Supporting Information

Cocrystal of Apigenin with Higher Solubility, Enhanced Oral Bioavailability and Anti-inflammatory Effect

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CONTENTS

1. EXPERIMENTAL SECTION

1.1 Materials and synthetic methods.

Apigenin was purchased as anhydrous powders with >98% purity from Ci Yuan Bio-Tech Co. Ltd. (Shanxi, China). 4,4′-bipyridine (98.5%) was obtained from Aladdin Reagent Inc. (Shanghai, China). All of the other chemicals and solvents were commercially available and used without further purification.

1.2 Identification and Characterization

1.2.1 Powder X-ray diffraction (PXRD).

The PXRD data of the cocrystal was were collected on a Rigaku MiniFlex 600 diffractometer, equipped with Scintillation Counter detector, with Cu Kα radiation at 1.54060 Å with tube voltage and power at 40 kV and 15 mA, respectively. Each pattern was collected with a step size of 2 °/min in the 2θ range of 3-45 °.

1.2.2 Vibrational Spectroscopy (FT-IR and FT-Raman).

The FT-IR spectroscopy analysis was collected on a calibrated Nicolet Nexus 470 in diffuse-reflectance mode. The data set of the samples dispersed in a dry KBR pellet was collected over the range of 4000−400 cm^{-1}, with an average of 64 scans and a 2 cm^{-1} spectral resolution. The FT-Raman spectrum was measured by Lab RAM HR800 Laser Confocal Micro-Raman Spectroscopy. Samples were scanned in a range of 3000–50 cm^{-1} with 4 cm^{-1} resolution and using a 1064 nm laser light.

1.2.3 The Differential Scanning Calorimetry (DSC).

The DSC thermogram was obtained on a calibrated Thermo Plus EVO2 Rigaku DSC 8231 analyzer with a heating rate of 5 °C/min in the temperature range 30–500 ℃. Samples were all purged by a stream of dry N_2 gas.

1.2.4 Thermogravimetric Analysis (TG).

The TG analysis was conducted on a Labsys evo TG-DSC/DTA equipment, a heating rate of 10 ℃ min^{-1} in the temperature range 30−900 °C was applied. Samples were all purged by a stream of dry N_2 gas.

1.2.5 Nuclear Magnetic Resonance (NMR).

About 5 mg of dried sample powders was dissolved in an adequate amount of deuterated methanol. Around 0.8 mL of the sample solution was added into an NMR tube having a 5 mm in outer diameter and 178 mm in length for solution ^{1}H NMR analysis (Bruker Ascend 600 MHz). Chemical shifts were relative to the one of methanol at 3.31 ppm. Resonance peak areas were integrated by MestReNova (Version 11.0.4) software from Mestrelab Research S. L.

1.2.6 Crystal Structure Determination. Single Crystal X-ray Diffraction (SC-XRD).

X-ray reflections were collected on a SuperNova CCD diffractometer, using Mo-Kα radiation (λ = 0.71073 Å). The empirical absorption corrections were applied by using the CrysAlisPro Program.[S1] The structures were solved by direct methods and refined on F^2 by full-matrix least-squares methods using the SHELX program

package.[S2,S3] Non-hydrogen atoms were refined anisotropically, and organic hydrogen atoms were generated geometrically. H atoms bonded to O were located by difference maps and the displacement factors were refined freely. Crystal data are listed in Table S1, hydrogen-bonding parameters are listed in Table S2 and selected bond lengths and angles are listed in Table S3.

1.3 Equilibrium Solubility Determination.

In this study, an excess of cocrystal was placed in vials containing 10 mL of ethanol and 10 mL a mixture of ethanol and water (50:50 v/v). This was agitated using a mechanical shaker for 24 h at room temperature. The solution was filtered, and the amount of drug dissolved was analyzed HPLC at 269 nm.

1.4 In Vitro Dissolution Studies.

In shaking an excess of sample (5 mg) in 10 mL of the buffer contained (pH=1.2 hydrochloric acid (HCl) buffer, pH=6.8 and pH=7.4 phosphate buffer) in stoppered conical flasks in a mechanical water bath shaker at constant rate of 100 rpm and bath temperature at 37 ℃. Sampling intervals were fixed at 5, 15, 30, 50, 60, 120, 180, 240, 360, 480, 720 min and 24 h with 0.7 mL aliquots withdrawn and replaced with equal amount of buffer and analyzed Agilent Cary 8454 UV/vis spectrophotometer at 269 nm.

1.5 Phase Solubility Studies.

An excess amount of APG was added to a test tube containing 10 mL of different

concentrations of BPY solution at 37 ℃ (phosphate buffer, pH=6.8). The suspensions were stirred at 500 rpm. After 24 h for equilibrium, the solutions were analyzed by Agilent Cary 8454 UV/vis spectrophotometer at 269 nm after filtration. The corresponding stability constant K_s is calculated by K_s= slope / [D_0 (1-slope)], D_0, solubility of APG without BPY, slope, slope of phase solubility diagram.[S4]

1.6 In Vivo Bioavailability Study.

1.6.1 Pharmacokinetic Study.

The pharmacokinetic parameters were evaluated in orally dosed, food but not water deprived (for 12 h) male SD rats, each weighing 200-250 g (n = 6 per group). The samples were delivered via oral gavage at a dose equivalent 500 mg of apigenin/kg body weight of animal as a suspension in aqueous 1 % sodium CMC. Subsequently, the blood withdrawn (approx. 500 μL) at intervals of 20, 40, 60, 90, 120, 150, 180, 210, 240, 360 and 420 min in heparinized tube. The plasma samples were separated by centrifugation (4000 rpm, 10 min) and stored at −80 °C until analysis. The determination of apigenin and cocrystal were achieved using an analyzed HPLC.[S5]

1.6.2 Plasma sample preparation.

Plasma samples were processed by protein precipitation method. Simply, 0.2 mL of plasma was combined with 0.8 mL methanol, then vortexed for 1 min. After centrifugation at 20000 rpm (15 min), the supernatant liquid after dry with nitrogen stream, dissolve with 0.75 mL methanol and 50 *u*L was injected into the HPLC for quantitative analysis.

1.6.3 Anti-Inflammatory Activity.

The anti-inflammatory estimation was done by the xylene-induced mouse ear edema model on kunming (KM) mice (20-25 g), which were obtained from the hunan. Animals were maintained on a 12 h light / a 12 h dark cycle under regulated temperature (20-25 ℃) and humidity (50 ± 10%) and fed with standard diet and clean water ad libitum. Animals of either sex were divided into 4 groups. Positive control aspirin (100 mg/kg, per animal, p.o.) and APG, APG-BPY cocrystal of them (100 mg/kg, per animal, p.o.) were given for 7 days. The blank group was used as a blank control. The animals were deprived of only food and not water for the night before the experiment. After 60 min the administration, each animal received 20 μL of xylene on the anterior and posterior surface of the right ear and left ear was considered as negative control. Another 60 min later both ears were sampled with a punch (5 mm diameter) and weighted. The extent of ear edema was evaluated by the weight difference between the right and the left ear biopsies of the same animal.[S6]

Percent inhibition = ($\Delta W_{control}$-$\Delta W_{treatment}$) / $\Delta W_{control}$

Where: $\Delta W = W_{right}$-W_{left} (W_{right} is the weight of right ear at the specific time, W_{left} is the weight of left ear at the specific time)

1.7 HPLC Analysis Method.

The quantification of APG in both the dissolution samples and the pharmacokinetic samples was done using the Agilent 1260 Infinity II HPLC Systems. The separation was carried out on C18 column (6 μm, 4.6 mm × 150 mm) by Agilent Corporation.

The mobile phase consisted of methanol and 2 % methanoic acid (55:45, v/v). The flow rate was set at 1 mL/min, and the inject volume was 10 μL. The absorbance was measured at a wavelength of 269 nm, and the column temperature was set at 30 ℃.

1.8 Repeated dose toxicity.

For repeated dose toxicity studies, the test material dose formulation was evaluated for concentration, stability, and homogeneity using a validated analytical method prior to dosing of the experimental animals. APG-BPY were administered by i.g. route daily for 28 days following the method described in OECD.[S7, S8] Four groups of 7 animals each were used at blank, low (250 mg/kg), medial (500 mg/kg) and high (1000 mg/kg). Doses were administered by gavage (0.2 mL/10g bw). The body weight of the animals and the food consumption were monitored daily for 28 days. After treatment time, animals were anesthetized and euthanatized. Immediately blood, internal organs (heart, liver, spleen, lungs and kidneys) were removed for further analysis. Blood was analyzed by using a commercial kit (Nanjing JianCheng Biology Engineering Institute Laboratory, China). The internal organs dissected and fixed in a 4% paraformaldehyde solution. The organ fixed were processed routinely, and then they were embedded in paraffin, sectioned at 4 mm thickness, deparaffinized, rehydrated and stained with hematoxylin and eosin (H.E) with the use of the standard techniques. The slides were observed and photos were taken using an optical microscope. All the animal experimental procedures were approved by Guangxi University of Chinese Medicine Institutional Animal Ethical and Welfare Committee (Approval NO. DW20180921-01).

2 RESULTS

Table S1. Crystallographic Parameters of APG-BPY

Compound	APG-BPY
Empirical formula	$C_{25}H_{18}N_2O_5$
Formula weight	426.41
Crystal system	Monoclinic
Space group	$P2_1/n$
a/Å	11.9198(4)
b/Å	11.6203(4)
c/Å	14.9912(4)
α/ °	90
β/ °	100.717(3)
γ/ °	90
Volume/Å^3	2040.24(11)
Z	4
ρcalcg/cm^3	1.388
μ/mm^{-1}	0.098
Crystal size/mm^3	0.3 × 0.25 × 0.1
Reflections collected	15714
Independent reflections	5044 [R_{int} = 0.0175, R_{sigma} = 0.0185]
Data/restraints/parameters	5044/0/292
Goodness-of-fit on F^2	1.041
Final R indexes [$I > 2\sigma(I)$]	R_1 = 0.0509, wR_2 = 0.1456
Final R indexes [all data]	R_1 = 0.0606, wR_2 = 0.1522
Largest diff. peak/ hole / e Å^{-3}	0.84/-0.22

Table S2. Selected Geometric Parameters of Hydrogen Bonds in APG-BPY

D–H ···A	d(H ···A)/Å	d(D ···A)/Å	D–H ···A /°	symmetry code
O1–H1 ···N2[1]	1.96	2.7592(2)	165.4	
O4–H4 ···O3	1.88	2.6061(2)	147.8	[1]-1/2+*x*,1/2-*y*,-3/2+*z*
O5–H5 ···N1	1.91	2.6954(2)	159.3	

Table S3. Selected bond lengths (Å) and angles (o) for APG-BPY.

O1-C1	1.3481(2)	O5-C13	1.3444(2)
O2-C7	1.3669(2)	N1-C16	1.308(2)
O2-C11	1.3703(2)	N1-C20	1.324(3)
O3-C9	1.2538(2)	N2-C21	1.327(2)
O4-C15	1.3496(2)	N2-C25	1.325(2)
C7-O2-C11	120.06(1)	O5-C13-C14	122.07(1)
O1-C1-C2	117.22(2)	O4-C15-C10	119.95(1)
O1-C1-C6	123.64(1)	O4-C15-C14	119.59(1)
O2-C7-C4	111.00(1)	C14-C15-C10	120.46(1)
C8-C7-O2	121.81(1)	C16-N1-C20	116.16(1)
O3-C9-C8	123.09(1)	C25-N2-C21	116.68(1)
O3-C9-C10	121.46(1)	N1-C16-C17	123.97(2)
O2-C11-C10	120.89(1)	N1-C20-C19	124.04(2)
O2-C11-C12	115.90(1)	N2-C21-C22	123.50(2)
O5-C13-C12	116.38(1)	N2-C25-C24	123.99(2)

Figure S1. Photos of powder sample of APG-BPY.

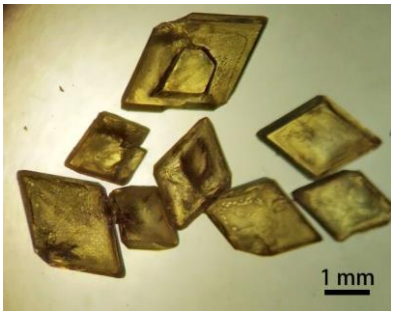

Figure S2. Photos of single crystals of APG-BPY.

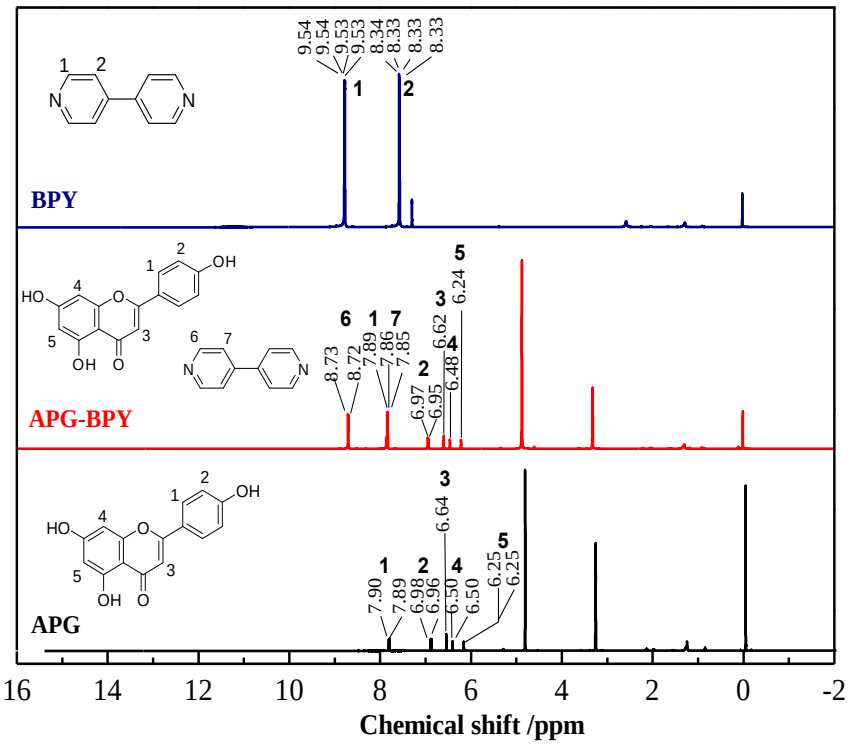

Figure S3. ^{1}H NMR (CD_3OD, 600 MHZ) of APG, BPY and APG-BPY.

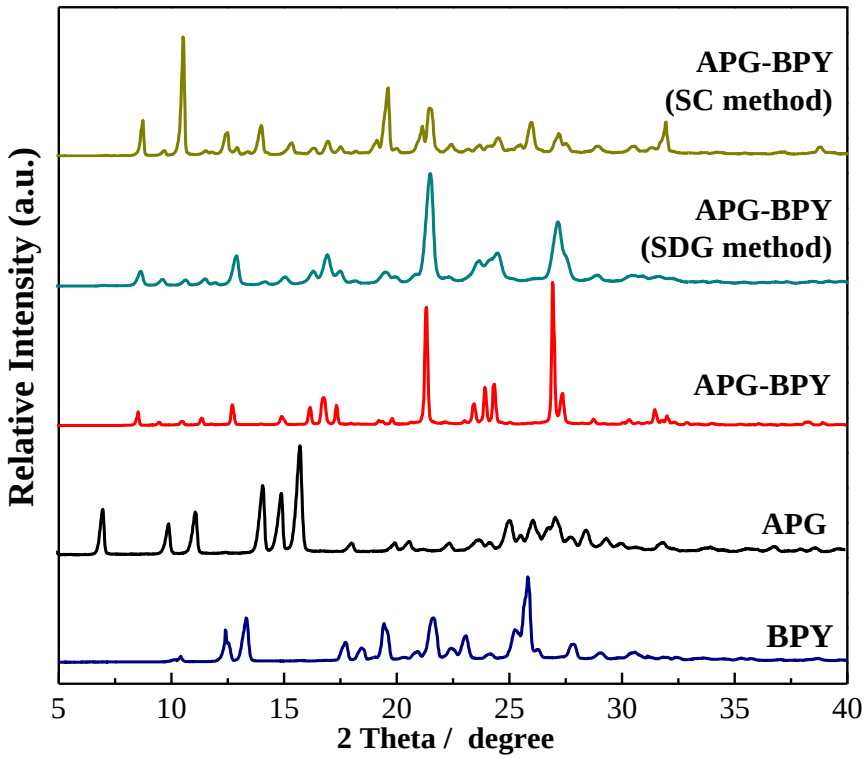

Figure S4. PXRD patterns of APG, BPY, APG-BPY, cocrystal products prepared by SDG and SC methods.

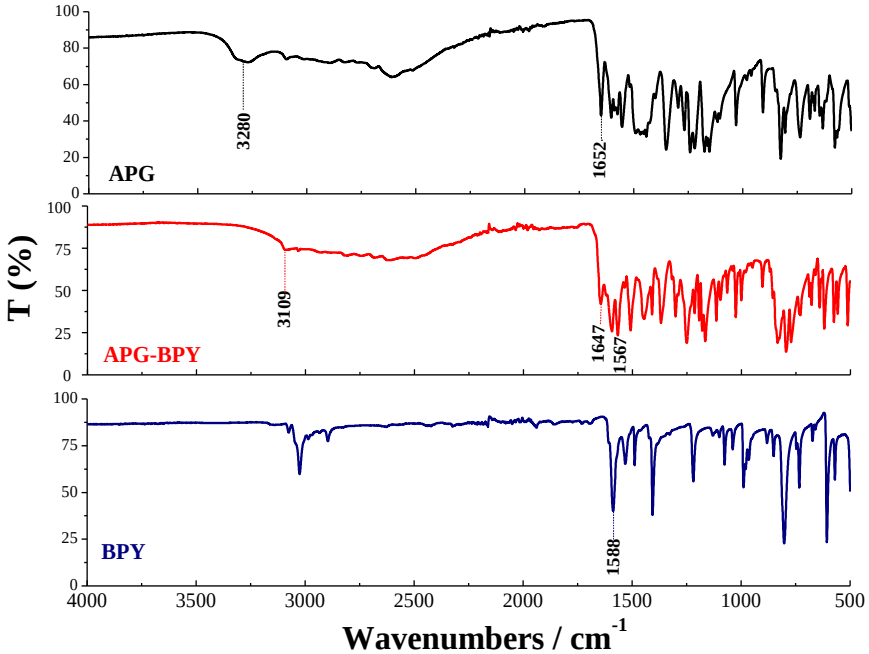

Figure S5. IR spectrums of APG, BPY and APG-BPY.

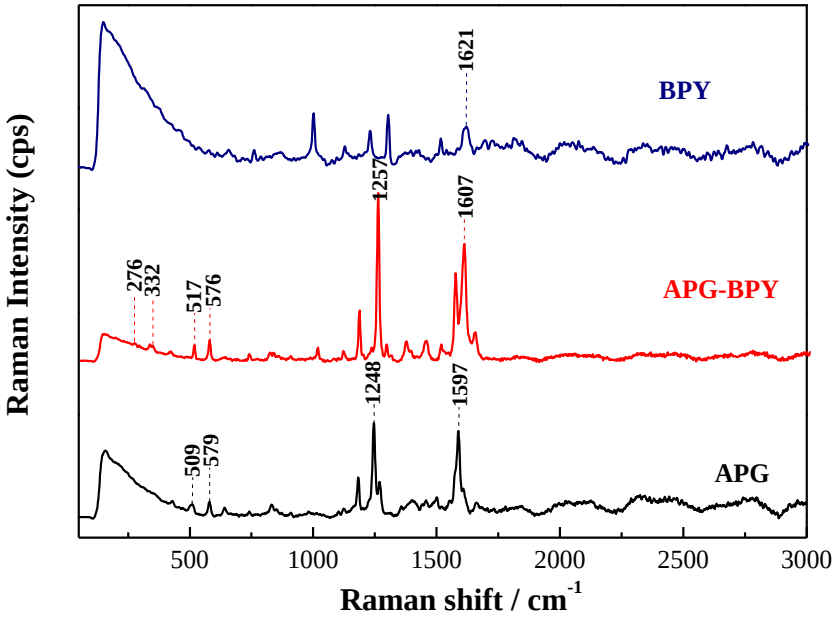

Figure S6. Raman spectrums of APG, BPY and APG-BPY.

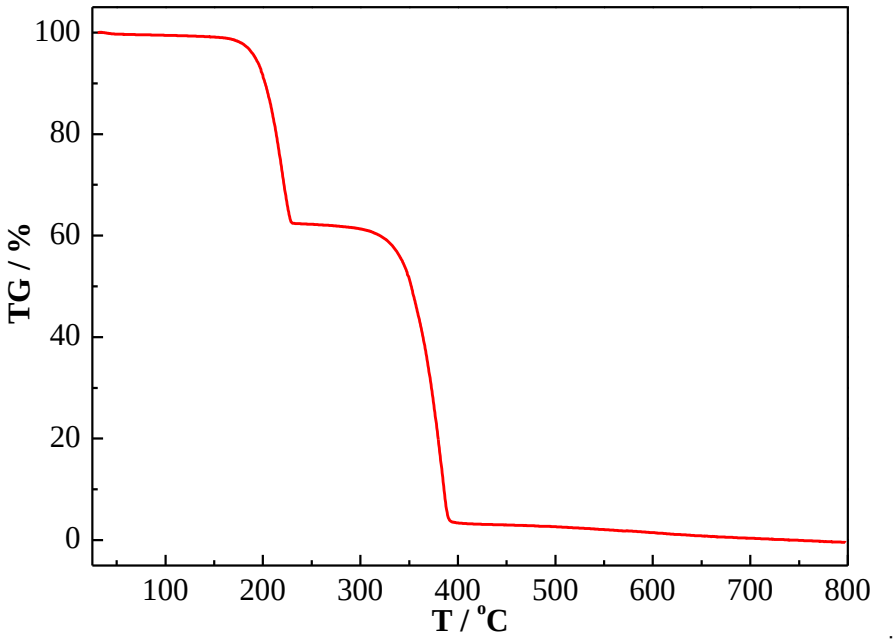

Figure S7. TG plot of APG-BPY.

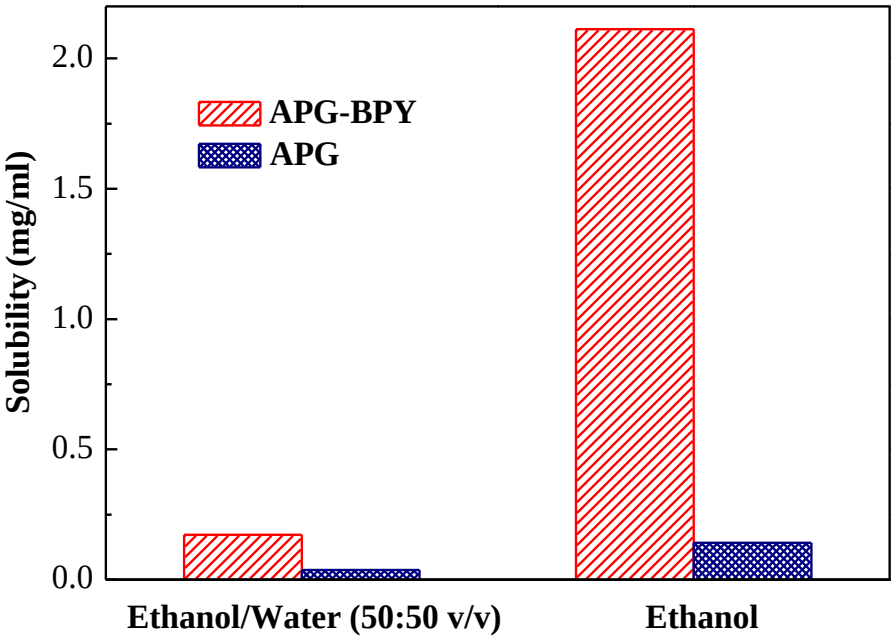

Figure S8. Solubility of APG in APG and APG-BPY.

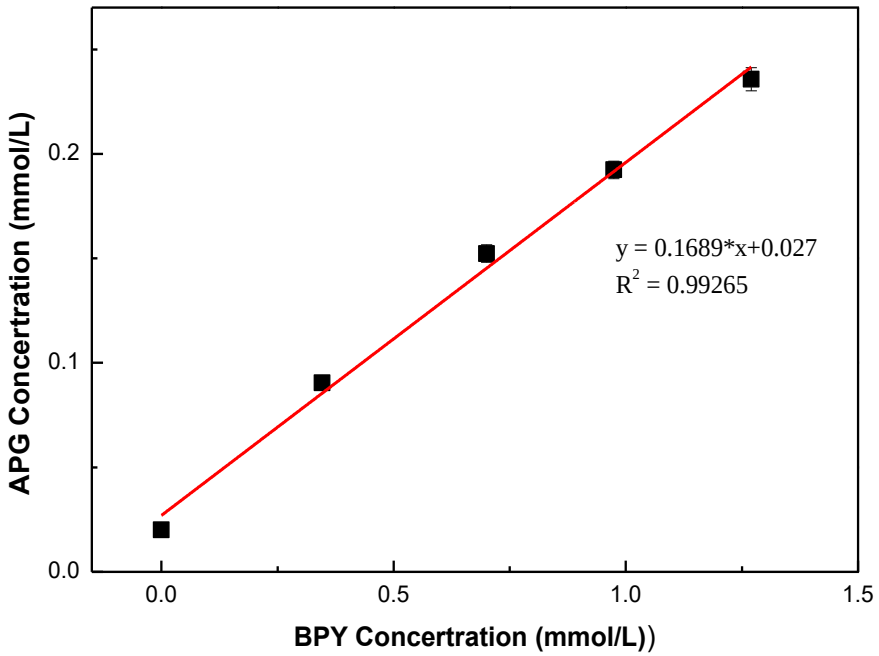

Figure S9 Phase solubility diagram for APG with BPY.

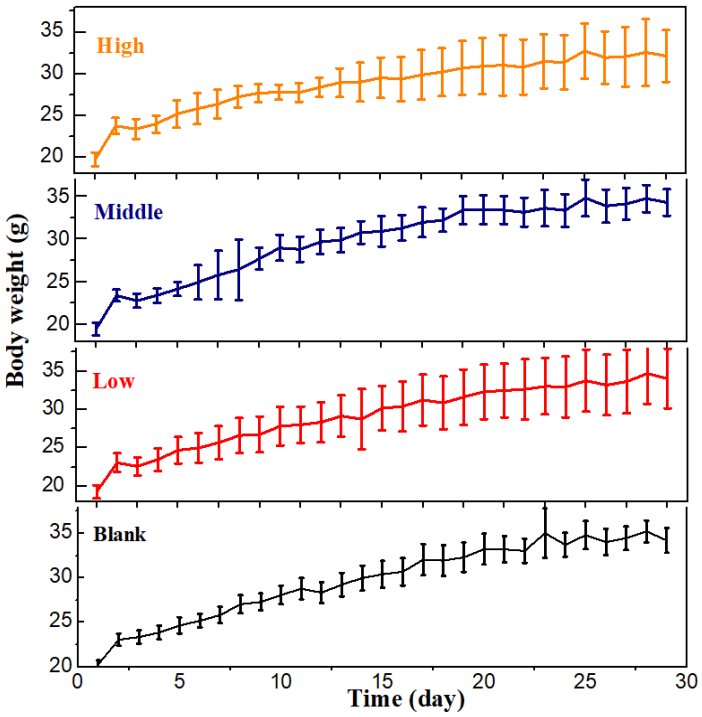

Figure S10. Weight of mice at different doses

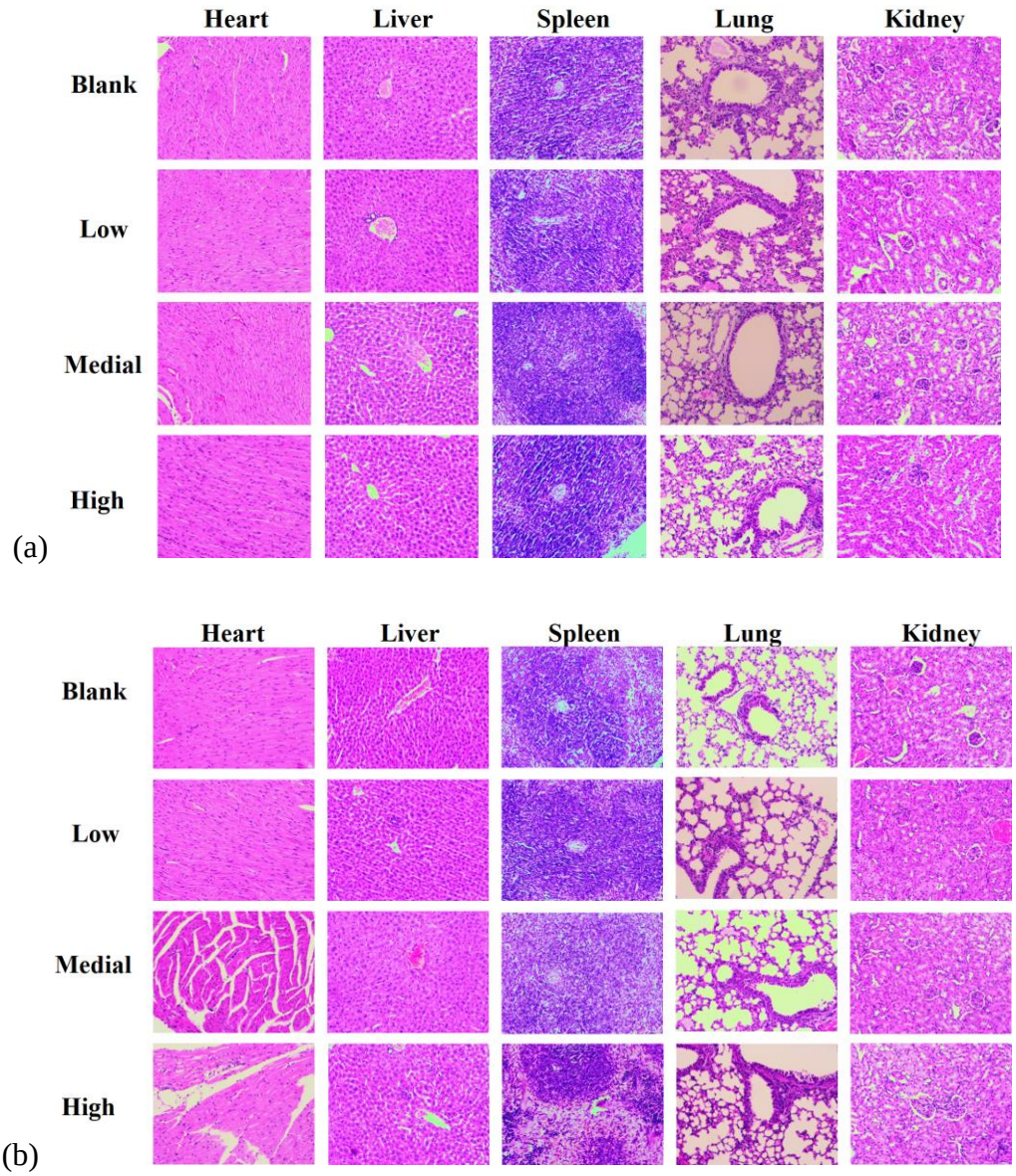

Figure S11 Effect of APG-BPY on organ tissues from mice during 28 days repeated dosage toxicity study (a is male; b is female). Photomicrographs representative of each treated and blank groups.

Table S4 Clinical blood chemistry parameters

Sex	Biochemical parameters	Study group			
		Blank	Low	Medial	High
Male	TG(mmol/L)	1.136±0.016	1.202±0.022	1.021±0.017	0.696±0.009
	TP(gprol/L)	39.205±0.046	51.205±0.09	40.623±0.077	41.200±0.038
	T-CHO(mmol/L)	5.213±0.025	5.694±0.017	5.646±0.020	4.777±0.021
	Alb(g/L)	42.949±0.009	45.215±0.007	40.703±0.009	47.858±0.017
	CRE(umol/L)	9.272±0.001	20.839±0.002	12.161±0.002	15.886±0.002
	BUN(mmol/L)	4.217±0.003	4.337±0.002	4.33±0.003	4.217±0.002
	AST/GOT(U/L)	6.191±0.005	6.480±0.018	7.714±0.007	10.483±0.012
	ALT/GPT(U/L)	26.876±0.002	28.220±0.013	28.605±0.015	30.676±0.013
Female	TG(mmol/L)	1.218±0.031	1.240±0.017	1.031±0.010	0.850±0.014
	TP(gprol/L)	50.756±0.072	47.563±0.044	43.217±0.045	36.767±0.104
	T-CHO(mmol/L)	3.761±0.012	4.836±0.021	4.201±0.017	4.656±0.012
	Alb(g/L)	45.592±0.009	42.571±0.009	45.781±0.006	35.152±0.009
	CRE(umol/L)	14.305±0.002	4.645±0.002	10.446±0.001	3.461±0.001
	BUN(mmol/L)	3.373±0.001	3.554±0.002	7.349±0.007	5.903±0.003
	AST/GOT(U/L)	3.335±0.005	6.402±0.008	7.652±0.003	5.739±0.007
	ALT/GPT(U/L)	21.725±0.005	24.199±0.008	42.991±0.003	34.596±0.007

Data are expressed as mean ± S.D. Triglycerides (TG); total protein (TP); total cholesterol (T-CHO); albumin (Alb); Creatinine (CRE); blood urea nitrogen (BUN); glutamic oxalacetic transaminase (AST/GOT); glutamic-pyruvic transaminase (ALT/GPT)

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