Supporting Information

Covalent Bridging of Corilagin Improves Antiferroptosis Activity: Comparison with 1,3,6-Tri-*O*-galloyl-*β*-D-glucopyranose

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CONTENTS

Methods and Materials

Animals, biological kits, and chemicals

Sprague-Dawley rats (4 weeks) were obtained from the Animal Centre of Guangzhou University of Chinese Medicine. The complete medium with glucose for SD rat bone marrow mesenchymal stem cells was purchased from Cyagen Biosciences (CA, USA); fetal bovine serum (FBS) and trypsin were from Molecular Probes (Carlsbad, USA). The probe C11-BODIPY was purchased from Molecular Probes (CA, USA). An annexin V/propidium iodide (PI) assay kit was purchased from *BD Biosciences (*NJ, *USA). Cell Counting Kit-8* kit Was purchased from Dojindo Chemistry Research Institute (Kumamoto, Japan). Erastin was from MedChemExpress (Monmouth Junction, NJ, USA). Fetal bovine serum (FBS), rat bone mesenchymal stem cell Basal medium, and trypsin were purchased from Gibco (Grand Island, NY, USA). 4,4-Difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (C11-BODIPY) was obtained from Invitrogen (Carlsbad, CA, USA). Percoll was obtained from GE Healthcare Life Sciences (Pittsburgh, PA, USA). Trypsin was from Promega Co. (Madison, WI, USA). Ferrostatin-1 (Fer-1) was purchased from Selleck Chemicals (Houston, TX, USA).

Corilagin ($C_{27}H_{22}O_{18}$, CAS number: 23094-69-1, M.W. 634.5, purity 98%) and 1,3,6-tri-*O*-galloyl-*β*-D-glucopyranose ($C_{27}H_{24}O_{18}$, CAS number: 18483-17-5, M.W. 636.5, purity 98%) were obtained from Chengdu Biopurify Phytochemicals, Ltd. (Chengdu, China). The 3-(2-pyridyl)-5, 6-bis (4-phenylsulfonicacid)-1,2,4-triazine (ferrozine) and (±)-6-hydroxyl-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) were obtained from Sigma-Aldrich (Shanghai, China). The 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide radical (PTIO$^{\bullet}$) was from TCI Chemical Co. (Shanghai, China). 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH$^{\bullet}$, $C_{18}H_{12}N_5O_6$) was obtained from Aladdin Chemical, Ltd (Shanghai, China). Water and acetonitrile were of HPLC grade. $FeCl_3 \cdot 6H_2O$ and the other reagents of analytical grade were purchased from Guangdong Guanghua Chemical Plants Co., LTD (Shantou, China).

Extraction and culture of bone marrow-derived mesenchymal stem cells (bmMSCs)

The bmMSCs were extracted and cultured using our routine experimental protocols.[1] Briefly, male Sprague-Dawley rats were collected, and the adherent soft tissues were removed. Both ends of the bones were cut away from the diaphysis with bone scissors. The bone marrow plugs were hydrostatically expelled from the bones by insertion of needles fastened to 10-mL syringes filled with complete medium; the needles were inserted into the distal ends of femora and proximal ends of tibiae, and the marrow plugs expelled from the opposite ends. The cells were centrifuged and resuspended twice in complete medium; 5×10^7 cells in 7-10 mL of complete medium were then introduced into 100-mm culture dishes. Two days later, the medium was changed and the nonadherent cells were discarded. The adherent cells were cultured in SD rat bone marrow mesenchymal stem cells complete medium with glucose, supplemented with 10 % (v/v) fetal bovine serum. The cultured cells were seeded and grouped to study the prevention of erastin-induced ferroptosis of corilagin and TGG.

Prevention of erastin-induced ferroptosis in bmMSCs

The erastin-induced ferroptosis model of bmMSCs was created based on the recent literature,[2] and with modifications. To measure the antiferroptosis bioactivities of corilagin and TGG, three assays referred as C11-BODIPY assay, flow cytometric assay, and CCK-8 assay.

The C11-BODIPY assay was used to characterize the degree of lipid peroxidation, and was performed using previously published method.[3] Briefly, the cultured bmMSCs were seeded at 1×10^6 cells per well into 12-well plates. After adherence for 24 h, bmMSCs were divided into control, model, and sample groups. In the control group, bmMSCs were incubated for 12 h in Stel Basal medium. In the model and sample groups, bmMSCs were incubated in the presence of erastin (20 μM). After incubation for 12 h, the mixture of erastin and medium was removed. The bmMSCs in the model group were incubated for 12 h in Stel Basal medium, while BMSCs in the sample group were incubated for 12 h in Stel Basal medium with the indicated 3 μg/mL sample concentrations and the positive control group with 1.0 μM Fer-1. The incubated cells were analyzed using the fluorescent probe C11-BODIPY (Invitrogen, Molecular Probes). Cells were incubated for 30 min prior to analysis with C11-BODIPY (2.5 μM). The images were taken using a fluorescence microscope.

The CCK-8 assay was conducted following previous literature,[4] with minor modifications. Briefly, the cultured bmMSCs were seeded at 1×10^6 cells per well into 96-well plates. After adherence for 12 h, BMSCs were divided into control, model, positive control (Fer-1), and sample groups. The incubated cells were treated as above by adding 10 μL CCK-8, and the culture was incubated for an additional 3 h. The culture medium was discarded. Absorbance was measured at 450 nm on a Bio-Kinetics reader (Multiskan FC, Thermo Scientific, Shanghai, China). According to the $A_{450\ nm}$ values, the viability was calculated.

The flow cytometric assay was conducted according to the previous methods.[5] Briefly, the cultured bmMSCs were seeded at 1×10^6 cells per well into 96-well plates. They were washed twice with cold PBS, and then resuspend cells in 1×Binding buffer at a concentration of 1×10^6 cells/mL. Then, 100 μL of the solution (1×10^5 cells) was transferred to a 5 mL culture tube, and 5 μL of FITC Annexin V and 5 μL PI were added. The cells were vortexed gently and were incubated for 15 min at room temperature in the dark, and 400 mL of 1×Binding Buffer was added to each tube after adherence for 12 h, BMSCs were divided into control, model, positive control (Fer-1), and sample groups. The three groups were analyzed by flow cytometry within 1 h. Each sample test was repeated in three independent wells.

Fe^{2+}-chelation assays

The Fe^{2+}-chealting activity of corilagin and TGG were preliminarily investigated using UV-vis spectra method.[6] Briefly, 100 μL sample in methanol solution (3.17 mg/mL) and 100 μL $FeCl_2{\bullet}4H_2O$ aqueous solution (100 mg/mL) were added to 1800 μL of methanol-water (1:1, v/v), and mixed well. The resulting mixture was subsequently scanned using a UV-Vis spectrophotometer (Unico 2600A, Shanghai, China) from 400-900 nm in 30 min. Methanol/water (1:1, v/v) served as a blank. Next, 200 μL of the supernatant was transferred to a 96-well plate and photographed using a camera. The remaining solution were diluted and scanned from 200-400 nm.

Furthermore, the quantitative evaluation was conducted using a previously published method.[7] Briefly, 0.05 mg/mL sample solution (30-150 μL) was added to a solution of 250 μM $FeCl_2$ (100 μL). The reaction was initiated by the addition of 500 μM ferrozine (150 μL). The total volume of the systems was adjusted to 1000 μL with methanol. Then, the mixture was shaken vigorously and left at room temperature for 5 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm (Unico 2100, Shanghai, China). The percentage of Fe^{2+}-chelation effect was calculated by using the formula given bellow:

$$Fe^{2+}\text{ - chelation \%} = \frac{A_0 - A}{A_0} \times 100\%$$

Where A is the absorbance in the presence of the sample or positive controls, while A_0 is the absorbance in the absence of the sample and positive controls.

PTIO•-scavenging assay

The PTIO•-scavenging assay was conducted based on our previously published method.[8] Briefly, PTIO• radical was dissolved in phosphate buffers (pH 4.5 and 7.4) to prepare a PTIO• solution; the samples were prepared using methanol (2 mg/mL). Various volumes (x = 2-10 μL) of samples were mixed with phosphate buffers at pH 4.5 and 7.4 (20 – x μL) and treated with PTIO• solution (80 μL). After reaction at 37°C for 1 h, the product mixture was analyzed by measuring the absorbance at 560 nm on a microplate reader (Multiskan FC, Thermo Scientific, Shanghai, China). The PTIO• inhibition percentage was calculated as follows:

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

Where A_0 is the absorbance value at 560 nm wavelength for the control sample (without test agent), and A is the absorbance value at 560 nm of the reaction mixture (with sample). The above experiment was repeated using phosphate buffers at different pH (including pH 4.5 and 7.4).

Fe^{3+}-reducing antioxidant power (FRAP) assay

The FRAP assay was carried out as per the method described by Benzie,[9] and slightly modified in our previous studies.[10] Briefly, the FRAP reagent was freshly prepared by mixing 10 mM TPTZ, 20 mM $FeCl_3$, and 0.25 M acetate buffer (pH 3.6) at a ratio of 1:1:10. Samples (0.1 mg/mL, x = 4-20 μL) were added to (20 − x) μL of methanol and treated with 80 μL of FRAP reagent. After reaction for 30 min, the absorbance of the mixture was measured at 593 nm wavelength (A_{593nm}) on a microplate reader (Multiskan FC, Thermo Scientific, Shanghai, China). The relative reducing antioxidant power of the sample as compared to the maximum absorbance was calculated by the following formula:

$$\text{Relative reducing power\%} = \frac{A - A_{min}}{A_{max} - A_{min}} \times 100\%$$

Where A_{min} is the lowest A_{593nm} value in the experiment, A is the A_{593nm} value of the reaction mixture with sample, and A_{max} is the greatest A_{593nm} value in the experiment.

DPPH•-scavenging assay

The DPPH• radical scavenging activity was determined as previously described.[11] Briefly, 80 μL of DPPH•-methanolic solution (0.1 M) was mixed with sample-methanolic solution (x = 1-5 μL, 0.02 mg/mL) and (20 – x) μL methanol. The mixture was maintained at room temperature for 5 min, and the absorbance was measured at 519 nm on the microplate reader. The percentage of DPPH•-scavenging activity was calculated based on the formula presented for *PTIO•-scavenging assay*, wherein A_0 is the absorbance at 519 nm of the control and A is the absorbance at 519 nm of the test.

UHPLC-ESI-Q-TOF-MS analysis of DPPH• reaction products with corilagin and TGG

The reaction of DPPH• with corilagin and TGG proceeded under the conditions described in a previous method.[12] Briefly, a methanol solution of sample was mixed with a methanol DPPH• solution with a

molar ratio of 1:2, and the resulting mixture was kept for 24 h at room temperature. Subsequently, the product was passed through a 0.22-μm filter for UHPLC-ESI-Q-TOF-MS analysis.

The UHPLC-ESI-Q-TOF-MS analysis was based on the method described in our previous study.[13] The UHPLC-ESI-Q-TOF-MS analysis system was equipped with a Phenomenex Luna C_{18} column (2.1 mm i.d. × 100 mm, 1.6 μm, Phenomenex Inc., Torrance, CA, USA). The mobile phase was employed for the elution of the system and consisted of a mixture of methanol (phase A) and 0.1% formic acid water (phase B). The column was eluted at a flow rate of 0.2 mL/min with the following gradient elution program: 0–2 min, maintained at 30% B; 2–10 min, 30–0% B; 10–12 min, 0–30% B. The sample injection volume was set at 3 μL for the separation of the different components. The Q-TOF-MS analysis was performed on a Triple TOF 5600^{plus} mass spectrometer (AB SCIEX, Framingham, MA, USA) equipped with an ESI source, which was run in the negative ionization mode. The scan range was set at 100–2000 Da. The system was run with the following parameters: ion spray voltage, −4500 V; ion source heater temperature, 550 °C; curtain gas pressure (CUR, N_2), 30 psi; nebulizing gas pressure (GS1, Air), 50 psi; Tis gas pressure (GS2, Air), 50 psi. The declustering potential (DP) was set at −100 V, whereas the collision energy (CE) was set at −45 V with a collision energy spread (CES) of 15 V.

Preferential conformation analysis by computational chemistry and molecular weight calculation

The preferential conformation was analyzed based on force fields by computational chemistry. Briefly, the energy minimization of both corilagin and TGG were, respectively, calculated through molecular mechanics II (MM2) using the Chem3D Pro14.0 program (PerkinElmer, Waltham, MA, USA). The preferential conformation has been expressed using the molecular models in **Figure 1C–D**. The Q-TOF-MS analysis is characterized by highly accurate *m/z* values, particularly molecular weights. The molecular weight calculation based on the formula is vital for comparison with the *m/z* values from the Q-TOF-MS analysis. In the present study, the molecular weight calculations of corilagin and TGG were conducted based on the accurate relative atomic masses. The relative atomic masses of C, H, O, and N were 12.0000, 1.007825, 15.994915, and 14.003074, respectively.

Statistical analysis

Each experiment was performed in triplicates; the data were recorded as mean ± standard deviation (SD). The dose-response curves were plotted using Origin 2017 professional software (OriginLab, Northampton, MA, USA). The IC_{50} value was defined as the final concentration of 50% radical inhibition (or relative reducing power). Statistical comparisons were carried out with one-way analysis of variance (ANOVA) to detect significant differences using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA) for Windows. A value of $p < 0.05$ was considered statistically significant.

REFERENCES

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Figure S1. The certificate of analysis of corilagin

产品分析证书
Certificate of Analysis

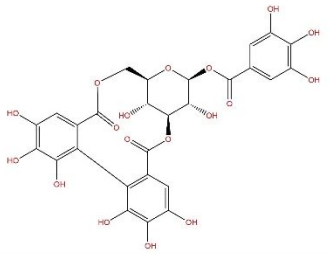
中文名称：柯里拉京

English Name：Corilagin
别名(Alias):

产品编码(Cat. No.):BP0393
CAS Number: 23094-69-1
分子式(M. F.): C27H22O18
分子量(M. W.): 634.455

批号(Batch No.): PRF7102406
报告日期(Report date): 2016/10/26

Structure



检验结果（Analytical result）：

检验项目（Test Item）	检验指标（Specifications）	检验结果（Results）
外观Appearance	Grey powder	Grey powder
干燥失重Loss on drying	＜3.0%	1.34%
纯度Purity (HPLC-DAD, 276nm)*	≥98.0%	99.71%
质谱Mass	634.455±1	Conforms
核磁NMR	Comply with the structure	Conforms

*** 色谱图见附件（Please find HPLC chromatography attached.）**

贮存条件（Storage）:2~8℃
复测期（Retest date）:two years (2018-10-25) under conditions list above.
备注（Remarks）：如遇质量问题，请于收到产品之日起 15 日内与我们联系。
In case of quality issue, please contact us within 15 days after receipt of the product.

QC: Zhang Ling　　QA: Wu Qi

Chengdu Biopurify Phytochemicals Ltd. 质检专用章 Quality Approval Seal

Date: 2016年10月26日　　Date: 2016年10月26日

Tel：+86-28-82633987　Fax：+86-28-82633165
http://www.biopurify.com　Email：sales@biopurify.com　biopurify@gmail.com

Figure S2. The HPLC analysis of corilagin

SAMPLE INFORMATION

Sample Name:	Corilagin PRF7102406	Acquired By:	System
Sample Type:	Unknown	Sample Set Name:	
Vial:	10	Acq. Method Set:	Corilagin
Injection #:	1	Processing Method:	Samples
Injection Volume:	10.00 ul	Channel Name:	276.0nm
Run Time:	25.0 Minutes	Proc. Chnl. Descr.:	PDA 276.0 nm
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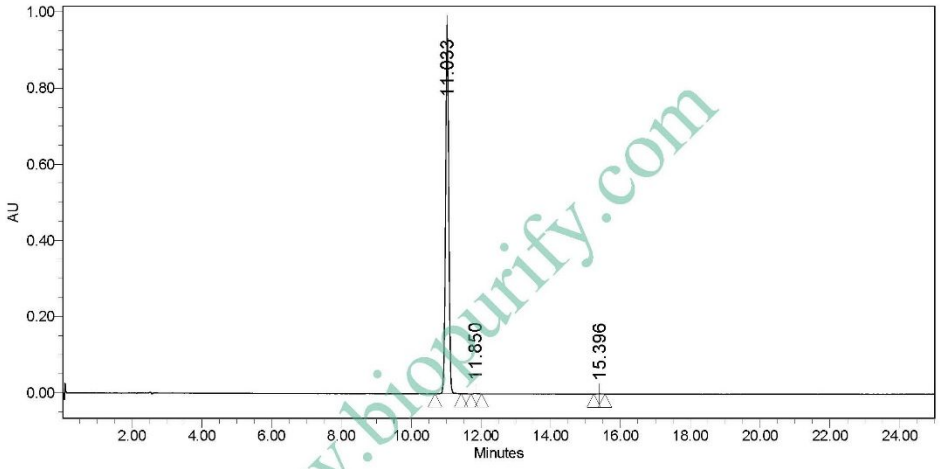
Auto-Scaled Chromatogram



Peak Results

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3		15.396	9845	0.16	47331.14	15.93

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Page: 1 of 1

Project Name: 2016
Date Printed:
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Figure S3. The MS spectra of corilagin

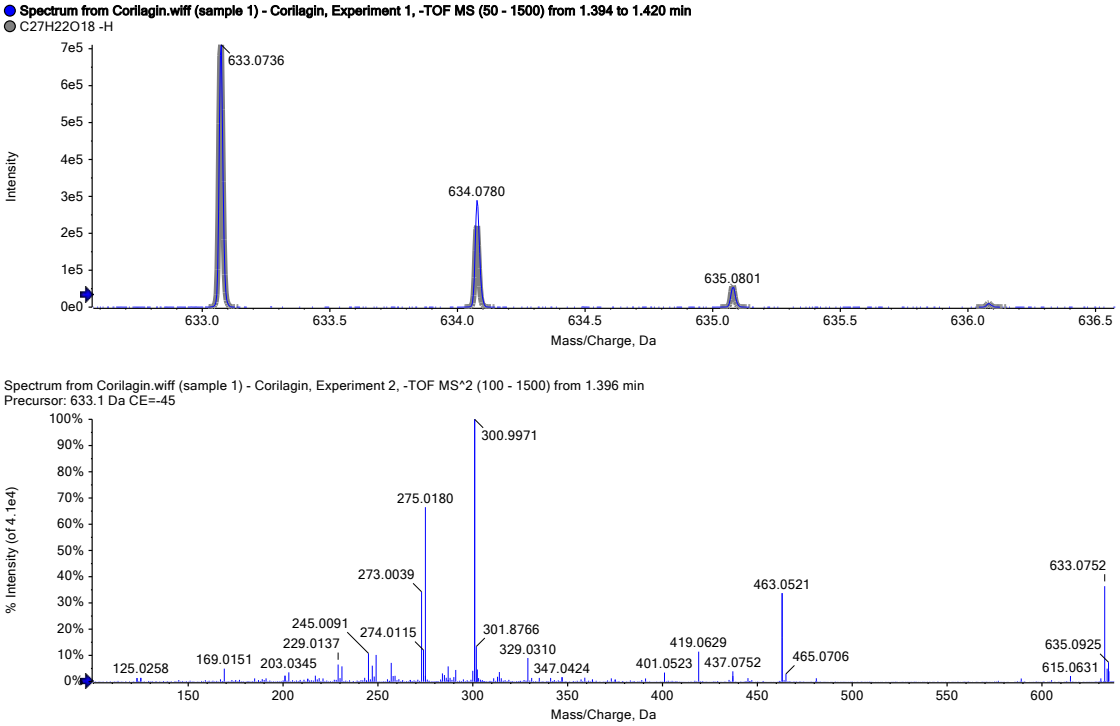
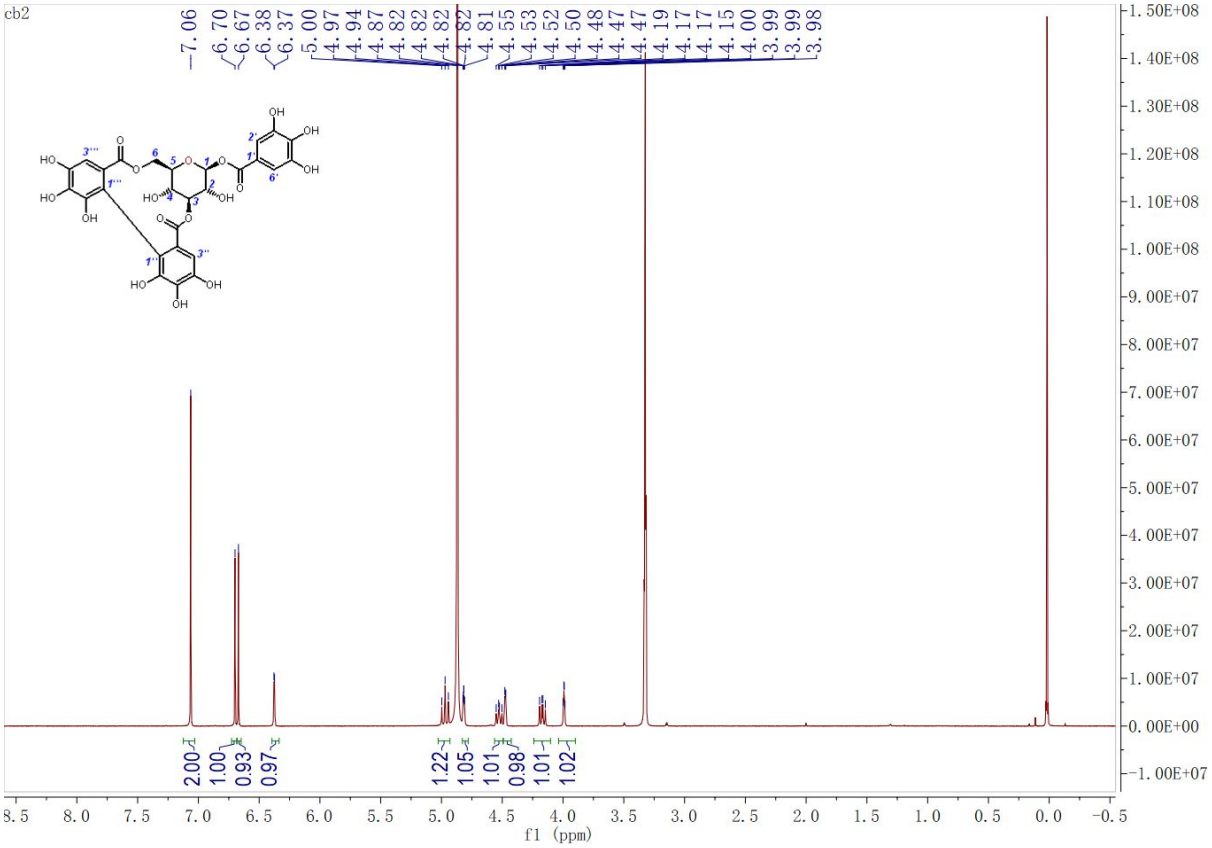


Figure S4. The ^{1}H-NMR of corilagin measured in CD_3OD



Corilagin ^{1}H NMR (400 MHz, CD3OD): δ 7.06 (s, 2H, H-2',6'), 6.70, 6.67 (each 1H, s, H-3", H-3"'), 6.38 (d, *J* = 2.1 Hz, 1H, H-1), 4.97 (dd, *J* = 10.7, 8.1 Hz, 1H, H-6a), 4.83-4.78 (m, 1H, H-3), 4.53 (t, *J* = 10.9 Hz, 1H, H-5), 4.49-4.43 (m, 1H, H-4), 4.17 (dd, *J* = 11.0, 8.0 Hz, 1H, H-6b), 3.99 (dd, *J* = 3.6, 2.2 Hz, 1H, H-2).

Figure S5. The appearance of corilagin

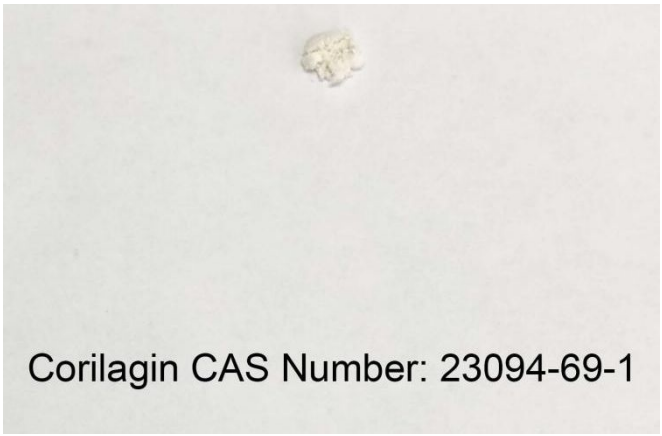


Figure S6. The certificate of analysis of TGG

产品分析证书
Certificate of Analysis

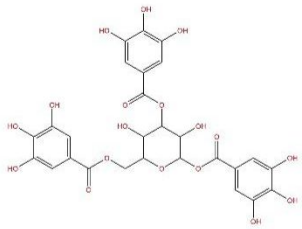
中文名称：**1,3,6-三-O-没食子酰葡萄糖**

English Name：1,3,6-Tri-O-galloyl-beta-D-glucose
别名(Alias): 1,3,6-Tri-O-galloyl-beta-D-glucopyranose

产品编码(Cat. No.):BP0004
CAS Number: 18483-17-5
分子式(M. F.): C27H24O18
分子量(M. W.): 636.471

批号(Batch No.): PRF7093022
报告日期(Report date): 2016/9/30

Structure



检验结果（Analytical result）：

检验项目（Test Item）	检验指标（Specifications）	检验结果（Results）
外观Appearance	Light purple powder	Light purple powder
干燥失重Loss on drying	＜3.0%	1.07%
纯度Purity (HPLC-DAD, 275nm)*	≥95.0%	95.55%
质谱Mass	636.471±1	Conforms
核磁NMR	Comply with the structure	Conforms

*** 色谱图见附件（Please find HPLC chromatography attached.）**

贮存条件（Storage）:2~8℃
复测期（Retest date）:two years (2018-09-29) under conditions list above.
备注（Remarks）：如遇质量问题，请于收到产品之日起 15 日内与我们联系。
In case of quality issue, please contact us within fifteen days after receipt of the product.

QC: Zhang Ling　　QA: Wu Qi

质检专用章 Quality Approval Seal

Date: 2016年9月30日　　Date: 2016年9月30日

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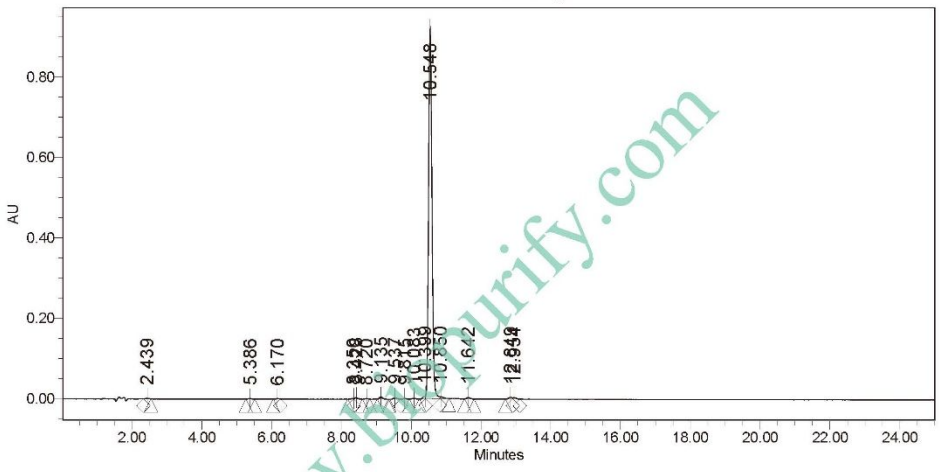
http://www.biopurify.com　Email：sales@biopurify.com　biopurify@gmail.com

Figure S7. The HPLC analysis of TGG

SAMPLE INFORMATION

Sample Name:	PRF7093022	Acquired By:	System
Sample Type:	Unknown	Sample Set Name:	
Vial:	93	Acq. Method Set:	1 3 6 Tri O galloyl beta D glu
Injection #:	1	Processing Method:	Samples
Injection Volume:	5.00 ul	Channel Name:	275.0nm
Run Time:	25.0 Minutes	Proc. Chnl. Descr.:	PDA 275.0 nm
Date Acquired:	2016-9-30 11:35:21 CST		
Date Processed:	2016-9-30 12:36:44 CST		

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	% Area	USP Plate Count	USP Resolution
1		2.439	10452	0.16	5636.74	
2		5.386	6454	0.10	6519.90	19.03
3		6.170	5950	0.09	14779.06	4.35
4		8.350	6918	0.11		11.80
5		8.428	25852	0.39	28307.99	0.36
6		8.720	7404	0.11	22694.74	1.34
7		9.135	38058	0.58	41665.84	2.28
8		9.537	27457	0.42	38078.34	2.18
9		9.815	11659	0.18	59778.49	1.55
10		10.083	14569	0.22	49948.42	1.54
11		10.399	29722	0.45		1.75

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Page: 1 of 2

Project Name: 2016
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Figure S8. The MS spectra of TGG

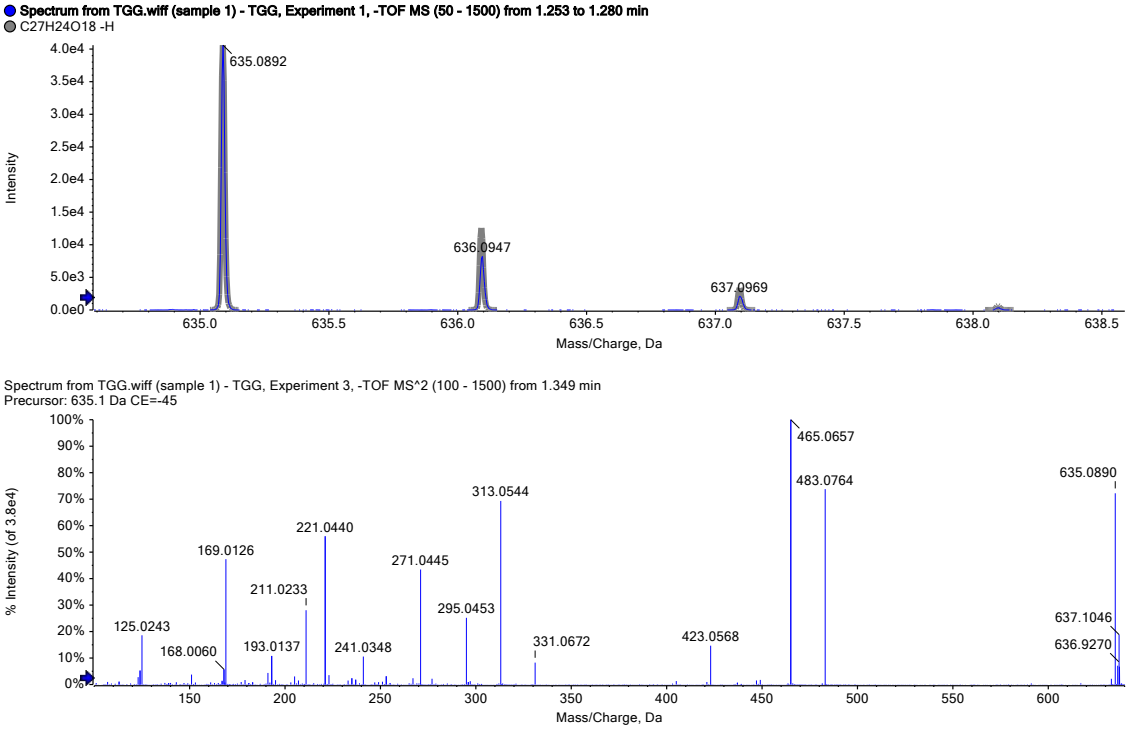
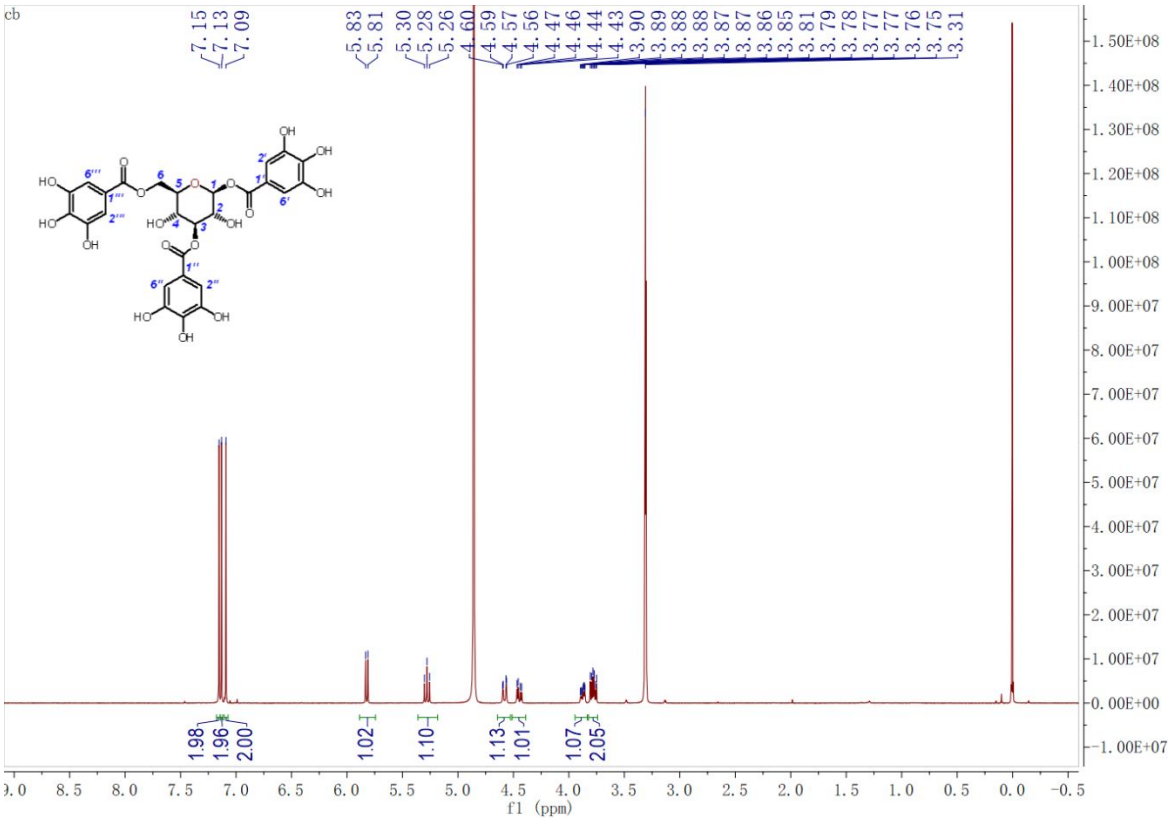


Figure S9. The ^{1}H-NMR of TGG measured in CD_3OD



TGG ^{1}H NMR (400 MHz, CD3OD): δ 7.15 (s, 2H, H-2',6'), 7.13 (s, 2H, H-2'',6''), 7.09 (s, 2H, H-2''',6'''), 5.82 (d, *J* = 8.2 Hz, 1H, H-1), 5.28 (t, *J* = 9.3 Hz, 1H, H-3), 4.58 (dd, *J* = 12.1, 1.9 Hz, 1H, H-6a), 4.45 (dd, *J* = 12.2, 4.8 Hz, 1H, H-6b), 3.88 (ddd, *J* = 9.8, 4.7, 1.9 Hz, 1H, H-5), 3.82 – 3.74 (m, 2H, H-2,4).

Figure S10. The appearance of TGG

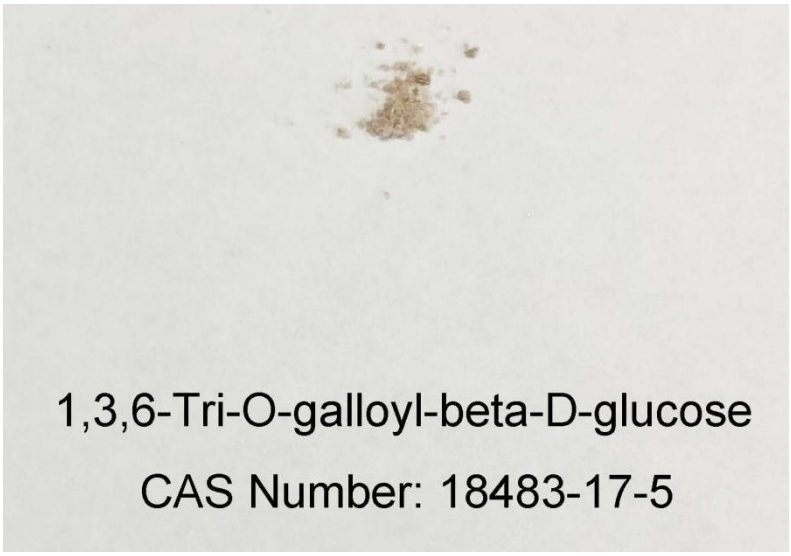
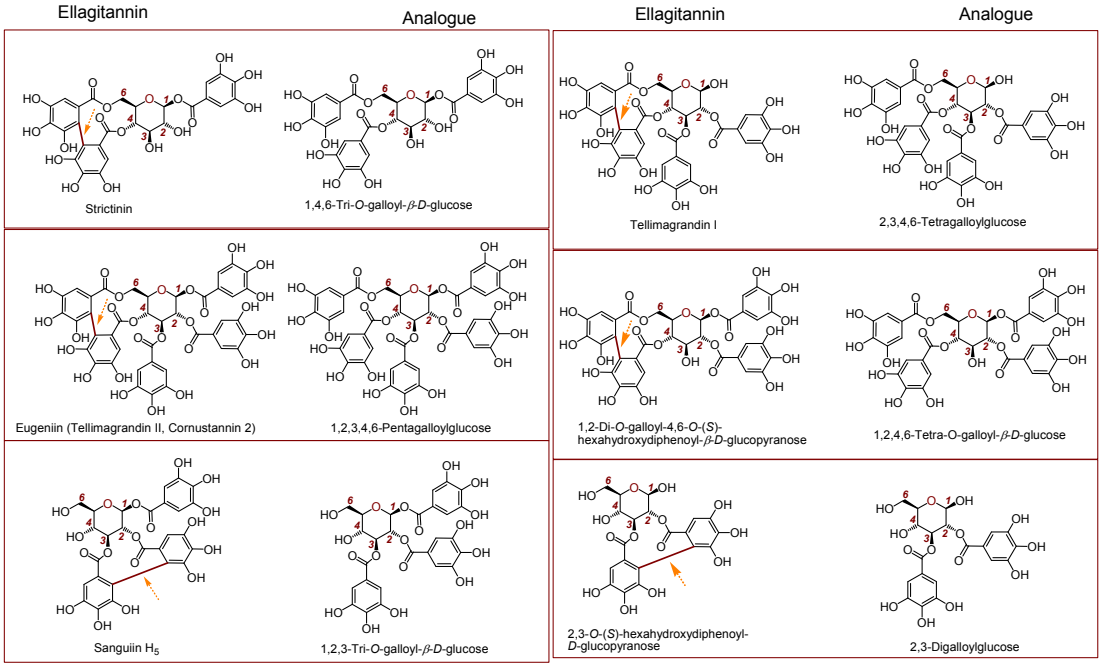


Figure S11. Structures of some typical covalent-bridged egallotannins and their analogues.



Eugeniin[1] *vs* 1,2,3,4,6-pentagalloylglucose[2]; tellimagrandin I[3] *vs* 2,3,4,6-tetragalloylglucose[4]; sanguiin H_5[5] *vs* 1,2,3-tri-*O*-galloyl-*β*-D-glucose[6]; 1,2-di-*O*-galloyl-4,6-*O*-(*S*)-hexahydroxydiphenoyl-*β*-D-glucopyranose[7] *vs* 1,2,4,6-tetra-*O*-galloyl-*β*-D-glucose[8]; strictinin[9] *vs* 1,4,6-tri-*O*-galloyl-*β*-D-glucose[10]; 2,3-*O*-(*S*)-hexahydroxydiphenoyl-D-glucopyranose[7] *vs* 2,3-digalloylglucose[11].

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Figure S12. The does response curves of corilgin, TGG, and the positive control in Fe^{2+}-chelating assay

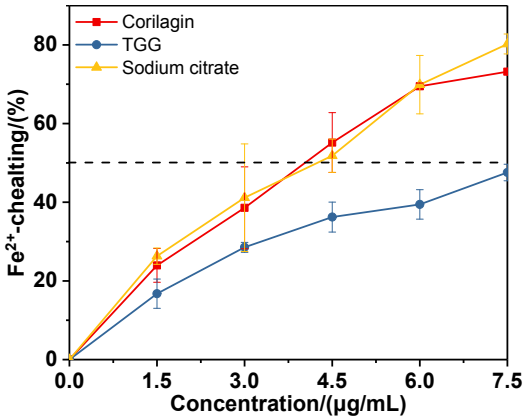

Figure S13. The does response curves of corilgin, TGG, and the positive control in PTIO•-inhibiting (pH 7.4) assay

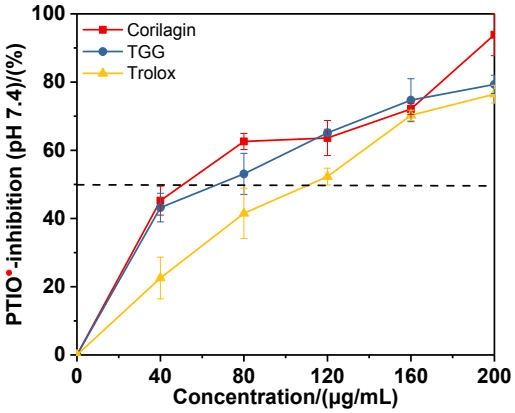

Figure S14. The does response curves of corilgin, TGG, and the positive control in PTIO•-inhibiting (pH 4.5) assay

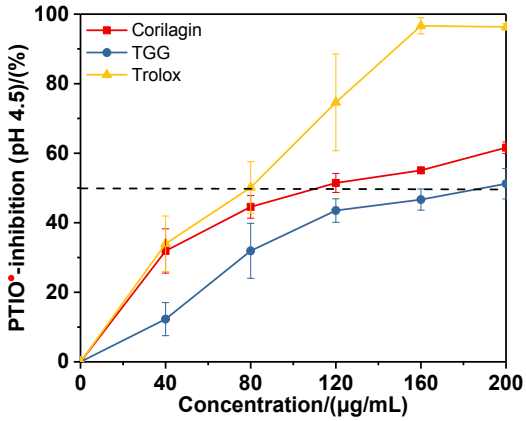

Figure S15. The does response curves of corilgin, TGG, and the positive control in Fe^{3+}-reducing assay

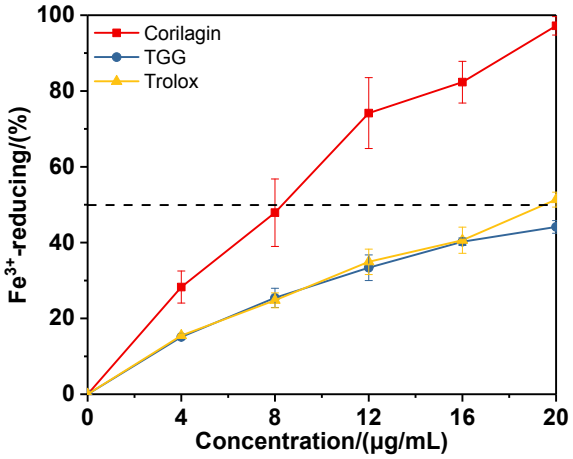

Figure S16. The does response curves of corilgin, TGG, and the positive control in DPPH•-inhibiting assay