


 Cite this: *RSC Adv.*, 2020, 10, 32043

 Received 23rd May 2020
 Accepted 12th August 2020

DOI: 10.1039/d0ra04565a

rsc.li/rsc-advances

Neuroprotective constituents from the aerial parts of *Cannabis sativa* L. subsp. *sativa*†

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Five new compounds including three new cannabinoids, cannabissativas A–C (1–3), two new phenolic acids, (7Z,9Z)-cannabiphenolic acid A (4) and (8S,9Z)-cannabiphenolic acid B (5), together with twelve known compounds (6–17), were isolated from the aerial parts of *Cannabis sativa* L. subsp. *sativa*. The structures of 1–5 were established on the basis of extensive 1D, 2D NMR and HRESIMS analysis. The absolute configurations were determined by comparison between their experimental and calculated spectra of electronic circular dichroism (ECD) or the modified Mosher's method. The neuroprotective effects of the compounds 1–17 were evaluated on PC 12 cells. Compounds 12, 13 and 15 showed potential protective effects against H₂O₂-induced damage.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease characterized by impairment in progressive cognition and memory. The main pathological changes in the brains of AD patients include plaques from the deposition of amyloid- β (A β), neurofibrillary tangles induced by hyperphosphorylation of microtubule-associated protein-Tau protein, and neuronal degeneration or loss.^{1–3} The pathological mechanism of AD is too complicated to be clarified. Hypotheses including β -amyloidogenesis,⁴ cholinergic dysfunction,⁵ tau hyperphosphorylation,^{6,7} and oxidative stress^{8,9} have been proposed. Among them, oxidative stress injury was demonstrated to be associated with the aggregation of A β , the increase in hyperphosphorylation of tau, and neuronal degeneration.^{8,9} The present drugs in clinics can alleviate some clinical symptoms, but are unable to prevent the disease from progressing.¹⁰

Cannabis sativa L. subsp. *sativa* is a member of the genus *Cannabis* of Cannabaceae. The fruits of *C. sativa* are popular food of Bama Yao Autonomous County in Guangxi province, which is well known as “The Village of Longevity” in China. It has been indicated that long term intake of fruits of *C. sativa*

benefited for the health and longevity of local people.^{11,12} The extracts of *C. sativa* have been demonstrated to have analgesic, antiemetic, and anxiolytic activities.^{13–15} A number of chemical constituents, e.g., cannabinoids (CBDs), mono- and sesquiterpenes, steroids, flavonoids, and nitrogenous compounds, were reported from *C. sativa*.¹⁶ Early studies confirmed CBDs possessed anticonvulsant activity, analgesic and neuroprotective effects.^{17–19} CBDs produced neuroprotection through activating the receptors-mediated signal transduction pathways.²⁰ In our previous study, the ethyl acetate extracts from the aerial parts of *C. sativa* were proved to significantly improve the spatial learning and decrease memory impairment of dementia rats.²¹ To discover new anti-AD active constituents from the aerial parts of *C. sativa*, we conducted a deep investigation on the ethyl acetate extracts from the aerial parts of *C. sativa*. As a result, three new cannabinoids, two new phenolic acids, and twelve known compounds were isolated and structurally determined. In addition, compounds 1–17 were *in vitro* evaluated for their neuroprotective activities.

Results and discussion

Chromatographic separation of the EtOAc extracts from the aerial parts of *C. sativa* yielded five new compounds (1–5) and twelve known compounds (6–17), named Δ^9 -*trans*-tetrahydrocannabivarin (6),²² cannabinal (7),²³ cannabispiron (8),²⁴ erythrodiol (9),²⁵ oleanolic acid (10),²⁶ maslinic acid (11),²⁷ *p*-hydroxybenzaldehyde (12),²⁸ (*E*)-methyl *p*-hydroxycinnamate (13),²⁹ (*Z*)-methyl *p*-hydroxycinnamate (14),³⁰ ferulic acid (15),³¹ phylligenol (16),³² and skullcapflavone II (17)³³ (Fig. 1).

Cannabissativa A was obtained as yellowish oil. Based on HRESIMS, its molecular formula was determined as C₂₀H₂₈O₄ containing 7 degrees of unsaturation. The ¹H NMR spectrum

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/d0ra04565a



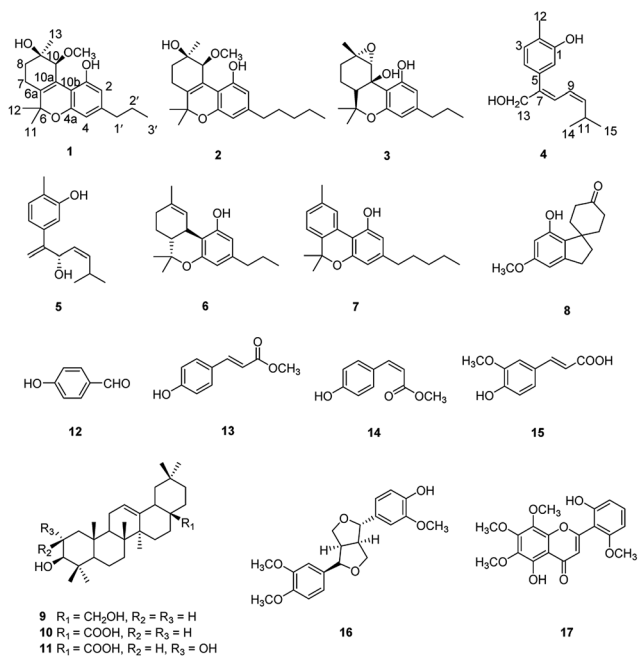


Fig. 1 Structures of the isolated compounds 1–17.

(Table 1) showed a phenolic hydroxyl at δ_{H} 9.40 (1H, br s), *meta*-coupled aromatic protons at δ_{H} 6.35 and 6.28 (each 1H, d, $J = 1.6$ Hz), a methoxy group at δ_{H} 3.34 (3H, s) and four methyl groups at δ_{H} 1.50, 1.41, 1.30 and 0.93. ^{13}C NMR and DEPT spectral analyses

revealed the presence of eight aromatic/olefinic carbons, two sp^3 quaternary carbons, one sp^3 methine, four sp^3 methylenes, four methyls and one methoxy (Table 1). The ^1H and ^{13}C NMR spectra of **1** were quite similar with those of cannabiteriol-C₃ (NMR data see Table 1), except that one additional methoxyl at δ_{C} 51.9 was present in **1**. It was further confirmed by the 2D NMR experiments. The ^1H - ^1H COSY correlations from H-2' to H-1' and H-3', and from H-7 to H-8 confirmed the substructures of C_{1'}-C_{2'}-C_{3'} and C₇-C₈ (Fig. 2). The key HMBC correlations of H-10/C-6a, C-10a, C-10b, C-8; H-7/C-6a; H-13/C-9; H-12/C-4a, C-6, and H-11/C-6a, C-6 permitted the establishment of a cannabiteriol structure. The methoxy and the C_{1'}-C_{2'}-C_{3'} unit was found to be attached to C-10 and C-3 according to HMBC correlation observed between 10-OCH₃/C-10 and H-1'/C-3, respectively.

The relative configuration of **1** ($9R^*$, $10S^*$) were supported by observed correlation of H-10/H-13 in ROESY spectrum. To determine the absolute configuration, ECD calculation method using time-dependent density functional theory (TDDFT) and metal rhodium salt method were both applied. The experimental ECD spectrum of **1** matched well with the calculated spectrum for the $9R,10S$ configuration (Fig. 3). The absolute configuration was also verified by testing CD difference spectrum after the reaction of C-9 hydroxyl group with metal rhodium salt.³⁴ The CD difference spectrum showed a negative Cotton effect at 350 nm (Fig. 4), suggesting the absolute configuration at C-9 was inferred to be *R*-form. Thus, **1** was identified as ($9R,10S$)-9-hydroxy-10-methoxy- $\Delta^{6a(10a)}$ -tetrahydrocannabiterin.

Table 1 NMR spectroscopic data of compounds 1–3 and cannabiteriol-C₃ (in CDCl₃)

No.	1		2		3		Cannabiteriol-C ₃	
	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)	δ_{C}
1		153.4		153.4		155.4		152.2
2	6.35, d (1.6)	111.2	6.36, d (1.6)	111.1	6.17, d (1.4)	109.4	6.34, d (1.6)	110.9
3		144.8		145.1		144.5		144.5
4	6.28, d (1.6)	108.7	6.28, d (1.6)	108.6	5.95, d (1.4)	108.3	6.30, d (1.6)	109.3
4a		153.5		153.5		153.7		153.7
6		76.3		76.3		75.7		76.6
6a		138.1		138.0	1.49, m	46.9		136.1
7	2.20, dd (19.3, 6.2) 2.45, m	22.1	2.20, dd (19.3, 6.0) 2.46, m	22.1	1.35, m 1.67, m	17.1	2.14, dt (19.0, 4.7) 2.39, m	22.6
8	1.75, m 1.89, dd (14.2, 7.3)	30.9	1.75, m 1.89, dd (14.2, 7.3)	30.9	1.81, ddd (14.5, 13.0, 4.6) 2.06, dt (14.5, 3.2)	29.7	1.78, m	29.1
9		70.1		70.1		61.6		70.5
10	4.27, br s	77.6	4.27, br s	77.6	3.87, s	66.3	4.19, br s	72.4
10a		117.8		117.8		68.7		122.2
10b		108.4		108.3		109.0		109.0
11	1.30, s	23.7	1.30, s	23.7	1.39, s	25.8	1.23, s	23.5
12	1.50, s	25.4	1.50, s	25.4	1.40, s	27.7	1.45, s	25.2
13	1.41, s	26.3	1.41, s	26.3	1.45, s	22.6	1.39, s	25.1
1'	2.45, m	37.7	2.46, m	35.6	2.27, m	37.6	2.45, t (7.2)	37.7
2'	1.60, m	23.8	1.58, m	30.4	1.48, m	23.7	1.60, m	23.9
3'	0.93, t (7.3)	13.9	1.30, m	31.5	0.87, t (7.3)	14.0	0.92, t (7.3)	13.8
4'			1.31, m	22.5				
5'			0.88, t (6.9)	14.0				
1-OH	9.40, br s		9.40, br s		7.38, br s		8.57, br s	
10-OCH ₃	3.34, s	51.9	3.34, s	51.9				

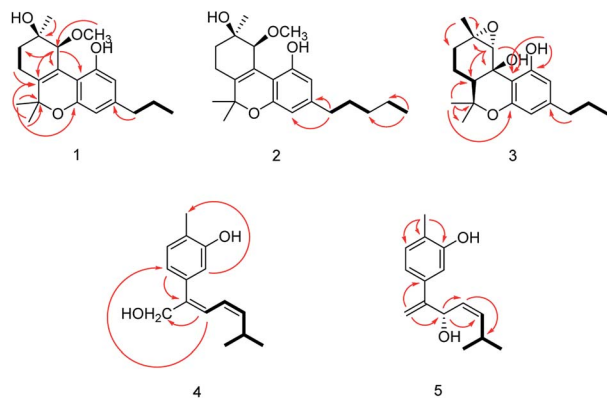


Fig. 2 Key ¹H-¹H COSY —, HMBC → correlations of compounds 1-5.

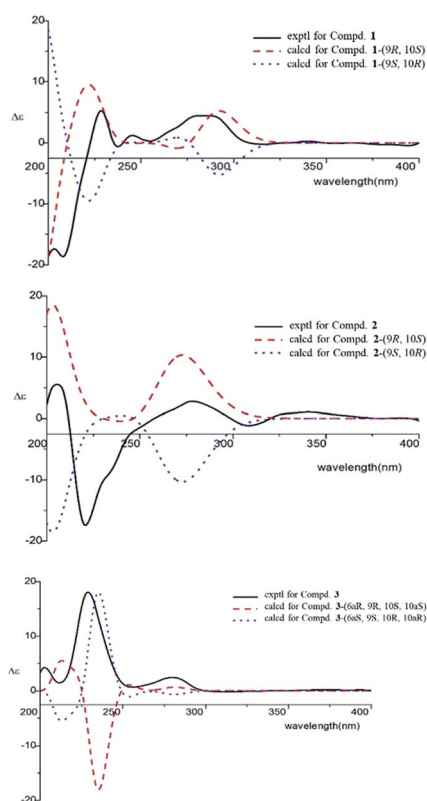


Fig. 3 ECD spectra for compounds 1-3.

Cannabisativa B was obtained as yellowish oil. The molecular formula of $C_{22}H_{32}O_4$ was assigned on the basis of HRESIMS ion peak at m/z 361.2383 $[M + H]^+$, requiring 7 degrees of unsaturation. Comparisons of the ¹H NMR and ¹³C NMR spectroscopic data of 2 with those of 1, additional δ_H 1.30 (2H, m), 1.31 (2H, m); δ_C 30.4, 31.5 were observed in 2. These signals were positioned at C-3 based on the ¹H-¹H COSY (H-5'/H-4'; H-2'/H-3', H-1') and HMBC (H-5'/C-3', C-4'; H-1'/C-3, C-4) correlations (Fig. 2). The configuration of 2 was determined as 9*R*,10*S* using the same method of 1 (Fig. 3). Accordingly, 2 was established as (9*R*,10*S*)-9-hydroxy-10-methoxy- Δ^6 (10*a*)-tetrahydrocannabinol.

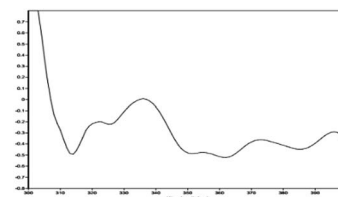


Fig. 4 The CD difference spectrum of $Rh_2(OCOCF_3)_4$ bind with compound 1.

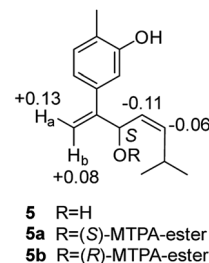
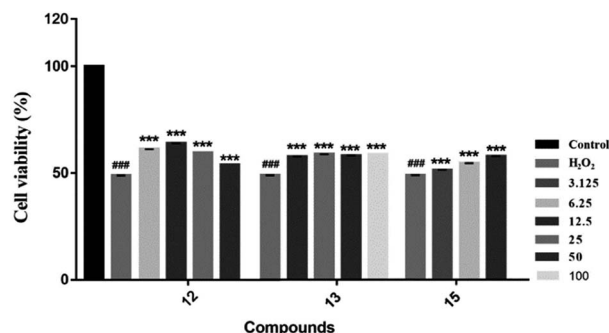
Cannabisativa C was obtained as yellowish oil, and showed an HRESIMS peak at m/z 317.1758 $[M-H]^-$, indicating a molecular formula of $C_{19}H_{26}O_4$ and 7 degrees of unsaturation. The ¹H NMR spectrum (Table 1) of 3 showed two *meta*-coupled aromatic protons (δ_H 6.17, 5.95 (each 1H, d, $J = 1.4$ Hz)), two sp^3 methine protons (δ_H 1.49, 3.87), four sets of methylenes and four methyls (δ_H 1.39, 1.40, 1.45 and 0.87). The ¹H-¹H COSY correlations from H-2' to H-1' and H-3', and from H-7 to H-8 and H-6a confirmed the substructures of $C_{1'}-C_{2'}-C_{3'}$ and $C_{6a}-C_7-C_8$ (Fig. 2). The key HMBC correlations of 1-OH/C-1, C-10b; H-10/C-6a, C-9, C-10a, C-10b; H-11/C-4a, C-6, C-6a, C-12; H-12/C-4a, C-6, C-6a, C-11; H-13/C-8, C-9, C-10 permitted the establishment of a cannabitril structure. The $C_{1'}-C_{2'}-C_{3'}$ unit was found to be attached to C-3 according to HMBC correlation observed between H-1'/C-3. In addition, the chemical shifts of C-9 and C-10 and the molecular formula of 3 indicated the presence of an oxirane ring between C-9 and C-10.

The relative configuration was established by NOESY experiment. Correlations of H-13/H-10, H-7b; H-7b/OH-10a suggested that they were situated in the axial position. Correlation of H-6a/H-8a suggested that they were in the equatorial position. Moreover, the calculated ECD spectrum of (6*aS*,9*S*,10*R*,10*aR*) were matched well with the experimental ECD spectrum of 3 (Fig. 3). Therefore, the structure of 3 was defined as (6*aS*,9*S*,10*R*,10*aR*)-9,10-epoxy-10a-hydroxy-tetrahydrocannabivarin.

Compound 4 was obtained as yellowish oil and had a molecular formula of $C_{15}H_{20}O_2$ as judged from HRESIMS m/z 231.1380 $[M-H]^-$ (calcd. for $C_{15}H_{19}O_2$ 231.1385), indicative of 6 degrees of unsaturation. The ¹³C NMR (Table 2) showed three methyls, one methylene, seven methines and four quaternary carbons. In the ¹H NMR spectrum, the characteristic signals indicated a 1,3,4-trisubstituted benzene ring at δ_H 7.11 (1H, d, $J = 7.7$ Hz), 6.76 (1H, br d, $J = 7.7$ Hz), 6.71 (1H, br s), two double bonds at δ_H 6.58 (1H, d, $J = 11.5$ Hz), 5.95 (1H, dd, $J = 11.5, 11.2$ Hz), 5.28 (1H, dd, $J = 11.2, 10.3$ Hz), a hydroxymethyl group at δ_H 4.40 (2H, m) and three methyl groups at δ_H 2.25 (3H, s), 1.00 (3H, d, $J = 6.6$ Hz) and 0.99 (3H, d, $J = 6.6$ Hz). The ¹H-¹H COSY correlations of H-11/H-10/H-14 (H-15), and H-9/H-8/H-10 combined with HSQC confirmed the $C_8-C_9-C_{10}-C_{11}-C_{14}(C_{15})$ moiety (Fig. 2). HMBC correlations from H-4 to C-7, H-12 to C-1, 2 and 3, and H-13 to C-5 and C-8 (Fig. 2) confirmed the planar structure. The geometry of Δ^7 double bond was determined by NOE difference spectra. Irradiation of H-13 resonance did not lead to a marked enhancement of the H-8 proton signal, and irradiation of the

Table 2 NMR spectroscopic data of compounds 4–5 (in CDCl₃)

No.	4		5	
	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)	δ_{C}
1		153.7		153.9
2		123.1		123.7
3	7.11, d (7.7)	131.0	7.04, d (7.6)	130.8
4	6.76, br d (7.7)	121.3	6.85, br d (7.6)	119.2
5		137.0		138.5
6	6.71, br s	115.5	6.87, br s	113.8
7		140.5		150.4
8	6.58, d (11.5)	122.4	5.36, overlap	69.9
9	5.95, dd (11.5, 11.2)	122.6	5.31, overlap	127.7
10	5.28, dd (11.2, 10.3)	141.6	5.29, overlap	140.6
11	2.88, m	27.0	2.62, br s	27.2
12	2.25, s	15.5	2.21, s	15.6
13	4.40, s	67.9	5.34, overlap	112.9
			5.26, br s	
14	1.00, d (6.6)	23.1	0.97, d (6.6)	23.2
15	0.99, d (6.6)	23.1	0.79, d (6.6)	22.6

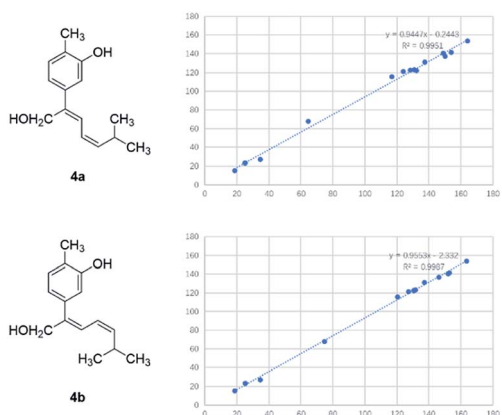
Fig. 6 $\Delta\delta$ ($\delta_{5a} - \delta_{5b}$ in ppm) obtained for the MTPA esters of compound 5.Fig. 7 Neuroprotective effects of compounds against H₂O₂-induced cell growth inhibition of PC12 cells. In the presence or absence of the tested compounds at different concentrations, MTT assay was used to examine the cell viability after H₂O₂ (200 μ M) treatment for 4 h. *** P < 0.001 vs. H₂O₂-treated group; ### P < 0.001 was considered statistically significant when compared with its enantiomer.

H-8 also did not cause an enhancement of the H-13. Next, we used ¹³C NMR calculation as well as DP4+ probability analyses to determine the geometry of Δ^7 double bond. The ¹³C NMR chemical shifts of **4a** and **4b** were calculated at the B3LYP/6-311 + G(d,p) level utilizing the polarizable continuum model (PCM) in methanol. The calculated results for **4b** ($R^2 = 0.9987$) were a better match with the experimental data than those of **4a** ($R^2 = 0.9951$) (Fig. 5). Moreover, according to the DP4+ probability analyses, **4b** was assigned with a 100% probability (Fig. S41†). Finally, the structure of compound 4 was identified and named as (7*Z*,9*Z*)-cannabiphenolic acid A.

Compound 5, obtained as yellowish oil, displayed an HRE-SIMS peak at m/z 231.1387 [M-H]⁻ corresponding to the molecular formula C₁₅H₂₀O₂, suggesting 6 degrees of unsaturation. Its ¹³C NMR and DEPT spectra (Table 2) displayed 15 carbon resonances, including three methyls, one methylene, seven methines and four quaternary carbons. The ¹H NMR spectrum showed an 1,3,4-trisubstituted benzene ring at δ_{H} 7.04 (1H, d, $J = 7.6$ Hz), 6.87 (1H, br s), 6.85 (1H, br d, $J = 7.6$ Hz), a double bond at δ_{H} 5.31 (1H, overlap) and 5.29 (1H, overlap), a terminal double

bond at δ_{H} 5.34 (1H, overlap) and 5.26 (1H, br s), and three methyl groups at δ_{H} 2.21 (3H, s), 0.97 (3H, d, $J = 6.6$ Hz) and 0.79 (3H, d, $J = 6.6$ Hz). The C₁₀-C₁₁-C₁₄(C₁₅) moiety was verified by the ¹H-¹H COSY correlations of H-11/H-10/H-14/H-15 (Fig. 2). The key HMBC (Fig. 2) correlations from H-8 to C-9, and C-10, H-9 to C-11, H-12 to C-1, C-2, and C-3, and H-13 to C-5, and C-8 connected the planar structure of compound 5. The geometry of Δ^9 was assigned as *Z* by the coupling constant ($J_{9,10} = 8.6$ Hz) in pyridine-*d*₅ (400 MHz). The absolute configuration of C-8 was identified by a modified Mosher's method (Fig. 6). Compound 5 was treated separately with (*R*)- and (*S*)-MTPA-Cl to obtain the respective (*S*)- and (*R*)-MTPA esters (**5a** and **5b**). The *S* configuration of C-8 was determined by the $\Delta\delta_{\text{H}(\text{S-R})}$ value of H-9, H-10, H-13a and H-13b. Based on the above findings, compound 5 was named as (8*S*,9*Z*)-cannabiphenolic acid B.

We evaluated the cytotoxicity of compounds 1–17 against PC12 cells using MTT method. Neuroprotective assays were performed at concentrations that had no significant effect on cell survival (compounds 1–5, 7, 8 and 12–16, cell survival rate > 90%) (Table S1†). We found that the novel compounds 1–5 can slightly improve the cell viability, but the significant difference is not obvious. Known compounds 12, 13 and 15 could attenuate H₂O₂-induced cytotoxicity in PC12 cells by the effect of antioxidant (Fig. 7).

Fig. 5 ¹³C NMR calculation results of two possible isomers of 4.

Conclusions

In summary, three new cannabinoids, cannabistativas A–C (1–3), two new phenolic acids, (7*Z*,9*Z*)-cannabiphenolic acid A (4) and (8*S*,9*Z*)-cannabiphenolic acid B (5), along with twelve known compounds (6–17), were identified from the aerial parts of *Cannabis sativa* L. subsp. *sativa*. All of the compounds were screened for their neuroprotective activity. The results indicated that compounds 1–5, can slightly improve the cell viability and 12, 13 and 15 showed potential protective effects against H₂O₂-induced damage.

Experimental section

General experimental procedures

UV spectra were recorded on a Shimadzu UV-1700 PharmaSpec UV-visible spectrophotometer. The ¹H, ¹³C, and 2D nuclear magnetic resonance (NMR) spectra were measured by a Bruker AVANCE-600 NMR spectrometer (Rheinstetten, Germany) with tetramethylsilane (TMS) as an internal standard. HRESIMS data were acquired using a Waters Synapt G2 QTOF mass spectrometer (Milford, CT, USA). ECD spectra were taken on a Biologic MOS-450. Optical rotations were measured using a JASCO VP-1020.

For column chromatography (CC), silica gel (100–200 and 200–300 mesh, Qingdao, China), Sephadex LH-20 (Uppsala, Sweden) and ODS (60–80 μm, Tokyo, Japan) was used. The analytical HPLC was obtained with an Agilent 1200 (CA, USA) with a DAD detector using a reversed-phase C18 column (5 μm, 250 × 4.60 mm). Semi-preparative HPLC was performed on a Shimadzu LC-6AD (Kyoto, Japan) equipped with a UV SPD-20A detector using a reversed-phase C18 column (5 μm, 250 × 10 mm).

Plant material

The *Cannabis sativa* L. subsp. *sativa* were collected from Bama Yao Autonomous County, Guangxi Province, China, in December 2014, and identified by Professor Liying Yu (Guangxi Botanical Garden of Medicinal Plants, Guangxi, China). A voucher specimen (YWGCS-2014) was deposited at the School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University, China.

Extraction and isolation

The dried aerial parts of *C. sativa* (20 kg) were extracted with 70% EtOH (200 L × 2) for 2 h to afford a crude extract (449.2 g), which was suspended in H₂O (5 L) and then partitioned with EtOAc (5 L × 3) and *n*-BuOH (5 L × 3). The EtOAc soluble extract (121.4 g) was subjected to CC over silica gel eluted with cyclohexane/EtOAc (from 100 : 0 to 0 : 100) to afford Fr. EA–EL.

Fr. EC (3.2 g) was subjected to fractionation on ODS column using a stepwise gradient of MeOH–H₂O to give Fr. EC1–EC7. Fr. EC2 (245.1 mg) was further purified by preparative TLC (cyclohexane–EtOAc, 8 : 2) and semi-preparative HPLC using 60% MeOH to afford compound 1 (6.6 mg) and 2 (3.5 mg). Fr. EC4 (723.2 mg) was purified by semi-preparative HPLC (71% MeOH) to yield compound 3 (12.8 mg) and 6 (189.0 mg).

Compound 7 (57.2 mg) was afforded from Fr. EC5 (67.5 mg) by purification with semi-preparative HPLC (80% MeOH). Fr. EG (2.4 g) was loaded onto a Sephadex LH-20 column to yield Fr. EG1–EG3. Fr. EG2 (483.3 mg) was subjected to ODS column eluted with the gradient solvent system of MeOH/H₂O, and Fr. EG25 (123.8 mg, 90% MeOH) was purified using semi-preparative HPLC (80% MeOH) to furnish compound 9 (23.7 mg). Fr. EG3 (1.2 g) was divided into two subfractions by ODS gel CC eluted with MeOH/H₂O (from 30% to 50%). Fr. EG31 (62.8 mg) was subjected to purification by a semi-preparative HPLC using 25% MeOH–H₂O to afford compound 12 (31.8 mg). Fr. EG32 (857.2 mg) was subjected to purification by a semi-preparative HPLC using 50% MeOH to afford compound 13 (641.0 mg) and compound 14 (11.8 mg). Fr. EH (2.7 g) was fractionated on CC of Sephadex LH-20 (CH₂Cl₂–MeOH, 1 : 1) to give Fr. EH1–EH3. Fr. EH2 (1.3 g) further separated to five fractions by CC of silica gel. Fr. EH22 (1.0 g, eluted with cyclohexane–EtOAc, 9 : 1) was subjected to ODS CC using 90% MeOH and then purified by semi-preparative HPLC (80% MeOH) to give compound 10 (32.1 mg). Fr. EH3 (506.6 mg) was purified by ODS CC, then Fr. EH323 (109.0 mg, eluted with 50% MeOH) was purified by semi-preparative HPLC (57% MeOH) to give compound 8 (81.2 mg). Fr. EH324 (108.4 mg, eluted with 60% MeOH) was purified by semi-preparative HPLC (55% MeOH) to give compound 5 (5.6 mg). Fr. EI (5.8 g) was applied to a Sephadex LH-20 column using CH₂Cl₂–MeOH (1 : 1) as the eluent to gain two subfractions. Fr. EI2 (4.7 g) was fractionated over silica gel CC to give five subfractions. Fr. EI23 (1.0 g, eluted with cyclohexane–EtOAc, 8 : 2) was further subjected to an ODS column and separated by semi-preparative HPLC (35% MeOH) to afford compound 4 (5.6 mg). Fr. EI24 (2.5 g, eluted with cyclohexane–EtOAc, 7 : 3) was further subjected to an ODS column and separated by semi-preparative HPLC (70% MeOH) to afford compound 17 (110.8 mg). Fr. EK (4.5 g) was subjected to fractionation on Sephadex LH-20 column to yield Fr. EK1–EK3. Fr. EK2 (2.3 g) was fractionated over silica gel CC to give seven subfractions. Fr. EK25 (1.0 g eluted with cyclohexane–EtOAc, 7 : 3) was subjected to ODS CC. Fr. EK253 (392.8 mg, 40% MeOH) was purified by semi-preparative HPLC (42% MeOH) to give compound 16 (218.3 mg). Fr. EK256 (174.7 mg, 50% MeOH) was purified by semi-preparative HPLC (70% MeOH) to give compound 11 (36.1 mg). Fr. EK3 (3.2 g) was fractionated over silica gel CC and further separated by ODS CC and purified by semi-preparative HPLC (20% MeOH) to give compound 15 (92.9 mg).

Cannabistativa A (1). Yellowish oil; $[\alpha]_D^{20} -21.0$ (*c* 1.0, MeOH); UV (MeOH) λ_{\max} (log ϵ): 228 (3.47) nm, 279 (3.18) nm; ¹H and ¹³C NMR data see Table 1; HRESIMS *m/z* 333.2071 [M + H]⁺ (calcd 333.2066, C₂₀H₂₉O₄).

Cannabistativa B (2). Yellowish oil; $[\alpha]_D^{20} -23.0$ (*c* 1.0, MeOH); UV (MeOH) λ_{\max} (log ϵ): 228 (3.43) nm, 279 (3.10) nm; ¹H and ¹³C NMR data see Table 1; HRESIMS *m/z* 361.2383 [M + H]⁺ (calcd 361.2379, C₂₂H₃₃O₄).

Cannabistativa C (3). Yellowish oil; $[\alpha]_D^{20} +35.0$ (*c* 1.0, MeOH); UV (MeOH) λ_{\max} (log ϵ): 230 (3.33) nm, 282 (3.80) nm; ¹H and

^{13}C NMR data see Table 1; HRESIMS m/z 317.1758 $[\text{M}-\text{H}]^-$ (calcd 317.1753, $\text{C}_{19}\text{H}_{25}\text{O}_4$).

(7Z,9Z)-Cannabiphenolic acid A (4). Yellowish oil; UV (MeOH) λ_{max} (log ϵ): 247 (2.92) nm, 284 (2.62) nm; ^1H and ^{13}C NMR data see Table 2; HRESIMS m/z 231.1380 $[\text{M}-\text{H}]^-$ (calcd 231.1385, $\text{C}_{15}\text{H}_{19}\text{O}_2$).

(8S,9Z)-Cannabiphenolic acid B (5). Yellowish oil; UV (MeOH) λ_{max} (log ϵ): 226 (3.14) nm, 275 (2.99) nm; ^1H and ^{13}C NMR data see Table 2; HRESIMS m/z 231.1387 $[\text{M}-\text{H}]^-$ (calcd 231.1385, $\text{C}_{15}\text{H}_{19}\text{O}_2$).

ECD calculations

The absolute configurations of compounds **1–3** were determined by using time-dependent density functional theory (TDDFT) calculations carried out with the Gaussian 09 package. First, they were built in GaussianView and subjected to systematic conformational search by CONFLEX. Conformations whose energy was within 3 kcal mol $^{-1}$ from the conformation with the lowest energy were selected for subsequent calculations. Next, the geometries of the compounds were optimized at the B3LYP/6-31G (d) level and the ECD of the conformers was calculated at the B3LYP/6-311++G (2d, p) level with the CPCM solvation model, where MeOH was used as the solvent to match the experimental conditions. The calculated ECD curve was generated using SpecDis 1.51 and compared with the experimental ECD curve to determine their absolute configurations.

Metal rhodium salt method

1.0 mg of compound **1** was dissolved in a dry solution of the stock $[\text{Rh}_2(\text{OCOCF}_3)_4]$ complex (6.0 mg) in CH_2Cl_2 (200 mL) and tested its CD spectrum immediately, then obtained the CD spectrum of Compd-Rh-CD1. After the reaction, the CD spectrum was tested again, and the CD spectrum of Compd-Rh-CD2 was obtained. After subtracting Compd-Rh-CD1 from Compd-Rh-CD2, we got the CD difference spectrum, and observed the Cotton effect at 350 nm.

NMR calculations

The plausible conformers of compounds **4a** and **4b** were performed by Gaussian 09 software. All obtained conformers were subsequently optimized at the B3LYP/6-31+G(d) level in a methanol solvent model.³⁵ The Boltzmann-weighted conformer population was calculated by the Gibbs free energy from the geometry optimization step. Then, Boltzmann-weighted averages of the chemical shifts were calculated to scale them against the experimental values. The DP4+ probability was applied to compute the chemical shift errors.

Modified Mosher's method

Compound **5** (0.5 mg) was reacted with *R*-MTPA-Cl (10 μL) in pyridine (0.5 mL) under the protection of nitrogen. The mixture was heated at 50 $^\circ\text{C}$ for 4 hours to obtain *S*-MTPA ester. Using the same procedure as described above to obtain the *R*-MTPA ester. ^1H -NMR was used to analyze the absolute configuration of the compound.

S-MTPA ester of 5. ^1H NMR (400 MHz, pyridine- d_5) δ 5.58 (br s, H-13), 5.55 (br s, H-13), 5.49 (overlap, H-10), 5.40 (overlap, H-9).

R-MTPA ester of 5. ^1H NMR (400 MHz, pyridine- d_5) δ 5.55 (overlap, H-10), 5.51 (overlap, H-9), 5.47 (br s, H-13), 5.45 (br s, H-13).

Cell culture

PC 12 cells (ATCC, Manassas, VA, USA) were cultured on RPMI-1640 (Roswell Park Memorial Institute) medium with 10% fetal bovine serum, 2 mM l-glutamine, 100 units per mL penicillin and 0.1 mg mL $^{-1}$ streptomycin in a humidified incubator at 37 $^\circ\text{C}$ and in 5% CO_2 .

Neuroprotective activity assay

The PC12 cells were seeded in 96-well plates at a density of 1×10^5 cells per mL in 90 μL of medium for 24 h. Then treated with different concentrations of compounds or H_2O_2 (200 μM) for 24 h. Cell viability was estimated by MTT colorimetric assay. 10 μL of MTT (5 mg mL $^{-1}$) was added to each well for 4 h culture. Subsequently, the medium was removed and the formazan crystals were dissolved by dimethyl sulfoxide. The absorbance of formazan solution was measured at 490 nm (Bio-Rad Model 680, Bio-Rad, Hercules, CA, USA).

Conflicts of interest

The authors declare no competing financial interest.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant No. 31970374) and the China Postdoctoral Science Foundation (Grant No. 2016M602697, No. 2018T110988).

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