Supporting Information

Luteolin prevents irinotecan-induced intestinal mucositis in mice through antioxidant and anti-inflammatory properties.

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Supporting Method

Method S1. Evaluation of digestive motility rate

Diarrheal state

The Swiss mice were divided into three groups, including naive, vehicle (Veh: water plus 1% Tween, 10 ml/kg) and luteolin (30 mg/kg, p.o), of 6 animals each, that were orally treated by gavage for 14 days, except for naive which did not receive any treatment. From the 7th to 10th day, irinotecan was intraperitoneally given at the dose of 75 mg/kg per day to the Veh and Lut groups. On the 15^{th} day of experiment, the animals received 0.5 ml of the semisolid marker solution (0.05% phenol red plus 1.5% carboxymethyl cellulose). After 20 min, the mice were euthanized and small intestine was removed. The distance traveled by phenol red (A) and the total length of the small intestine (B) were measured to further calculate the digestive motility rate (DM %) through equation $DM = A \div B \times 100$ (Boeing et al., 2018).

Resting state

The Swiss mice were divided into three groups of 6 animals each, including vehicle (Veh: water plus 1% Tween, 10 ml/kg, p.o.), atropine (Atp: 3 mg/kg, s.c.), and luteolin (30 mg/kg, p.o.). Thirty minutes after Atp or one hour after Veh or luteolin treatments, the animals received 0.5 ml of the semisolid marker solution (0.05% phenol red plus 1.5% carboxymethyl cellulose). After 20 min, the mice were euthanized, and digestive motility rate was evaluated as described above.

Method S2. Effect of Luteolin treatment during 7 days on irinotecan-induced intestinal mucositis.

The Swiss mice were divided into three groups, including naive, vehicle (Veh: water plus 1% Tween, 10 ml/kg) and luteolin (30 mg/kg), of 10 animals each, that were orally treated by gavage for 7 days, except for naive which did not receive any treatment.

From the 1st to the 4th day, irinotecan was administered intraperitoneally at a dose of 75 mg/kg per day to the Veh and Lut groups to induce intestinal mucositis (Ikuno et al., 1995). The body weight was daily measured, and diarrhea score and weight/length ratio of intestine were measured in the 7th day of experiment as previously described.

Method S3. Mechanical withdrawal threshold evaluation

The Swiss mice were divided into three groups, including naive, vehicle (Veh: water plus 1% Tween, 10 ml/kg) and luteolin (30 mg/kg), of 10 animals each, that were orally treated by gavage for 14 days, except for naive which did not receive any treatment.

From the 7th to 10th day, irinotecan was intraperitoneally given at a dose of 75 mg/kg per day to the Veh and Lut groups and the mechanical hypersensitivity was measured.

For the evaluation of mechanical hypersensitivity, mice were individually placed in Plexiglas boxes on elevated wire mesh platforms to allow access to the ventral surface of the right hind paw. The mice were previously habituated in the compartments for 30 min daily for 2 weeks before the beginning of the experiment. The withdrawal response frequency was measured following 10 applications (duration of 1 s each) of von Frey filaments (Stoelting, Chicago, IL, USA). Stimuli were delivered from below, to the plantar surface of the right hind paw. The 0.6 g filament produces a mean withdrawal

frequency of about 15% in naive mice, which is considered to be an adequate value for the measurement of mechanical hypersensitivity (Quintão et al., 2019).

Mechanical threshold was defined as the minimum weight of the filament that induced at least 5 responses (fast paw withdrawal, flinching, or licking/biting of the stimulated paw) out of 10 stimulations (Cobos et al., 2012). The comparison between groups was done by the line graph and also by the area under the curve of the respective group.

Method S4. Biochemical analyses

Blood samples collected at the end of the experiment were separated by centrifugation at 3000g for 15 min at 4 °C. The plasma levels of creatinine, urea, aspartate transaminase (AST) and alanine transaminase (ALT) were assessed using colorimetric test (Bioclin, Belo Horizonte, MG, Brazil), following the manufacturer's instructions.

Method S5. Evaluation of antitumor activity

C57BL/6 female mice were exclusively used to perform this study, once this mouse strain allows tumor development by B16F10 cells (RRID:CVCL_0159) injection. Briefly, B16F10 (melanoma cells) were injected subcutaneously in the flank of mice at a concentration of 2×10^5 following the protocol previously described (Arifa *et al*., 2016). After 21 days, mice that developed the tumor were randomly divided into groups (6-10) which were treated with vehicle (Veh: water plus 1% Tween, 10 ml/kg, p.o.), irinotecan (Iri: 75 mg/kg, i.p.) luteolin (Lut: 30 mg/kg, p.o.) or Lut plus Iri for 7 days. The tumor area was measured daily using a pachymeter and after euthanasia on the 8th day, weight and volume were also evaluated.

Method S6. Cell viability

Murine intestinal epithelial cells (IEC-6, RRID:CVCL_0343) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Vitrocell Embriolife, Brazil), supplemented with 10% foetal bovine serum (Vitrocell Embriolife, Brazil) and 0.1% penicillin-streptomycin (Gibco) at 37 °C in 5% CO2 atmosphere until reaching 80% of confluence. Thereafter, cell viability was evaluated through MTT assay.

Briefly, 5×10^4 cells / well were seeded in 96-well plate and after 24 hours the following treatments were incubated: Basal (DMEM plus DMSO 0.1%), DMSO (10%), irinotecan (Iri 3-30 µM), luteolin (lut 3-30 µM in DMEM plus DMSO 0.1%), or lut 30 µM plus Iri 30 µM. After 21h, 10 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/ml) were added in each well for 3 hours, then 250 µl of the supernatant was removed and replaced for 150 µl of DMSO. Each solution was homogenized, and the absorbance was measured at 570 nm. The percentage of cell viability was determined considering the means of the basal group as 100%.

Method S7. DPPH· radical scavenging assay

The antioxidant effect of luteolin on DPPH· radical was estimated according to the procedure described by Brand-Williams *et al*. with a few modifications. An aliquot (750 µl) of different concentrations of luteolin (0.1 – 10 µg/ml) was mixed to 250 µl of DPPH· 6.0×10^{-5} M in methanol solution. The decrease in the absorbance was determined at 515 nm when the reaction reached a plateau. The absorbance of the DPPH· radical without any antioxidant was measured (Veh), and ascorbic acid (AA: 50 µg/ml) was used as a positive control. The individual values of absorbance were interpolated to a standard curve of DPPH (0–60 µM) and the results were expressed as µM of DPPH (Brand-Williams et al., 1995).

Supporting Results

Figure S1

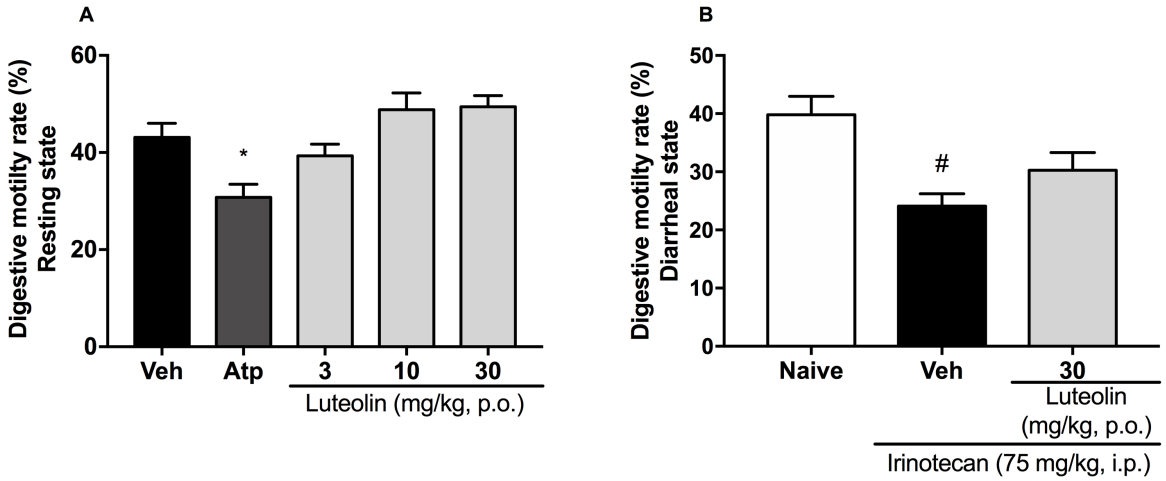
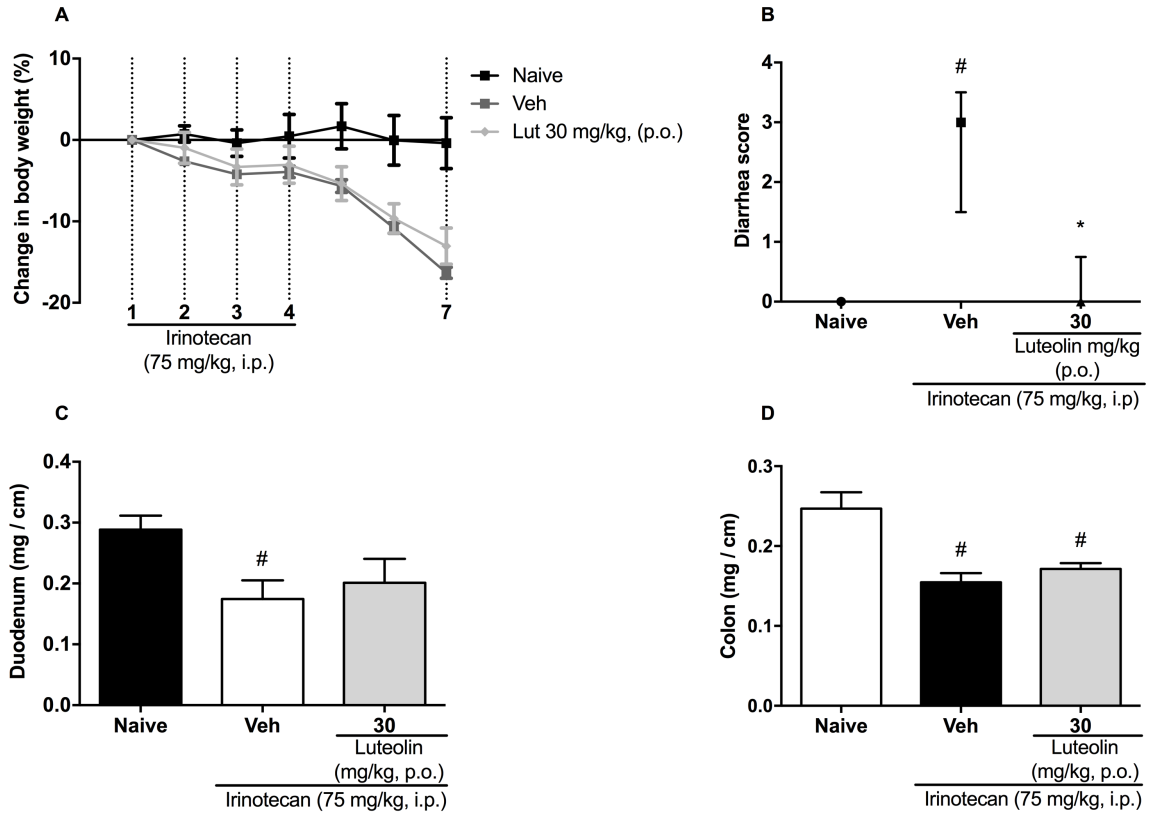


Figure S1. Effect of luteolin on digestive motility rate (%). (A) Resting state. The results are expressed as mean ± SEM (n = 6). Statistical analysis was performed using one-way ANOVA followed by Bonferroni's post hoc test. * $p < 0.05$ compared with the vehicle group (Veh: water plus 1% tween. 10 ml/kg. p.o.); Atp: Atropine (3 mg/kg. s.c.). (B) Diarrheal state. Female mice were treated for 14 days by oral route with vehicle (Veh: water plus Tween 1%, 10 ml/kg) or luteolin (30 mg/kg). Irinotecan was given by intraperitoneal route (75 mg/kg, from 7th to 10th day) for Veh and Luteolin-treated groups. Digestive motility rate was evaluated on the 15th day of experiment. The results are expressed as mean ± SEM (n = 6). Statistical analysis was performed using one-way ANOVA followed by Bonferroni's post hoc test. # $p < 0.05$ compared to the naive.

137 **Figure S2**



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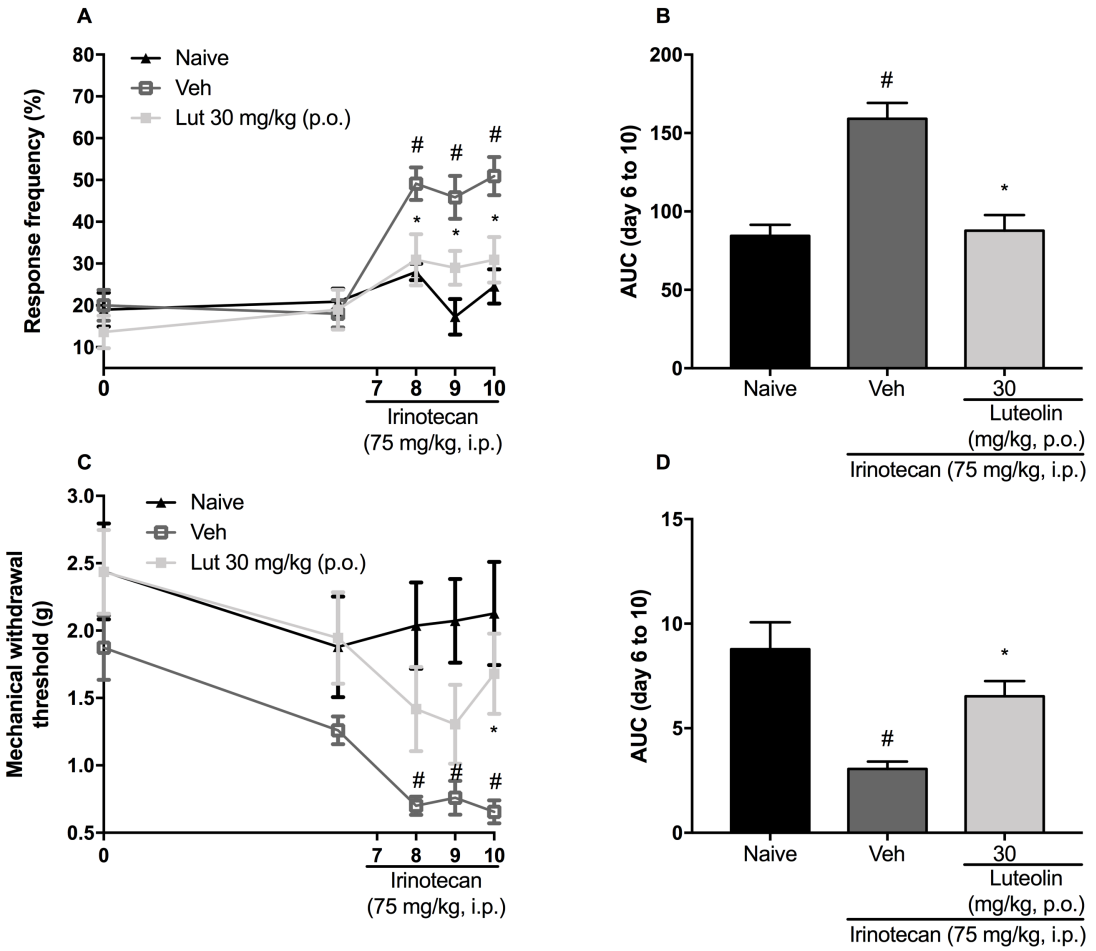
139 **Figure S2. Effect of luteolin treatment by oral route on irinotecan-induced intestinal**
140 **mucositis**. Female mice were treated for 7 days by oral route with vehicle (Veh: water
141 plus Tween 1%, 10 ml/kg) or luteolin (Lut 30 mg/kg). Irinotecan was given by
142 intraperitoneal route (75 mg/kg, from 1st to the 4th day) for Veh and Luteolin-treated
143 groups. (A) The weight of the animals was measured daily. Weight loss was significantly
144 different from the 5th to 7th day of experiment in both treated groups compared to naive.
145 The results are expressed as mean ± SEM (n = 10). Statistical analysis was performed
146 using two-way ANOVA followed by Bonferroni's post hoc test. (B) The diarrhea score
147 was evaluated on the 7th day of treatment. The results are expressed as median with
148 interquartile range (n = 10). Statistical analysis was performed by Kruskal-Wallis
149 followed by Dunn's test. *p < 0.05 compared to vehicle. # p < 0.05 compared to naive

group. (C and D) Weight/length ratio of duodenum and colon. The results are expressed as mean ± SEM (n = 10). Statistical analysis was performed using one-way ANOVA followed by Bonferroni's post hoc test. # $p < 0.05$ compared to the naive group.

159 **Figure S3.**



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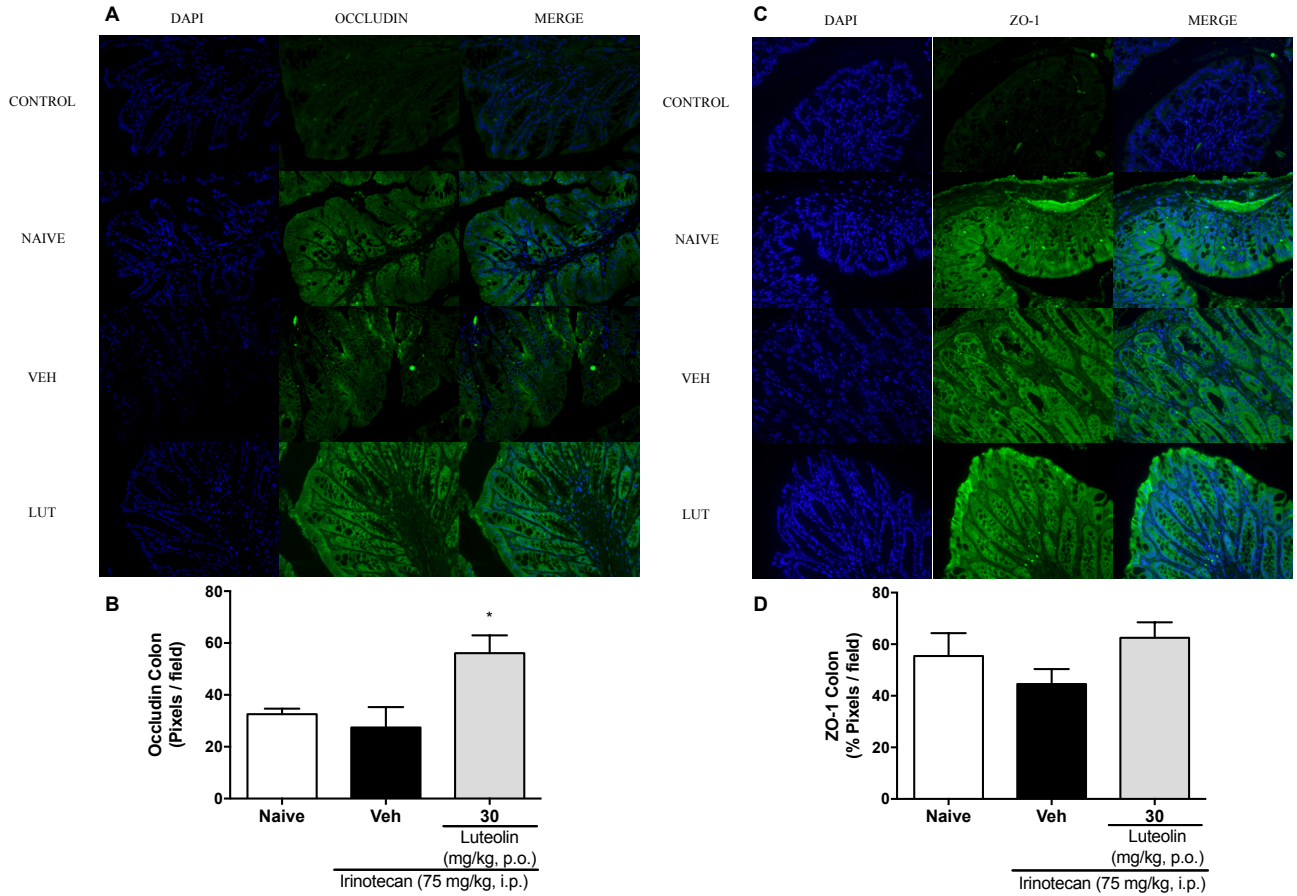
161 **Figure S3. Effect of luteolin by oral route in the mechanical hypersensitivity of**
162 **irinotecan-exposed mice.** Female mice were treated by oral route with vehicle (Veh:
163 water plus Tween 1%, 10 ml/kg) or luteolin (Lut 30 mg/kg). From the 7th to 10th day
164 irinotecan was intraperitoneally given at a dose of 75 mg/kg per day to the Veh and Lut
165 groups and the mechanical hypersensitivity was measured by von Frey filament. (A)
166 Response frequency (%) and (C) mechanical withdrawal threshold (g) are presented
167 followed their respective A.U.C. (B and D). The results are expressed as mean ± SEM (n
168 = 10). Statistical analysis was performed using two-way ANOVA followed by
169 Bonferroni's post hoc test. * $p < 0.05$ compared to vehicle. # $p < 0.05$ compared to naive
170 group.

171 **Figure S4**



183 **Figure S4. Effect of luteolin on tight junctions (TJs) expression in the colon of irinotecan-exposed mice.** Immunofluorescence staining of the

184 colon for Occludin (A) and ZO-1 (C) protein was performed (green). Nuclei are stained with DAPI (blue). Non-relevant IgG was used as control.

Magnification ×20. (B, D) Fluorescence was quantified with ImageJ® program. Six fields per animal were analyzed (n = 5). The results are expressed as mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's post hoc test. * $p < 0.05$ compared to vehicle group.

Figure S5.

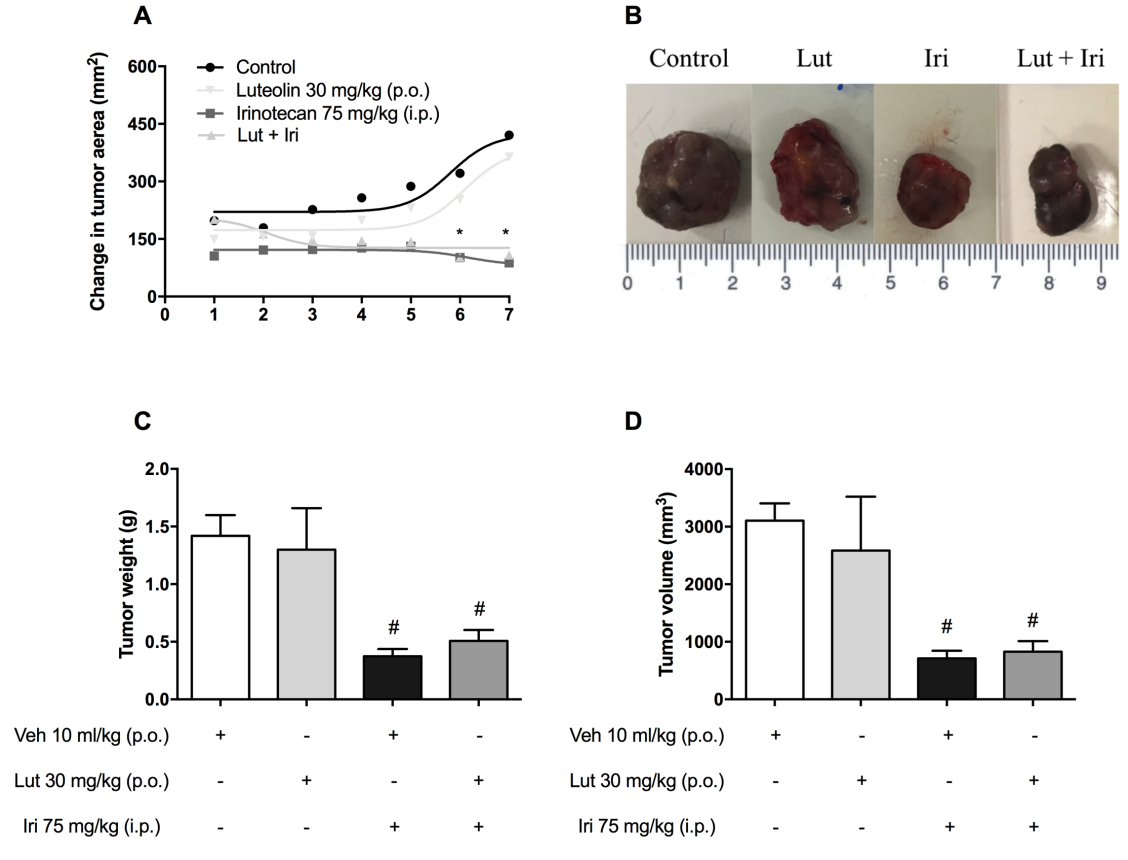


Figure S5. Luteolin does not interfere with the antitumor activity of Irinotecan. Melanoma cells (B16F10) were injected in the flank of C57BL/6 mice and after the tumor development, the animals were treated for 7 days with control (water plus Tween 1%, 10 ml/kg, p.o.), luteolin (Lut 30mg/kg, p.o.), irinotecan (Iri 75 mg/kg, i.p.) or luteolin plus irinotecan (Lut + Iri). Tumor area (A) was measured daily. Tumor weight (C) and tumor volume (D) were measured after euthanasia of mice. The results are expressed as mean ± SEM (n = 10). Statistical analysis was performed using one-way ANOVA followed by Bonferroni's post hoc test. (A) * $p < 0.05$ compared Irinotecan and Lut + Iri to control group. (C and D) # $p < 0.05$ compared to vehicle group without irinotecan.

Figure S6

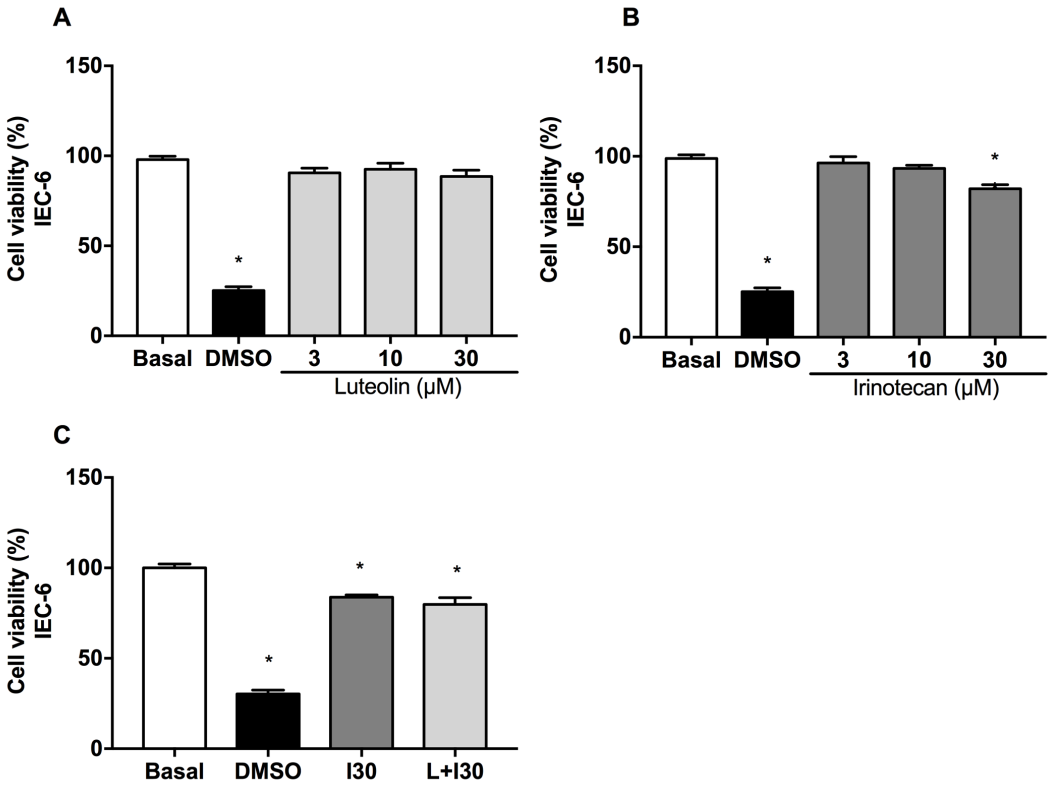


Figure S6. Luteolin does not affect intestinal epithelial cell viability and does not alter the irinotecan-induced cytotoxicity. Murine epithelial cells (5×10^4 cells/well) were seeded in 96-well plate and cell viability was measured using the MTT assay. Basal (DMEM plus DMSO 0.1%); DMSO (DMEM plus DMSO 10%); irinotecan (I: 3-30 μM); luteolin (L: 3-30 μM). The results are expressed as mean ± SEM (n = 3 replicates). Statistical analysis was performed using one-way ANOVA followed by Bonferroni's post hoc test. $*$ $p < 0.05$ when compared to basal group.

Figure S7

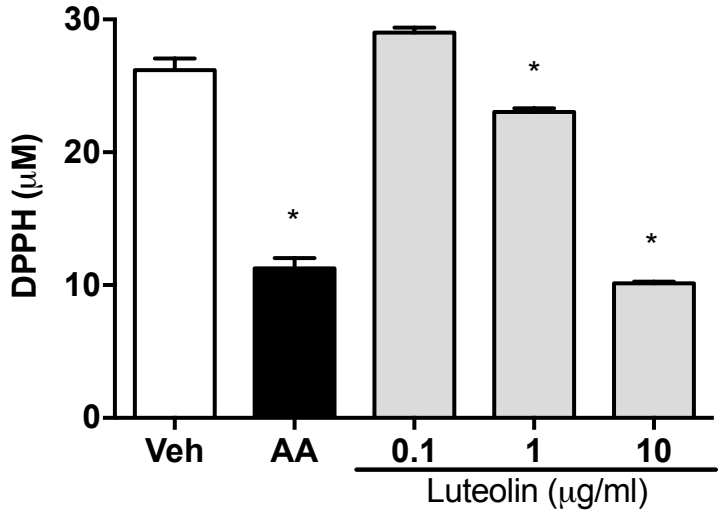


Figure S7. Effect of Luteolin on *in vitro* ability to scavenge the free-radical DPPH. The results are expressed as mean ± SEM. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Bonferroni test. IC50 of ~1.84 μg/ml. *$p < 0.05$ compared to the vehicle group. Vehicle (Veh: water). Ascorbic acid (AA: 50 μg/ml).

Table S1. Effects of Luteolin on relative organ weights of irinotecan-exposed mice.

Organ weight (g/10g body weight)	Groups			
	Naive	**Veh + Iri**	**Lut 30 mg/kg + Iri**	**Lut 30 mg/kg**
Heart	0.038 ± 0.002	0.039 ± 0.002	0.043 ± 0.002	0.041± 0.004
Lung	0.067 ± 0.007	0.061 ± 0.003	0.069 ± 0.004	0.056 ± 0.004
Spleen	0.044 ± 0.003	0.020 ± 0.002[a]	0.054 ± 0.008[b]	0.041 ± 0.004
Liver	0.488 ± 0.022	0.385 ± 0.017[a]	0.510 ± 0.023[b]	0.391 ± 0.027
Right Kidney	0.065 ± 0.002	0.057 ± 0.003	0.067 ± 0.003	0.062 ± 0.002
Left Kidney	0.058 ± 0.001	0.062 ± 0.003	0.064 ± 0.003	0.058 ± 0.001

Female mice were treated for 14 days by oral route with vehicle (Veh: water plus Tween 1%, 10 ml/kg) or luteolin (30 mg/kg). Irinotecan was given by intraperitoneal route (75 mg/kg, from 7th to 10th day) for Veh and Lut-treated groups (Veh + Iri and Lut + Iri). The results are expressed as mean ± SEM (n = 6). Statistical analysis was performed using one-way ANOVA followed by Bonferroni's post hoc test. [a]$p < 0.05$ compared to the naive. [b]$p < 0.05$ compared to Veh + Iri.

Table S2. Effects of Luteolin on plasma biochemistry of irinotecan-exposed mice.

Test	Groups			
	Naive	**Veh + Iri**	**Lut 30 mg/kg + Iri**	**Lut 30 mg/kg**
Urea (mg/dL)	57.1± 5.9	45.8 ± 3.9	49.9 ± 5.0	43.1 ± 2.3
Creatinine (mg/dL)	0.12 ± 0.02	0.09 ± 0.01	0.11 ± 0.01	0.10 ± 0.01
AST (UI/L)	165.1± 58.1	410.2± 35.8[a]	390.4 ± 28.1[a]	350.8 ± 49.8
ALT (UI/L)	60.1 ± 10.4	119.4 ± 6.8[a]	67.5 ± 12.6[b]	51.6 ± 8.3

Female mice were treated for 14 days by oral route with vehicle (Veh: water plus Tween 1%, 10 ml/kg) or luteolin (30 mg/kg). Irinotecan was given by intraperitoneal route (75 mg/kg, from 7th to 10th day) for Veh and Lut-treated groups (Veh + Iri and Lut + Iri). The results are expressed as mean ± SEM (n = 6). Statistical analysis was performed using one-way ANOVA followed by Bonferroni test. [a]$p < 0.05$ compared to the naive. [b]$p < 0.05$ compared to the Veh + Iri.

Supporting References

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