SUPPLEMENTARY MATERIAL

Nucleosides and amino acids, isolated from Cordyceps sinensis, protected against cyclophosphamide-induced myelosuppression in mice

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The material basis of *Cordyceps sinensis (Berk.) Sacc* has not yet been well understood and natural *C. sinensis* resources are very rare. The present study aimed to clarify the substance basis and compare the protective effect of natural and artificially-cultivated *C. sinensis* against cyclophosphamide (CTX)-induced myelosuppression. Both natural and artificially-cultivated *C. sinensis* effectively improved CTX-induced decrease of peripheral blood counts and hemopoietic growth factors, pathological changes, and apoptosis of bone marrow. Importantly, artificially-cultivated *C. sinensis* showed similar capacity compared with natural *C. sinensis*. Uridine (1), adenosine (2), L-pyroglutamic acid (3), lysinonorleucine (4), 1,3,5-trimethoxybenzene (5), D-mannitol (6), L-pyroglutamic acid methyl ester (7), tryptophan (8), and phenylalanine (9) were isolated from bioactivity-guided fraction and identified to attenuate CTX-induced myelosuppression in mice. In conclusions, nucleosides and amino acids represented the effective chemical components in *C. sinensis*. Artificial cultivation can be used as an effective substitute for natural *C. sinensis.*

Keywords: natural *C. sinensis*, artificially-cultivated *C. sinensis*, cyclophosphamide, myelosuppression

Experimental section

1 Materials

Natural and artificially-cultivated *Cordyceps sinensis* (Lot: B1810A, B1810B) were provided by Dongguan Dongyangguang Cordyceps R&D Co., Ltd. (Dongguan, China). They were identified by Prof. Xiaochi Ma and were deposited in the Department of Medicinal Chemistry, Dalian Medical University. CTX (Lot: M0915A), adenosine (Lot: M0515A), uridine (Lot: J0701A), mannitol (Lot: MB3542), tryptophan (Lot: D1010A), phenylalanine (Lot: A0725A), and pyroglutamic acid (Lot: M0108A) were purchased from Dalian Meilun Biotechnology Co., Ltd. (Dalian, China). Diyu Shengbai Tablets (Lot: 170704) were purchased from Chengdu Di'ao Group Tianfu Pharmaceutical Co., Ltd. (Chengdu, China). Erythropoietin (EPO, Lot: P125ZH5INR), Thrombopoietin (TPO, Lot: 5974RF3YVE), and Granulocyte colony-stimulating factor (G-CSF, Lot: TLR8FM9PBE) ELISA kit were purchased from Elabscience Biotechnology co., ltd. (Wuhan, China).

2 Animals

Male Kunming mice (6-8 weeks, 18-25 g) were provided by the SPF Experimental Animal Center of Dalian Medical University [certificate serial number: SCXK (Liao) 2018-0003]. The mice were kept in an environmentally controlled room (23–25°C, 45–65% relative humidity and 12-hr light/dark cycle) with free access to standard mice food and pure water. The animal experiments were performed in accordance with the care and use of laboratory animals and were approved by the ethics committee of Dalian Medicine University. The animals were housed for 1 week before the experiments.

3 Preparation of C. sinensis extract

Natural and artificially-cultivated *C. sinensis* were crushed, screened, and then suspended in water and used for administration.

To track the active fraction, the powders of artificially-cultivated *C. sinensis* were successively extracted with petroleum ether, ethanol, and water. The solvent was removed to afford the extract of active fractions.

To isolate the chemical composition, the powders of artificially-cultivated *C. sinensis* were extracted with 50% EtOH for 3 times and 2 h each time, and removed the solvent to afford the residue. The residue was subjected to silica gel column chromatography and eluted with a solution of CH_2Cl_2-MeOH (from 100:1 to 1:1), resulting in the production of eight fractions (C1-C8). Fraction C3 was separated by a silica gel column eluted with CH_2Cl_2-MeOH (from 50:1 to 1:1) to obtain nine subfractions (C31-C39). Purification of subfractions C34 and C39 through preparative HPLC (10% MeOH) afforded compounds 1, 2, and 7. Separation of fraction C5 by silica gel column chromatography (CH_2Cl_2-MeOH, from 50:1 to 1:1) yielded eight subfractions (C51-C58). Subfractions C51 and C52 were isolated by preparative HPLC (5%-10% MeOH) to afford compounds 3, 4, and 6. In addition, compounds 5, 8, and 9 were isolated from fraction C7 through silica gel column chromatography (CH_2Cl_2-MeOH, from 50:1 to 1:1) and preparative HPLC (15%-28% MeOH) (Supplementary Figure 7).

4 Animal treatment

Preliminary evaluation of natural and artificially-cultivated *C. sinensis*. Mice were

randomly divided into 9 groups (n = 6), including (1) control group, (2) CTX group, (3) positive control group (Diyu Shengbai Tablets, 200 mg/kg), (4-6) natural *C. sinensis* treatment groups with low, middle, or high dosage (25, 50, 100 mg/kg), (7-9) artificially-cultivated *C. sinensis* treatment groups with low, middle, or high dosage (25, 50, 100 mg/kg). Mice were orally administered vehicle (water), Diyu Shengbai Tablets, natural or artificially-cultivated *C. sinensis* (suspended in water, 0.1 ml/10 g body weight) for 10 days. From the 8th to 10th day, mice were intraperitoneally injected with saline or CTX (100 mg/kg, dissolved in saline, 0.1 ml/20 g) once a day to induce myelosuppression. Mice were observed for the mortality, behavioral changes, and changes in body weight. All the mice were killed by cervical dislocation after 10 days of drug administration.

Evaluation of *C. sinensis* fractions by different extract solvents. Mice were randomly divided into 6 groups (n = 6), including (1) control group, (2) CTX group, (3) petroleum ether extract group (16 mg/kg), (4) ethanol extract group (3.7 mg/kg), (5) water extract group (30.5 mg/kg), (6) residue group (43.6 mg/kg). Mice were orally administered vehicle (water), petroleum ether extract, ethanol extract, water extract, or residue of *C. sinensis* (suspended in water, 0.1 ml/10 g body weight) for 10 days. The dosage of each extract was equal to 100 mg/kg of *C. sinensis*. From the 8th to 10th day, mice were intraperitoneally injected with saline or CTX (100 mg/kg, dissolved in saline, 0.1 ml/20 g) once a day.

Evaluation of active components isolated from *C. sinensis*. Mice were randomly divided into 8 groups (n = 6), including (1) control group, (2) CTX group, (3)

Adenosine group (50 mg/kg), (4) Uridine group (50 mg/kg), (5) Mannitol group (50 mg/kg), (6) Tryptophan group (50 mg/kg), (7) Phenylalanine group (50 mg/kg), (8) Pyroglutamic acid group (50 mg/kg). Mice were orally administered vehicle (water), or components (suspended in water, 0.1 ml/10 g body weight) for 10 days. From the 8th to 10th day, mice were intraperitoneally injected with saline or CTX (100 mg/kg, dissolved in saline, 0.1 ml/20 g) once a day.

5 Determination of peripheral hemogram

After sacrifice, blood was collected and the counts of white blood cells (WBC), red blood cells (RBC), hemoglobin (HGB), platelets and lymphocytes (%) (LYM%) were analyzed using a BC-6800 Plus automatic hematology analyzer (Mindray, Shenzhen, China).

6 Determination of organ index

After sacrifice, spleen and thymus weights and body weight were measured. Thymus and spleen indices were expressed as relative organ weight.

7 Determination of serum levels of hemopoietic growth factors

EPO, TPO, and G-CSF levels were measured by ELISA kits (Elabscience biotechnology co., Ltd, China) according to the manufacturer's instructions.

8 Bone marrow cell count and apoptosis detection

Left femurs were removed and soaked into RPMI-1640 medium. Bone marrow cells were obtained by bone marrow puncture and PBS flushing. After filtering with a sieve, the red blood cell lysate was added and mixed evenly, and stood for 5 min. The single bone marrow cell suspension was centrifuged at 1000 r/min for 5 min. The cells were

then washed and resuspended in pre-cooled PBS. A 50 μL aliquot of cell suspension was stained with trypan blue and counted under a light microscope. The remaining cells were stained with AnnexinV/PropidiumIodide for apoptosis detection by flow cytometry.

9 H&E staining of femur

Right femurs were fixed in 4% paraformaldehyde, decalcified, and embedded in paraffin and then sectioned at a thickness of 4 μm. After stained with hematoxylin & eosin (HE), the slices were then observed under a light microscope.

10 Quantitative determination of active components

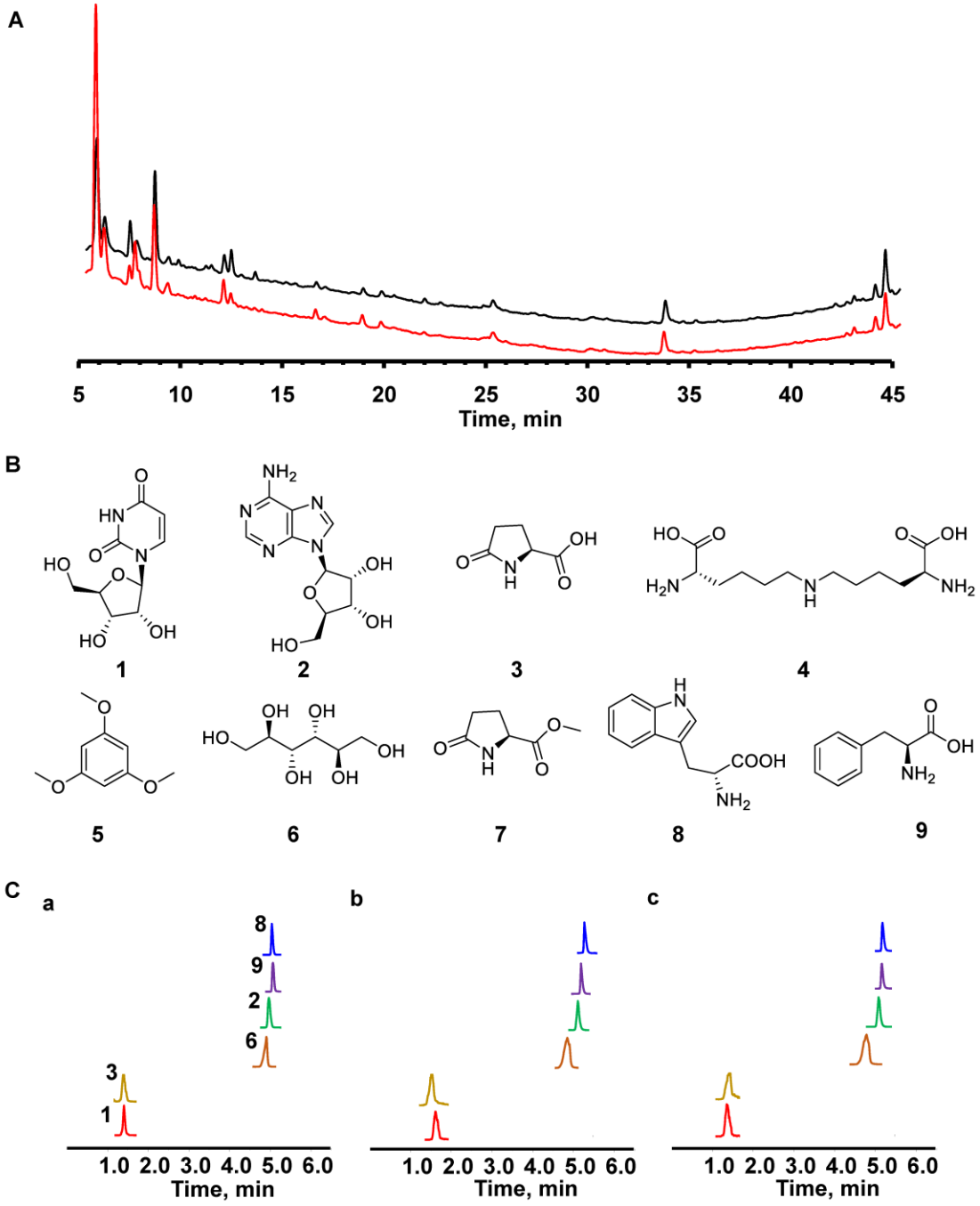
Adenosine, uridine, mannitol, tryptophan, phenylalanine, and pyroglutamic acid in natural and artificially-cultivated *C. sinensis* were determined by liquid chromatography tandem mass spectrometry (LC-MS/MS, AB SciexQtrap® 5500 LC-MS/MS system, USA). Chromatographic separation was performed on a Welch Ultimate HILIC Silica column (150 × 2.1 mm, 5.0 μm) at 30 °C. The mobile phase was composed of A (water containing 0.1% formic acid) and B (acetonitrile) with a gradient elution of 100% B at 0–2 min, 10% B at 5–7 min, 100% B at 8–10 min. The flow rate was 0.4 mL/min. The detection was performed by multiple reaction monitoring (MRM) in negative and positive ionization modes. The source parameters were set as follows: Ion spray voltage, -4.5 kV for negative ionization mode and 5.5 kV for positive ionization mode; Temperature, 500 °C; Gas source 1, 40 psi; Gas source 2, 50 psi; curtain gas, 30 psi; CAD, Medium. The MRM transitions and the related optimized declustering potential (DP), entrance potential (EP), collision

energy (CE), and collision cell exit potential (CXP) for the different analytes are listed in Supplementary Table 2. Analyst 1.6.3 software (Applied Biosystems, USA) was used to control the equipment and for data acquisition and analysis.

11 Statistical Analysis

All data were expressed as mean ± SEM. Significant differences were analyzed using one-way ANOVA. Statistical difference was considered significant when $P < 0.05$.

Supplementary Figures



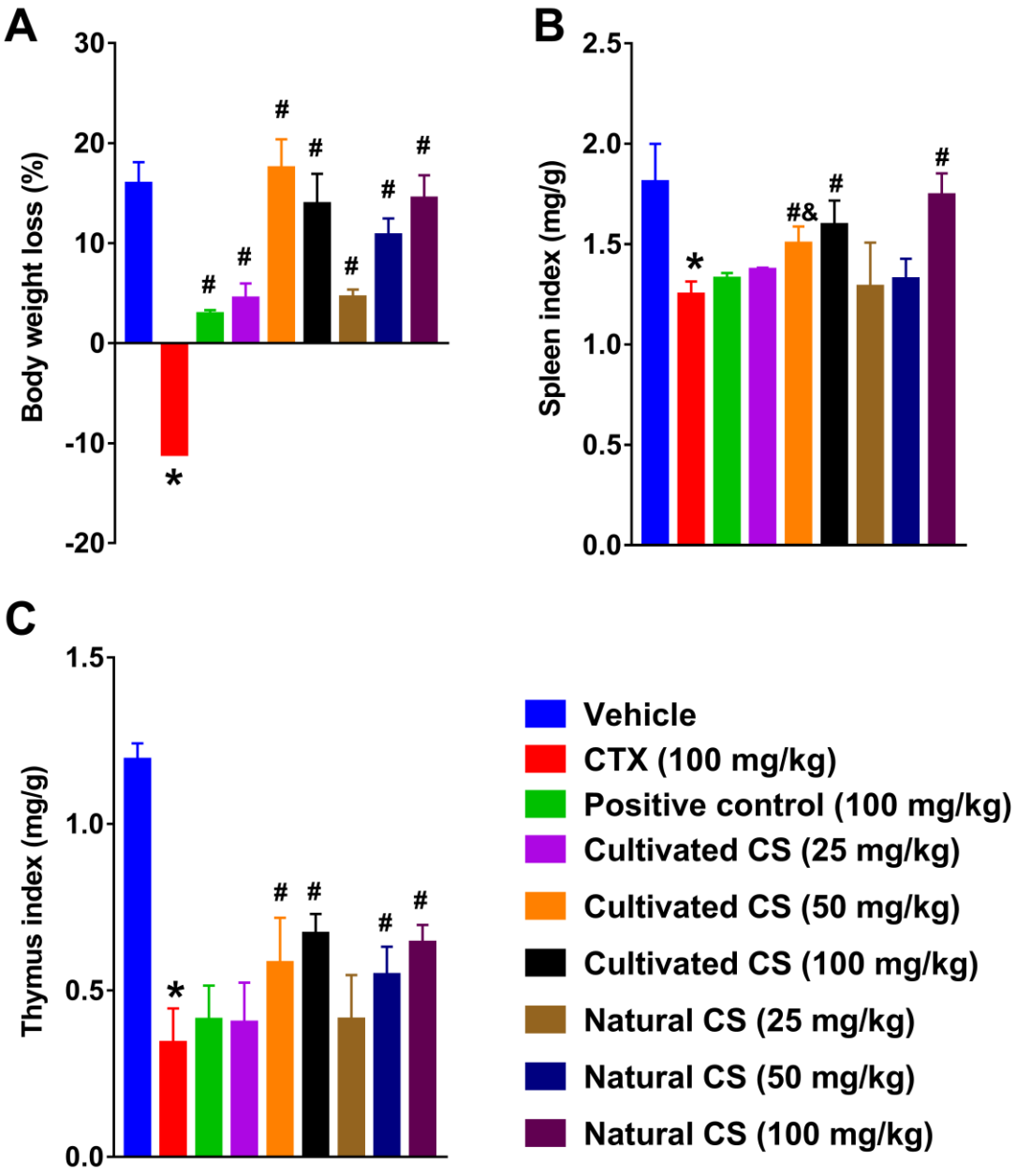
Supplementary Fig. 1 Chemical composition *C. sinensis*

A, Typical HPLC chromatogram of natural and artificially-cultivated *C. sinensis*. Blank line, natural *C. sinensis*. Red line, artificially-cultivated *C. sinensis*.

B, Chemical Structure of active components isolated from *C. sinensis*.

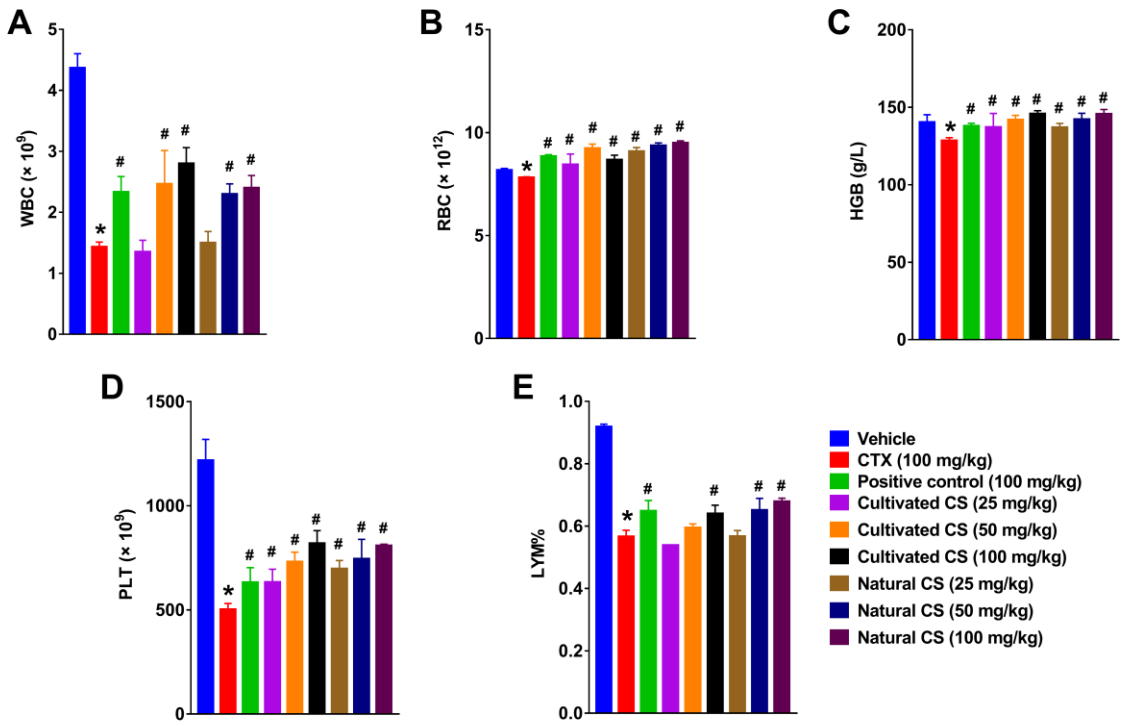
C, LC-MS/MS profile of active components isolated from *C. sinensis*. a, standard references. b, natural *C. sinensis*. c, artificially-cultivated *C. sinensis*. 1, uridine. 2,

adenosine. 3, L-pyroglutamic acid. 6, D-mannitol. 8, tryptophan. 9, phenylalanine.



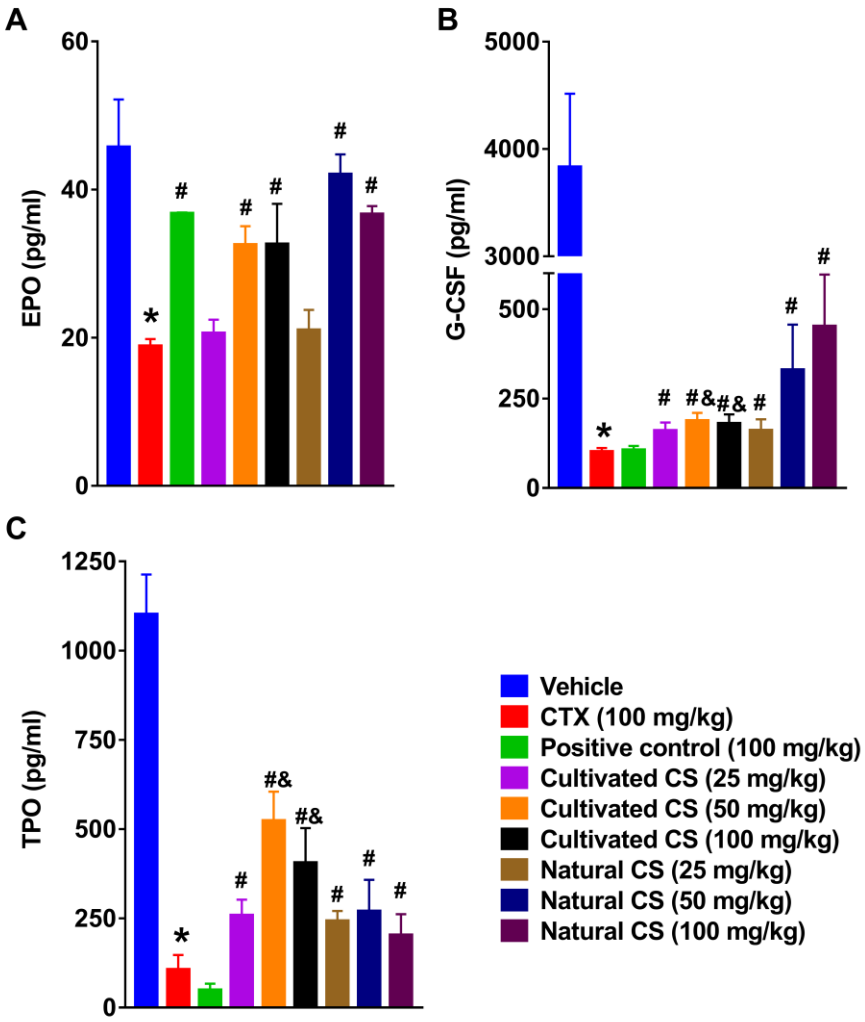
Supplementary Fig. 2 Effects of natural and artificially-cultivated *C. sinensis* on body weight, thymus and spleen index in CTX-induced mice

A, body weight. B, thymus index. C, spleen index. *, $p < 0.05$ compared with control group. #, $p < 0.05$ compared with CTX (100 mg/kg) group. &, $p < 0.05$ compared with natural *C. sinensis* group at equal dose. Data represented the mean ± S.E. (n=6). CS, *C. sinensis*.



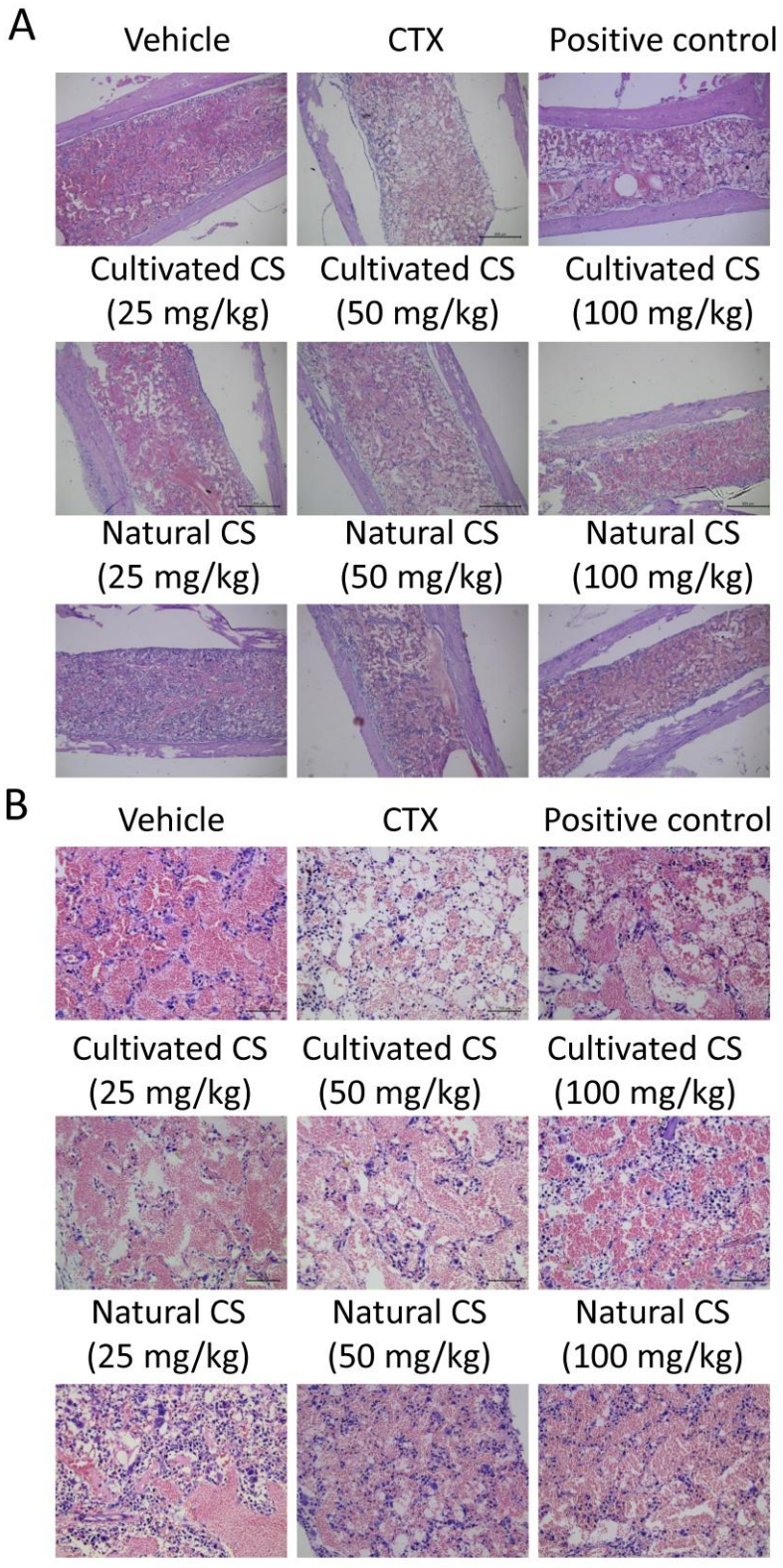
Supplementary Fig. 3 Effects of natural and artificially-cultivated *C. sinensis* on the counts of WBC, RBC, HGB, PLT and LYM% in CTX-induced mice

A, WBC. B, RBC. C, HGB. D, PLT. E, LYM%. *, $p < 0.05$ compared with control group. #, $p < 0.05$ compared with CTX (100 mg/kg) group. Data represented the mean ± S.E. (n=6). CS, *C. sinensis*. WBC, white blood cells. RBC, red blood cells, HGB, hemoglobin. PLT, platelets. LYM%, lymphocytes (%).



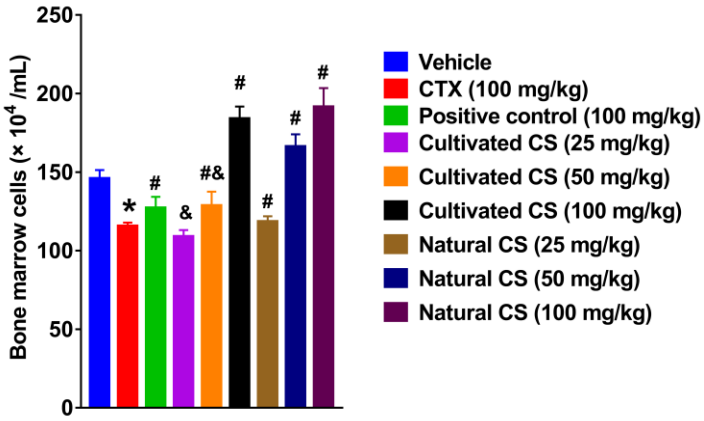
Supplementary Fig. 4 Effects of natural and artificially-cultivated *C. sinensis* on serum levels of EPO, G-CSF, and TPO in CTX-induced mice

A, EPO. B, G-CSF. C, TPO. *, $p < 0.05$ compared with control group. #, $p < 0.05$ compared with CTX (100 mg/kg) group. &, $p < 0.05$ compared with natural *C. sinensis* group at equal dose. Data represented the mean ± S.E. (n=6). CS, *C. sinensis*. EPO, erythropoietin. TPO, thrombopoietin. G-CSF, granulocyte colony-stimulating factor.



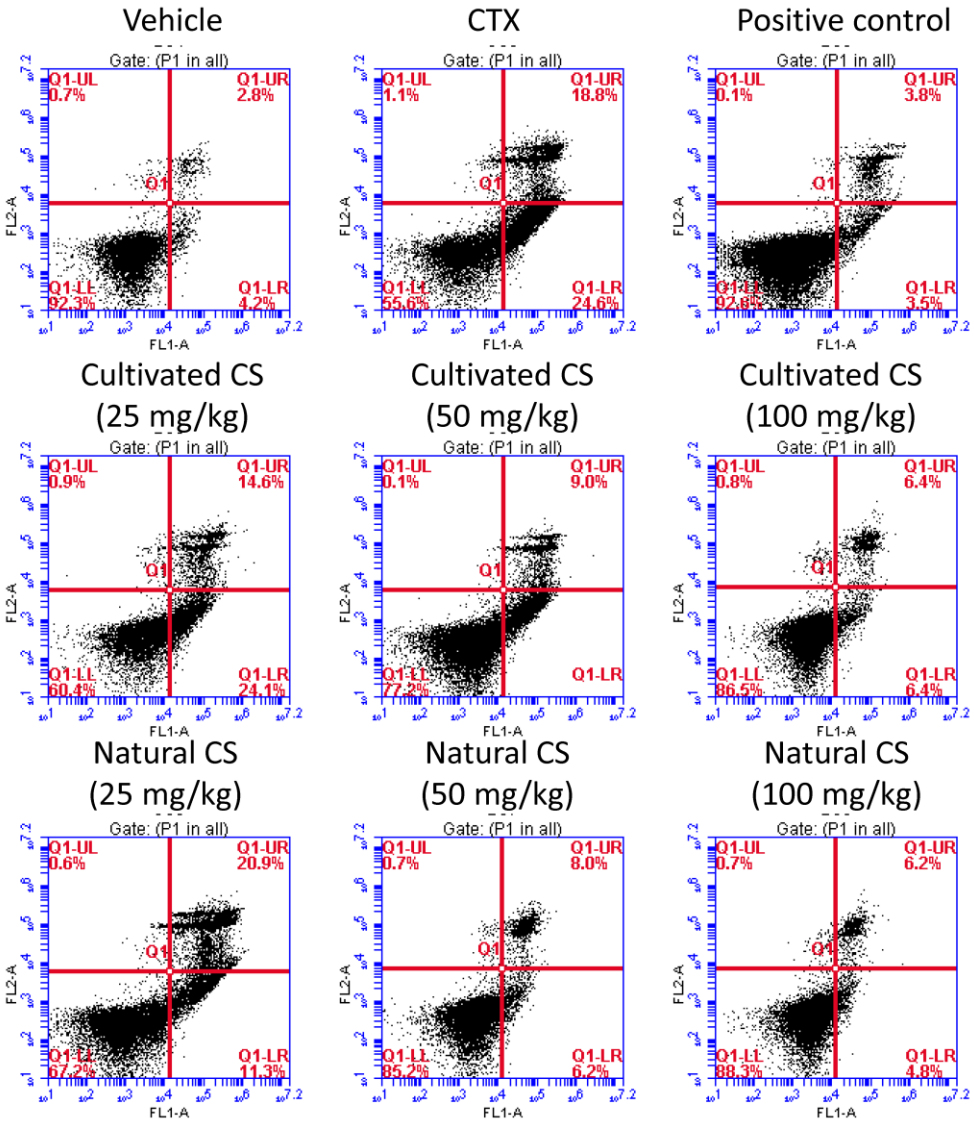
Supplementary Fig. 5 Effects of natural and artificially-cultivated *C. sinensis* on femur histopathology in CTX-induced mice

A, 50 ×. B, 200 ×. CS, *C. sinensis*.



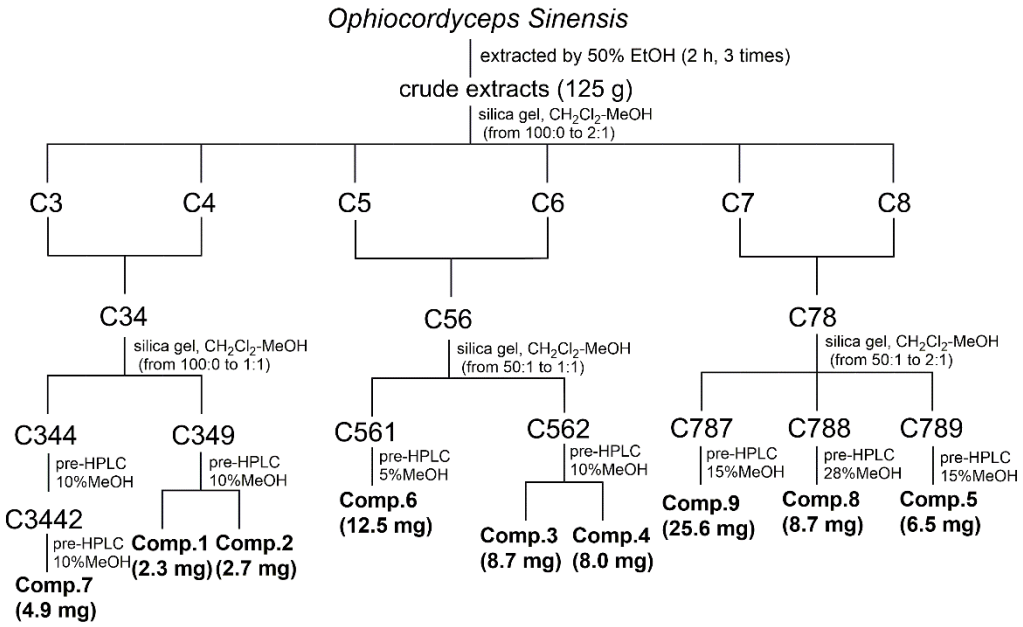
Supplementary Fig. 6 Effects of natural and artificially-cultivated *C. sinensis* on bone marrow cell counts of femur in CTX-induced mice

*, $p < 0.05$ compared with control group. #, $p < 0.05$ compared with CTX (100 mg/kg) group. &, $p < 0.05$ compared with natural *C. sinensis* group at equal dose. Data represented the mean ± S.E. (n=6). CS, *C. sinensis*.

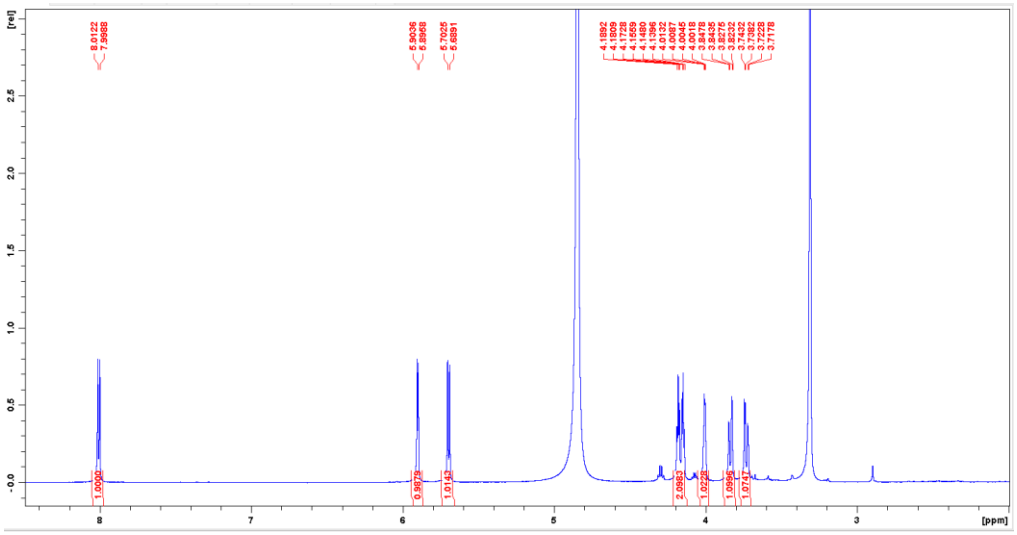


Supplementary Fig. 7 Effects of natural and artificially-cultivated *C. sinensis* on cell apoptosis of femur bone marrow in CTX-induced mice

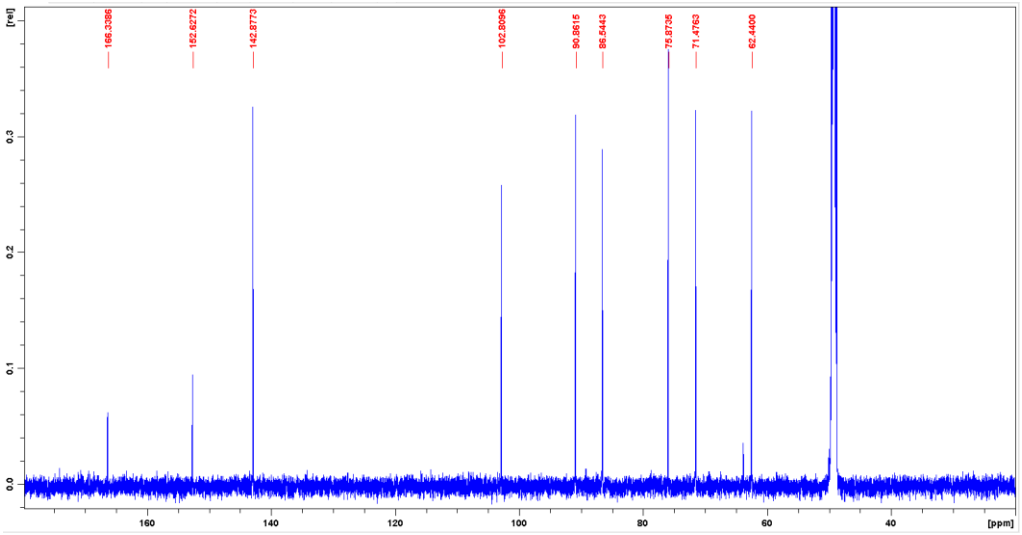
CS, *C. sinensis*.



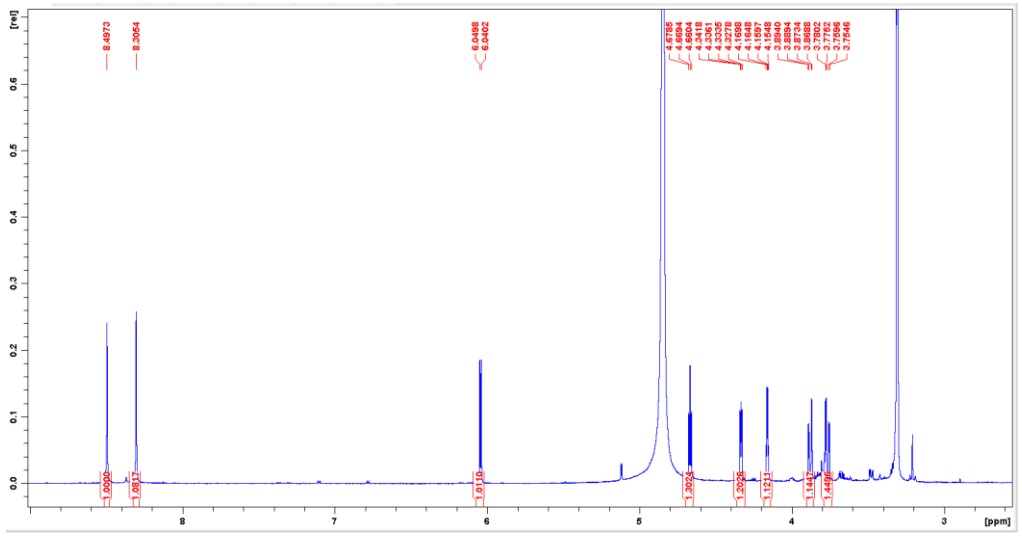
Supplementary Fig.8. Extraction and separation of Cordyceps sinensis



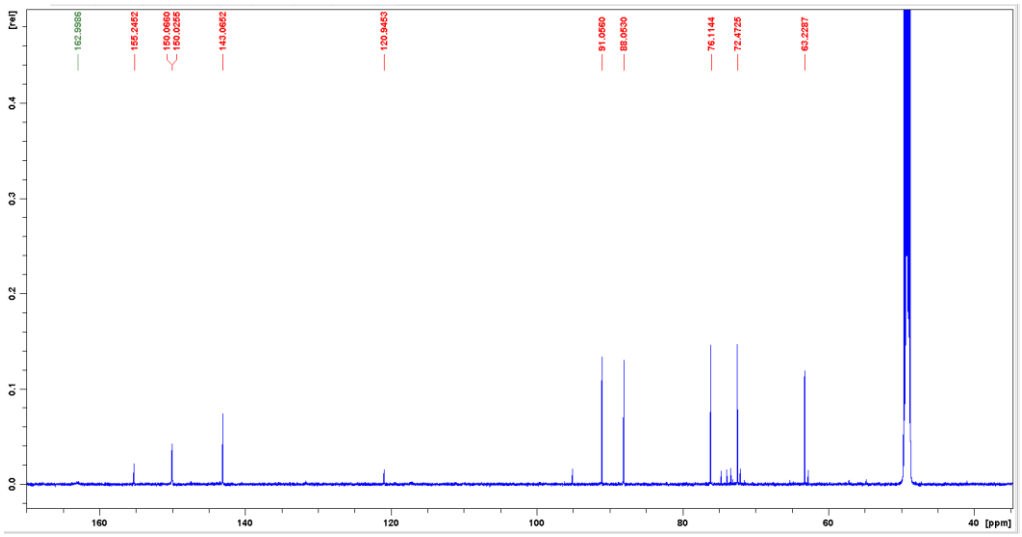
Supplementary Fig.9. ^{1}H-NMR spectrum (600 MHz, CD_3OD) of Compound (1)



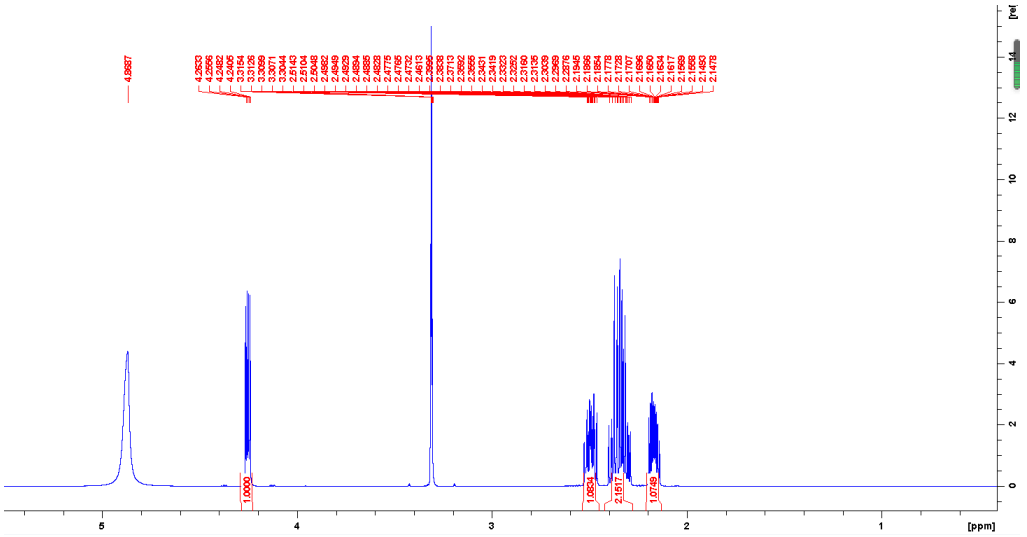
Supplementary Fig.10. ^{13}C-NMR spectrum (150MHz, CD_3OD) of Compound (1)



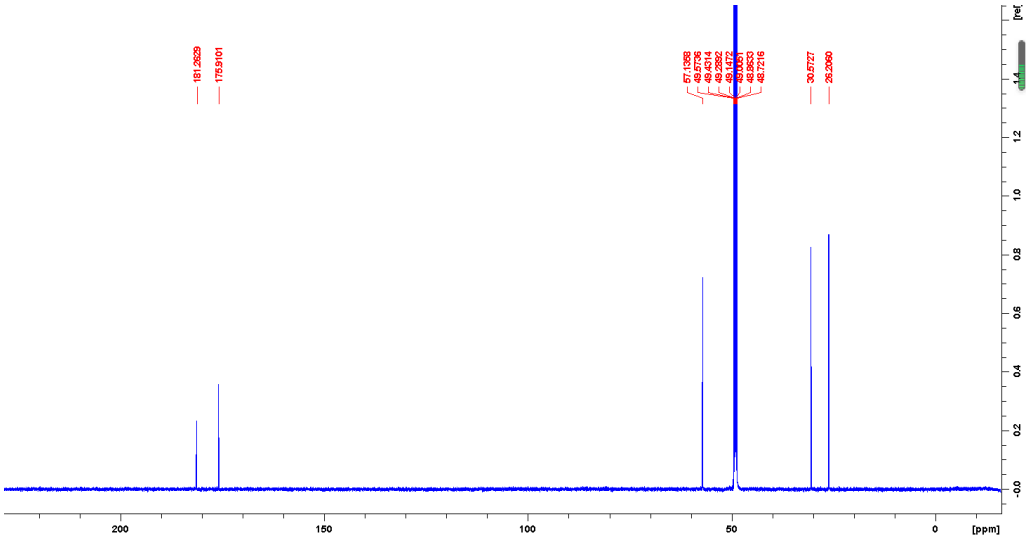
Supplementary Fig.11. ^{1}H-NMR spectrum (600 MHz, CD_3OD) of Compound (2)



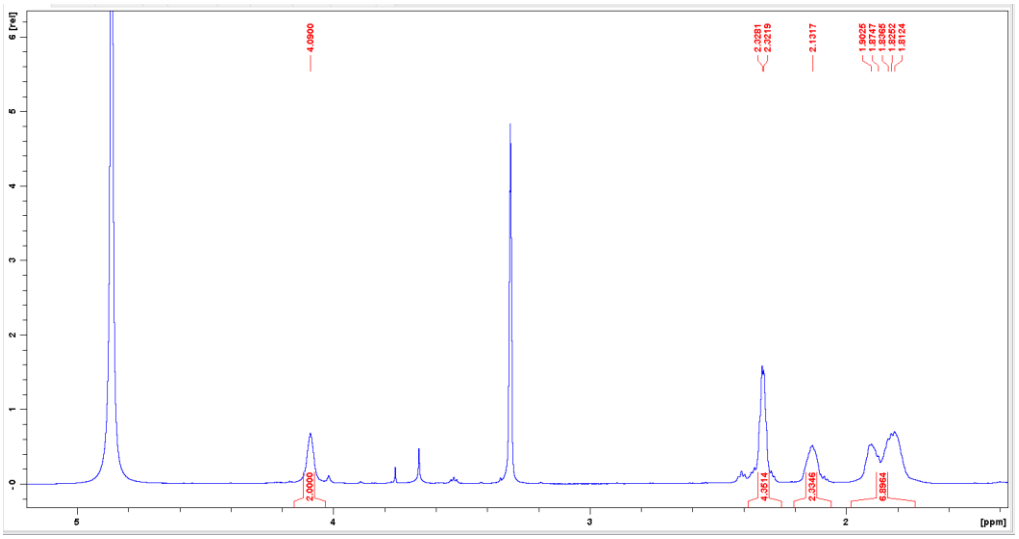
Supplementary Fig.12. ^{13}C-NMR spectrum (150MHz, CD_3OD) of Compound (2)



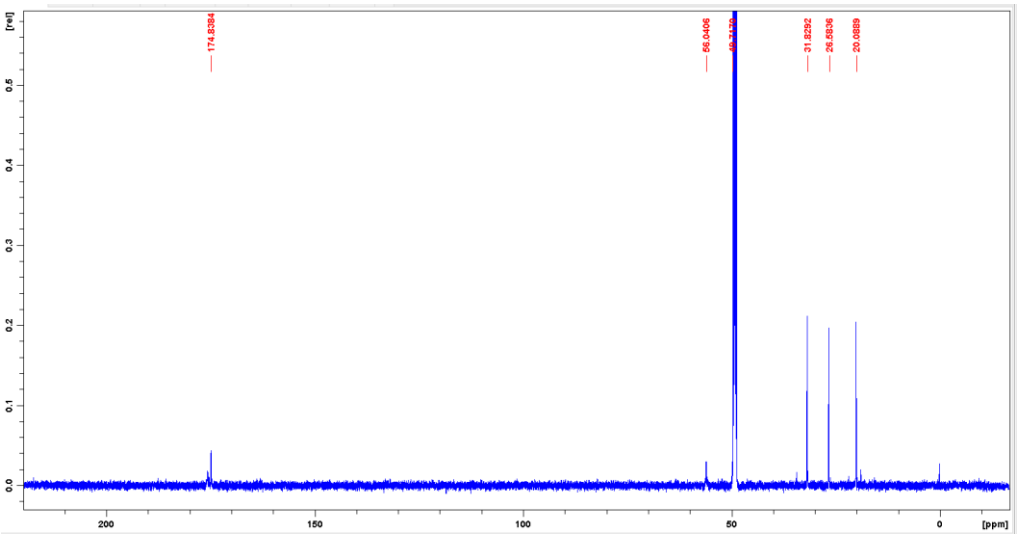
Supplementary Fig.13. ^{1}H-NMR spectrum (600 MHz, CD_3OD) of Compound (3)



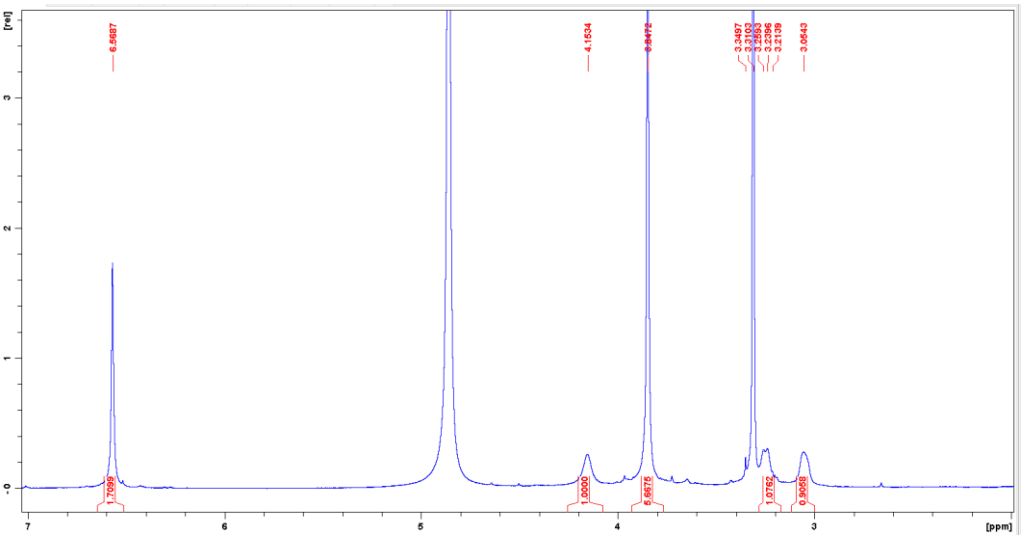
Supplementary Fig.14. ^{13}C-NMR spectrum (150MHz, CD_3OD) of Compound (3)



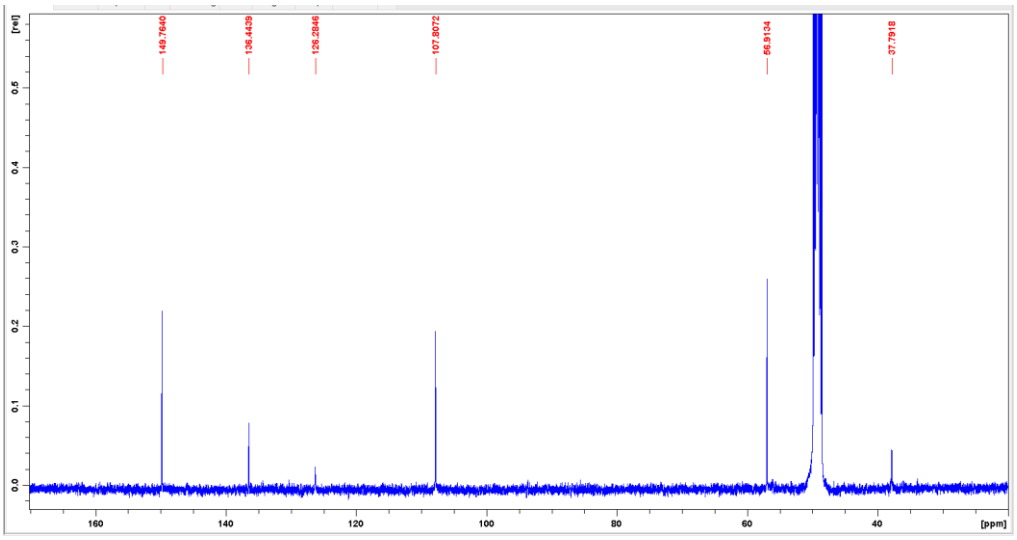
Supplementary Fig.15. ^{1}H-NMR spectrum (600 MHz, CD_3OD) of Compound (4)



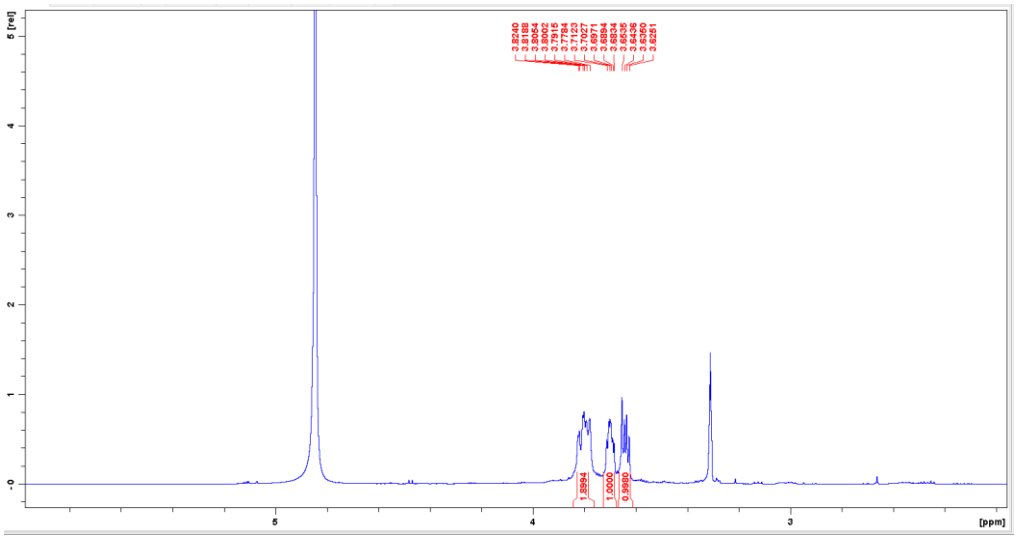
Supplementary Fig.16. ^{13}C-NMR spectrum (150MHz, CD_3OD) of Compound (4)



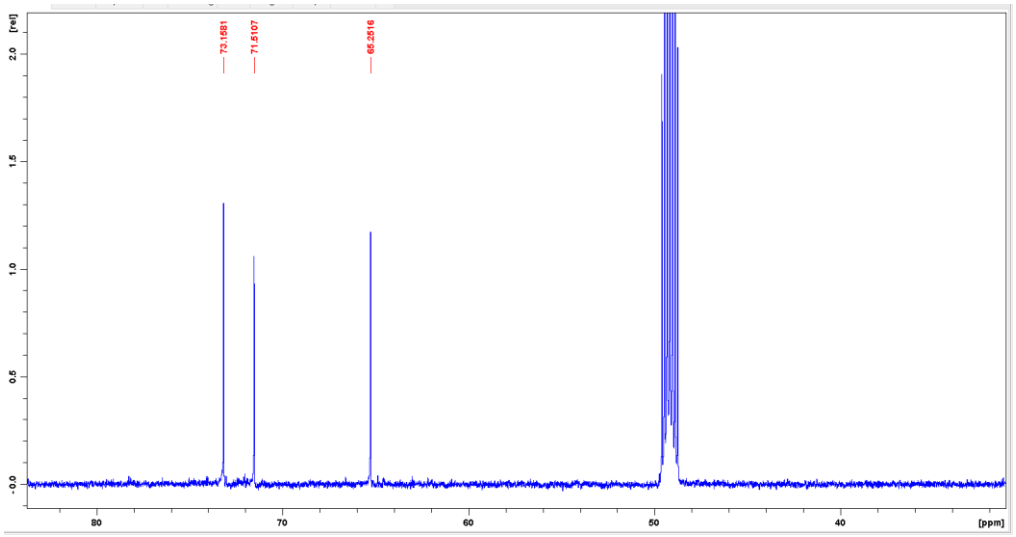
Supplementary Fig.17. ^{1}H-NMR spectrum (600 MHz, CD_3OD) of Compound (5)



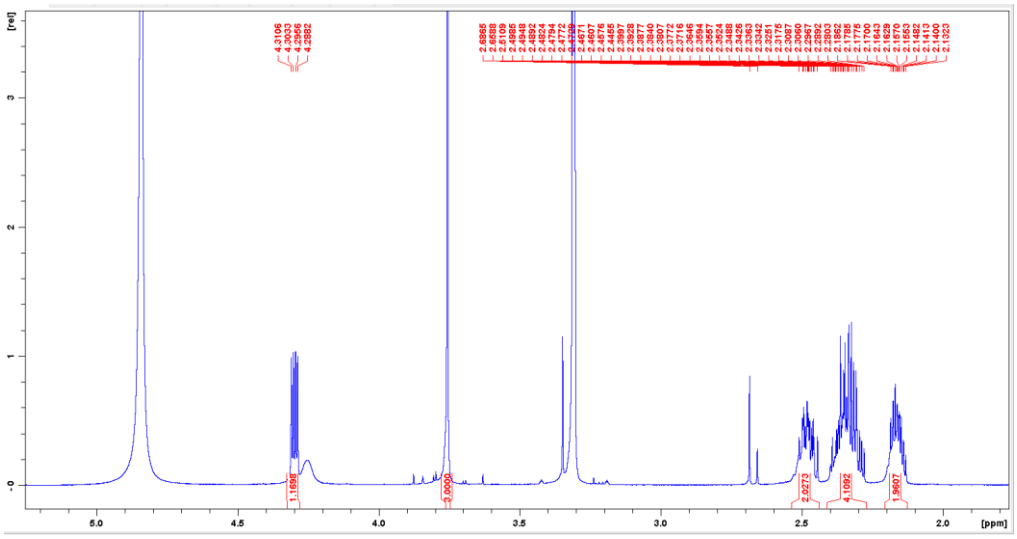
Supplementary Fig.18. ^{13}C-NMR spectrum (150MHz, CD_3OD) of Compound (5)



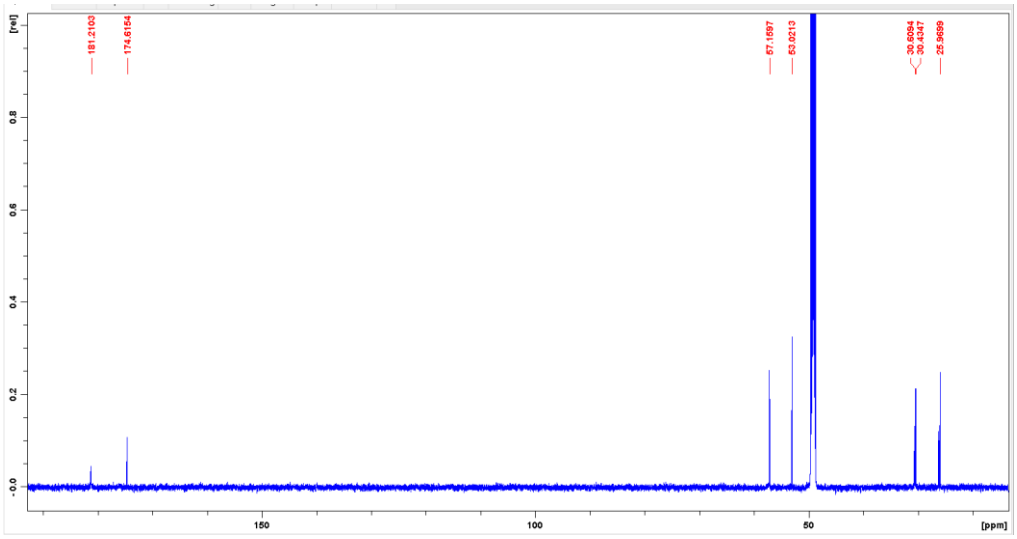
Supplementary Fig.19. ^{1}H-NMR spectrum (600 MHz, CD_3OD) of Compound (6)



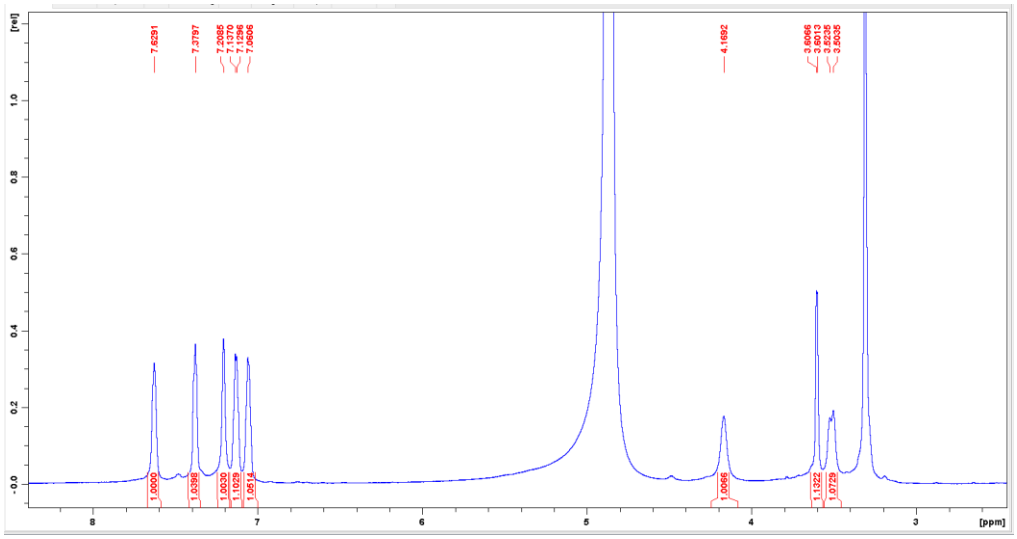
Supplementary Fig.20. ^{13}C-NMR spectrum (150MHz, CD_3OD) of Compound (6)



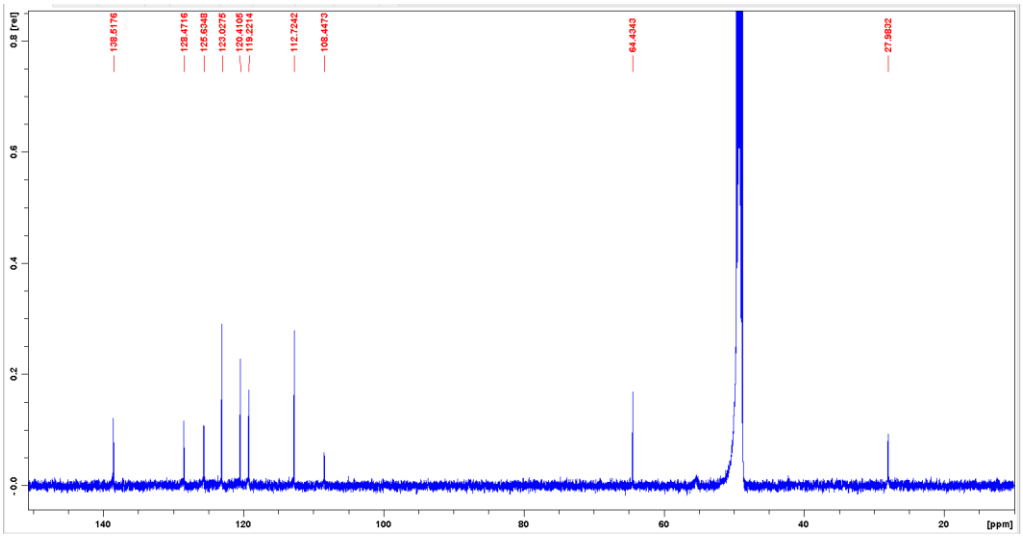
Supplementary Fig.21. ^{1}H-NMR spectrum (600 MHz, CD_3OD) of Compound (7)



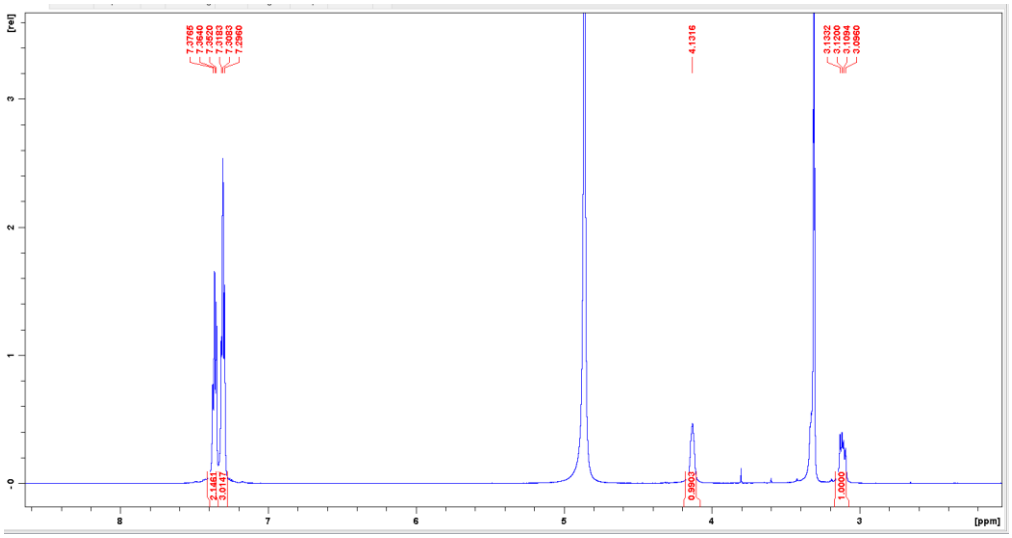
Supplementary Fig.22. ^{13}C-NMR spectrum (150MHz, CD_3OD) of Compound (7)



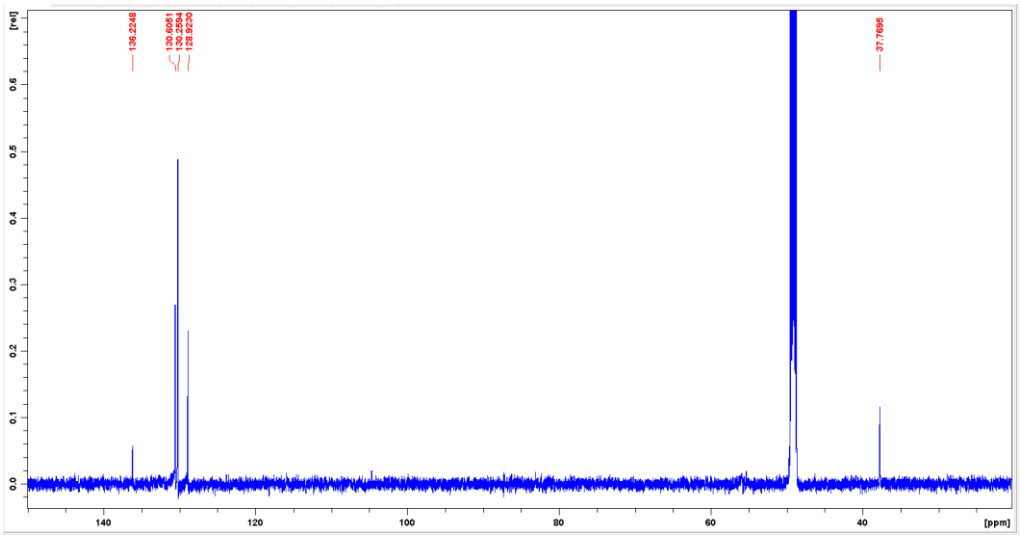
Supplementary Fig.23. ^{1}H-NMR spectrum (600 MHz, CD_3OD) of Compound (8)



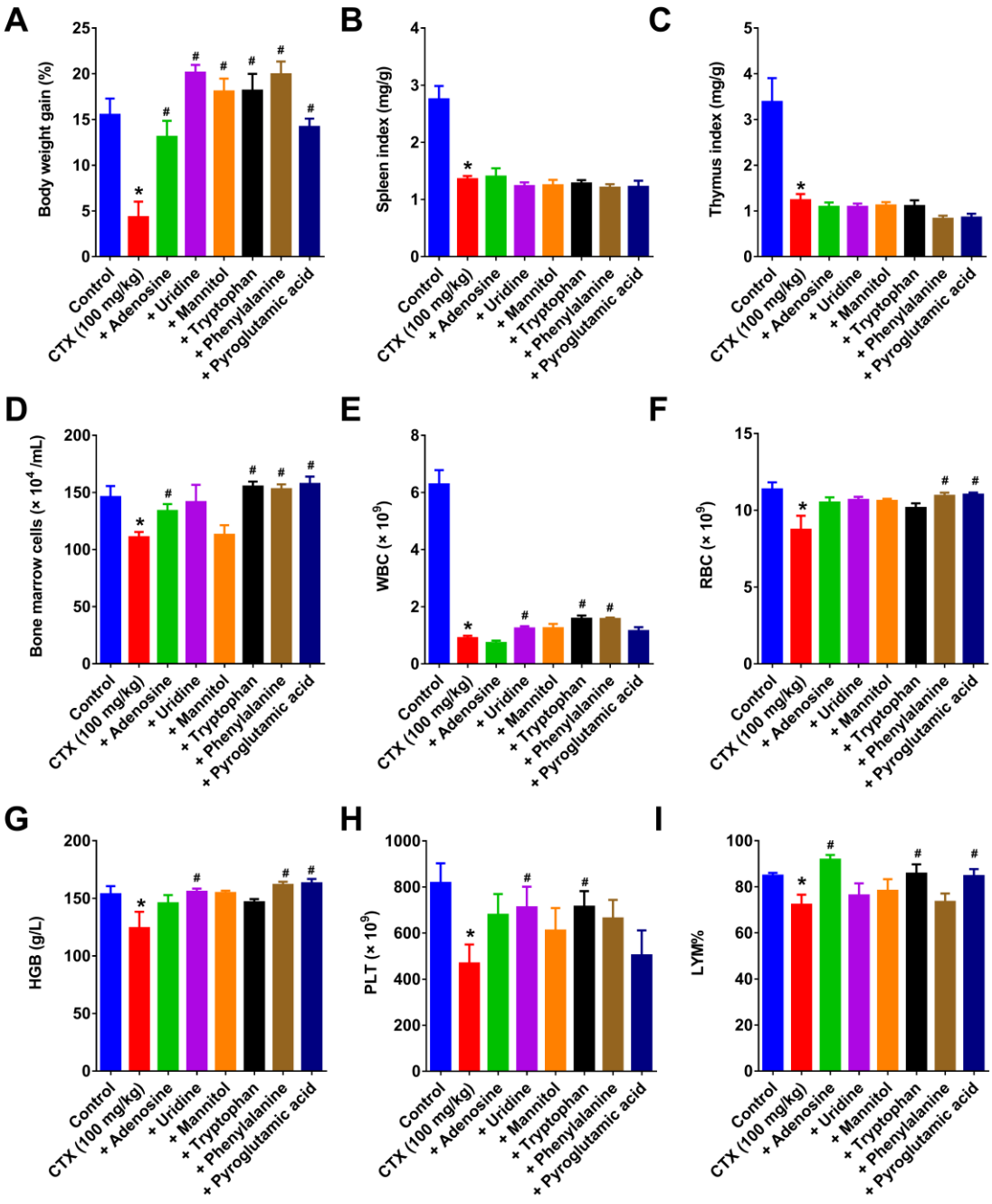
Supplementary Fig.24. ^{13}C-NMR spectrum (150MHz, CD_3OD) of Compound (8)



Supplementary Fig.25. ^{1}H-NMR spectrum (600 MHz, CD_3OD) of Compound (9)



Supplementary Fig.26. ^{13}C-NMR spectrum (150MHz, CD_3OD) of Compound (9)



Supplementary Fig. 27 Effects of *C. sinensis* fractions on hematopoietic function in CTX-induced mice

A, body weight. B, thymus index. C, spleen index. D, bone marrow cells of femur. E, WBC. F, RBC. G, HGB. H, PLT. I, LYM%. *, $p < 0.05$ compared with control group. #, $p < 0.05$ compared with CTX (100 mg/kg) group. Data represented the mean ± S.E. (n=6). WBC, white blood cells. RBC, red blood cells, HGB, hemoglobin. PLT, platelets. LYM%, lymphocytes (%).

Supplementary Tables

Supplementary Table 1 Effects of *C. sinensis* fractions on the counts of WBC, RBC, HGB, PLT and LYM% in CTX-induced mice

	Control	CTX (100 mg/kg)	Petroleum ether	ethanol	water	Residue
RBC	8.93 ± 0.51	8.04 ± 1.01*	9.04 ± 0.88#	9.22 ±1.06#	9.43 ± 0.48#	9.23 ± 0.70#
WBC	6.13 ± 1.27	1.76 ± 0.64*	2.26 ± 0.60	3.14 ± 1.06#	3.48 ± 0.93#	2.48 ± 1.13
HGB	153 ± 6	134 ± 17*	152 ± 12#	154 ± 16#	157 ± 8#	152 ± 10#
PLT	958 ± 197	569 ± 184*	849 ± 213#	898 ± 183#	904 ± 246#	885 ± 200#
LYM%	0.69 ± 0.046	0.56 ± 0.11*	0.55 ± 0.10	0.56 ± 0.08	0.66 ± 0.10#	0.63 ± 0.05

*, $p < 0.05$ compared with control group. #, $p < 0.05$ compared with CTX (100 mg/kg) group. Data represented the mean ± S.E. (n=6). WBC, white blood cells. RBC, red blood cells, HGB, hemoglobin. PLT, platelets. LYM%, lymphocytes (%).

Supplementary Table 2. Mass spectrum parameters of analytes.

Analytes	Precursor ion (*m/z*)	Product ion (*m/z*)	DP	EP	CE	CXP
uridine	243.1	200.0	-70	-10	-15	-13
adenosine	268.0	136.1	40	10	24	13
L-pyroglutamic acid	128.1	81.9	-30	-10	-13	-13
D-mannito	181.1	101.0	-70	-10	-19	-13
tryptophan	205.2	188.1	40	10	14	13
phenylalanine	166.1	120.1	50	10	18	13

Supplementary Table 3. the contents of adenosine, uridine, mannitol, tryptophan, phenylalanine and pyroglutamic acid in natural and artificially-cultivated *C. sinensis*

	Unit	artificially-cultivated *C. sinensis*	natural *C. sinensis*
uridine	μg/g	530 ± 40.1	298 ± 9.87
adenosine	μg/g	254 ± 6.03	531 ± 18.6
L-pyroglutamic acid	mg/g	3.10 ± 0.27	6.48 ± 0.45
D-mannitol	mg/g	98.5 ± 0.91	125 ± 2.08
tryptophan	μg/g	20.4 ± 4.33	13.2 ± 10.4
phenylalanine	μg/g	308 ± 4.62	225 ± 15.7