids, sterols, phenols, and glycosides, with sesquiterpenoids being the major constituents (Ma et al., 2008; Zhang et al., 2015; Wang et al., 2019; Feng et al., 2022).

The genus *Carpesium* is rich in sesquiterpenoids. In the past two years, scholars worldwide have isolated 51 sesquiterpenoid compounds from this genus, including five new germacrane-type sesquiterpenoids, and investigated their antitumor and anti-inflammatory activities (Wu et al., 2021; Yan et al., 2021; Yang et al., 2021; Zhong et al., 2022; Wang et al., 2022; Yang et al., 2022; Shen et al., 2022; Chen et al., 2022). Our research group previously isolated and identified five new germacrane-type sesquiterpenoids from *C. cernuum* using various chromatographic and spectroscopic techniques, and activity tests showed they possessed certain antitumor activities, particularly strong inhibitory effects against leukemia cells (Yan et al., 2018; Yan et al., 2021). However, the new compound discovered in this study differs structurally from the germacrane-type sesquiterpenoids reported recently and from our previous isolates, mainly in having an oxygen bridge outside the ring. Additionally, activity tests revealed that this new compound showed no significant antitumor activity.

The development of highly effective and low-toxicity antitumor drugs is a critical issue that the medical industry urgently needs to address. Based on our previous research, to further elucidate the antitumor substance basis of *C. cernuum*, this study focused on this plant species, leveraging the Guizhou ethnic medicine research and development platform and modern advanced instrumentation. Using multiple chromatographic separation techniques, modern spectroscopic identification methods, and modern pharmacological technologies, we aimed to address the following questions: (1) the chemical constituents in the ethyl acetate fraction of *C. cernuum*; and (2) the *in vitro* inhibitory effects of some isolated compounds on leukemia cells.

Materials and Methods

1.1 Materials Plant material: *Carpesium cernuum* was collected in Zhenning County, Anshun City, Guizhou Province in October 2016 and identified by Professor Sun Qingwen of the College of Pharmacy, Guizhou University of Traditional Chinese Medicine. A voucher specimen (No. Assrmyy201608) was deposited in the Pharmacy Laboratory of Anshun City People' s Hospital.

Cell lines: Human leukemia cells (K562 and HEL) were purchased from the ATCC cell bank and maintained in the laboratory of Professor Li Yanmei at the Key Laboratory of Chemistry for Natural Products of Guizhou Province and Chinese Academy of Sciences.

1.2 Instruments and Reagents Instruments: INOVA-400 MHz and INOVA-500 MHz superconducting NMR spectrometers (Varian, USA); Bruker HCT/Esquire and Waters Autospec Premier P776 mass spectrometers (Bruker, Germany; Waters, USA); Bruker Tensor-27 FT-IR spectrometer (Bruker, Germany); Shimadzu UV-2401PC UV-Vis spectrophotometer (Shimadzu, Japan); JASCO P-1020 polarimeter (JASCO, Japan); Waters 1525 EF HPLC system (Waters, USA); 0SB-2200 rotary evaporator (Shanghai Ailang Instrument Co., Ltd.); inverted optical microscope (Zeiss); benchtop high-speed refrigerated centrifuge (Beckman); adjustable micropipettes (Eppendorf); cell culture incubator (Thermo Scientific Forma); and multifunctional microplate reader (Gene).

Reagents: Sephadex LH-20 (Mitsubishi Chemical, Japan); silica gel for column chromatography (300–400 mesh, Qingdao Marine Chemical Factory); silica gel GF_{254} TLC plates (Qingdao Marine Chemical Factory); HPLC-grade acetonitrile and methanol (Sahn Chemical Technology Co., Ltd.); analytical-grade acetone and chloroform (Chuandong Chemical Group Co., Ltd.); dimethyl sulfoxide (DMSO, Sangon Biotech); MTT (Beijing Solarbio); fetal bovine serum (FBS, HyClone); RPMI 1640 medium (Gibco).

2.1 Extraction and Isolation

Dried whole herbs of *C. cernuum* (20.0 kg) were powdered and extracted three times with 95% ethanol under reflux at 80°C (3 h each). The combined extracts were concentrated to recover ethanol, yielding 2.0 kg of crude extract. After reserving 1.0 g for sampling, the remaining extract was suspended in water and partitioned with ethyl acetate to obtain ethyl acetate and aqueous layers. The ethyl acetate fraction (960.0 g) was mixed with 40–80 mesh silica gel, dried, and subjected to silica gel column chromatography (300–400 mesh) eluted with petroleum ether-acetone (60:1$\rightarrow$1 : 1).*Theeluatesweremonitoredby TLC under UV light and visualized with* 10*{2}SO*{4}$ in ethanol. Similar fractions were combined and finally washed with methanol to afford seven fractions (Fr.1–Fr.7).

Fraction Fr.1 (64.0 g) was applied to a silica gel column eluted with petroleum ether-ethyl acetate (60:1$→1 : 1)*togivefoursubfractions*(*Fr*.1 − 1–*Fr*.1 − 4).*Fr*.1−1*wasfurtherpurifiedbysilicagelcolumnchromatographyusingpetroleumether*− *acetone*(60 : 1→10 : 1)*aseluent*, *followedbyrecrystallization*, *toyieldcompound*∗ ∗11∗∗(16.5*mg*).*Fr*.1−2*wasseparatedbysilicagelcolumnchromatographywithpetroleumether*− *ethylacetate*(30 : 1→$1:1) and further purified by Sephadex LH-20 column chromatography with methanol to obtain compound **6** (12.4 mg).

Fraction Fr.4 (103.5 g) was subjected to macroporous adsorption resin column chromatography eluted with methanol-water (20%, 40%, 60%, 80%, 95%) to give five subfractions (Fr.4-1-Fr.4-5). Fr.4-3 was separated by silica gel column chromatography using petroleum ether-acetone (15:1$→3 : 1)*toaffordthreesubfractions*(*Fr*.4 − 3 − 1–*Fr*.4 − 3 − 3).*Fr*.4 − 3 − 2*wasfurtherpurifiedbysilicagelcolumnchromatographywithpetroleumether*− *acetone*(10 : 1→3 : 1)*toyieldcompound*∗∗1∗∗(2.0*mg*).*Fr*.4−4*waschromatographedonsilicagelelutedwithpetrol* *acetone*(8 : 1→1 : 1)*togivefivesubfractions*(*Fr*.4−4−1–*Fr*.4−4−5).*Fr*.4−4− 2*wassubjectedtoSephadexLH* − 20*columnchromatographywithchloroform* − *methanol*(1 : 1)*toobtainsixsubfractions*(*Fr*.4 − 4 − 2 − 1–*Fr*.4 − 4 − 2 − 6).*Fr*.4−4−2−3*wasrepeatedlychromatographedonsilicagel*(*petroleumether* : *acetone* = 10 : 1→3 : 1)*andSephadexLH* − 20(*CHCl{3}*: *MeOH* = 1 : 1)*toaffordcompounds*∗∗5∗∗(5.0*mg*), ∗∗7∗∗(6.0*mg*), *and*∗∗10∗∗(4.5*mg*).*Fr*.4− 4 − 2 − 4*wasrepeatedlychromatographedonsilicagel*(*petroleumether* : *acetone* = 10 : 1→3 : 1)*andSephadexLH*−20(*MeOH*), *followedbypreparativeHPLC*(*CH*{3}*CN* : *H*_{2}*O* = 40→1 : 1)*togivefivesubfractions*(*Fr*.4−5−1–*Fr*.4−5−5).*Fr*.4− 5−1*wasfurtherpurifiedbysilicagelcolumnchromatographyusingpetroleumether* : *acetone*(10 : 1→$1:1) to obtain compounds **8** (3.5 mg) and **9** (2.0 mg).

2.2 *In Vitro* Anti-Leukemia Activity of Isolated Compounds

The anti-leukemia activity of compounds 1–10 against K562 and HEL cells was evaluated using the MTT assay. Logarithmic-phase K562 and HEL cells were counted under an inverted microscope and seeded in 96-well plates at 8$×10^{4}$ cells/well. After 4–6 h incubation for cell stabilization, compounds at concentrations of 2.5, 5, 10, and 20 μmol·L^{-1} were added, with DMSO as the control group (five replicates per group). Following 72 h incubation at 37°C with 5% CO_2, 10 μL of MTT solution (5 mg·mL^{-1}) was added to each well. After 4 h, the medium was replaced with 100 μL of solubilization buffer (containing 50 g SDS, 25 mL isobutanol, and 0.5 mL concentrated HCl per 500 mL) and incubated overnight. Cell viability was determined by measuring absorbance at 570 nm using a multifunctional microplate reader, and IC_{50} values were calculated.

2.3 Statistical Analysis

All data were processed using Excel 2013 and analyzed statistically with SPSS 20.0. Graphs were prepared using GraphPad Prism 9.0. Results are expressed as mean ± standard deviation ($\bar{x}\pm s$). Inter-group differences were compared using Student's t-test, with $P<0.05$ considered statistically significant (*$P<0.05$, **$P<0.01$).

3.1 Structure Identification

Compound 1 was obtained as a white powder with $[\alpha]^{20}$ -50.7 (c 0.1, MeOH); UV (MeOH) λ : 195 nm; IR (KBr) 3,423, 1,753, 1,717, 1,643 cm^{-1}. HRESIMS showed a quasi-molecular ion peak at m/z 433.1830 $[M+Na]^+$ (calcd. for $C_{21}H_{30}O_8Na$, 433.1833), suggesting the molecular formula $C_{21}H_{30}O_8$ with seven degrees of unsaturation. The ^1H NMR spectrum displayed four methyl signals at δ 3.38 (3H, s, H-16), 1.99 (3H, m, H-4), 1.84 (3H, m, H-5), and 1.01 (3H, d, J = 6.0 Hz, H-14), including one oxygenated methyl; and two olefinic proton signals at δ 6.15 (1H, q, J = 7.5 Hz, H-3), 5.39 (1H, s, H-15α), and 5.30 (1H, s, H-15β). The ^{13}C NMR spectrum exhibited 21 carbon signals, which according to the DEPT spectrum included four methyl carbons at δ 59.3 (C-16), 16.0 (C-4), 18.9 (C-14), and 20.5 (C-5) (one oxygenated), two double bond carbons at δ 141.8 (C-4), 141.4 (C-3), 126.1 (C-2), and 123.4 (C-15), and two carbonyl carbons at δ 176.5 (C-12) and 166.1 (C-1).

Comprehensive analysis of 1D and 2D NMR data allowed full assignment of all proton and carbon signals (Table 1). The ^1H-^1H COSY spectrum revealed four structural fragments: H-14/H-10/H-1/H-2/H-3, H-5/H-6/H-7/H-8, H-7/H-11/H-13, and H-3 /H-4 (Figure 2 [Figure 2: see original paper]). In the HMBC spectrum, correlations of H-14 (δ 1.01) with C-1 (δ 45.6), C-9 (δ 106.9), and C-10 (δ 34.7) indicated that Me-14 was attached to C-10. Correlations of H-2 (δ 4.20) with C-9 (δ 106.9) and H-13α (δ 3.81) with C-16 (δ 59.3), combined with HRESIMS data, suggested oxygen bridges between C-2 and C-9 and between C-13 and C-16. HMBC correlations of H-3α (δ 2.40) with C-4 (δ 141.8), C-5 (δ 80.6), and C-15 (δ 123.4), and of H-5 (δ 4.22) with C-4 (δ 141.8) and C-15 (δ 123.4) located the exocyclic double bond at C-4. Correlations of H-6 (δ 5.05) with C-7 (δ 37.5) and C-12 (δ 176.5) connected the five-membered γ-lactone ring to the ten-membered carbocycle. HMBC correlations of H-4 (δ 1.99) with C-2 (δ 126.1) and C-3 (δ 141.4), H-5 (δ 1.84) with C-1 (δ 166.1), C-2 (δ 126.1), and C-3 (δ 141.4), and H-8 (δ 5.34) with C-1 (δ 166.1) indicated an angeloyl group attached at C-8. These data were similar to those of carpescernolide C (Yan et al., 2021), suggesting a germacrane-type sesquiterpene lactone skeleton.

The relative configuration was determined by ROESY spectroscopy. ROESY correlations of H-15α with H-5, H-5 with H-7, H-3β with H-6, H-6 with H-8, H-8 with H-11, and H-2 with H-14 established that H-2, H-6, H-8, H-11, and

H-14 were β-oriented, while H-5 and H-7 were α-oriented. Comparison of the experimental and calculated ECD spectra confirmed the absolute configuration (Figure 3). Therefore, compound 1 was identified as 2,9-epoxy-5,9-dihydroxy-8-angeloyloxy-11-methoxymethyl-4(15)-germacraen-6,12-olide.

Table 1 ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data of compound 1 (in $CDCl_3$)

Position	δ	δ (J in Hz)
1	45.6	1.80, m; 2.54, m
2	79.1	4.20, m
3	44.7	2.40, d (13.5); 2.89, d (13.5)
4	141.8	-
5	80.6	4.22, m
6	77.2	5.05, dd (8.5, 4.0)
7	37.5	2.81, m
8	73.2	5.34, dd (12.0, 5.0)
9	106.9	-
10	34.7	2.44, m; 2.54, m
11	40.8	2.54, m
12	176.5	-
13	73.9	3.81, dd (9.0, 2.5); 3.42, dd (9.0, 3.0)
14	18.9	1.01, d (6.0)
15	123.4	5.39, s; 5.30, s
16	59.3	3.38, s
1	166.1	-
2	126.1	-
3	141.4	6.15, q (7.5)
4	16.0	1.99, d (7.5)
5	20.5	1.84, s

Figure 1 [**Figure 1: see original paper**] Key HMBC, ^{1}H-^{1}H COSY, and ROESY correlations of compound 1.

Figure 2 Comparison of experimental and calculated ECD spectra of compound 1.

Compound 2 (cardivin D) was obtained as a white powder with molecular formula $C_{23}H_{34}O_9$. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.48 (1H, d, J = 2.0 Hz, H-13β), 5.67 (1H, d, J = 1.6 Hz, H-13α), 5.05 (1H, d, J = 7.2 Hz, H-9), 4.72 (1H, s, H-6), 4.34 (1H, s, H-8), 3.79 (1H, m, H-2), 3.02 (1H, m, H-7), 2.70 (1H, m, H-2), 2.70 (1H, s, H-2), 2.26 (2H, m, H-1), 1.96 (1H, d, J = 6.4 Hz, H-4), 1.85 (2H, m, H-3), 1.32 (3H, s, H-14), 1.26 (3H, d, J = 5.8 Hz, H-3), 1.26 (3H, d, J = 5.8 Hz, H-3), 1.26 (3H, d, J = 5.8 Hz, H-4), 1.26 (3H, d, J = 5.8 Hz, H-4), 1.02 (3H, d, J = 6.8 Hz, H-15). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 214.7 (C-5), 178.1 (C-1), 178.1 (C-1), 168.2 (C-12), 132.6 (C-11), 124.6 (C-13), 81.2

(C-10), 79.0 (C-8), 78.6 (C-6), 78.5 (C-9), 71.8 (C-2), 42.1 (C-7), 34.3 (C-2), 34.1 (C-2), 33.9 (C-1), 29.6 (C-4), 25.6 (C-3), 25.3 (C-14), 20.8 (C-15), 19.3 (C-3), 19.0 (C-3), 18.9 (C-4), 18.9 (C-4). These data matched those reported in the literature (Kim et al., 1997), confirming compound 2 as cardivin D.

Compound 3 (cernuumolide I) was obtained as a white powder with molecular formula $C_{23}H_{34}O_8$. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.46 (1H, d, J = 1.2 Hz, H-13β), 5.89 (1H, d, J = 0.8 Hz, H-13α), 5.26 (1H, d, J = 1.2 Hz, H-8), 4.76 (1H, dd, J = 4.4, 1.2 Hz, H-6), 4.60 (1H, d, J = 4.4 Hz, H-5), 3.81 (1H, m, H-7), 2.83 (1H, m, H-10), 2.67 (1H, m, H-2), 2.64 (1H, m, H-2), 1.80 (1H, m, H-1β), 1.79 (1H, m, H-2β), 1.70 (1H, m, H-1α), 1.58 (1H, m, H-3α), 1.35 (1H, m, H-3β), 1.21 (3H, m, H-3), 1.21 (3H, m, H-3), 1.21 (3H, m, H-4), 1.21 (3H, m, H-4), 1.18 (3H, d, J = 6.4 Hz, H-14), 1.15 (3H, s, H-15), 1.04 (1H, m, H-2α). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 208.5 (C-9), 177.0 (C-1), 175.9 (C-1), 168.6 (C-12), 135.0 (C-11), 124.1 (C-13), 80.8 (C-8), 77.6 (C-5), 73.1 (C-4), 71.4 (C-6), 44.2 (C-7), 43.7 (C-10), 34.8 (C-3), 33.9 (C-2), 33.7 (C-2), 32.9 (C-1), 24.6 (C-15), 23.1 (C-2), 20.0 (C-14), 18.8 (C-3), 18.8 (C-4), 18.7 (C-4), 18.6 (C-3). These data matched those reported in the literature (Dang et al., 2019), confirming compound 3 as cernuumolide I.

Compound 4 (cernuumolide J) was obtained as a white powder with molecular formula $C_{24}H_{34}O_8$. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.45 (1H, d, J = 1.2 Hz, H-13α), 6.14 (1H, m, H-3), 5.89 (1H, d, J = 1.2 Hz, H-13β), 5.33 (1H, d, J = 1.2 Hz, H-8), 4.75 (1H, dd, J = 4.4, 1.2 Hz, H-6), 4.59 (1H, d, J = 4.4 Hz, H-5), 3.81 (1H, m, H-7), 2.84 (1H, m, H-10), 2.67 (1H, m, H-2), 1.95 (3H, dd, J = 4.8, 1.2 Hz, H-4), 1.87 (3H, m, H-14), 1.80 (1H, m, H-2β), 1.70 (2H, m, H-1), 1.57 (1H, m, H-3α), 1.36 (1H, m, H-3β), 1.25 (1H, s, H-2α), 1.23 (3H, d, J = 4.8 Hz, H-4), 1.21 (3H, s, H-3), 1.20 (3H, s, H-15), 1.14 (3H, s, H-5). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 208.5 (C-9), 177.0 (C-1), 168.7 (C-12), 166.3 (C-1), 140.7 (C-3), 135.0 (C-11), 126.6 (C-2), 124.2 (C-13), 81.2 (C-8), 77.7 (C-5), 73.1 (C-4), 71.5 (C-6), 44.2 (C-7), 43.8 (C-10), 34.8 (C-3), 33.9 (C-2), 32.8 (C-1), 24.6 (C-15), 23.1 (C-2), 20.3 (C-14), 20.0 (C-5), 18.9 (C-4), 18.8 (C-3), 15.8 (C-4). These data matched those reported in the literature (Dang et al., 2019), confirming compound 4 as cernuumolide J.

Compound 5 was isolated as a yellow oil with molecular formula $C_{18}H_{26}O_6$. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.89 (1H, d, J = 5.6 Hz, H-5), 6.68 (1H, s, H-2), 6.63 (1H, d, J = 5.6 Hz, H-6), 4.47 (1H, d, J = 8.0 Hz, H-9α), 4.47 (1H, d, J = 8.0 Hz, H-10α), 4.27 (1H, d, J = 8.0 Hz, H-9β), 4.27 (1H, d, J = 8.0 Hz, H-10β), 2.55 (1H, m, H-2), 2.55 (1H, m, H-2), 2.25 (3H, s, H_3-7), 1.12 (3H, s, H-3), 1.12 (3H, s, H-3), 1.10 (3H, s, H-4), 1.10 (3H, s, H-4). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 177.4 (C-1), 177.4 (C-1), 156.4 (C-3), 140.0 (C-1), 126.5 (C-5), 120.4 (C-6), 119.0 (C-4), 118.4 (C-2), 78.4 (C-8), 67.1 (C-9), 67.1 (C-10), 33.9 (C-2), 33.9 (C-2), 20.9 (C-7), 18.8 (C-3), 18.8 (C-4), 18.8 (C-3), 18.8 (C-4). These data matched those reported in the literature (Liu et al., 2020; Chen et al., 2021), confirming compound 5 as 8-hydroxy-9,10-diisobutyryloxythymol.

Compound 6 was isolated as a yellow oil with molecular formula $C_{22}H_{36}O_4$.

^{1}H NMR (400 MHz, $CDCl_3$) δ: 5.42 (1H, t, J = 8.0 Hz, H-2), 5.40 (1H, t, J = 8.0 Hz, H-6), 5.38 (1H, t, J = 8.0 Hz, H-10), 5.08 (1H, t, J = 8.0 Hz, H-14), 4.59 (2H, s, H-19), 4.14 (2H, d, J = 8.0 Hz, H-1), 3.97 (1H, t, J = 8.0 Hz, H-12), 2.22 (2H, s, H-5), 2.20 (2H, s, H-13), 2.13 (2H, s, H-9), 2.09 (2H, s, H-4), 2.08 (3H, s, H-AcO), 2.07 (2H, s, H-8), 1.72 (3H, s, H-16), 1.64 (3H, s, H-17), 1.63 (3H, s, H-20), 1.62 (3H, s, H-18). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 171.2 (COO-AcO), 137.3 (C-11), 137.2 (C-3), 134.7 (C-15), 133.6 (C-7), 130.4 (C-6), 125.3 (C-10), 124.0 (C-2), 120.1 (C-14), 77.0 (C-12), 61.9 (C-19), 59.3 (C-1), 39.3 (C-4), 34.7 (C-13), 34.5 (C-8), 26.1 (C-5), 25.9 (C-9), 25.8 (C-16), 20.0 (CH_3-AcO), 18.0 (C-17), 16.2 (C-20), 11.8 (C-18). These data matched those reported in the literature (Gao et al., 2008), confirming compound 6 as (2E,6Z,10E,12R)-7-[(acetyloxy)methyl]-3,11,15-trimethylhexadeca-2,6,10,14-tetraene-1,12-diol.

Compound 7 was obtained as a white powder with molecular formula $C_{19}H_{38}O_4$. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.67 (3H, s, OCH_3), 3.40 (1H, m, OH), 2.30 (2H, t, J = 5.2 Hz, H-2), 1.62 (2H, s, H-3), 0.88 (3H, t, J = 4.4 Hz, H-18). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 174.3 (C-1), 74.5 (C-9), 74.4 (C-10), 51.4 (OCH_3), 34.1 (C-11), 33.7 (C-8), 33.6 (C-2), 31.8 (C-16), 29.6 (C-15), 29.5 (C-14), 29.4 (C-13), 29.3 (C-6), 29.1 (C-5), 29.0 (C-4), 25.7 (C-12), 25.5 (C-7), 24.9 (C-3), 22.6 (C-17), 14.1 (C-18). These data matched those reported in the literature (Dailey et al., 2009; Wu et al., 2019), confirming compound 7 as 9,10-dihydroxyoctadecanoate.

Compound 8 was obtained as an orange-yellow powder with molecular formula $C_{15}H_{10}O_5$. ^{1}H NMR (400 MHz, CD_3OD) δ: 7.96 (1H, s, H-7), 7.87 (1H, m, H-2), 7.87 (1H, m, H-4), 7.82 (1H, m, H-5), 7.40 (1H, m, H-3), 4.78 (2H, s, $HOCH_2$). ^{13}C NMR (100 MHz, CD_3OD) δ: 194.1 (C-9), 183.1 (C-10), 164.0 (C-1), 163.4 (C-6), 152.8 (C-8a), 138.3 (C-5a), 135.1 (C-3), 134.9 (C-4a), 125.5 (C-2), 122.2 (C-7), 120.7 (C-8), 118.6 (C-4), 117.2 (C-1a), 113.2 (C-5), 64.1 ($HOCH_2$-8). These data matched those reported in the literature (Cui et al., 2008), confirming compound 8 as 1,6-dihydroxy-8-hydroxymethyl-anthraquinone.

Compound 9 was obtained as a white powder with molecular formula $C_{15}H_{10}O_5$. ^{1}H NMR (500 MHz, DMSO) δ: 12.08 (1H, s, OH-1), 12.01 (1H, s, OH-8), 7.48 (1H, s, H-5), 7.16 (1H, s, H-7), 7.11 (1H, d, J = 2.4 Hz, H-4), 6.59 (1H, d, J = 2.4 Hz, H-2), 2.41 (3H, s, -CH_3). ^{13}C NMR (125 MHz, DMSO) δ: 190.7 (C-9), 182.3 (C-10), 166.5 (C-3), 165.4 (C-1), 162.3 (C-8), 149.2 (C-6), 136.0 (C-14), 133.8 (C-11), 125.1 (C-7), 121.4 (C-5), 114.3 (C-12), 110.0 (C-13), 109.7 (C-4), 108.9 (C-2), 22.4 (-CH_3). These data matched those reported in the literature (Knut et al., 1992), confirming compound 9 as emodin.

Compound 10 was isolated as a yellow oil with molecular formula $C_{13}H_{20}O_2$. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 5.85 (1H, s, H-4), 2.54 (1H, m, H-8α), 2.53 (1H, m, H-8β), 2.37 (1H, d, J = 11.6 Hz, H-2α), 2.16 (3H, s, H-10), 2.04 (1H, d, J = 11.6 Hz, H-2β), 1.99 (3H, s, H-13), 1.98 (1H, m, H-7α), 1.89 (1H, t, J = 3.6 Hz, H-6), 1.71 (1H, m, H-7β), 1.06 (3H, s, H-12), 1.02 (3H, s, H-11). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 207.2 (C-9), 198.8 (C-3), 164.5 (C-5), 125.6 (C-4), 50.1 (C-6), 47.0 (C-2), 42.6 (C-1), 36.3 (C-8), 30.1 (C-10), 28.8 (C-11), 27.3 (C-12), 24.6

(C-7), 23.5 (C-13). These data matched those reported in the literature (Xiao et al., 2016), confirming compound 10 as 4-megastigmen-3,9-dione.

Compound 11 was obtained as white flaky crystals (from methanol) with mp 135–137°C and molecular formula $C_{29}H_{50}O$. TLC analysis in three solvent systems (petroleum ether-acetone, petroleum ether-ethyl acetate, and chloroform-methanol) showed no fluorescence under UV 254 nm, but turned purple-red with 10% H_2SO_4 in ethanol. The Rf value was identical to that of an authentic β-sitosterol standard, confirming compound 11 as β-sitosterol.

Figure 3 [**Figure 3: see original paper**] Chemical structures of compounds 1–11.

3.2 Activity Test Results

As shown in Figure 4 [Figure 4: see original paper], treatment with different concentrations of compounds 1–10 for 72 h significantly inhibited the viability of K562 and HEL cells ($P<0.05$). The inhibitory effect became more pronounced with increasing compound concentration. Compared with the DMSO control group, compounds 1–10 showed certain inhibitory effects on both K562 and HEL cells at 20 $\mathrm{mol \cdot L^{-1}}$, with compounds 2–4 exhibiting the most significant inhibition ($P<0.01$).

Figure 4 Effects of compounds 1–10 on leukemia cell viability. Compared with the DMSO group, *$P<0.05$, **$P<0.01$.

As shown in Table 2 , compounds cardivin D (2), cernuumolide I (3), and cernuumolide J (4) demonstrated potent inhibitory effects against K562 and HEL cells, with compound 2 being the most active (IC_{50} values of (2.27$±0.46)$\mu mol \cdot L\hat{}${-1}$ against K562 and (1.84$±0.14)$\mu mol \cdot L\hat{}${-1}$ against HEL).

Table 2 IC_{50} values of compounds 1–10

Compound	IC_{50} ($\mathrm{mol \cdot L^{-1}}$)
1	>20
2	2.27 ± 0.46 (K562); 1.84 ± 0.14 (HEL)
3	5.53 ± 0.41 (K562); 2.36 ± 0.90 (HEL)
4	3.90 ± 0.80 (K562); 2.31 ± 1.17 (HEL)
5–10	>20
Imatinib (positive control)	0.09 ± 0.01 (K562); 4.24 ± 0.04 (HEL)

Note: Imatinib was used as the positive control.

Discussion and Conclusion

This study investigated the chemical constituents of *Carpesium cernuum* using phytochemical methods, leading to the isolation of 11 compounds including five sesquiterpenoids, one diterpenoid, two anthraquinones, one sterol, one aromatic compound, and one alkane. Among these, compound 1 is a new germacrane-type sesquiterpenoid. The anti-leukemia activity results demonstrated that cardivin D (2), cernuumolide I (3), and cernuumolide J (4) exhibited potent inhibitory effects against K562 and HEL cells with IC_{50} values ranging from 1.84 to 5.53 $mol \cdot L^{-1}$. Notably, their inhibitory effects against HEL cells were superior to the positive control imatinib, suggesting their potential as anti-leukemia drug candidates. These findings provide new insights and directions for developing novel and effective anti-leukemia agents.

However, efficient and large-scale enrichment of these active compounds remains a challenge that requires further investigation. Future studies should focus on network pharmacology, cellular transcriptomics, *in vivo* anti-leukemia activity, and mechanism of action studies for compounds 2-4 to explore their signaling pathways and molecular targets, thereby providing modern pharmacological evidence for the application of *C. cernuum* in anti-leukemia therapy.

Literature reports indicate that cernuumolide I (3) and cernuumolide J (4) also exhibit inhibitory effects against various tumor cell lines including cervical cancer HeLa, hepatoma Hep G2, lung cancer A549, and colon cancer HCT116 cells, with IC_{50} values ranging from 0.87 to 42.73 $mol \cdot L^{-1}$ (Liu et al., 2016; Zhang et al., 2019). This supports the broad-spectrum antitumor activity of germacrane-type sesquiterpenoids from *C. cernuum*. Therefore, in-depth investigation of the chemical constituents of *C. cernuum*, an important ethnic medicine in Guizhou, not only enriches its chemical diversity but also provides a valuable source of compounds for antitumor drug research, laying a foundation for the comprehensive development of this medicinal plant.

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Note: Figure translations are in progress. See original paper for figures.