

Therapeutic effects of a lipid transfer protein isolated from *Morinda citrifolia* L. (noni) seeds on irinotecan- induced intestinal mucositis in mice

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Abstract

This work aimed to evaluate the activity of a lipid transfer protein isolated from *Morinda citrifolia* L. seeds, *McLTP*₁, on the development of intestinal mucositis following irinotecan administration. *McLTP*₁ (0.5, 2 and 8 mg/kg, i.v.) was injected into mice 1h before irinotecan administration (75 mg/kg, i.p.; 4 days), and then for additional 6 days. Seven days after the first dose of irinotecan, diarrhea was assessed and the intestine was removed for histological evaluation, assessment of intestinal over-contraction, measurement of myeloperoxidase (MPO), glutathione (GSH), malondialdehyde (MDA), proinflammatory cytokines and chemokine (IL-1, IL-6, and KC levels - a murine homolog of human IL-8 chemokine), analysis of cyclooxygenase 2 (COX-2), nuclear factor kappa B (NF-κB) and nitric oxide synthase (iNOS) expression. At the two highest doses, *McLTP*₁ administration decreased mortality and diarrhea. *McLTP*₁ (8 mg/kg, i.v.) significantly prevented irinotecan-induced intestinal damage and led to a reduction in over-contraction of the intestinal muscle ($p < 0.05$). Moreover, *McLTP*₁ decreased the MPO, IL-1β, IL-6, and KC levels significantly by 74.7%, 42%, 92.9%, and 95.9%, respectively. Also, the expression of COX-2, NF-κB, and iNOS was reduced. Pretreatment with *McLTP*₁ (8mg/kg; i.v.) significantly reduced the MDA level and increased the duodenum homogenates' GSH level. Our study provides a potential new therapeutic for preventing irinotecan-induced mucositis, improved clinical parameters, reduced inflammation, and oxidative damage.

Introduction

Irinotecan is a semisynthetic analog of the natural alkaloid camptothecin. It acts by inhibiting topoisomerase I, and is one of the main chemotherapeutic agents used in the treatment of colorectal cancer (Fujita et al. 2015). The clinical advantage of irinotecan is limited by its side-effects, including mucositis and severe diarrhea, which affect approximately 40% of patients under treatment (Bleiberg and Cvitkovic 1996). Mucositis reduces the survival of colorectal cancer patients and diminishes chemotherapy's efficiency due to the need to decrease treatment doses or discontinue therapy (Merrouche et al. 1997).

Intestinal mucositis is associated with histological damage and polymorphonuclear proliferation (Logan et al. 2008a). These events trigger an immunogenic response through the activation of nuclear factor kappa B (NF-κB) (Logan et al. 2008b), which, in turn, upregulates the expression of inflammatory mediators including tumor necrosis factor-alpha (TNF-α) (Melo et al. 2008), interleukin-1 beta (IL-1β) (Melo et al. 2008; Arifa et al. 2014) and interleukin-6 (IL-6) (Logan et al. 2008b), as well as the enzymes cyclooxygenase 2 (COX-2) (Wong et al. 2015) and nitric oxide synthase (iNOS) (Lima-Júnior et al. 2012). Moreover, the production of reactive oxygen species (ROS) and reactive nitrogen species, which induce cell death, are involved in intestinal mucositis mechanisms (Arifa et al. 2014). Currently, mucositis management is only supportive; therefore, new interventions to reduce mucositis are needed (Ribeiro et al. 2016).

The search for substances purified from plants for the prevention and treatment of intestinal mucositis is promising, and several of these have been described previously (de Alencar et al. 2017; Cechinel-Zanchett et al. 2019; Boeing et al. 2020). Proteins with therapeutic potential have several advantages over low molecular weight compounds due to their high specificity and low level of interference with normal biological processes, causing fewer adverse effects (Leader et al. 2008).

Morinda citrifolia L. is a fruit-bearing tree (Rubiaceae), more commonly known as noni. Traditionally, noni has been studied due to its range of pharmacological effects, including analgesia (Basar et al. 2010), antibacterial (Cruz-Sánchez et al. 2019), antidiabetic (Nerurkar et al. 2015), liver-protective (Wang et al. 2008), anticancer (Sharma et al. 2016) and immunomodulatory (M et al. 2019) activities. Recently, a thermostable lipid transfer protein, named *McLTP*₁ (9.4 kDa), was isolated from its seeds (UniProtKB accession number: C0HJH5). This protein was shown to be resistant to pepsin, trypsin, and chymotrypsin digestion and displayed potent analgesic and anti-inflammatory activities when orally administered to mice (Campos et al. 2016, 2017).

Previous studies have demonstrated that the anti-inflammatory effects of *McLTP*₁ are related to the modulation of proinflammatory cytokines. Given the involvement of these cytokines in intestinal mucositis pathogenesis, we decided to investigate the effects of *McLTP*₁ in the present study. Therefore, this work aimed to evaluate the effect of *McLTP*₁ on irinotecan-induced intestinal mucositis, seeking to characterize a potential natural compound as an efficient approach to preventing chemotherapy-induced adverse reactions.

Material And Methods

Plant material and purification of *McLTP*₁

Noni (*Morinda citrifolia* L.) seeds were finely ground and thoroughly defatted with petroleum ether (1:10 w/v), air-dried for 24 h at 25°C, then stored at -20°C until used for *McLTP*₁ purification. This protein was isolated from defatted noni seed flour according to a previously delineated protocol (Campos et al. 2016). For purity analysis, lyophilized samples of *McLTP*₁ were submitted to electrophoresis under non-reducing conditions (15% SDS-PAGE) following the method described by Laemmli (Laemmli 1970). For this study, *McLTP*₁ was solubilized in sterile saline (0.9% NaCl) immediately before administration to mice. The total protein concentration of the *McLTP*₁ samples was estimated using the Bradford method (Bradford 1976).

Animals

Adult male Swiss mice (*Mus musculus*), weighing 25 ± 3.0 g, were obtained from the Central Animal House of the Federal University of Ceará. The animals were housed in a controlled environment (24 ± 2°C, 12-h light/dark cycle) with *ad libitum* food and water. The mice were randomized into experimental groups of 8–10 animals each. All experimental procedures were conducted following the National

Experimental irinotecan-induced mucositis

Experimental intestinal mucositis was induced in mice as previously described (Ikuno et al. 1995), with modifications (Melo et al. 2008). Briefly, mice were administered 0.15 M NaCl (0.1 mL/10 g, i.v.), representing the vehicle group, or *MdLTP*₁ (i.v.) at a dose of 0.5, 2, or 8 mg/kg one day before the administration of irinotecan (75 mg/kg i.p., Trebyxan®, Bergamo, São Paulo, Brazil). From the first to the fourth day, the animals received irinotecan. Besides, from days 1 to 7, the animals were administered daily *MdLTP*₁ (i.v.) or 0.15 M NaCl 1 h before irinotecan administration. The naïve group received only NaCl daily (0.1 mL/10 g, i.p.). During the experimental period, the survival of the animals was measured daily. On the seventh day, all animals were euthanized, and the total length of the small intestine was measured. The duodenum's segments were collected, fixed in 10% neutral buffered formalin, and processed for histological and immunohistochemical analyses. Moreover, portions of the duodenum were quickly collected for the evaluation of in vitro contractility and stored at -80°C for the measurement of myeloperoxidase (MPO) activity and tissue levels of cytokines (IL-1 β , IL-6, and KC), thiobarbituric acid reactive substances (TBARS) and glutathione (GSH).

Diarrhea assessment

The severity of diarrhea was evaluated on the seventh day and scored as follows: 0, normal (normal stool or absent), 1, mild (slightly wet and soft stool), 2, moderate (wet and unformed stool with moderate perianal staining of the coat), and 3, severe (watery stool with severe perianal staining of the coat) (Kurita et al. 2000).

Histopathological analysis and intestinal morphometry

Duodenal segments were fixed in 10% (v/v) neutral buffered formalin, dehydrated, and fixed in paraffin. Sections (5 μ m thick) were stained with hematoxylin and eosin (H&E) for subsequent light microscopy examination. The severity of mucositis was graded according to previously described criteria (Woo et al. 2000). The length of the intestinal villi and depth of the crypts were measured using ImageJ software (version 1.4, NIH, USA). Between 5 and 10 villi and crypts were measured per slice of duodenum, and 5–8 slices were analyzed for each experimental group.

In vitro contractility of duodenum preparations

Duodenum segments (0.6 cm long) were dissected and processed to investigate in vitro contractility (Araujo-Neto et al. 2010). Briefly, the duodenal strips were placed in Petri dishes containing cold modified Tyrode's solution (composition in mM: NaCl, 136.0, KCl, 5.0, MgCl₂, 0.98, CaCl₂, 2.0, NaH₂PO₄, 0.36, NaHCO₃, 11.9, and glucose, 5.5). Then, each segment was placed in a glass organ bath

containing 5 mL of Tyrode's solution at 37°C and continuously bubbled with carbogen (5% CO₂ and 95% O₂) to maintain the pH 7.4. The lower end of the strip was fastened to a fixed hook at the bottom of the organ bath, and the upper end was connected with a steel hook to a force transducer (model MLT0201, AD Instruments, BellaVista, NSW, Australia) under 1-g baseline tension. A concentration-response curve to acetylcholine was generated using increasing and cumulative concentrations ranging from 1×10^{-10} to 1×10^{-4} M, which were added into the organ bath every 5 min. The data registered from the cholinergic concentration-response curve were analyzed as the percentage contractile response compared to the mean of two standard contractions of 60 mM KCl.

Myeloperoxidase (MPO) activity assay

The activity of MPO was measured in the duodenum segments. Briefly, duodenum tissues were collected, homogenized in 0.02 M NaPO₄ buffer (pH 4.7), and centrifuged at 3000 rpm for 15 min at 4°C. After lysis with 0.2% NaCl hypotonic solution the pellet was centrifuged again at 3000 rpm for 15 min at 4°C. The pellet was resuspended in 300 µL of 0.05M NaPO₄ buffer, pH 5.4, with 0.5% hexadecyltrimethylammonium bromide (HTAB, Sigma, St Louis, MO, USA). The supernatant was assayed by measuring the change in absorbance at 450 nm using tetramethylbenzidine (1.6 mM) and H₂O₂ (0.5 mM), and stopped with a 2 N H₂SO₄ solution. The results were expressed as neutrophils $\times 10^2$ per milligram of tissue (Alves-Filho et al. 2006).

Cytokines (IL-1 β , IL-6 and KC) level determination

Concentrations of cytokines IL-1 β , IL-6 and KC were measured in the duodenum homogenates. Stored duodenum tissue was triturated and homogenized to 10% (mg tissue/µL) at 4°C (POLYTRON®, Biosystems, Paraná, Brazil) in PBS solution (pH 7.4). Then the pellet was removed after centrifugation at 5000 rpm for 5 min. Cytokines were measured using specific ELISAs (R&D Systems, Minneapolis, MN, USA), which were performed according to the manufacturer's recommendations. The results are expressed as picograms cytokines per milliliter of homogenate.

Immunohistochemistry

Segments of duodenum were incubated with goat anti-COX-2 (1:200, SC-1747, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-NF- κ B p65 (1:200, ab194926, Abcam) or rabbit anti-iNOS (1:200, ab15323, Abcam, Eugene, USA) antibodies. The quantitative estimation of DAB products from immunostaining was performed using digital images of at least 10 different areas of each section. Image J software was used to quantify the immunostaining area. The result was expressed as a percentage of the marked area.

Quantification of antioxidant parameters: glutathione (GSH) and thiobarbituric acid reactive substances (TBARS)

Glutathione, a water-soluble antioxidant, was measured in the duodenum segments from mice in different experimental groups. The tissue was triturated in ethylenediaminetetraacetic acid solution (EDTA, 0.02 M). This homogenate (40 μ L) was mixed with 60 μ L 10% trichloroacetic acid and then centrifuged. The supernatant was mixed with Tris buffer (25 mL, 0.4 M) and 5,5-dithio-bis-2-nitrobenzoic acid (DTNB, 650 μ L, 0.01 M), stirred for 3 min, then analyzed by measuring the absorbance at 412 nm. The results are expressed in μ g of GSH per miligram of tissue (Sedlak and Lindsay 1968).

Levels of MDA in duodenum homogenates were quantified. The tissue samples were homogenized in potassium chloride solution (KCl, 0.15 M), the mixture was stirred and heated in a heating bath (37°C) for 60 min. Perchloric acid 35% (400 μ L) was added to stop the peroxidation, and the mixture was centrifuged at 14,000 rpm for 15 min at 4°C. The supernatant was mixed with 0.8% thiobarbituric acid (200 μ L) and heated in a boiling bath (95°C) for 30 min. After cooling, samples were assayed by measuring the absorbance at 532 nm. The results are expressed as micromoles of MDA per miligram of tissue (μ M/mg of tissue) (Draper and Hadley 1990).

Statistical analysis

Data are expressed as the mean and standard error of the mean (SEM) or the median (nonparametric data) of at least three independent observations. Analysis of variance (ANOVA) followed by Bonferroni's post hoc test was used to compare the means, the Kruskal–Wallis test followed by Dunn's test was used to compare medians, for survival analyses, the log-rank test (Mantel-Cox) was used. Statistical significance was accepted when $P < 0.05$. All data were analyzed using GraphPad Prism software version 6.0 (Graph-Pad Software, Inc., La Jolla, CA, USA).

Results

Mc LTP₁ reduces the clinical parameters of experimental irinotecan-induced mucositis

The Irinotecan administration produced a mortality rate of 34% of the animals compared to the naïve group. Mice that received *McLTP*₁ treatment presented 80%, 90%, and 100% survival at the doses of 0.5, 2, and 8 mg/kg, respectively (Fig. 1a). The lowest dose of *McLTP*₁ (0.5 mg/kg) was not able to reduce diarrhea induced by irinotecan (score 3 [range 1–3]). However, when administered at the doses of 2 and 8 mg/kg, *McLTP*₁ reduced the severity of diarrhea (scores 0 [range 0–2] and 0 [range 0–2], respectively) when compared to the irinotecan group (score 3 [range 2–3]; Fig. 1b).

Corroborating with the reduction in diarrhea, the data presented in Fig. 1c demonstrate that irinotecan induced a significant increase in intestinal contractility compared to the naïve group. On the other hand, the treatment of mice with *McLTP*₁ (8 mg/kg; i.v.) significantly reduced by 56.2% (114.2 ± 29.19) the intestinal over-contraction compared to the maximum contraction induced by acetylcholine.

Mc LTP₁ reduces the histological damage and intestinal shortening induced by irinotecan

Irinotecan administration provoked significant histological damage characterized by reduced villus height, loss of crypt architecture, necrosis, apoptosis, intense inflammatory cell infiltration in the lamina propria, and vacuolization of the lining epithelium cells when compared to the naïve group. However, a repairing effect of intestinal damage was observed following treatment with *McLTP1* (8 mg/kg, i.v.) administration (Fig. 2a). As depicted in Fig. 2b the intestinal damage scores were significantly improved after *McLTP1* (8 mg/kg, i.v.) administration.

Irinotecan injection also provoked a significantly ($p < 0.05$) reduction in the length of the small intestine by 35.7% when compared to the naïve group. This effect was restored by *McLTP1* (8 mg/kg, i.v.), resulting in a significantly lengthened small intestine compared with irinotecan controls (Fig. 2c). At the doses of 0.5 and 2 mg/kg, *McLTP1* did not show any significant effect when compared to the vehicle group (Fig. 2c).

Irinotecan markedly decreased (by 60.3%) the villus height and the crypt depth (by 35.8%) when compared to the naïve group. Animals treated with 8 mg/kg *McLTP1* (i.v.) showed a significant reduction (48.4%, $p < 0.05$) in intestinal damage based on the villus height. Furthermore, the crypt depth was normalized compared to the irinotecan group (Fig. 3a and b). Changes in villus height to crypt depth ratio was similar to that of villus height. Irinotecan significantly decreased the ratio in duodenal sections compared to the naïve group (2.5 ± 0.1 vs 5.8 ± 0.3 , $p < 0.05$). On the other hand, the *McLTP1* 8 mg/kg (i.v.) treatment significantly ameliorated the alterations promoted by irinotecan, displaying a villus/crypt ratio of 3.2 ± 0.2 ($p < 0.05$, Fig. 3c).

***McLTP1* decreased the inflammatory response after mucositis induction**

The inflammatory response induced by irinotecan was characterized by an increase in neutrophil influx to the duodenum (6398 ± 849 neutrophils $\times 10^2$ /mg of tissue) compared to the naïve group (1689 ± 300 neutrophils $\times 10^{-2}$ /mg of tissue). *McLTP1* 8 mg/kg (i.v.) significantly reduced the neutrophil influx (1618 ± 79 neutrophils $\times 10^2$ /mg of tissue) compared to the irinotecan group (Fig. 4a).

Mice in irinotecan group had significantly higher proinflammatory cytokine levels (IL-1 β : 21.15 ± 2.97 pg/mg of tissue, IL-6: 14.89 ± 5.20 pg/mg of tissue, and KC: 77.76 ± 21.23 pg/mg of tissue) in comparison to the those observed in the naïve group. Interestingly, the treatment with *McLTP1* (8 mg/kg, i.v.) significantly reduced the proinflammatory cytokines levels to 12.37 ± 2.01 pg/mg of tissue for IL-1 β , 1.055 ± 0.44 pg/mg of tissue for IL-6 and 5.450 ± 2.22 pg/mg of tissue for KC (Fig. 4b–d), attenuating the severity of intestinal mucositis.

***McLTP1* reduces the immunochemical detection of COX-2, NF- κ B and iNOS**

The immunochemical analysis of COX-2 in duodenum sections of mice undergoing irinotecan treatment showed intensive immunostaining, mainly in the epithelium, including the lamina propria and the mucosal muscle layer, represented by a 51.4% increase in the stained area. *McLTP1* (8 mg/kg) treatment

decreased the duodenum tissue's stained area by 62% compared to animals treated with irinotecan (Fig. 5).

Like the results for COX-2 immunostaining, sections of mice duodenum subjected to treatment with irinotecan (75 mg/kg) showed intensive NF- κ B immunostaining, represented by a 33.8% increase in the stained area. *McLTP1* treatment decreased the duodenum tissue's marked area by 51.6% compared to animals treated with irinotecan (Fig. 6).

Sections of duodenum subjected to treatment with irinotecan showed intensive iNOS immunostaining with more prevalent stained areas on the epithelium's surface, represented by a 61% increase in the stained area. *McLTP1* treatment decreased the duodenum tissue's marked area by 44.2% compared to animals treated with irinotecan (Fig. 7).

Mc LTP₁ decreased oxidative stress

In this study, TBARS was measured as a marker of oxidative stress and lipid peroxidation products. In the group treated with irinotecan (75 mg/kg), a significant increase in the level of tissue MDA (91.20 ± 4.508 μ M/mg of tissue) was observed compared to the naïve group (72.67 ± 7.999 μ M/mg of tissue). *McLTP₁* treatment normalized the concentration of intestinal MDA (58.35 ± 3.732 μ M/mg of tissue), the levels of lipid peroxidation and tissue damage caused by irinotecan (Fig. 8a).

NPSH (non-protein thiols) groups are representative of the level of the antioxidant cellular defense agent GSH. Treatment with irinotecan significantly decreased the GSH concentration in the duodenum (153.6 ± 29.83 μ g/mg of tissue) compared to the naïve group (534.3 ± 32.39 μ g/mg of tissue). *McLTP₁* treatment significantly increased the endogenous antioxidant level (1225 ± 378.1 μ g/mg of tissue), thereby minimizing the negative effects of irinotecan (Fig. 8b).

Discussion

Morinda citrifolia L (noni) is considered an alternative medicine which stands out due to its diverse therapeutic effects such as anti-inflammatory, antinociceptive, analgesic (Basar et al. 2010), antioxidant (Serafini et al. 2011), antitumor (Sharma et al. 2016), antibacterial (Zhang et al. 2016), antidiabetic (Algenstaedt et al. 2018), antidyslipidemic (Mandukhail et al. 2010), antiangiogenic (García-Vilas et al. 2017), antifungal (Barani et al. 2014), anti-ulcer, antiesophagitis (Mahattanadul et al. 2011), antinausea (Prapaitrakool and Itharat 2010) and anxiolytic effects (Kannan et al. 2014). However, studies on substances isolated from noni that focus on the characterization and elucidation of their action mechanisms are scarce. This study demonstrated for the first time the effect of an isolated protein of the LTP class, *McLTP1*, in an intestinal mucositis model.

Gastrointestinal mucositis is a severe side effect of both anticancer chemotherapy and radiation therapy (Keefe et al. 2000). Currently, mucositis is managed using clinically used drugs such as loperamide, octreotide, antibiotics, and probiotics (Schmittl et al. 2004; Osterlund et al. 2007; Regnard et al. 2011;

Sun and Yang 2014). However, these medicines only control diarrhea without addressing the underlying pathogenesis of this side effect; therefore, it is imperative to search for drugs that effectively control inflammation and reduce damage to the intestinal mucosa, promoting continuity of treatment and a better quality of life.

Previous studies reported the pharmacological effects of *MdLTP₁* after oral administration in mice. However, in the present study, *MdLTP₁* was administered intravenously. When administered by the intravenous route, the drug does not need to be absorbed; thus, the onset of its therapeutic effect is faster. Besides, as the drug does not undergo first-pass metabolism, its bioavailability is more significant when compared to oral administration. The intravenous route is also chosen for prophylactic treatment with corticosteroids and antiemetics to prevent nausea and vomiting induced by chemotherapy. This major adverse effect is associated with a significant deterioration in the quality of life perceived by patients (Bloechl-Daum et al. 2006; Navari et al. 2013; Wang et al. 2018).

Irinotecan causes severe or continuous diarrhea or vomiting in 60–87% of patients (Rougier et al. 1997), and therapy to manage this adverse effect fails in 10–15% of patients (Lenfers et al. 1999; Hoff et al. 2014). Interestingly, *MdLTP₁* treatment has been found to reduce diarrhea; thus, we assessed whether this effect is related to lowering intestinal contractility and confirmed that *MdLTP₁* treatment reduces the contraction induced by irinotecan.

Irinotecan is a direct inducer of mucosal damage, mainly intestinal crypt cell apoptosis (Ribeiro et al. 2016), which aggravates diarrhea. Pretreatment with *MdLTP₁* significantly reduced the shortening of the villus, the erosive and ulcerative areas at the crypt, and the inflammatory infiltrate. These results corroborate a similar study that assessed the use of a protein fraction extracted from *Calotropis procera* in a model of irinotecan-induced intestinal mucositis, which showed antidiarrheal activity and preserved the architecture of the intestinal mucosa (de Alencar et al. 2017). Amelioration of the clinical parameters and intestinal damage was associated with a decreased neutrophil influx, as evidenced by a reduction in MPO activity. Mucositis triggers a series of events culminating in cytokine maturation and leading to the release of IL-1 β , IL-6, and KC (analogous to human IL-8) (Melo et al. 2008; Ribeiro et al. 2016). Nevertheless, *MdLTP₁* attenuated this inflammatory process, shown by a reduction in the levels of these cytokines in the duodenum. This mechanism is similar to the effect of *MdLTP₁* in a carrageenan-induced paw edema model (Campos et al. 2017).

The activation of IL-1 β occurs via NOX-2-derived oxidative stress, which mediates inflammasome activation (Arifa et al. 2014). This cytokine plays a significant role in the pathogenesis of intestinal mucositis, and depletion of IL-1 β results in a reduced inflammatory response and attenuated intestinal injury. The effect of *MdLTP₁* on attenuating the release of IL-1 β suggests that its modulatory activity is multifactorial.

Administration of *MdLTP₁* was also associated with a reduction in NF- κ B, a transcription factor activated by radiotherapy or chemotherapy agents such as irinotecan. Once activated, NF- κ B leads to the

upregulation of several genes, including those that result in the production of proinflammatory cytokines TNF- α , IL-1 β , and IL-6 (Sonis et al. 2004). These results support the idea that *MdLTP₁* induces anti-inflammatory effects by reducing the production of NF- κ B and proinflammatory cytokines. In addition, upregulation of the COX-2 pathway activated by irinotecan injection is associated with an increase in the severity of the mucosal injury and prolonged inflammation (Trifan et al. 2002; Wong et al. 2015). In this study, the immunochemical analysis of COX-2 showed that treatment of mice with *MdLTP₁* decreased the duodenum tissue's-stained area compared to animals treated with irinotecan.

Because *MdLTP₁* modulated inflammatory cytokines and transcription factors, we decided to investigate the effectiveness of *MdLTP₁* on nitric oxide, which is another essential inflammatory mediator in irinotecan-induced mucositis. That said, the immunohistochemistry results obtained in the current study suggest a reduction in iNOS, confirming the involvement of nitric oxide in the anti-inflammatory effects of *MdLTP₁*. Another mechanism involved in intestinal mucositis induced by irinotecan is ROS production from enterocytes exposed to chemotherapy, considered a starting step in the pathogenesis of mucositis (Lima-Júnior et al. 2012; Arifa et al. 2014; Rtibi et al. 2017). Additionally, ROS generated by NADPH oxidase 2 is involved in caspase-1 activation and consequent IL-1 β release. The production of NF- κ B, COX-2, TNF- α , and IL-6 is also associated with increased neutrophil recruitment and tissue damage (Ribeiro et al. 2016). Therefore, as *MdLTP₁* modulates IL-1 β , this suggests that the protein could act as an antioxidant. Corroborating this hypothesis, *MdLTP₁* was shown to reduce MDA content and increase GSH level.

In summary, these data support the idea that the administration of *MdLTP₁* prevented the intestinal damage induced by irinotecan by acting as a potent inhibitor of NF- κ B, iNOS, COX-2 and proinflammatory cytokine activation. Besides, *MdLTP₁* also diminished ROS production from enterocytes exposed to irinotecan.

Conclusion

Currently, there is no research on the modulatory effects of isolated proteins in this intestinal mucositis model. The current study expands the therapeutic potential of noni and provides evidence that the mechanism of action of *MdLTP₁* occurs through the modulation of proinflammatory cytokines. Additionally, *MdLTP₁* not only has anti-inflammatory effects, but also has antidiarrheal and antioxidant activities. Thus, treatment with *MdLTP₁* is a promising alternative to reduce the side effects of chemotherapy and increase patients' quality of life, while allowing the continuity of chemotherapy treatment and favoring adequate therapy in the treatment of cancer.

Declarations

Author contributions

NMNA, HDO had the idea for the article and provided the study materials, reagents, laboratory samples, animals, and instrumentation. ASC and DCOC purified the protein used in the experiments (*MdLTP₁*). LDC, GFPR, LMAR, TFGS, RCLJr, DVTW, RFCL, AAVS, and PJCM collected the data, performed the literature search and data analysis, drafted and critically revised the published work. All authors read and approved the manuscript and all data were generated in-house and no paper mill was used.

Data availability All data generated or analyzed during this study are included in this published article.

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Ethical approval

This study was approved by the "Animal Use Ethics Committee (CEUA)" of the Federal University of Ceará vide protocol no. 93/15. The guidelines of "National Council for the Control of Animal Experimentation (CONCEA)" were followed for all animal experiments and care of animals.

Consent to participate Not applicable.

Consent to publish All authors have agreed to publish this study.

Conflict of interest The authors declare that they have no conflict of interest.

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Figures

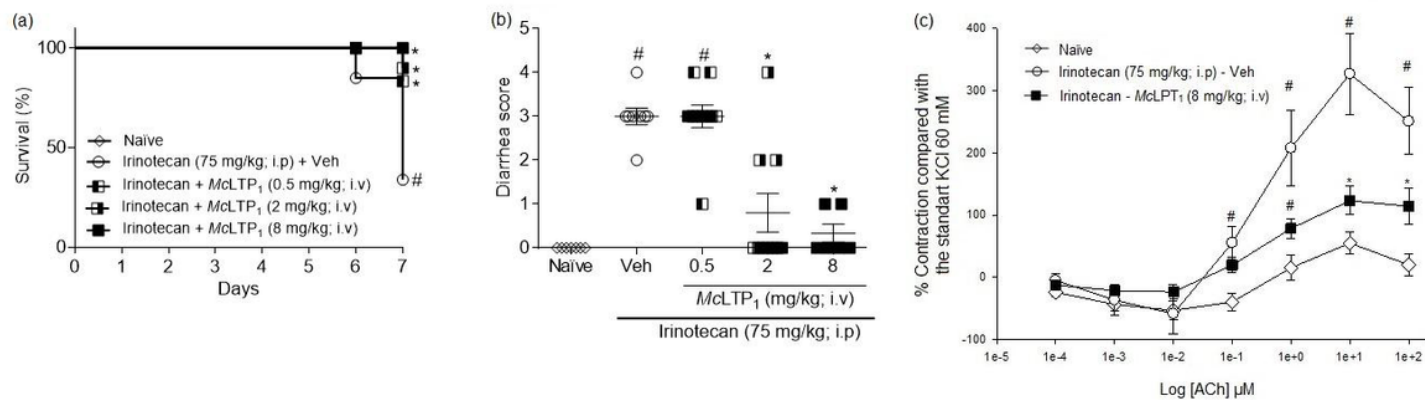


Figure 1

Effects of McLTP1 (0.5, 2, and 8 mg/kg) on (a) survival rate, (b) diarrhea, and (c) duodenal contractility in mice with intestinal irinotecan-induced mucositis. #p < 0.05 compared to the naïve group and *p < 0.05 compared to the irinotecan-treated group (n = 6-10 animals per group, Mantel-Cox log-rank test, Kruskal–Wallis with Dunn’s test, and ANOVA with Bonferroni’s test, respectively)

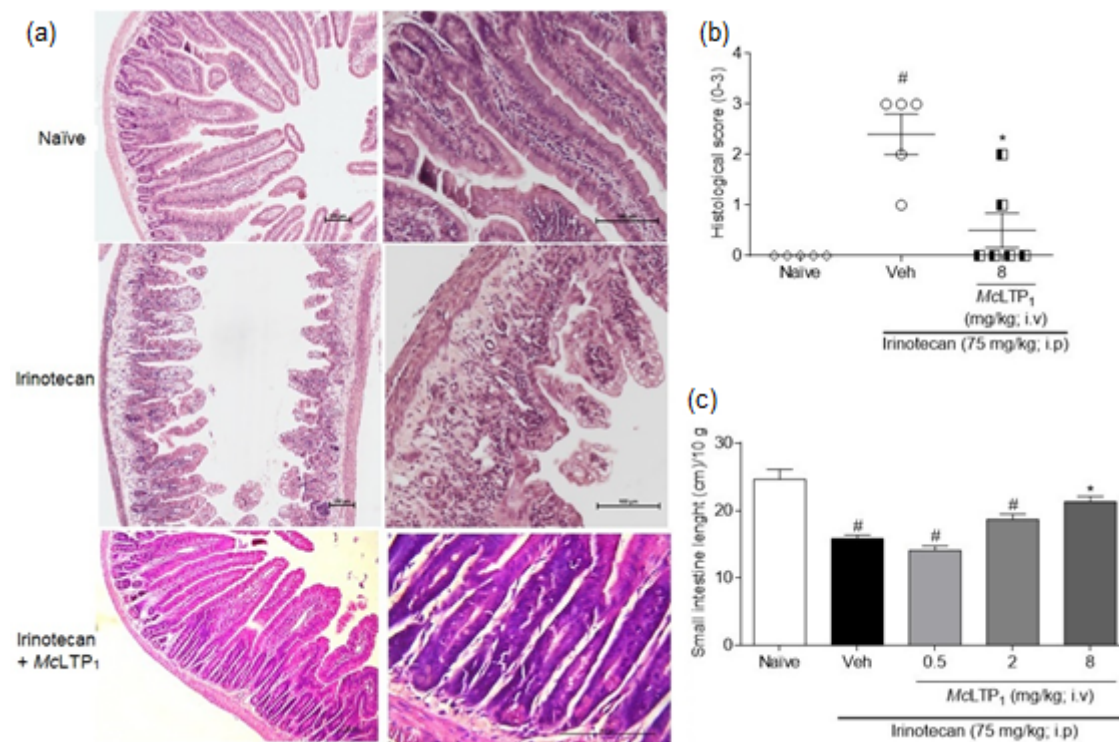


Figure 2

(A) Representative histology of duodenum with hematoxylin and eosin stain in mice from the naïve group and from irinotecan administered animals (×100 and ×400 magnifications). Scale bar: 100 μm. (B) Histological scores of the animals submitted to treatment with saline, irinotecan or McLTP₁ 8 mg/kg (i.v.). (C) Small intestine length of mice with irinotecan-induced intestinal mucositis. The values are expressed

as the means $\pm$ SEM (parametric data) or median (minimum-maximum) for non-parametric data. #p < 0.05 compared to the naïve group and *p < 0.05 compared to the irinotecan-treated group (n = 6-10 animals per group). Parametric and non-parametric data were analyzed using ANOVA followed by Bonferroni's post hoc testing or Kruskal-Wallis/Dunn's test, respectively

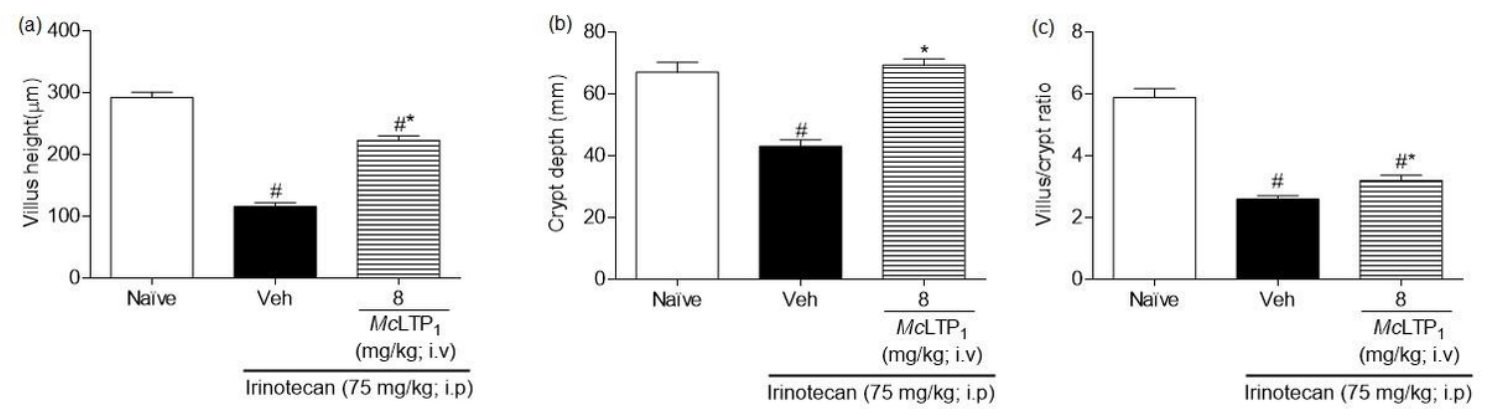


Figure 3

Morphometric parameters of mice with irinotecan-induced intestinal mucositis: villus height (a), crypt depth (b), and villus/crypt ratio (c). The results are expressed as the mean $\pm$ SEM. #p < 0.05 compared to the naïve group and *p < 0.05 compared to the irinotecan-treated group (n = 10 animals per group, ANOVA with Bonferroni's test)

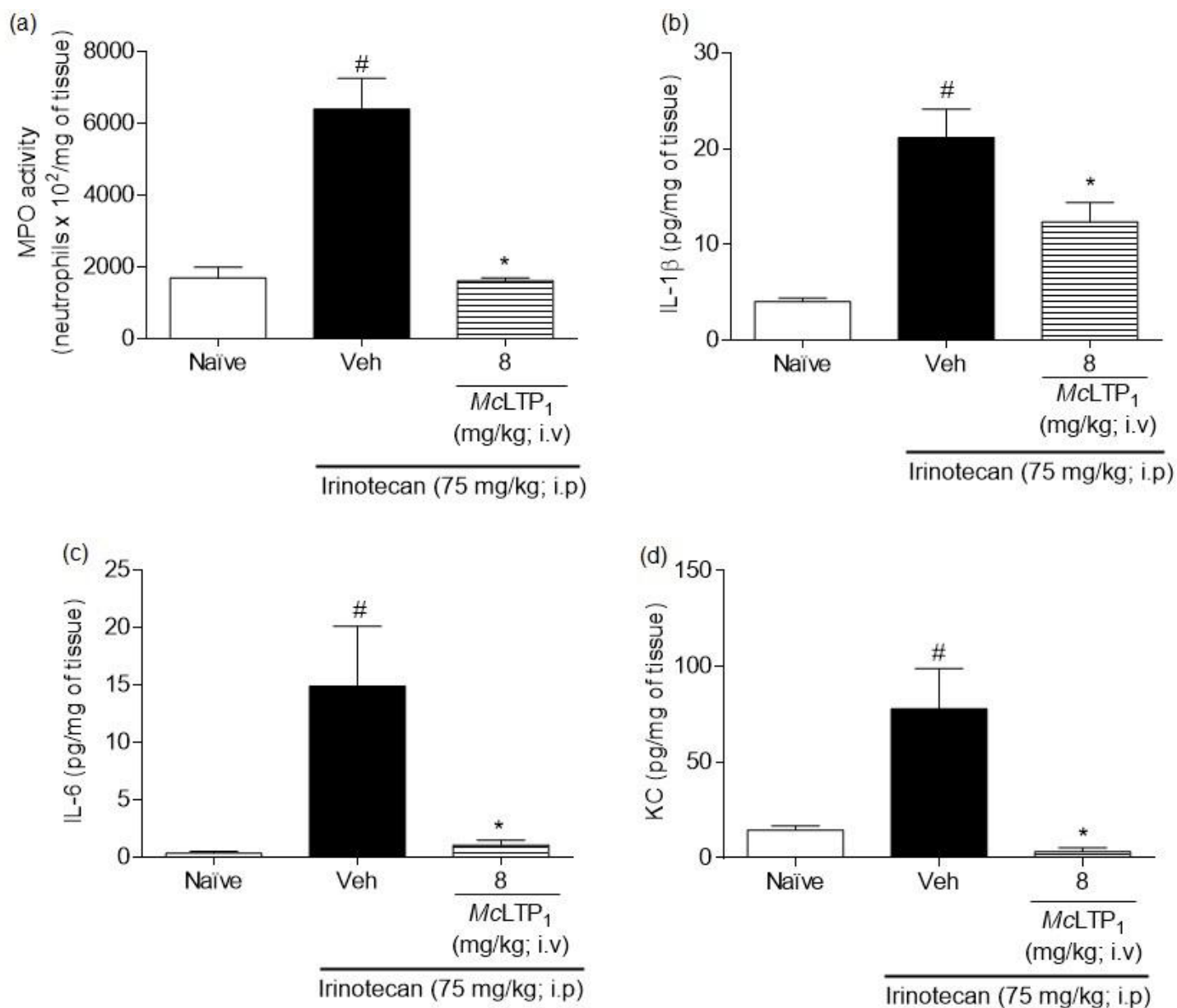


Figure 4

*McLTP*₁ treatment reduced (a) MPO, (b) IL-1 β , (c) IL-6 and (d) KC activities in mice with irinotecan-induced intestinal mucositis. The results are expressed as the mean $\pm$ SEM. #*p* < 0.05 compared to the naïve group and **p* < 0.05 compared to the irinotecan-treated group (n = 6-10 animals per group, ANOVA with Bonferroni's test)

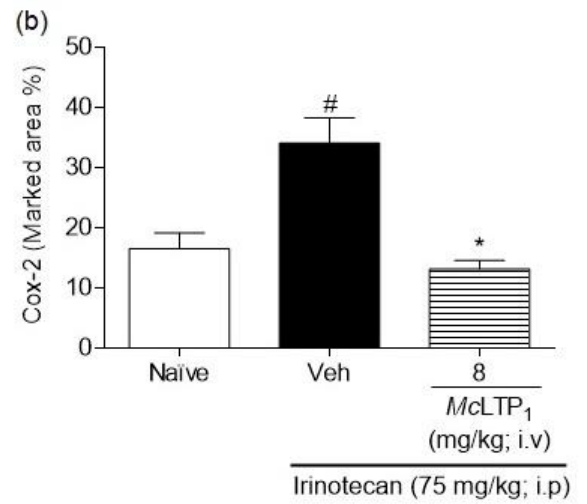
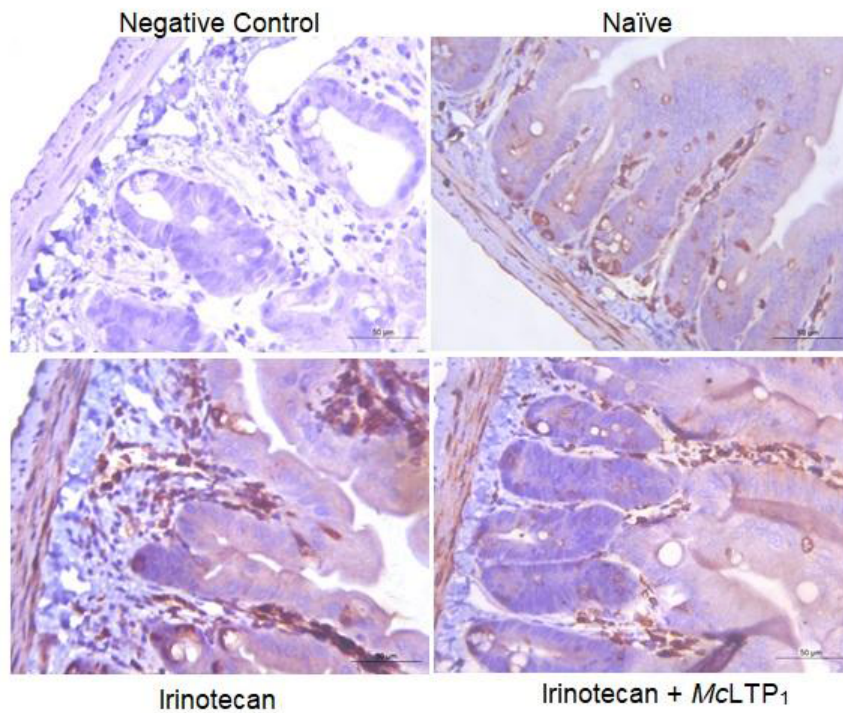


Figure 5

COX-2 immunohistochemistry following *McLTP*₁ (8 mg/kg, i.v.) treatment in mice with irinotecan-induced intestinal mucositis (75 mg/kg, i.p.). Increased COX-2 immunostaining in the duodenum of animals submitted to irinotecan-induced intestinal mucositis compared to the naïve group. *McLTP*₁ decreased the immunostaining of this enzyme when compared to those animals only treated with irinotecan, demonstrated by a reduction in the percentage of the marked area following *McLTP*₁ treatment (b). Scale bars is 50 µm at 100× magnification

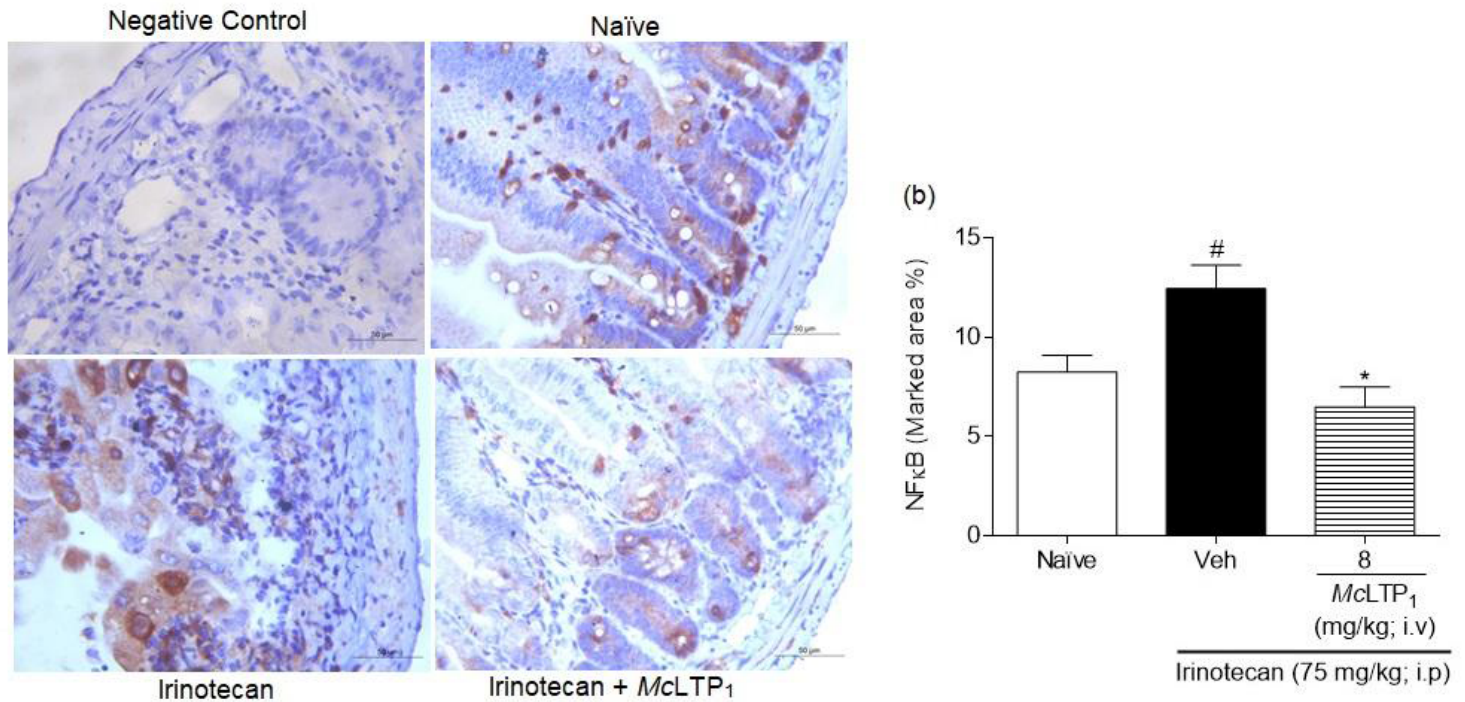


Figure 6

NF-κB immunohistochemistry following *McLTP*₁ treatment in mice with irinotecan-induced intestinal mucositis. NF-κB p65 immunostaining in the duodenum of animals submitted to irinotecan-induced intestinal mucositis was increased compared to the naïve group. *McLTP*₁ decreased the immunostaining of this transcription factor when compared to irinotecan, demonstrated by a reduction in the percentage of the marked area following *McLTP*₁ treatment (b). Scale bars in panels is 50 μm at 100× magnification

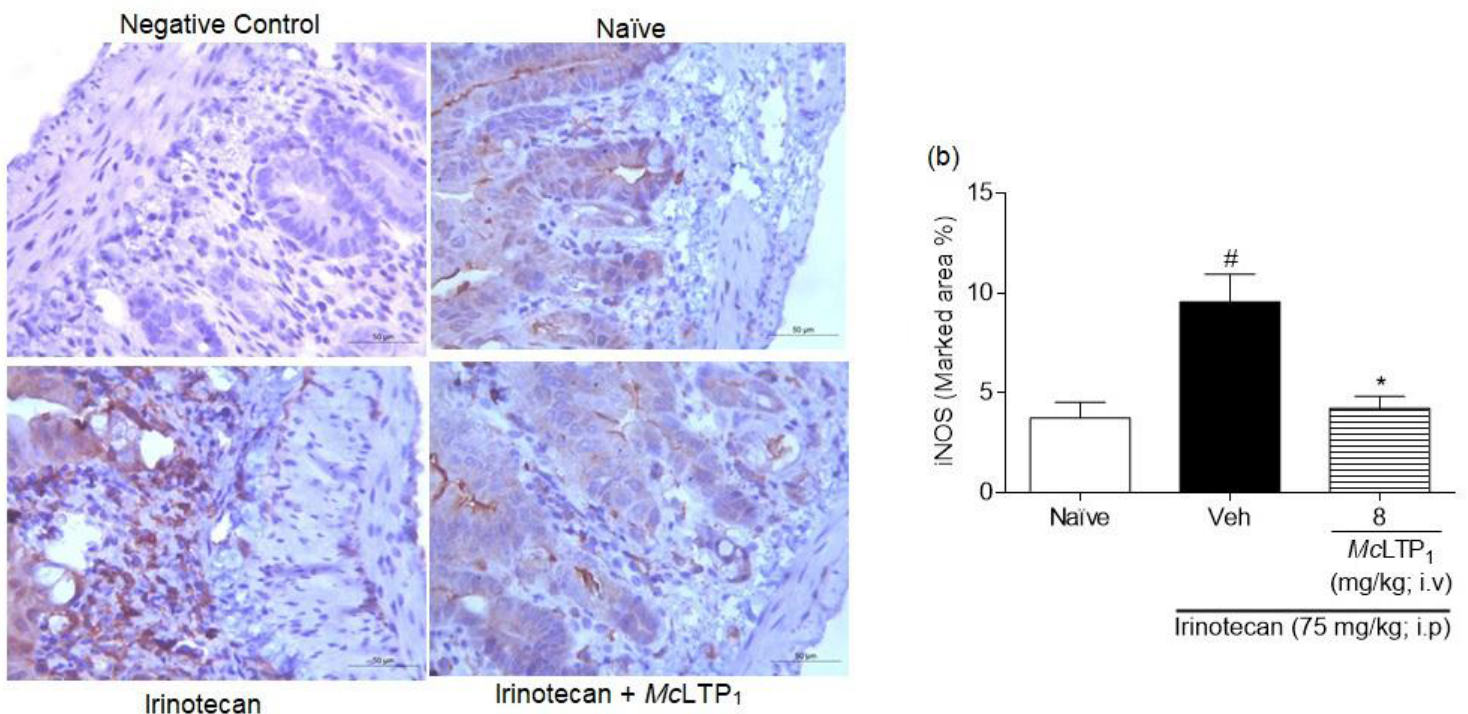


Figure 7

Nitric oxide synthase (iNOS) immunohistochemistry following *McLTP*₁ treatment in mice with irinotecan-induced intestinal mucositis. iNOS immunostaining in the duodenum of animals submitted to irinotecan-induced intestinal mucositis was increased compared to the naïve group. *McLTP*₁ decreased the immunostaining of this enzyme when compared to irinotecan, demonstrated by a reduction in the percentage of the marked area following *McLTP*₁ treatment (**b**). Scale bars is 50 μ m at 100 $\times$ magnification

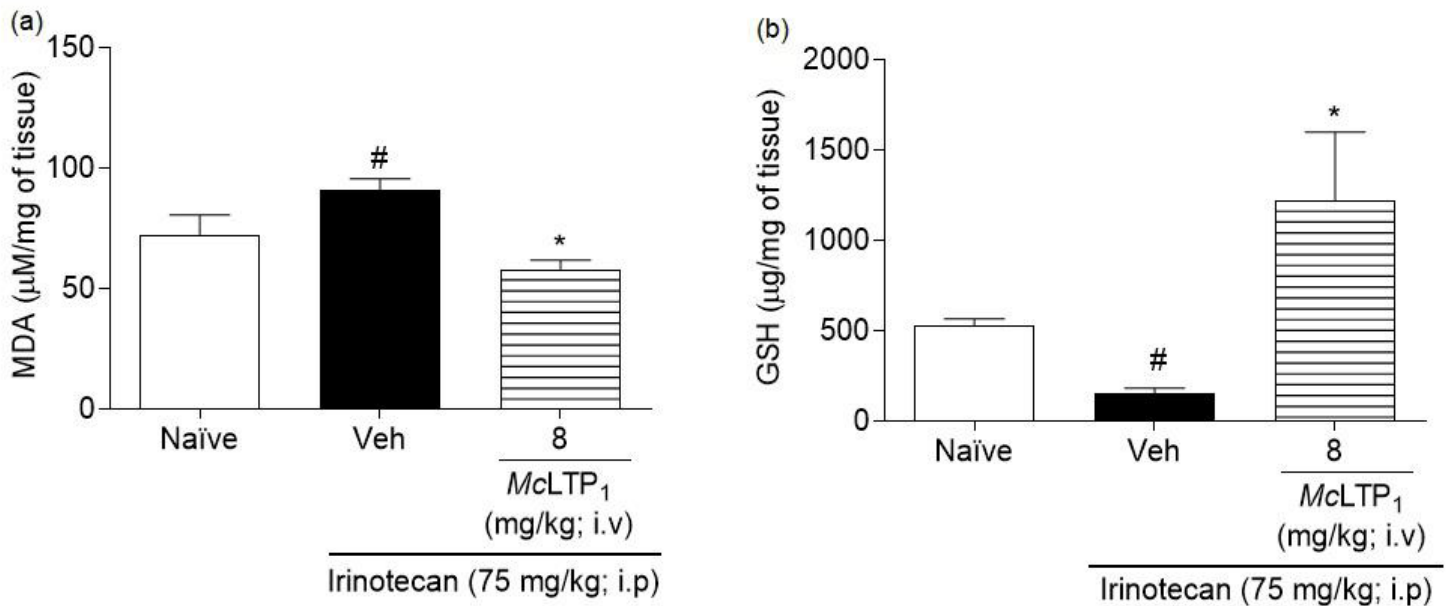


Figure 8

Effects of *McLTP*₁ (8 mg/kg, i.v.) treatment on (a) MDA levels and (b) GSH activity in mice with irinotecan-induced intestinal mucositis. The results are presented as the mean $\pm$ SEM. #p < 0.05 compared to the naïve group and *p < 0.05 compared to the irinotecan-treated group (n = 5 animals per group, ANOVA with Bonferroni's test)