Electronic Supplementary Information (ESI) for

CO release with ratiometric fluorescence changes: A promising visible-light-triggered metal-free CO-releasing molecule

Weiyong Feng, Shumin Feng and Guoqiang Feng*

Key Laboratory of Pesticide and Chemical Biology of Ministry of Education, Chemical Biology Center, College of Chemistry, Central China Normal University (CCNU), 152 Luoyu Road, Wuhan 430079, P. R. China.

*Corresponding author. E-mail: gf256@mail.ccnu.edu.cn (G. Feng).

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1. Experimental

1.1. Materials and instruments

Unless otherwise stated, all chemicals and reagents were purchased from commercial suppliers and used as received. Carboxy-hemoglobin (COHb) detection kit was purchased from Leagene biotechnology company (Beijing, China) and was used in accordance with the instructions provided. Reactive oxygen species and reactive nitrogen species (ROS/RNS) were prepared and used freshly according to our published procedures.[1-3] The wild zebrafish (3 days old) were purchased from China Zebrafish Resource Center, Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China). Kunming mice (weight 20 ± 2 g) were purchased from Wuhan Center for Disease Control and Prevention. All animal experiments were proceeded with the permission of Wuhan Animal Ethics Committee and were carried out strictly in accordance with the requirements of the National Act on the use of experimental animals (China). NMR spectra were recorded on a Varian 400 NMR spectrometer. Mass spectra were collected on a Thermo Scientific DSQ II GC/MS spectrometer and a Bruker micro TOF-Q instrument. UV-Vis and fluorescence spectra were recorded on an Agilent Cary-100 UV-Vis spectrophotometer and an Agilent Cary Eclipse fluorescence spectrophotometer, respectively. The spectra of gas chromatography (GC) were recorded on a Fuli 9790IIA-2 GC spectrometer with a flame ionization detector (FID) detector and a Nickel conversion furnace. Cell and zebrafish imaging were performed on TCS SP8 Laser Scanning Confocal Microscopy (Leica, Germany) with 40× and 10× objective lens, respectively. In vivo imaging experiments were carried out in a Bruker In Vivo-Xtreme imaging system.

1.2. Synthetic procedures for compound 2, Cou-Flavone and Cou-Carboxylate
Scheme S2. Synthesis route for compound 2, Cou-Flavone and Cou-Carboxylate.

Synthesis of compound 2. To a solution of 2-hydroxy acetophenone (272 mg, 2 mmol) and 7-(diethylamino)-2-oxo-2H-chromene-3-carbaldehyde (coumarin 1[4], 490 mg, 2 mmol) in ethanol (15 mL) was added 200 μL of piperidine. The mixture was then heated to reflux for 24 h. After cooling, the red solid product was collected by filtration, and then recrystallized from methanol to afford compound 2 as red crystals (550 mg, 76%). Mp: 203-204 °C. TLC (silica plate): *Rf ~0.7* (dichloromethane). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 8.32 (d, $J = 15.1$ Hz, 1H), 8.01 (d, $J = 7.8$ Hz, 1H), 7.77 (s, 1H), 7.67 (d, $J = 15.0$ Hz, 1H), 7.47 (t, $J = 7.7$ Hz, 1H), 7.32 (d, $J = 8.8$ Hz, 1H), 6.99 (d, $J = 8.3$ Hz, 1H), 6.94 (t, $J = 7.6$ Hz, 1H), 6.59 (d, $J = 9.1$ Hz, 1H), 6.48 (s, 1H), 5.52 (br s, 1H), 3.43 (q, $J = 6.7$ Hz, 4H), 1.23 (t, $J = 7.1$ Hz, 6H). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 194.39, 163.51, 160.22, 156.70, 152.18, 147.15, 140.82, 136.15, 130.27, 121.11, 120.29, 118.88, 118.28, 114.44, 109.71, 108.95, 96.83, 45.12, 12.53. EI-MS: m/z found 362.78 (M$^+$).

Synthesis of Cou-Flavone. Compound 2 (363 mg, 1 mmol) was dissolved in methanol (10 mL) and followed by addition of 50 mL of NaOH aqueous solution (10%). Then 1 mL of hydrogen peroxide (30%) was added dropwise to the reaction mixture. The resulting mixture was then stirred under room temperature for 24 h. After that, the pH of the reaction solution was adjusted to 6-7 with diluted HCl. Water (10 mL × 3) was used to wash the resulting solution for three times, and the dichloromethane phase was dried over Na$_2$SO$_4$. After filtered and removal of the organic solvent, the crude product was purified by silica column chromatography to afford Cou-Flavone as a
yellow solid (168 mg, 45 %). Mp: 216-217 °C. TLC (silica plate): $R_f \approx 0.65$ (dichloromethane:methanol, 10:1, v/v). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 8.24 (d, $J = 5.4$ Hz, 2H), 7.66 (t, $J = 7.8$ Hz, 1H), 7.54 (d, $J = 8.5$ Hz, 1H), 7.39 (q, $J = 8.2$, 7.4 Hz, 2H), 7.01 (s, 1H), 6.66 (d, $J = 8.4$ Hz, 1H), 6.52 (s, 1H), 3.46 (q, $J = 7.0$ Hz, 4H), 1.25 (t, $J = 7.0$ Hz, 6H). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 173.37, 160.02, 157.11, 155.56, 152.25, 145.73, 143.49, 138.95, 133.45, 130.49, 125.57, 124.42, 121.59, 118.30, 110.43, 109.91, 108.21, 96.85, 45.13, 12.48. EI-MS: m/z found 377.1 (M$^+$). ESI-MS: m/z found 378.27 (M + H$^+$). HR-MS: calcd for C$_{22}$H$_{20}$NO$_5^+$ (M + H$^+$): 378.1336; found 378.1335 and calcd. for C$_{22}$H$_{19}$NO$_5$Na$^+$ (M + Na$^+$): 400.1155; found 400.1158.

**Synthesis of Cou-Carboxylate.** Cou-Flavone (38 mg, 0.1 mmol) was dissolved in 50 mL of 10 mM HEPES buffer (pH 7.4, with 10 % DMSO). Then the mixture was stirred under a portable 460 nm light irradiation (7 w, 12 mW /cm$^2$) at room temperature for 6 h. After the reaction finished, 50 mL of dichloromethane was added and the solution was washed by water (10 mL $\times$ 3). Finally, the organic phase was dried over Na$_2$SO$_4$. After filtered and removal of the organic solvent, the crude solid product was collected and further purified by silica column chromatography to afford Cou-Carboxylate as a light-yellow solid (25 mg, 66 %). Mp: 235-236 °C. TLC (silica plate): $R_f \sim 0.35$ (dichloromethane:methanol, 10:1, v/v). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 10.58 (s, 1H), 8.67 (s, 1H), 7.93 (d, $J = 8.0$ Hz, 1H), 7.52 (t, $J = 7.8$ Hz, 1H), 7.46 (d, $J = 8.9$ Hz, 1H), 7.01 (d, $J = 8.3$ Hz, 1H), 6.93 (t, $J = 7.4$ Hz, 1H), 6.71 (d, $J = 8.9$ Hz, 1H), 6.53 (s, 1H), 3.50 (q, $J = 7.0$ Hz, 4H), 1.27 (t, $J = 7.0$ Hz, 6H). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 174.01, 165.59, 165.05, 162.16, 158.11, 153.87, 150.47, 136.72, 132.09, 130.90, 119.51, 117.74, 111.56, 111.00, 108.61, 105.29, 96.84, 45.40, 12.43. EI-MS: m/z found 381.1 (M$^+$). ESI-MS: m/z found 382.36 (M + H$^+$). HR-MS: calcd. for C$_{22}$H$_{20}$NO$_6^+$ (M + H$^+$): 382.1285; found 382.1222.

**1.3. Optical studies**

The optical studies of Cou-Flavone (10 µM) were performed in HEPES buffer (10 mM, pH 7.4, with 10% DMSO, v/v) and in a quartz cuvette with 10 mm of lightpath. A portable UV-light (460 nm, 7 w, 12 mW /cm$^2$) was used as the light source for
irradiation of \textbf{Cou-Flavone} to release CO. The UV-Vis or fluorescent spectra of the \textbf{Cou-Flavone} solution were recorded after irradiation at 460 nm or addition of an analyte at room temperature. $\lambda_{ex} = 420$ nm and slit width: $d_{ex} = d_{em} = 5$ nm were set for fluorescence measurements. The fluorescence quantum yield was reported with fluorescein as the reference.

1.4. Cytotoxicity and anti-proliferation experiments

All of the cytotoxicity and anti-proliferation experiments were measured by standard MTT assays (MTT: 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyltetrazolium bromide). The cytotoxicity of \textbf{Cou-Flavone} and its product \textbf{Cou-Carboxylate} were measured using living MCF-7 cells and HeLa cells. The cytotoxicity of 460 nm irradiation (7w, 12 mW/cm$^2$) were measured using living MCF-7 cells. The anti-proliferation experiments were measured using living MCF-7 cells.

For cytotoxicity experiments, the cells were cultured in Dulbecco Modified Eagle Medium (DMEM) with high glucose medium, supplemented with 10% fetal bovine serum (FBS), 100 $\mu$g/mL penicillin and 100 $\mu$g/mL streptomycin in a 5% CO$_2$, water saturated incubator at 37 °C. After the cells were treated with various concentrations of \textbf{Cou-Flavone}, \textbf{Cou-Carboxylate}, and different times of irradiation (460 nm, 7w, 12 mW/cm$^2$), then with MTT, the absorbance of the resulting solution dissolved with DMSO was measured at 490 nm with a microplate reader. The cell viability was expressed as the optical density ratio of the treatment to control.

For anti-proliferation experiments, the cells were cultured in Dulbecco Modified Eagle Medium (DMEM) with high glucose medium, supplemented with 10% fetal bovine serum (FBS), 100 $\mu$g/mL penicillin and 100 $\mu$g/mL streptomycin in a 5% CO$_2$, water saturated incubator at 37 °C. After 12 h, the blank group of cells were treated with MTT, the absorbance of the resulting solution dissolved with DMSO was measured at 490 nm with a microplate reader. The experimental groups were continue cultured in the absence and in the presence of different concentrations of \textbf{Cou-Flavone} (0, 10, 20, 30, 40, and 50 $\mu$M) with 15 min irradiation (460 nm, 7w, 12 mW/cm$^2$) for another 24 h, then with MTT and measured by the absorbance. The relative numbers of cells was expressed as the optical density ratio of the experimental group to blank
group.

1.5. CO-releasing and imaging with **Cou-Flavone** in living cells, zebrafish and mice

**CO-releasing and Imaging with Cou-Flavone in Living Cells.** MCF-7 cells were seeded in a 24-well culture plate for one night before cell imaging experiments. In the experiment of cell imaging, living cells were incubated with **Cou-Flavone** (10 μM) at 37 °C for 15 min as the control. For imaging of CO-releasing, cells were incubated with **Cou-Flavone** (10 μM) for 15 min followed with an illumination (460 nm, 7w, 12 mW/cm²) for 5, 10, or 15 min, respectively, and then imaged after incubation with our recently published NIR fluorescent CO probe system[^5] (10 μM **Probe 1** + 10 μM PdCl₂) for another 15 min at 37 °C. All images were taken after washing the cells with PBS buffer for three times.

**CO-releasing and Imaging with Cou-Flavone in Living Zebrafish.** The wild type zebrafish (3 dpf) were cultivated in a 6-well culture plate and costained with **Cou-Flavone** (10 μM) for 15 min. After washed with PBS for three times, the wild type zebrafish were given an illumination (460 nm, 7 w, 12 mW/cm2) by using a portable corn lamp for 0, 5, and 15 min, respectively. The imaging of CO-releasing was then carried out after incubation with our NIR fluorescent CO probe system (10 μM **Probe 1** + 10 μM Pd²⁺) for another 15 min. The images were obtained with excitation at 405, 458 and 670 nm, and emissions were collected at 420–510 nm, 520–620 nm, and 670–750 nm for blue channel, yellow channel, and red channel, respectively.

**CO-releasing and Imaging with Cou-Flavone in Living Mice.** For in vivo imaging of CO-releasing, Kunming mice were used as model. Before the mice experiments, all of the mice were anesthetized by intraperitoneal (ip) injection of 1% pelltobarbitalummatricum solution (0.01 mL/g). The mice were then divided into three groups. The first group was only given an ip injection of 100 µL of saline for 5 min as the blank group. The second group was given an ip injection of 100 µL of our **probe 1** system (10 μM **Probe 1** + 10 μM Pd²⁺) for 5 min and imaged as another control. The third group was first given an ip injection of 100 µL of **Cou-Flavone** (20 μM) for 15 min. Then the injection area of the abdominal cavity was given an illumination (460 nm, 7w, 12 mW/cm2) with a portable corn lamp for 0, 5, and 20 min, respectively.
Finally, 100 µL of the **probe 1** system (10 µM **Probe 1** + 10 µM Pd²⁺) was ip injected to the same area and the mouse was then imaged after 5 min. Imaging was carried out in a Bruker In Vivo-Xtreme imaging system with three different channels for fluorescence imaging. The blue channel has an excitation filter of 420 nm and an emission filter of 470 nm, which was used for detection of **Cou-Carboxylate** (the photolysis product of **Cou-Flavone**). The yellow channel has an excitation filter of 460 nm and an emission filter of 550 nm, which was used for detection of **Cou-Flavone**. The red channel has an excitation filter of 670 nm and an emission filter of 710 nm, which was used for detection of the released CO with the **probe 1** system.

Note: For living zebrafish studies, the illumination was given to the whole body of zebrafish due to their small sizes. For illumination in living mice, we covered the mouse with an opaque black cloth with a square hole (~3×3 cm) which was exposed to the injection area of abdomen for illumination.

1.6. Detection of CO released from **Cou-Flavone** by gas chromatography

The CO released from **Cou-Flavone** was also recorded by gas chromatography with a FID detector. In this experiment, 1.5 mL of transparent glass bottles with sealing ring were prepared at first, followed with addition of 1 mL of **Cou-Flavone** (200 µM) solution (dissolved in 10 mM HEPES buffer, pH 7.4, with 10% DMSO, v/v). The bottles were capped immediately and the air inside was removed by vacuum. Then the bottles were given different times (1, 5, 10, and 15 min) of irradiation with a portable light (460 nm, 7 w, 12 mW /cm²), respectively, After that, the bottles were placed in a 70 °C water bath for 10 min to release CO into the vial headspace. Analysis of the released CO was completed by injecting 500 µL from the headspace of each bottle into the GC system after the bottle content was cooled to room temperature.

1.7. Detection of CO released from **Cou-Flavone** in vivo by colorimetry

For further confirm the CO release with **Cou-Flavone** in living systems, we measured the COHb production in vivo by colorimetric method. Kunming mice were used as model. Before the experiments, all of the mice were anesthetized by intraperitoneal (ip) injection of 1% pelltobarbitalumnatricum solution (0.01 mL/g). The mice were then divided into two groups. The first group was only given an ip injection of 100 µL of
saline as the blank group. The second group was given an ip injection of 100 µL of **Cou-Flavone** (200 µM), then the injection area of the abdominal cavity was illuminated by a portable corn lamp (460 nm, 7w, 12 mW/cm²) for 0, 5, 10 and 20 min, respectively. After 1 h, the fresh blood was collected from the heart of mice. 0.1 mL of blood was then quickly added to an Alkaline Buffer (2 mL) followed by addition of Na₂S₂O₄ (20 mg). The Alkaline Buffer and Na₂S₂O₄ were used from the purchased COHb detection kit. After mixing well, 0.2 mL of the resulting solution was added to a 96-well plate. The absorbance at 538 nm and 578 nm was then measured immediately with a microplate reader. The amount of COHb in the total amount of Hb (ω in %) was calculated based on the method of COHb (%) = \{2.44*(A538/A578)-2.68\}*100% in accordance with the instructions of the detection kit provided.

**References**


2. **Table S1. Comparison of metal-free fluorescent CORMs**

<table>
<thead>
<tr>
<th>Reference</th>
<th>Structure</th>
<th>Conditions</th>
<th>Ex/Em (nm)</th>
<th>Ratiometric Fluorescence changes</th>
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<td><strong>This work</strong></td>
<td><img src="image1" alt="Structure" /> HEPES buffer (10 mM, 10% DMSO, pH 7.4).</td>
<td>Ex 420 Em 475/555</td>
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<td>Nat. Chem. 2018, 10, 787–794</td>
<td><img src="image2" alt="Structure" /> Click and release</td>
<td>20% of DMSO/PBS</td>
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<tr>
<td>Angew. Chem., Int. Ed. 2018, 57, 12415–12419</td>
<td><img src="image3" alt="Structure" /> PBS buffer solution (50 mM, pH 7.4) with 10% CH₃CN</td>
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<td>Description</td>
<td>Compound Structure</td>
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<td>J. Am. Chem. Soc.</td>
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<td>ACS Chem. Biol.</td>
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<td>CH₃CN·DMSO (10:1)</td>
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<td>Org. Lett.</td>
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<td>Aqueous phosphate buffer solutions (pH = 7.4).</td>
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<td>410/460(582)</td>
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\textbf{H NMR spectrum of compound 2 in CDCl}_3.

\textbf{C NMR spectrum of compound 2 in CDCl}_3.
EI-MS spectrum of compound 2

$^1$H NMR spectrum of Cou-Flavone in CDCl$_3$. 
$13^\text{C}$ NMR spectrum of \textbf{Cou-Flavone} in CDCl$_3$.

EI-MS spectrum of \textbf{Cou-Flavone}

ESI-MS spectrum of \textbf{Cou-Flavone}
HR-MS spectrum of Cou-Flavone

$\text{CO}_2\text{H}_2\text{NO}_2^+ + \text{H}^+$

Exact Mass: 378.1336

$\text{C}_2\text{H}_5\text{NNaO}_5^+$

Exact Mass: 400.11554

$^1\text{H}$ NMR spectrum of Cou-Carboxylate in CDCl$_3$. 
$^{13}$C NMR spectrum of Cou-Carboxylate in CDCl$_3$.

El-MS spectrum of Cou-Carboxylate

LC-MS spectrum of Cou-Carboxylate
Figure S1. (a) The fluorescent intensity changes at 555 and 475 nm of the Cou-Flavone (10 µM) solution with 460 nm light irradiation. (b) Abs changes at 470 and 434 nm of the Cou-Flavone (10 µM) solution with 460 nm light irradiation. All data were collected in HEPES buffer (10 mM, pH 7.4, 10% DMSO, v/v) at 25 °C and each data was collected after 1 min irradiation (460 nm, 7 w, 12 mW/cm²). For fluorescent measurements, \( \lambda_{ex} = 420 \) nm, slit width: \( d_{ex} = d_{em} = 5 \) nm.
Figure S2. The fluorescent intensity changes of the Cou-Flavone (10 µM) solution at 555 nm during light irradiation (460 nm, 7 w, 12 mW/cm²) in HEPES buffer (10 mM, with 10% DMSO, v/v) at 25 °C. λ<sub>ex</sub> = 420 nm, slit width: d<sub>ex</sub> = d<sub>em</sub> = 5 nm. The data was fitted (red line) by a first order reaction scheme.

Figure S3. (a) The fluorescent intensity changes of the Cou-Flavone (10 µM) solution at 555 and 475 nm in the absence of irradiation. (b) Time-dependent of fluorescent intensity changes of the Cou-Flavone (10 µM) solution at 555 and 475 nm after the periodic irradiation every 5 min (460 nm, 7 w, 12 mW/cm²). All data were collected in HEPES buffer (10 mM, pH 7.4, 10% DMSO, v/v) at 25 °C. λ<sub>ex</sub> = 420 nm, slit width: d<sub>ex</sub> = d<sub>em</sub> = 5 nm.
Figure S4. The structure of **Probe 1** for NIR fluorescent turn-on detection of CO (see Feng, W.; Feng, G. *Sens. Actuators B*. 2018, 255, 2314–2320.).

![Diagram of Probe 1](image)

**Figure S5.** The release of CO from **Cou-Flavone** was proved by **probe 1**. (a) The NIR fluorescence spectrum of **Cou-Flavone** (10 μM) after irradiated (460 nm, 7w, 12 mW/cm²) for 15 min as blank. (b) The fluorescence spectrum of 5 μM **Probe 1** and 5 μM Pd^{2+} as control. (c) The fluorescence spectrum of **Cou-Flavone** (10 μM) after irradiated (460 nm, 7w, 12 mW/cm²) for 15 min and then indicated with 5 μM **Probe 1** and 5 μM Pd^{2+}. All spectra were measured in HEPES buffer (10 mM, with 10% DMSO, v/v) at 25 °C with λ<sub>ex</sub> = 670 nm, slit width: d<sub>ex</sub> = 5 nm; d<sub>em</sub> = 10 nm.
Figure S6. (a) Gas chromatographic peak of CO and (b) amount of CO (ppm) released from **Cou-Flavone** (200 µM) with an irradiation (460 nm, 7w, 12 mW/cm²) in HEPES buffer (10 mM, pH 7.4, 10% DMSO, v/v) at 25 ºC for different times (0, 1, 5, 10, and 15 min). CO gas (3 ppm) was used as reference.

Figure S7. LC-MS analysis of the reaction mixture of **Cou-Flavone** (50 µM) after irradiation (460 nm, 7w, 12 mW/cm²) for different times (0, 1, and 5 min) in HEPES buffer (10 mM, pH 7.4, with 10 % DMSO, v/v) at room temperature. With **Cou-Carboxylate** as reference, this data showed that **Cou-Carboxylate** was generated and
Figure S8. HR-MS analysis of the reaction mixture of Cou-Flavone (10 μM) with irradiation (460 nm, 7w, 12 mW/cm²) in HEPES buffer (10 mM, pH 7.4, with 10 % DMSO, v/v) for 5 min at room temperature. This result showed that Cou-Carboxylate was generated.

Figure S9. The (a) fluorescence and (b) UV-vis spectra of Cou-Flavone, Cou-Flavone after irradiation (460 nm, 7w, 12 mW/cm²), and Cou-Carboxylate in HEPES buffer (10 mM, pH 7.4, 10 % DMSO, v/v) at 25 ℃. For fluorescent measurements, λ<sub>ex</sub> = 420 nm, slit width: Δ<sub>ex</sub> = 5 nm, Δ<sub>em</sub> = 5 nm.
**Figure S10.** Percentage of viable HeLa and MCF-7 cells after treated with different concentrations of the (a) Cou-Flavone and (b) Cou-Carboxylate for 24 hours, respectively. The cell viability was obtained via MTT assay.

**Figure S11.** (a) Percentage of viable MCF-7 cells after irradiation (460 nm, 7w, 12 mW/cm²) for different times. (b) The result of anti-proliferation experiments (the green column means the blank control cells without treatment of Cou-Flavone. The red columns mean the cell viability after treatment of different concentrations of Cou-Flavone with 15 min irradiation (460 nm, 7w, 12 mW/cm²). The cell viability of MCF-7 cells were obtained via MTT assay.
Figure S12. The COHb production in vitro. For groups A-E: (A) normal mouse as control group. (B) **Cou-Flavone** (100 µL, 200 µM in saline with 10% DMSO) treated mouse without irradiation as blank group. (C) **Cou-Flavone** (100 µL, 200 µM in saline with 10% DMSO) treated group with irradiation for 5 min (460 nm, 7w, 12 mW/cm²). (D) **Cou-Flavone** (100 µL, 200 µM in saline with 10% DMSO) treated group with irradiation for 10 min (460 nm, 7w, 12 mW/cm²). (E) **Cou-Flavone** (100 µL, 200 µM in saline with 10% DMSO) treated group with irradiation for 20 min (460 nm, 7w, 12 mW/cm²). Results are presented as mean ± s.d. n = 5 per group. **P < 0.01, versus group A.