Received: 4 February 2019 | Revised: 21 November 2019 | Accepted: 8 January 2020

DOI: 10.1111/bph.14987

RESEARCH PAPER

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

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Funding information
Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

Background and Purpose: Intestinal mucositis refers to mucosal damage caused by cancer treatment, and irinotecan is one of the agents most associated with this condition. Focusing on the development of alternatives to prevent this important adverse effect, we evaluated the activity of the flavonoid luteolin, which has never been tested for this purpose despite its biological potential.

Experimental Approach: The effects of luteolin were examined on irinotecan-induced intestinal mucositis in mice. Clinical signs were evaluated. Moreover, histological, oxidative, and inflammatory parameters were analysed, as well as the possible interference of luteolin in the anti-tumour activity of irinotecan.

Key Results: Luteolin (30 $mg \cdot kg^{-1}$; p.o. or i.p.) prevented irinotecan-induced intestinal damage by reducing weight loss and diarrhoea score and attenuating the shortening of the duodenum and colon. Histological analysis confirmed that luteolin (p.o.) prevented villous shortening, vacuolization, and apoptosis of cells and preserved mucin production in the duodenum and colon. Moreover, luteolin treatment mitigated irinotecan-induced oxidative stress, by reducing the levels of ROS and LOOH and augmenting endogenous antioxidants, and inflammation by decreasing MPO enzymic activity, TNF, IL-1β, and IL-6 levels and increasing IL-4 and IL-10. Disruption of the tight junctions ZO-1 and occludin was also prevented by luteolin treatment. Importantly, luteolin did not interfere with the anti-tumour activity of irinotecan.

Conclusion and Implications: Luteolin prevents intestinal mucositis induced by irinotecan and therefore could be a potential adjunct in anti-tumour therapy to control this adverse effect, increasing treatment adherence and consequently the chances of cancer remission.

1 | INTRODUCTION

Intestinal mucositis is a toxic manifestation resulting from many anticancer treatments, including radiation or chemotherapy, and is characterized by extensive damage to the gut mucosa, leading to symptoms such as diarrhoea, bleeding, nausea, vomiting, abdominal pain, malnutrition, infections, and sepsis related to bacterial translocation (Vanhoecke et al., 2015). Reaching high incidence, 40% of patients receiving standard doses, and 100% of patients receiving high doses (Cinausero et al., 2017), the main consequence of this condition is

Abbreviations: CPT-11, irinotecan; DCFH-DA, dichlorodihydrofluorescein diacetate; DPPH, 2,2-diphenyl-1-picrylhydrazyl; DSS, sodium dextran sulfate; HE, haematoxylin and eosin; LOOH, lipid hydroperoxides; MPO, myeloperoxidase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PAS, periodic acid-Schiff; ZO-1, zonula occludens.

failure of the chemotherapy. When this adverse effect occurs, it is generally necessary to decrease the dose or to interrupt the chemotherapeutic regime, decreasing the chances of cancer remission and increasing health care costs and death rates, either by cancer or by the complications resulting from intestinal damage (Ribeiro et al., 2016).

Irinotecan (CPT-11) is a semisynthetic analogue of the natural alkaloid camptothecin and is one of the main agents used in the treatment of colorectal cancer. Although the combinations containing irinotecan significantly increase the survival rates of patients, it is, unfortunately, one of the main agents that cause intestinal mucositis (Fujita, Kubota, Ishida, & Sasaki, 2015).

Particularly with this drug, acute diarrhoea may occur due to activation of the parasympathetic pathway, which is clinically controlled by **atropine** (Andreyev et al., 2014). On the other hand, late diarrhoea is associated with intestinal mucositis, but its pathological mechanisms remain controversial because clinical management has had limited success (Ribeiro et al., 2016). As far as we know, irinotecan-induced DNA strand breaks result in direct cellular injury that targets cells in the basal epithelium, as well as those within the submucosa, where it activates several apoptosis-related pathways (Bowen et al., 2006). In addition, inflammation of the mucosal membranes and production of ROS and reactive nitrogen species, which induce cell death, seem to be involved (Arifa et al., 2014; Ribeiro et al., 2016; Sonis, 2004). Indeed, oral rehydration, electrolyte replacement, and pharmacological agents to reduce fluid loss or decrease intestinal motility, such as opioid agonists, are the few treatments employed in clinical practice (Andreyev et al., 2014).

The importance of seeking new therapeutic strategies for treatment of, or even more efficiently to prevent, the development of intestinal mucositis induced by irinotecan is evident, as the chemotherapy is given in fixed cycles of treatment, generally as 350 $mg \cdot m^{-2}$ every 3 weeks or 125 $mg \cdot m^{-2}$·per week (Stein, Voigt, & Jordan, 2010). Thus, in this situation, we have focused on the secondary bioactive metabolites obtained from natural products, of which polyphenols are the most likely candidates, as they have been extensively studied for the prevention and treatment of several oxidative stress and inflammation-related conditions (Nunes, Almeida, Barbosa, & Laranjinha, 2017).

Luteolin (3′,4′,5,7-tetrahydroxyflavone) is a flavonoid of the flavone subclass found in different medicinal plants, vegetables, and fruits (Ross & Kasum, 2002), and several reports have highlighted its biological properties, such as antioxidant and cytoprotective (Seelinger, Merfort, & Schempp, 2008), anti-proliferative (Pandurangan & Esa, 2014), anti-diabetic (Salib, Michael, & Eskande, 2013), diuretic (Boeing, da Silva, Mariott, Andrade, & de Souza, 2017), antihypertensive (Lv et al., 2013), and neuroprotective (Nabavi et al., 2015). In addition, the intestinal anti-inflammatory activity of luteolin in vivo on dextran sulfate sodium (DSS)-induced colitis (Li, Shen, & Luo, 2016; Nishitani et al., 2013) and in vitro on intestinal epithelial cells stimulated with cytokines (Nunes et al., 2017) or LPS (Kim & Jobin, 2005) have also been demonstrated. Therefore, considering the beneficial properties of luteolin, the lack of effective treatments for intestinal mucositis, and the abundance of this compound in nature, we have studied, here, the effect of luteolin on irinotecan-induced intestinal mucositis, seeking to characterize a promising natural compound as a useful approach to the prevention of this severe adverse effect of chemotherapy.

What is already known

- The drugs currently used to treat intestinal mucositis have low clinical efficacy.
- Luteolin has already been shown to be effective in experimental models of ulcerative colitis.

What this study adds

- Luteolin attenuated intestinal mucositis, decreased inflammation and oxidative stress and increased protective factors in mucosa.
- At the same time, luteolin did not affect the anti-tumour activity of irinotecan.

What is the clinical significance

- By preventing intestinal mucositis, luteolin can increase the chances of cancer remission.
- Luteolin could be a new therapeutic or adjunctive agent in the management of intestinal mucositis.

2 | METHODS

2.1 | Animals

All animal care and experimental procedures complied with international standards, following all the ethical guidelines on animal welfare and were approved by the institutional animal ethics committee of UNIVALI (number 021/16p). Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010) and with the recommendations made by the *British Journal of Pharmacology*. Female Swiss mice (25-30 g) were provided by the animal facility of the Universidade do Vale do Itajaí (UNIVALI). The mice were housed under standard laboratory conditions (12-hr light/dark cycle, temperature of 22 ± 2°C). The mice were randomly assigned according to their body weights, and all experiments were carried out in a blinded manner, compliant with Curtis et al. (2018).

2.2 | Experimental irinotecan-induced intestinal mucositis

The mice were divided into five groups, including naive, vehicle (Veh: water plus 1% Tween, 10 $ml \cdot kg^{-1}$), and luteolin (Lut 3, 10 and 30 $mg \cdot kg^{-1}$), of 10 animals each. The size of the experimental group follows the recommendations of previously published studies to

ensure the reproducibility of the data in this experimental model (see Arifa et al., 2014, 2016). The mice were treated by gavage for 14 days, except for the naive group which did not receive any treatment.

From the 7th to 10th day, irinotecan was given i.p. at a dose of 75 $mg \cdot kg^{-1} \cdot day^{-1}$ to the Veh and Lut groups to induce intestinal mucositis (Ikuno, Soda, Watanabe, & Oka, 1995). The body weight of the animals was evaluated daily, and the diarrhoea score was based on the criteria described by Arifa et al. (2016) as normal (0), mild perianal soiling (1), moderate perianal soiling (2), severe perianal soiling (3), severe perianal soiling with presence of blood, and signs of morbidity (4).

On the 15th day of the experiment, the mice were anaesthetized with **ketamine** (80 $mg \cdot kg^{-1}$) and **xylazine** (10 $mg \cdot kg^{-1}$), and the blood was collected from the superior vein cava. The total leukocyte number was counted (with Neubauer chambers), and biochemical analysis was performed. The animals were killed, and the small intestine and colon were excised. The weight/length ratio of tissue was measured, and samples of the duodenum and colon were collected for the analysis of histological, oxidative, and inflammatory parameters. In addition, one part of each tissue was dried for 24 hr at 60°C to measure tissue water content. The relative weight of the heart, lung, spleen, liver, right kidney, and left kidney were determined as g per 10 g of body weight. In a different set of experiments, vascular permeability or leukocytes recruitment assay was performed.

To evaluate the possible systemic effects of luteolin, Swiss mice were divided into the same five groups described above, including Naive, Vehicle (Veh: PBS pH 7.3, 10 $ml \cdot kg^{-1}$) and luteolin (Lut 3, 10 and 30 $mg \cdot kg^{-1}$), that were treated i.p. for 14 days, except for naive which did not receive any treatment. Irinotecan was given once a day from the 7th to the 10th day, and the body weight, diarrhoea score, and weight/length ratio of intestine were measured as previously described.

2.3 | Evaluation of vascular permeability

Vascular permeability was assessed by measuring extravasation of **Evans blue dye** (Saria & Lundberg, 1983) after performing the entire experimental protocol of irinotecan-induced intestinal mucositis described above. Evans blue dye (20 $mg \cdot kg^{-1}$) was injected via the retro-orbital plexus into the naive-, vehicle-, and luteolin-treated (30 $mg \cdot kg^{-1}$, p.o.) groups, 20 min before killing. Segments of the duodenum and colon were cut into small pieces and mixed with formamide (0.3 $ml \cdot cm^{-1}$), incubated for 4 hr at 37°C, and then the absorbance of the formamide was read at 620 nm. The experimental values were interpolated on a standard curve of Evans blue (1–100 $\mu g \cdot ml^{-1}$). The results were expressed as μg Evans blue per 100 mg of tissue.

2.4 | Assay of leukocyte recruitment

The leukocyte recruitment assay was performed on the last day of the irinotecan-induced intestinal mucositis protocol described above. Briefly, mice were injected i.p. with 1 ml of 7.5% casein sodium solution. After 4 hr, the mice were anaesthetized with ketamine (80 $mg \cdot kg^{-1}$) and xylazine (10 $mg \cdot kg^{-1}$). An abdominal incision was made, and 2 ml of PBS were injected. The peritoneal exudate was then collected, and the number of cells was determined in the Neubauer chamber. The duodenum and colon of the same mice were excised to determine myeloperoxidase (MPO) activity.

2.5 | Histological analysis

Tissue samples of the duodenum and colon were fixed in Alfac (85% ethanol 80%; 10% formaldehyde and 5% acetic acid) for 24 hr. They were subsequently dehydrated with alcohol and xylene, embedded in paraffin wax, sectioned at 5 μm and stained with haematoxylin/eosin (HE), or for histochemical analysis of mucin content and goblet cells number with periodic acid-Schiff (PAS) and Alcian blue, respectively.

The morphometric analyses of the intestine, as well as the histological score, were performed as described by Soares et al. (2008). Briefly, microscopic slides were reviewed by a histologist blinded to the experimental groups and damage extent. Histological score was considered as (0) normal histological findings, (1) villus blunting, loss of crypt architecture, sparse inflammatory cell infiltration, vacuolization, and oedema in the mucosa and normal muscular layer, (2) villus blunting with fattened and vacuolated cells, crypt necrosis, intense inflammatory cell infiltration, vacuolization, and oedema in the mucosa and normal muscular layer, (3) villus blunting with fattened and vacuolated cells, crypt necrosis, intense inflammatory cell infiltration, vacuolization, and oedema in the mucosa, and oedema, vacuolization, sparse neutrophil infiltration in the muscular layer.

Villus height measurements (from villus tip to villus–crypt junction) and crypt depths (defined as invagination depth between adjacent villi) were measured using ImageJ® (RRID:SCR_003070) program. Ten intact and well-oriented villi and crypts were measured per animal, from 5 animals).

Mucin-like glycoproteins were quantified using ImageJ® program and expressed as pixels per field. Goblet cells were counted in crypts and villi under 40× magnification. For both assays, six fields per animal were analysed from 5 animals ($n = 5$).

2.6 | Immunofluorescence

The immuno-related procedures used comply with the recommendations made by the *British Journal of Pharmacology* (Alexander et al., 2018). Tissue sections were deparaffinized, rehydrated, and permeabilized in 0.1% Triton X-100 PBS for 10 min at room temperature. Then, the slides were incubated with blocking buffer (5% BSA in 0.05% Triton X-100 PBS) for 30 min. After that, primary antibodies ZO-1 (dilution 1:200 in 1% BSA, 0.05% Triton X-100 PBS) or occludin (dilution 1:200 in 1% BSA, 0.05% Triton X-100 PBS) were incubated overnight at 4°C. Finally, incubation with secondary antibody (goat anti-rabbit Alexa 488; dilution 1:400 in 0.05% Triton X-100 PBS) was

performed for 1 hr at room temperature, and nuclei were stained with DAPI solution. Samples were visualized under a fluorescence microscope (Leica, Bensheim, Germany). Fluorescence was quantified with ImageJ® program. Six fields per animal were analysed (n = 5).

2.7 | Determination of oxidative and inflammatory parameters

Samples of the duodenum and colon were collected and immediately homogenized with 200-mM potassium phosphate buffer (pH 6.5) to determine the levels of reduced GSH and lipid hydroperoxides (LOOH). The remaining homogenate was centrifuged at 7,000× g for 20 min at 4°C, and the supernatant was frozen at −80°C for further determination of SOD and catalase (CAT) activity. Protein concentrations were determined by the Bradford method (Bio-Rad, Hercules, CA, USA). For a detailed protocol of these measurements, see Boeing et al. (2016).

Myeloperoxidase (MPO) activity and the levels of ROS, cytokines, and nitrite were measured in the supernatant of the duodenum and colon samples that were homogenized with 80-mM potassium phosphate buffer (pH 5.4) containing 0.5% hexadecyltrimethylammonium and centrifuged at 7,000× g for 20 min at 4°C.

Briefly, the MPO activity was determined at 620 nm in the presence of H_2O_2 and 3,3′,3,5′-tetramethylbenzidine (Bradley, Priebat, Christensen, & Rothstein, 1982). The results were expressed as relative units that denote the activity of MPO relative to casein-elicited murine peritoneal leukocytes determined as described before.

For ROS determination, 100 μl of supernatant were incubated with 30 μl of 2′,7′-dichlorodihydrofluorescein diacetate (10-μM DCFH-DA) for 40 min in the dark. Fluorescence was measured using a spectrofluorometer microplate reader with excitation wavelength at 480 nm and emission wavelength at 530 nm.

Nitrite levels were quantified through Griess reaction following the protocol previously described by de Almeida et al. (2017). The amounts of TNF-α, IL-1β, IL-6, IL-4, IL-10, and PGE_2 were measured by ELISA using mouse cytokine ELISA kits from BD Biosciences (Franklin Lakes, New Jersey, USA) or RD systems (Minneapolis, MN, USA), according to the manufacturer's instructions.

2.8 | Data and statistical analysis

The data and statistical analysis comply with the recommendations of the British Journal of Pharmacology on experimental design and analysis in pharmacology (Curtis et al., 2018). The data were analysed by an investigator blinded to the experimental conditions. Each in vitro experiment was conducted independently at least three times, and more than five animals per group were needed for in vivo experiment. The Kolmogorov–Smirnov normality test was applied to verify the data normality. The data were expressed as mean ± SEM, and one- or two-way ANOVA followed by Bonferroni's post hoc test was applied to verify the differences between means. The non-parametric data were expressed as the median with interquartile range and analysed by Kruskal–Wallis followed by Dunn's test, while Mann–Whitney test was used to compare differences between two groups. Statistical analysis was performed using the software GraphPad Prism (RRID: SCR_002798) version 7.00 (GraphPad Software, La Jolla, CA, USA). Values of P < .05 were considered to show significant differences between means.

2.9 | Materials

Luteolin (≥98% purity, powder) was commercially obtained from Active-Pharmaceutica (Palhoça, SC, Brazil). Syntec (São Paulo, SP, Brazil) was the supplier of both ketamine and xylazine. All other drugs and reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) and Merck (Darmstadt, Germany). Tween-20 was purchased from Vetec (Rio de Janeiro, RJ, Brazil). The following antibodies were used: anti-occludin polyclonal antibody (Abcam, Cat# ab31721, RRID:AB_881773), anti-ZO1 polyclonal antibody (Thermo Fisher Scientific, Cat# 61-7300, RRID:AB_2533938), and rabbit secondary antibodies (Thermo Fisher Scientific, Cat# A-31566, RRID:AB_2536179).

Experimental solutions were prepared as follows; luteolin was solubilized in water plus Tween 1%, and irinotecan was dissolved in saline.

2.10 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander, Christopoulos et al., 2019; Alexander, Cidlowski et al., 2019; Alexander, Fabbro et al., 2019a, b).

3 | RESULTS

3.1 | Luteolin does not interfere in the digestive motility rate

In a resting state, luteolin given by gavage at doses of 3, 10, or 30 mg·kg^{-1} did not inhibit the digestive motility rate. Treatment with atropine (3 mg·kg^{-1}, s.c.), a muscarinic receptor antagonist, was used as the positive control and internal control for this experiment (Figure S1A). In a diarrhoeal state induced by irinotecan, digestive motility rate was significantly lower in the vehicle-treated mice, than that in the naïve group, while mice treated with luteolin (30 mg·kg^{-1}, p.o.) did not show significant changes in this parameter (Figure S1B).

3.2 | Luteolin attenuates clinical parameters and signs of irinotecan-induced intestinal mucositis

Oral administration of luteolin (30 mg·kg^{-1}) to mice over 7 days, starting at the same day as irinotecan, decreased the diarrhoea score, compared with the vehicle group (Figure S2B). However, luteolin treatment did not reverse the change in the body weight or weight/length ratio of the duodenum and colon, induced by irinotecan (Figure S2A,C,D). On the other hand, when luteolin was given to mice for 14 days, starting 7 days before the first dose of irinotecan, the compound was able to reverse all changes induced by the chemotherapy agent.

Figure 1a shows that 2 days after the first dose of irinotecan, the vehicle-only-treated group started to lose weight. At the end of the experimental period, mice of this group lost approximately 29% of weight compared with the naive group, while animals treated with luteolin (30 mg·kg^{-1} p.o.) lost approximately half that value. In the same way, when compared with vehicle group, the diarrhoea score in the final period of the experiment (i.e., on the 14th day) was decreased by luteolin treatment (Figure 1b). The damage induced by irinotecan was accompanied by decreased weight/length ratio of both the duodenum and the colon, as shown by the difference between vehicle and naive groups. Such decreases were not observed in luteolin-treated mice (Figure 1c,d).

Complementary to these results with treatment p.o., we also gave the same treatments by i.p. injection. Here, the vehicle-only-treated (i.p.) group lost approximately 26% of the body weight compared to the naive group, while luteolin treatment (30 mg·kg^{-1} i.p.) still reversed the changes of body weight (Figure 2a), diarrhoea score (Figure 2b), and weight/length ratio of the duodenum and colon (Figure 2c,d).

From these data, we standardized the treatment with luteolin for 14 days orally to continue the study. Histological examination of the intestine of irinotecan-exposed mice clearly showed the extensive damage, in the vehicle-treated group, caused by irinotecan in both the duodenum and colon. Changes such as villous shortening in the duodenum, disruption of crypts in the colon, apoptosis, vacuolization of cells, oedema, and infiltration of polymorphonuclear cells were the main changes seen, compared with normal tissue. Interestingly, all these abnormalities were less pronounced in mice treated with luteolin (Figure 3a), leading to a diminished total histological score in both parts of the intestine

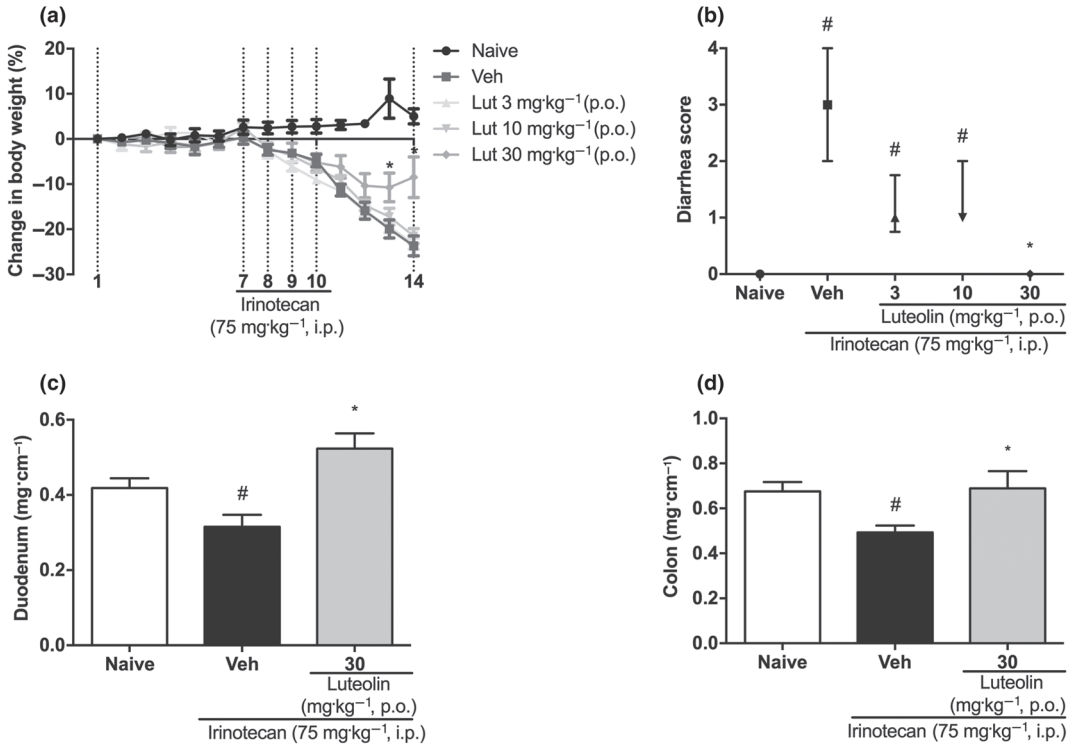

FIGURE 1 Effect of luteolin, given p.o., on irinotecan-induced intestinal mucositis. Female mice were treated for 14 days by gavage, with vehicle (Veh: water plus Tween 1%, 10 ml·kg^{-1}) or luteolin (Lut: 3, 10 or 30 mg·kg^{-1}). Irinotecan was given i.p. (75 mg·kg^{-1}, from the 7th to the 10th day) for Veh and Lut-treated groups. (a) The weight of the animals was measured daily. Weight loss was significantly different from the 9th to the 14th day of experiment in the treated groups compared to naive. The results are expressed as mean ± SEM (n = 10). *P < .05, significantly different from vehicle; two-way ANOVA followed by Bonferroni's post hoc test. (b) The diarrhoea score was evaluated on the 14th day of treatment. The results are expressed as median with interquartile range (n = 10). *P < .05, significantly different from vehicle; $^{\#}P$ < .05, significantly different from the naive group; Kruskal–Wallis followed by Dunn's test. (c and d) Weight/length ratio of the duodenum and colon. The results are expressed as mean ± SEM (n = 10). *P < .05, significantly different from vehicle; $^{\#}P$ < .05, significantly different from the naive group; one-way ANOVA followed by Bonferroni's post hoc test

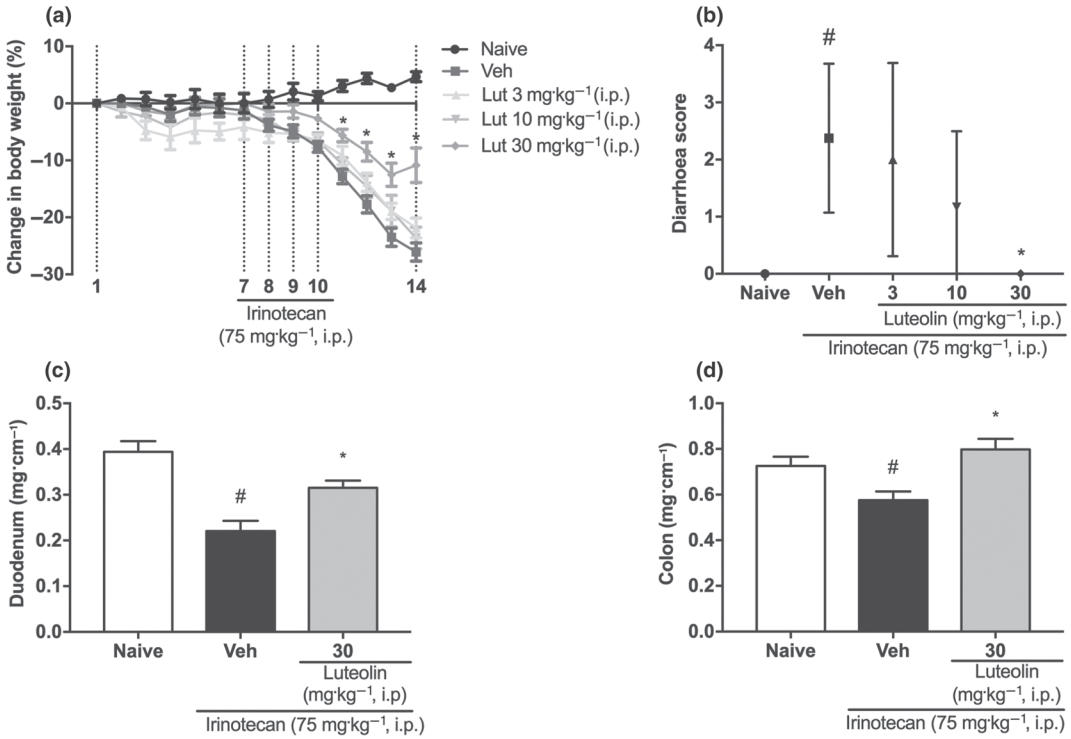

FIGURE 2 Effect of i.p. luteolin on irinotecan-induced intestinal mucositis. Female mice were treated for 14 days by i.p. injection of vehicle (Veh: PBS pH 7.0, 10 ml·kg^{-1}) or luteolin (Lut: 3, 10 or 30 mg·kg^{-1}). Irinotecan was given i.p. (75 mg·kg^{-1}, from the 7th to the 10th day) for Veh and Lut-treated groups. (a) The weight of the animals was measured daily. Weight loss was significantly different from the 9th to the 14th day of experiment in the treated groups compared to naive. The results are expressed as mean ± SEM (n = 10). *P < .05, significantly different from vehicle; two-way ANOVA followed by Bonferroni's post hoc test. (b) The diarrhoea score was evaluated on the 14th day of treatment. The results are expressed as median with interquartile range (n = 10). *P < .05, significantly different from vehicle. ; #P < .05, significantly different from the naive group; Kruskal–Wallis followed by Dunn's test. (c and d) Weight/length ratio of the duodenum and colon. The results are expressed as mean ± SEM (n = 10). *P < .05, significantly different from vehicle; #P < .05, significantly different from the naive group; one-way ANOVA followed by Bonferroni's post hoc test

(Figure 3b). Moreover, irinotecan treatment decreased the villus height and increased the depth of crypts, consequently decreasing the villus/crypt ratio of the duodenum, changes which were prevented by luteolin treatment (Figure 3c–e).

Besides, Figure S3 shows that irinotecan induced a secondary hypersensitization of mice compared to naive group, while luteolin treatment reduced the mechanical hypersensitivity, inhibiting approximately 20% of the response frequency and increasing the von Frey threshold compared to vehicle group.

The extent of oedema in the duodenum and colon was assessed by measuring the tissue water content (as % weight) and the vascular permeability, using Evans blue dye. Consistent with the macroscopic findings, intestinal tissue of mice that received irinotecan plus vehicle had a greater water content than that in the naive group, while intestinal tissue of mice treated with luteolin showed water content, similar to that at baseline (Figure 4a,b). Moreover, as shown in Figure 4c,d, while the vascular permeability in colonic tissue samples was unaltered between groups, this parameter was increased in the duodenum of mice from the irinotecan+vehicle group, but this effect was not present in the duodenal tissue obtained from animals treated with luteolin and then with irinotecan.

3.3 | Luteolin attenuates oxidative stress and inflammation in irinotecan-exposed mice

At the end of the experiment, the duodenum and colon of mice were collected and analysed for changes in oxidative and inflammatory parameters, as shown in Tables 1 and 2.

The intestinal mucositis induced by irinotecan clearly increased the ROS levels in the duodenum, as shown by the difference between vehicle and naive groups. Moreover, irinotecan also increased the LOOH levels, along with decreased levels of GSH and of catalase activity. Treatment with luteolin prevented all these changes, providing levels near those in the samples from naïve animals (Table 1). By contrast, in the colon, irinotecan did not induce significant changes in these oxidative parameters, except for a reduction in the amount of GSH.

However, in terms of inflammatory parameters, both tissues were clearly affected. Irinotecan caused a large increase in MPO activity and in the levels of pro-inflammatory cytokines (TNF, IL-1β, and IL-6), while the anti-inflammatory cytokines IL-4 and IL-10 appeared to be decreased (Table 2). Moreover, in duodenum, nitrite and PGE_2 levels were increased, effects which were not observed in the colon (Figure 5). Although exposure to irinotecan

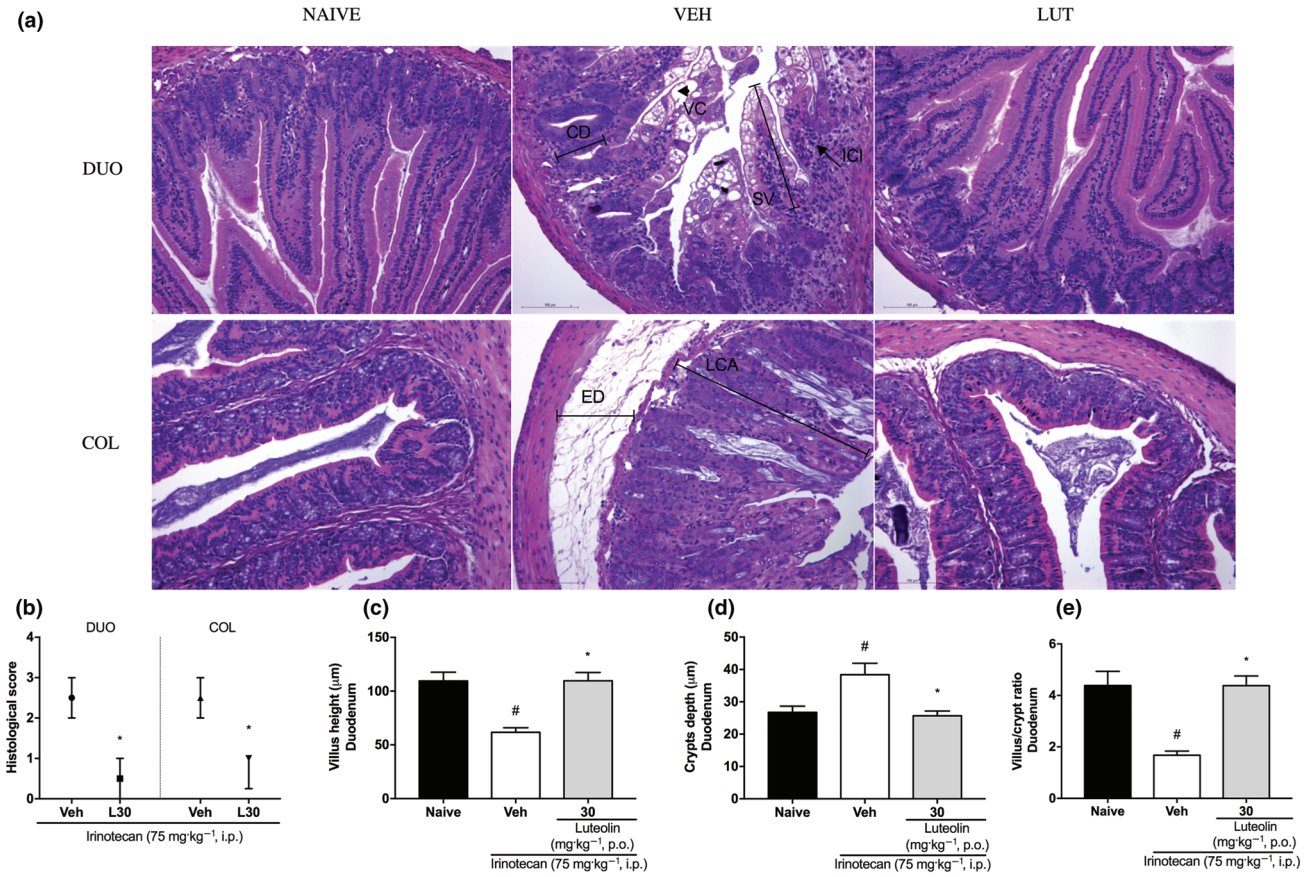

FIGURE 3 Histological sections of the duodenum and colon of irinotecan-exposed mice. Female mice were treated for 14 days by oral route with vehicle (VEH: water plus Tween 1%, 10 ml·kg^{-1}) or luteolin (LUT: 30 mg·kg^{-1}). Irinotecan was given by intraperitoneal route (75 mg·kg^{-1}, from the 7th to the 10th day) for Veh- and Lut-treated groups. Histological sections were stained with HE (a). NAIVE showing normal villi, crypts, and muscular layer. VEH DUO showing vacuolated cells (VC), shortened villi (SV), crypt depth (CD), and inflammatory cell infiltration (ICI). VEH COL showing loss of normal crypt architecture (LCA) and oedema (ED). Scale bars 100 µm, magnification 20×. DUO: duodenum, COL: colon. Histological score (b): The results are expressed as median with interquartile range (n = 5). *P < .05, significantly different from vehicle; Kruskal–Wallis followed by Mann–Whitney test. Villus height (c), crypt depth (d), villus/crypt ratio (e): The results are expressed as mean ± SEM (n = 5). *P < .05, significantly different from vehicle; #P < .05, significantly different from the naive group; one-way ANOVA followed by Bonferroni's post hoc test

induced a marked inflammatory reaction, the mice treated with luteolin showed decreased MPO activity and decreased TNF, IL-1β, and IL-6 levels in both the duodenum and the colon, along with an increase of IL-4 and IL-10 and a reduction of nitrite levels in the duodenum, compared with the vehicle-treated group.

3.4 | Luteolin prevents changes in the mucus production in the intestine of irinotecan-exposed mice

A regular balance in the mucus production in the intestine is fundamental to maintain the normal function of the organ. Accordingly, we measured goblet cells numbers in the duodenum by Alcian blue staining and the levels of mucin-like glycoproteins in the colon by PAS staining, as shown in Figure 6a. The number of intact goblet cells in the vehicle-treated group was significantly lower than in the naive group, as was the PAS staining in the colon. On the other hand, such changes were not seen in the intestinal tissues obtained from luteolin-treated mice (Figure 6b,c).

3.5 | Luteolin prevents changes in the expression of the tight junction in the duodenum of irinotecan-exposed mice

Immunofluorescence analysis of duodenum samples for the tight junction proteins, zonula occludens (ZO-1; Figure 7a,b) and occludin (OC; Figure 7c,d) showed alterations in the pattern of expression of these proteins induced by irinotecan in the duodenum of the vehicle group, compared with naïve mice, while the mice treated with luteolin showed expression similar to that in naive mice, both in location and in intensity. Although the expression of the tight junction proteins in the colonic tissue was not affected by irinotecan, the group treated with luteolin showed a higher fluorescence intensity for occluding, when compared with the vehicle-treated group (Figure S4).

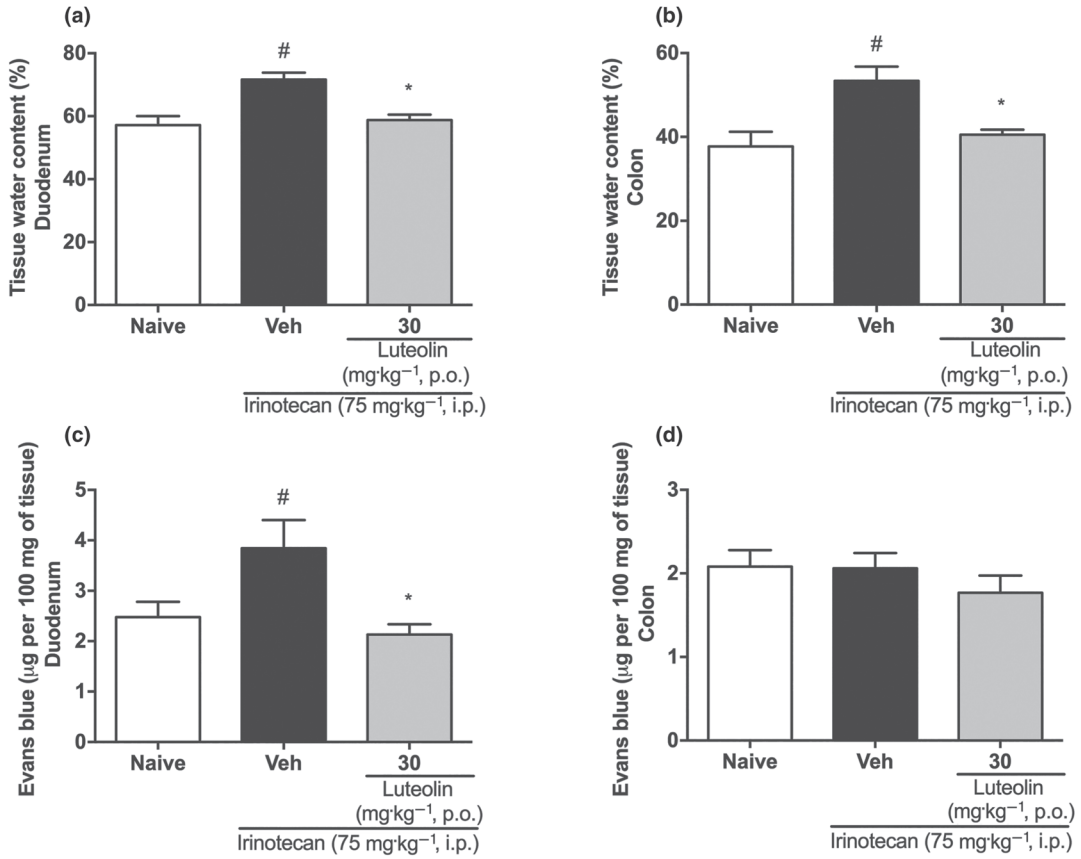

FIGURE 4 Effect of luteolin in the oedema of intestine from irinotecan-exposed mice. Female mice were treated for 14 days p.o. with vehicle (Veh: water plus Tween 1%, 10 ml·kg^{-1}) or luteolin (30 mg·kg^{-1}). Irinotecan was given i.p. (75 mg·kg^{-1}, from the 7th to the 10th day) for Veh- and Lut-treated groups. (a and b) Tissue water content. (c and d) Vascular permeability of tissue. The results are expressed as mean ± SEM (n = 10). *P < .05, significantly different from vehicle; #P < .05, significantly different from the naive group; one-way ANOVA followed by Bonferroni's post hoc test

TABLE 1 Effect of luteolin on oxidative parameters of irinotecan-exposed mice

Tissue	Duodenum			Colon		
Groups	Naïve	Vehicle	Luteolin	Naïve	Vehicle	Luteolin
ROS (fluorescence U·mg^{-1} of protein)	48.8 ± 6.0	69.7 ± 5.2*	46.6 ± 4.9#	49.6 ± 3.3	54.2 ± 3.1	55.2 ± 2.5
LOOH (mmol·mg^{-1} of tissue)	12.0 ± 0.5	17.9 ± 1.9*	13.6 ± 0.6#	331.0 ± 10.1	311.1 ± 10.2	328.3 ± 12.9
GSH (mg·g^{-1} of tissue)	1,385.0 ± 151.8	450.6 ± 60.1*	1,319.0 ± 393.8#	439.7 ± 47.1	267.3 ± 25.5*	361.3 ± 41.9
SOD (U·mg^{-1} of protein)	73.2 ± 3.1	69.3 ± 2.1	71.5 ± 1.8	66.9 ± 1.5	66.2 ± 2.9	73.4 ± 2.1
CAT (mmol·min^{-1} mg^{-1} of protein)	86.7 ± 21.8	25.7 ± 5.1*	85.9 ± 18.8#	36.1 ± 8.4	31.8 ± 5.1	32.4 ± 4.0

Note. Data shown are means ± SEM (n = 10).
*P < .05, significantly different from naive group; #P < .05, significantly different from vehicle-treated group; one-way ANOVA followed by Bonferroni test.
ROS, reactive oxygen species; CAT, catalase; LOOH, lipid hydroperoxides; SOD, superoxide dismutase; GSH, reduced glutathione.

3.6 | Luteolin does not reverse leukopenia or interfere with the antitumour activity of irinotecan

Irinotecan is known to induce leukopenia and we therefore measured the numbers of leukocytes in blood and in peritoneal exudate in our model of intestinal mucositis. As shown in Figure 8, the vehicle-treated group presented fewer leukocytes, compared with those in the naïve group, and this effect was not reversed by luteolin treatment, indicating that luteolin did not modify this effect of the chemotherapy.

In order to confirm, more directly, that luteolin did not modify the anti-tumour activity of the irinotecan, melanoma cells (B16F10) were injected in the flank of C57BL/6 mice and, after tumour development, the animals were treated with luteolin only, irinotecan only, and irinotecan plus luteolin. The results clearly showed that irinotecan decrease the tumour area, weight, and volume, while luteolin did not show any anti-tumour effect. Further, when luteolin was given in combination with irinotecan, this flavonoid did not interfere with the anti-tumour effect of the chemotherapeutic agent (Figure S5).

TABLE 2 Effect of luteolin on inflammatory parameters of irinotecan-exposed mice

Tissue	Duodenum			Colon		
Groups	Naive	Vehicle	Luteolin	Naive	Vehicle	Luteolin
MPO (relative units)	3.6 ± 0.7	50.3 ± 11.1*	19.7 ± 6.0#	10.9 ± 4.2	211.7 ± 73.5*	54.6 ± 18.2#
TNF (fg·mg^{-1} of protein)	54.6 ± 6.8	89.8 ± 10.0*	56.4 ± 7.8#	40.3 ± 5.4	64.4 ± 8.2*	38.3 ± 5.1#
IL-1β (fg·mg^{-1} of protein)	1.9 ± 0.2	4.7 ± 1.0*	1.3 ± 0.1#	0.9 ± 0.1	1.8 ± 0.2*	1.1 ± 0.1#
IL-6 (fg·mg^{-1} of protein)	30.5 ± 3.9	52.5 ± 5.4*	32.7 ± 4.6#	18.3 ± 2.1	25.6 ± 2.3*	15.6 ± 1.5#
IL-4 (fg·mg^{-1} of protein)	7.5 ± 1.6	3.6 ± 0.3*	4.8 ± 0.8#	61.7 ± 5.0	37.1 ± 1.9*	42.6 ± 3.6
IL-10 (fg·mg^{-1} of protein)	39.0 ± 5.5	16.2 ± 1.1*	41.2 ± 8.6#	294.7 ± 25.9	213.2 ± 13.9*	213.5 ± 14.7

Note. Data shown are means ± SEM (n = 10).
*P < .05, significantly different from naive group; #P < .05 significantly different from vehicle-treated group; one-way ANOVA followed by Bonferroni test.
MPO, myeloperoxidase.

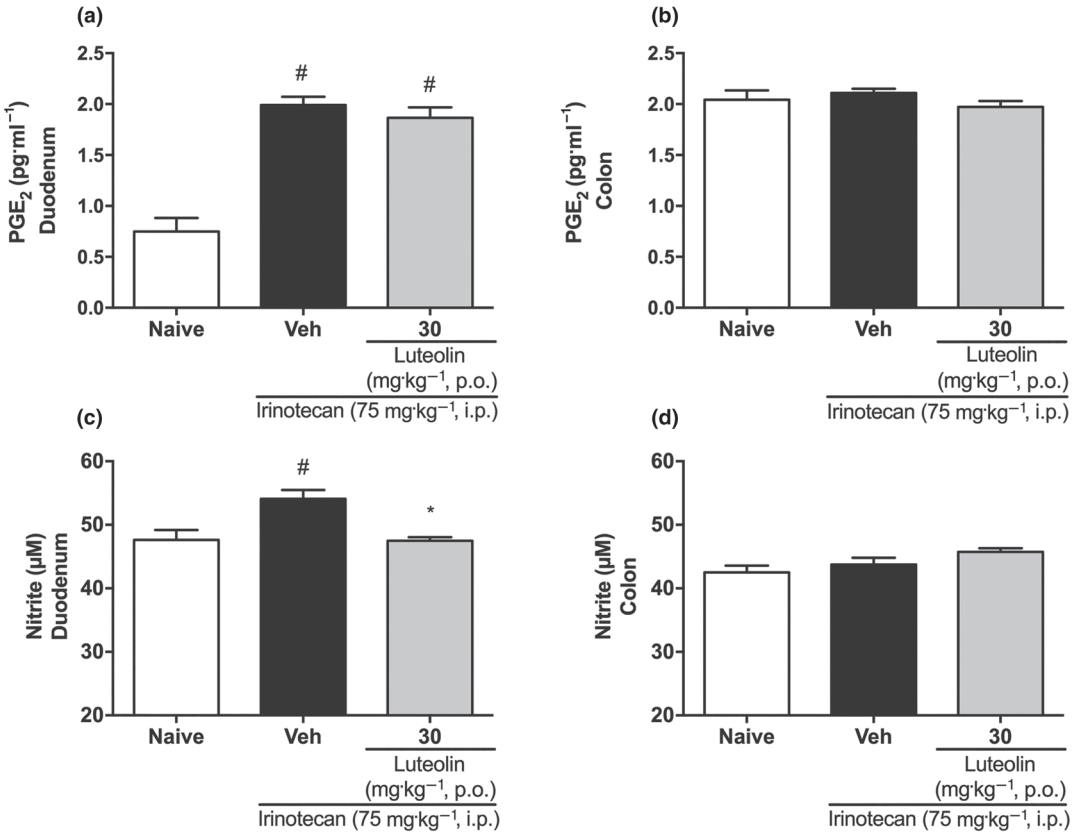

FIGURE 5 Effect of luteolin on PGE_2 and nitrite levels of irinotecan-exposed mice. Female mice were treated for 14 days p.o. with vehicle (Veh: water plus Tween 1%, 10 ml·kg^{-1}) or luteolin (30 mg·kg^{-1}). Irinotecan was given i.p. (75 mg·kg^{-1}, from the 7th to the 10th day) for Veh- and Lut-treated groups. PGE_2 (a and b) and nitrite levels (c and d). The results are expressed as mean ± SEM (n = 10). *P < .05, significantly different from vehicle; #P < .05, significantly different from the naive group; one-way ANOVA followed by Bonferroni's post hoc test

3.7 | Luteolin does not change the intestinal epithelial cells (IEC-6) toxicity induced by irinotecan

The cytotoxicity of luteolin and irinotecan was studied on IEC-6 cells, using the MTT assay. Luteolin did not alter cell viability when tested over a concentration range (3 - 30 μM), while irinotecan showed significant cytotoxicity effect at 30 μM. Importantly, when exposed concomitantly to the cells, luteolin did not interfere with the cytotoxic effects of irinotecan, consistent with the in vivo results described above (Figure S6).

3.8 | Luteolin did not present signs of toxicity in the experimental period of treatment

We evaluated the effect of luteolin and irinotecan on the organ weights of mice. As shown in Table S1, treatment with luteolin (30 mg·kg^{-1}, p.o.) for 14 days did not cause any change in the organ weights, compared with those in the naive group. However, mice treated with the vehicle (plus irinotecan) decreased the spleen (54%) and liver (21%) weights, while mice receiving irinotecan plus treatment with luteolin (30 mg·kg^{-1}, p.o.) did not show these changes.

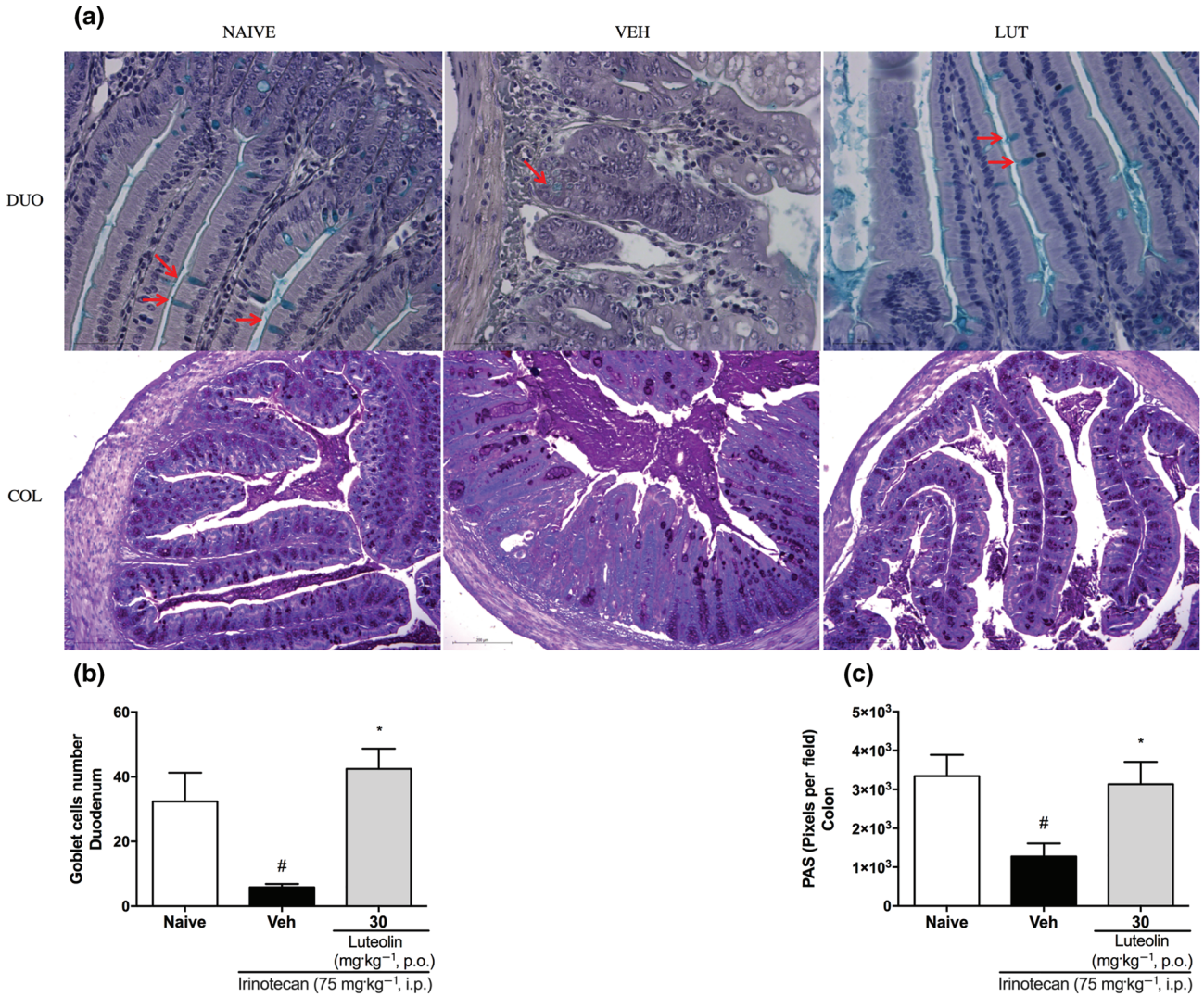

FIGURE 6 Effect of luteolin on mucus production in the intestine of irinotecan-exposed mice. Female mice were treated for 14 days p.o. with vehicle (VEH: water plus Tween 1%, 10 ml·kg^{-1}) or luteolin (LUT: 30 mg·kg^{-1}). Irinotecan was given i.p. (75 mg·kg^{-1}, from the 7th to the 10th day) for Veh- and Lut-treated groups. Goblet cells were stained with Alcian blue (a) in the duodenum (arrows) (DUO) and were counted (b) in crypts and villi under 40× magnification, scale bars 50 μm. Six fields per animal were analysed (n = 5). Mucin-like glycoproteins were stained by PAS (a) (COL) and quantified (c) with ImageJ® program. Scale bars 200 μm. Six fields per animal were analysed (n = 5). The results are expressed as mean ± SEM. $^{*}P < .05$, significantly different from vehicle; $^{\#}P < .05$, significantly different from the naive group; one-way ANOVA followed by Bonferroni's post hoc test. COL, colon; DUO, duodenum

Consistent with these findings, the plasma levels of urea, creatinine, aspartate transaminase (AST), and alanine transaminase (ALT) in mice treated with luteolin (30 mg·kg^{-1}) were not statistically different from the baseline levels, whereas vehicle-treated mice receiving irinotecan increased AST (248%) and ALT (198%) levels, when compared to naive. Luteolin-treated mice receiving irinotecan still showed increased AST levels but had ALT levels comparable to those in the naive group (Table S2).

4 | DISCUSSION

As previously mentioned, intestinal mucositis can be a dose-limiting effect of chemotherapy, and the absence of effective treatments for this side-effect highlights the necessity for discoveries in this therapeutic field. Apart from oral rehydration therapy, the pharmacotherapy of chemotherapy-induced diarrhoea includes mainly the use of drugs which decrease intestinal motility such as **loperamide** (synthetic opioid), **octreotide** (somatostatin analogue), and atropine (competitive inhibitor of muscarinic ACh receptors; Andreyev et al., 2014). Therefore, to evaluate whether luteolin could be a promising candidate for mucositis management, we first demonstrated that luteolin did not affect digestive motility rate, either in a resting state or in a diarrhoeal state, and thus acts differently from the first and ineffective treatment of late diarrhoea induced by chemotherapy.

Chemotherapy-induced diarrhoea appears to be a multifactorial process, including loss of intestinal epithelium, superficial necrosis, and inflammation of the bowel wall, and has thus been classified as

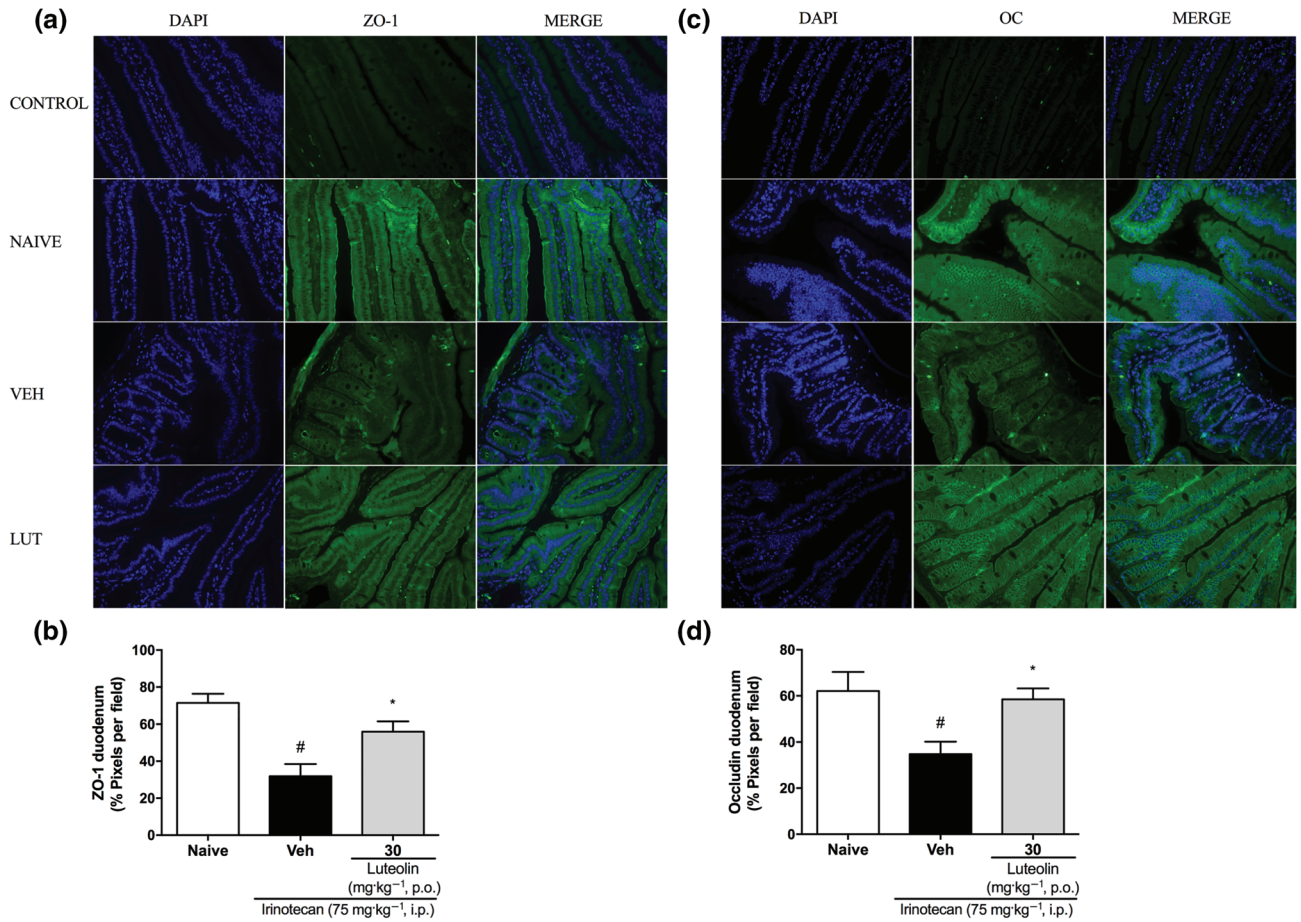

FIGURE 7 Effect of luteolin on tight junctions (TJs) expression in the duodenum of irinotecan-exposed mice. Female mice were treated for 14 days p.o. with vehicle (VEH: water plus Tween 1%, 10 ml·kg^{-1}) or luteolin (LUT: 30 mg·kg^{-1}). Irinotecan was given i.p. (75 mg·kg^{-1}, from the 7th to the 10th day) for Veh- and Lut-treated groups. Immunofluorescence staining of the duodenum for ZO-1 (a) and occludin (OC) (c) protein were performed (green). Nuclei are stained with DAPI (blue). (c and d) Non-relevant IgG was used as CONTROL. Magnification 20×. Fluorescence was quantified with ImageJ® program (b and d). Six fields per animal were analysed (n = 5). The results are expressed as mean ± SEM. *P < .05, significantly different from vehicle; $^{\#}P$ < .05, significantly different from the naive group; one-way ANOVA followed by Bonferroni's post hoc test

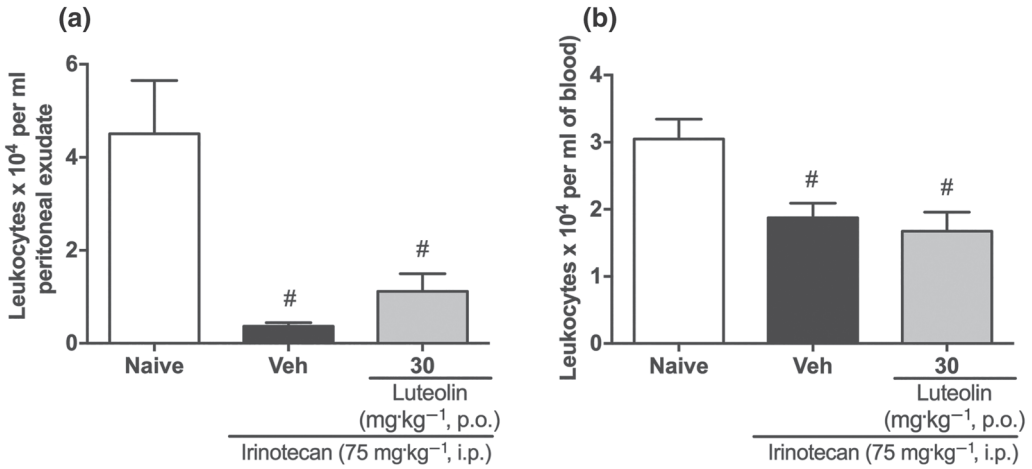

FIGURE 8 Luteolin does not reverse the leukopenia induced by irinotecan. (a) Leukocyte numbers in the peritoneal exudate. (b) Leukocyte numbers in blood. The results are expressed as mean ± SEM (n = 10). $^{\#}P$ < .05, significantly different from the naive group; one-way ANOVA followed by Bonferroni's post hoc test

inflammatory/secretory diarrhoea (Cherny, 2008). Interestingly, we observed that mice in our model of intestinal mucositis induced by irinotecan and treated with vehicle, had a decreased digestive motility rate, compared with that in normal (naïve) mice, which was not detected in the luteolin-treated group. Gastric motility is regulated by the neural circuits that affect the activity of the smooth muscles and also by hormones released from the stomach, intestines, pancreas, and other tissues (Goyal, Guo, & Mashimo, 2019). It is possible that a hormonal/neural feedback to decrease the digestive motility process occurred in an attempt to decrease secretory diarrhoea, which was very pronounced in the vehicle plus irinotecan-treated group.

After this first screening, we evaluated the effect of luteolin treatment on the irinotecan-induced intestinal mucositis, following the experimental protocol for mucositis most described in the literature, where treatment with the compound is started on the same day as the irinotecan treatment and continues for 7 days. Under these conditions, luteolin treatment (30 mg·kg^{-1}, p.o.) was able to diminish diarrhoea score but did not reduce the body weight loss of mice and weight/length ratio of the duodenum and colon. In order to obtain a better effect of the compound and bearing in mind that, in clinical practice, chemotherapy is delivered in fixed treatment cycles (Stein et al., 2010), it seemed that prevention of intestinal mucositis could be a better strategy for the management of this severe adverse effect, we have used a new experimental schedule, starting the luteolin administration 7 days before the first dose of irinotecan.

The first sign of irinotecan-induced intestinal mucositis in mice was body weight loss. However, at the end of the experimental period, animals treated with luteolin (30 mg·kg^{-1}, p.o.), lost less weight than the vehicle (irinotecan only) group. This beneficial effect was accompanied by a significant attenuation of diarrhoea, the main cause of morbidity and mortality in patients experiencing intestinal mucositis (Andreyev et al., 2014). In agreement, when luteolin was given i.p. at the same dose as used p.o., it showed the same response, indicating a systemic effect of the compound, as i.p. injection is used for small species for which i.v. access is challenging (Turner, Brabb, Pekow, & Vasbinder, 2011). In rats, luteolin administered by the oral route, is absorbed into the systemic circulation, and the plasma contains free luteolin, the glucuronide or sulfate conjugates of unchanged luteolin and *o*-methyl luteolin (Lin, Pai, & Tsai, 2015; Shimoi et al., 1998).

In addition to diarrhoea, luteolin also reversed the mechanical hypersensitivity induced by irinotecan. Indeed, the anti-nociceptive effects of luteolin have been described in several models of pain (Fan et al., 2018; Hashemzaei et al., 2017), highlighting the potential of the compound in pain management. Wardill et al. (2016) demonstrated that the hypersensitivity induced by irinotecan treatment is dependent on activation of **TLR4**. This finding leads us to suggest that the protective effect of luteolin in preserving the tight junctions could have decreased the activation of neuronal TLR4 by products of bacteria of the intestinal lumen, thus also reducing activation of the gut/CNS axis and consequently mechanical hypersensitivity.

Apart from the decreased body weight loss, mechanical hypersensitivity and diarrhoea following luteolin treatment, we have also presented histological evidence that this flavone significantly reduced the severity of the intestinal damage by decreasing structural disorder in the tissues, supported by the macroscopic analysis indicating that oedema, vascular permeability, and tissue weight loss were diminished.

Furthermore, irinotecan caused depletion of goblet cells in the duodenum and the quantity of mucin-like glycoproteins in the colon, similar to that reported by Stringer et al. (2009), who described that deficiencies in goblet cell numbers and mucin expression or secretion may contribute to chemotherapy-induced diarrhoea. However, as shown by immunohistochemical staining, treatment with luteolin significantly preserved goblet cells in the duodenum and the amount of mucin in the colon. As the mucus barrier is a crucial factor against mechanical and chemical stress of the epithelium, protecting against bacterial overgrowth and penetration, and essential to the correct transit of the luminal contents (Smirnov, Sklan, & Uni, 2004), this set of data provides further evidence for the protective effects of luteolin on the intestine.

The anti-tumour mechanism of irinotecan and its active metabolite SN-38 on inhibiting topoisomerase I adversely induces apoptotic death of intestinal cells (Ribeiro et al., 2016). Sonis (2004) has described that DNA strand breaks in basal epithelial cells and underlying tissue can result in cell death or injury, and non-DNA injury is initiated through a variety of mechanisms, some of them mediated by the generation of ROS.

Indeed, luteolin treatment significantly decreased the amount of ROS induced by irinotecan in the duodenum, thereby reducing oxidative damages to cell membranes as shown by the diminished levels of LOOH (Girotti, 1998). Moreover, this flavonoid completely restored the levels of GSH, which itself is a critical factor in maintaining the cellular redox balance and also has been involved in the regulation of cell signalling and repair pathways (Cnubben, Rietjens, Wortelboer, van Zanden, & van Bladeren, 2001). In addition, luteolin treatment prevented the loss of catalase activity, which is responsible for the conversion of H_2O_2 into H_2O and O_2 (Kwiecien et al., 2014), preserving values similar to those found in the naive group. Li et al. (2016) have evaluated the effect of luteolin on DSS-induced colitis and have found comparable results, suggesting that luteolin may activate the Nrf2 pathway. Nevertheless, luteolin itself is a potent antioxidant molecule (IC_{50} of ~1.84 μg·ml^{-1} was found in DPPH assay; Figure S7), and then, its directly scavenging properties on ROS formed during the pathophysiology of mucositis, at least in part, may contribute for the beneficial effects of the compound, as described here. On the other hand, we did not detect oxidative imbalance in the colon. In fact, Krajewski et al. (1994) have described that the colon is less affected than the small intestine during intestinal mucositis, probably due to a difference in the expression of the Bcl-2 and Bax proteins between these tissues, and here, we provide strong evidence that ROS formation is also lower in the colon, contributing to less aggressive damage.

Formation of ROS in the intestine is considered as an initiating step in mucositis that triggers a series of events, culminating in cytokine maturation (Ribeiro et al., 2016) and release of damage-associated molecular patterns (DAMPs). This sequence leads to the production of NF-κB, IL-1, IL-18, **COX-2** (Logan et al., 2008; Ribeiro

et al., 2016), TNF, and IL-6 (Logan et al., 2008), as well as an up-regulation of CXCR2 expression on neutrophils, thereby increasing neutrophil recruitment and further tissue damage and bacterial translocation (Guabiraba et al., 2014).

In fact, irinotecan significantly increased MPO activity, indicating neutrophil infiltration, as well as enhancing levels of TNF, IL-1β, and IL-6 and decreasing amounts of IL-4 and IL-10, compared with the naive group. The presence of TNF and IL-6 stimulates early damage in connective tissue and endothelium resulting in epithelial basal cell death and injury (Sonis, 2004). However, luteolin attenuated this inflammatory process as shown by the reduction in MPO activity and of TNF, IL-1β, and IL-6 levels in the duodenum and colon and by the increase of IL-4 and IL-10 in the duodenum, relative those in the vehicle group.

Besides, during intestinal mucositis, the process of inflammation amplification leads to increased PG and NO production, raising intestinal secretion and tissue damage (Ribeiro et al., 2016), as we have also seen in our results. Although luteolin did not reverse the increased PGE_2 levels, it significantly decreased the amounts of nitrite, showing a possible reduction in NO generation, a very important inflammatory mediator in irinotecan-induced mucositis (see Lima et al., 2012).

Although the clinical symptoms of mucositis result from epithelial damage, this condition is a consequence of a dynamic series of biological events involving different tissue compartments and mucosal cells, where leukocyte infiltrates are present even in late stages of the pathological process (Sonis, 2004). During the immune response, irinotecan is known to promote macrophage activation by activating the NF-κB, STAT1, and MAPK signalling pathways (Li et al., 2015). However, we observed that luteolin controlled the activation of classical macrophages by reducing M1 phenotype biomarkers, such as TNF, IL-1β, IL-6, and nitrite generation. In the same way, this effect was observed by Zhang, Li, Xu, Xiang, and Ma (2018) in RAW264.7 cells stimulated with LPS, where the authors have described that luteolin decreased the activation of the NLRP3 inflammasome.

Furthermore, increased TNF, IL-1β, IL-6, and decreased IL-10 levels have been associated with disruption of tight junctions (Suzuki, 2013; Wardill, Bowen, & Gibson, 2012). Tight junctions are multiple protein complexes which regulate intestinal permeability controlling the movement of fluids, nutrients, microbes and toxins across the epithelia (Suzuki, 2013). Peripherally located, zonular occludens proteins (ZO-1, ZO-2, and ZO-3) are cytosolic scaffold proteins which interact with anchor transmembrane proteins like occludin and claudin to the cytoskeleton (Suzuki, 2013). It is well established that ZO and occludin play central roles in maintaining the integrity of the tight junctions and the mucosal barrier function (Cummins, 2012; Fanning & Anderson, 2009). Thus, we evaluated the expression of these two proteins and found that, consistent with earlier reports, irinotecan treatment modified the expression of ZO-1 and occludin (Wardill et al., 2014). Conversely, treatment with luteolin efficiently maintained the levels and location of both proteins in a range similar to that in the naive mice, demonstrating that this flavone has a potential to protect intestinal mucosa from the late effects of mucositis, such as bacterial translocation, which are strongly correlated with death.

Finally, luteolin did not reverse the leukopenia induced by irinotecan, and it did not show interference in the anti-tumour activity of the drug when given in combination with the chemotherapy for mice with melanoma. Moreover, the treatment in its isolated form was shown to be ineffective in remission of the melanoma-type tumour. Similarly, when the effects of luteolin and irinotecan were evaluated on the cellular viability of intestinal cells, the treatment with luteolin did not show any signs of toxicity, whereas exposure with irinotecan significantly reduced the viability of these cells, which was not reversed by luteolin.

Indeed, no toxicity has been described in rats or mice to this flavonoid (Aziz, Kim, & Cho, 2018). However, because luteolin was given to mice for a substantial period, we monitored the organ weights of the animals, as this parameter is commonly used to evaluate toxicity (Sellers et al., 2007). No significant change in the collected organs was observed, after luteolin treatment. On the other hand, luteolin prevented the reduction in spleen and liver weights induced by irinotecan. Chemotherapy toxicity in organs not affected by cancer is one of its limitations (Aikemu et al., 2016), and the hepatotoxic side effects have been a serious clinical challenge in chemotherapy (Thatishetty, Agresti, & O'Brien, 2013). In our experiments, irinotecan increases the plasma levels of AST and ALT, whereas luteolin treatment prevented the increase in ALT, findings similar to those of other authors evaluating hepatoprotective effects of this compound (Liu et al., 2014; He et al., 2019).

In conclusion, the present study provides important evidence that luteolin prevents irinotecan-induced intestinal mucositis, an action which may be associated with a decrease of oxidative stress and of inflammatory processes, along with a preservation of mucosal protective factors, such as mucus and expression of tight junctions. Luteolin is a partial agonist of PPARγ (Puhl et al., 2012), which is a receptor known to promote anti-inflammatory effects by a variety of mechanisms, including NF-κB suppression (Ricote & Glass, 2007), suppression of the NLRP3 inflammasome (Cheng, Li, Wang, Cheng, & Liu, 2018; Meng et al., 2019) and modulation of macrophage function (Dai et al., 2017; Szanto et al., 2010), leading us to suggest that this pathway may mediate all the effects of the flavonoid described here, which prevent intestinal mucositis. However, further studies are needed to support this hypothesis. Moreover, it is important to note that luteolin did not reduce the effectiveness of irinotecan as an anti-cancer agent but only prevented the side effect of this drug. Thus, this flavonoid could be suggested as a potential adjuvant in anti-tumour therapy to control intestinal mucositis.

ACKNOWLEDGEMENTS

We are grateful to Dr. Luis Carlos Stoeberl who provided the irinotecan for this study. This research was supported in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Universidade do Vale do Itajaí (UNIVALI), Institut National de la Santé et de la Recherche (Inserm), and the Université de Lille.

AUTHOR CONTRIBUTIONS

T.B., P. de S., S.S., L.B.S., L.N.B.M., and B.J.C. carried out the pharmacological tests of mucositis. M.F. dos A. and N.L.M.Q. carried out the mechanical withdrawal threshold evaluation. T.B., P. de S., L.D., P.D., L.M. da S., and S.F. de A. performed the statistical analysis and wrote and corrected the manuscript. All authors have read and approved the manuscript.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Boeing T, de Souza P, Speca S, et al. Luteolin prevents irinotecan-induced intestinal mucositis in mice through antioxidant and anti-inflammatory properties. *Br J Pharmacol*. 2020;177:2393–2408. https://doi.org/10.1111/bph.14987