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Side-Effects of Irinotecan (CPT-11), the Clinically Used Drug for Colon Cancer Therapy, Are Eliminated in Experimental Animals Treated with Latex Proteins from *Calotropis procera* (Apocynaceae)‡

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Intestinal mucositis (IM) is the critical side effect of irinotecan (CPT-11), which is the front-line drug used for the treatment of colorectal cancer. This study aimed to evaluate the effectiveness of latex proteins (LP) from *Calotropis procera* to prevent IM and diarrhea in animals. Swiss mice were treated daily with saline or LP (1, 5, or 50 mg/kg, i.v.) 24 h prior to CTP-11 (75 mg/kg/4 days, i.p) and for additional 6 days. Animal survival, body weight variation, and diarrhea were registered. After animal sacrifice (day 7 post first injection of CPT-11), intestinal samples were collected to study morphology and inflammatory parameters. Animals given LP exhibited improved parameters (survival, body weight, and absence of diarrhea) as compared with the CPT-11 control. The severity of IM observed in animals given CPT-11 was reduced in animals treated with LP. Treatment with LP also prevented the reduction in the villus/crypt ratio promoted by CPT-11. The rise in MPO activity and pro-inflammatory cytokines, over-contractility of the smooth muscle, and diarrhea were all abrogated in LP-treated mice. Markedly reduced immunostaining intensity for COX-2, TNF-α, IL-1β, iNOS, and NF-κB was observed in the intestinal tissue of animals treated with LP. The side-effects of CPT-11 were eliminated by LP treatment in experimental animals and improved clinical parameters characteristic of IM All known biochemical pathogenesis. Copyright © 2016 John Wiley & Sons, Ltd.

Keywords: Calotropis procera; cytokines; irinotecan; latex proteins; mucositis.

INTRODUCTION

Drug resistance and the harmful side-effects observed with therapy are generally the main concerns associated with medicines. Both of these issues prevent adequate clinical progress in patients and thus are the main motivating causes of treatment cessation. In an effort to mitigate these side-effects, the use of natural medicines as alternatives to the medicine-based clinical protocols has been encouraged. This study addresses the management of intestinal mucositis (IM) and diarrhea associated with colorectal therapy using CPT-11.

A spectrum of signs and symptoms compromises the life quality of patients undergoing cancer therapy. Among them, mucositis is recognized worldwide as a challenge to be handled. Different types of human malignancies, such as advanced metastatic colorectal cancer, are clinically managed with a potent DNA topoisomerase I inhibitor known as irinotecan (CPT-11: 7-ethyl-10[4-[1-piperidino-1-piperidino] carbonyl-oxycamptothecin). CPT-11 is metabolized vis carboxylesterase-mediated hydrolysis in the liver and converted to SN-38, the active metabolite (Hebbar *et al*., 2009). Treatment based on CPT-11 chemotherapy results in harmful side-effects in patients. CPT-11 induces IM, which is characterized by an inflammatory and ulcerative process of the mucous membranes lining the digestive tract (Sonis, 2004). This status is clinically characterized by abdominal pain, vomiting, and persistent diarrhea, with secondary anti-nutritional consequences and weight loss. These effects are associated with intense IM resulting from SN-38 activity (Peterson *et al*., 2011).

The pharmacological potential of latex compounds has been progressively recognized and globally validated (Samy *et al*., 2012). Particularly, the latex-producing plant *Calotropis procera* (Apocynaceae), which is part of the Ayurveda, the Indian natural medical compendium, has had some of its claimed activities confirmed scientifically (Chaudhary *et al*., 2015). Further isolation of latex fractions and discarding the

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† in memoriam
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insoluble material (rubber) provides organic and protein fractions, which are both sources of pharmacologically active molecules. For instance, secondary metabolites and some of their chemical derivatives have been highlighted as promising anticancer drugs (Van Quaquebeke *et al*., 2005). Complementarily, the involvement of LP in the modulation of some inflammatory disorders is now well-established and partially characterized (Chaudhary *et al*., 2015). Thus, we examined the ability of the LP of *C. procera* to prevent oral mucositis induced in 5-fluorouracil treated animals, because LP have been proposed to down-regulate inflammatory mediators and to reestablish homeostasis in inflamed tissues (Freitas *et al*., 2012). In the present study, LP was assessed for its ability to eliminate the adverse clinical signs commonly observed in patients undergoing CPT-11 treatment. For this, experimental animals were used, and a panel of parameters were recorded and evaluated. The results reported in this study show that LP is a strong candidate for phytotherapy.

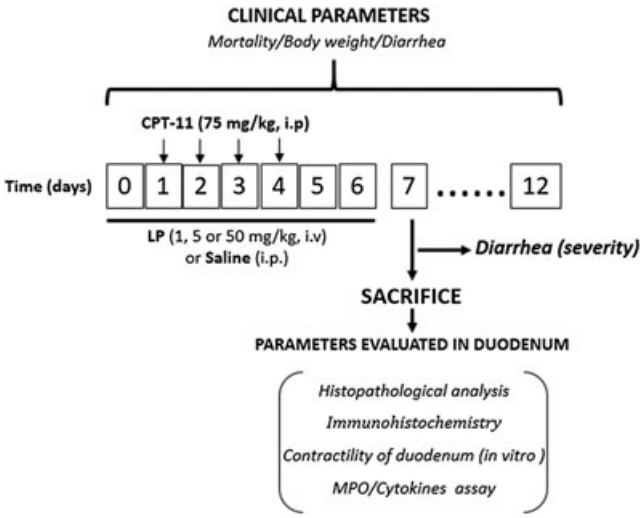

Figure 1. Experimental design. Schedule of administration of irinotecan or LP and parameters evaluated. Three independent experiments were performed: (1) to evaluate survival and record body weight progress; (2) to evaluate duodenal contractility, and (3) to collect duodenal samples to perform biochemical and histopathological analyses.

MATERIAL AND METHODS

Plant material and latex processing. The fresh latex was collected in tubes (1:1, v/v in distilled water) from the aerial parts of *C. procera* (Ait.) R.Br. growing in the vicinity of Fortaleza, Brazil in July 2012. The plant voucher (sample specimen no. 32663) was deposited at Prisco Bezerra Herbarium of Federal University of Ceará, Brazil, where the botanical material was identified by a local taxonomist. Latex/water mixtures were adjusted to a dilution ratio of 1:2 (v/v) and further processed (Freitas *et al*., 2012). Water soluble LP used in this study was lyophilized and stored at 25 °C until use. All known biochemical and pharmacological characterizations of this sample have been extensively reported (Freitas *et al*., 2012; Oliveira *et al*., 2010). Briefly, cysteine proteases, chitinases, peroxidases, and osmotin have been identified as the major proteins present in LP.

Induction of intestinal mucositis. Adult male Swiss mice (*Mus musculus*), weighing 23 ± 3.0 g, were obtained from the Central Animal House of the Federal University of Ceará, Brazil. The animals were housed in plastic cages under controlled laboratory conditions (temperature of 25 °C, humidity 55 ± 10% and 12/12 h light/dark cycles) and provided water and food *ad libitum* (commercial sterile diet; Purina, Paulínia, SP, Brazil). All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institute of Health (NIH 1985), and were approved by the ethical committee of the Federal University of Ceará (protocol n°24/2009). The experimental protocol was based on a previously described model (Ikuno *et al*., 1995) and adapted for the experimental conditions. Irinotecan hydrochloride (CPT-11) (irinotecan, Camptosar®, Pharmacia and Upjohn Co, Kalamazoo, MI, USA, 100-mg ampoule) was administered intraperitonally (i.p.) to healthy animals at a dose of 75 mg/kg, once a day for 4 days.

Experimental design. The animals were randomly arranged into five experimental groups ($n = 8$ animals/per group), as follows: (i) normal group: animals treated only with sterile saline (5 mL/kg, i.p.); (ii) CPT-11 group: animals treated with saline (5 mL/kg, i.p.) and 24 h later given CTP-11, as described above; and (iii) LP groups: animals treated (i.v.) with the laticifer proteins of *C. procera* (LP), dissolved in saline (vehicle), at doses of 1, 5, or 50 mg/kg 24 h prior to initiating CTP-11 treatment and continuing for six consecutive days (single daily dose). The schedule of administration of irinotecan or LP and the evaluated parameters are summarized in Fig. 1. For all experiments, data were recorded from eight animals per group. Reproducibility was confirmed by three independent experiments.

All efforts were made in order to minimize animal suffering. In the survival study, the animals were monitored twice daily for 10 days following the first injection of irinotecan. During the experiments, the animals succumb because of the treatment and its consequences, including diarrhea. Those animals that showed signs of imminent death, including piloerection, reduced locomotion, inability to maintain upright position, ataxia, tremor, and altered breath frequency were sacrificed by ketamine/xylazine overdose (>100/10 mg/kg, s.c., União Química, São Paulo, Brazil) followed by cervical dislocation. Pain relievers or anesthesia were not used in our experiments because those agents directly interfere with the production of inflammatory mediators and/or alter the gastrointestinal transit and mask the diarrheic events in this animal model. At the end of the survival experiment, live animals were sacrificed by ketamine/xylazine overdose (>100/10 mg/kg, s.c., União Química, São Paulo, Brazil) followed by cervical dislocation. The experimental protocol, including the mortality aspects of the protocol, was reviewed and approved by the Committee on the Ethics of Animal Experiments of the Federal University of Ceará.

Animals from all experimental groups were sacrificed by cervical dislocation on experimental day 7 after CPT-11 exposure. The proximal intestine (duodenum) of each was harvested, and tissue samples were immediately subjected to morphometric, immunohistochemistry analysis, or functional studies (*in vitro* contractility of the isolated duodenum). In other

experiments, the proximal intestines were harvested, weighed, and stored at −70 °C until required for the myeloperoxidase assay (MPO), measurement of cytokines (TNF-α and IL-1β), or assayed by Western blot for iNOS.

Clinical parameters. Animals from all experimental groups were observed daily for mortality, from the beginning of CPT-11 treatment and continuing for 12 days. The results are expressed as the percentage of survival. Throughout the experimental period, the animals were monitored for body weight loss and the severity of diarrhea. Each mouse was weighed at the beginning of the experiment, before treatment and daily throughout the experiment. The values are expressed as the percentage of weight loss and compared to the initial weight. On experimental day 7 after CPT-11 exposure, prior to animal euthanization, the severity of the diarrhea was scored (Kurita *et al*., 2000). All comparative scoring measures were done using a blinded method to prevent observer bias.

Histopathological analysis. The specimens were fixed in 10% (v/v) neutral-buffered formalin, dehydrated, and embedded in paraffin. The samples were cut into 5-μm sections, stained with hematoxylin–eosin (H&E) and examined by light microscopy in a blinded manner. All histopathological analyses were performed using random fields per slice. The severity of mucositis was graded using previously described criteria (Woo *et al*., 2000). The morphometric study was performed using ImageJ®1.36b software (National Institutes of Health, USA). The height of the villus was determined from the top to the bottom, which corresponds to the crypt/villus junction. The depth of the crypts was defined as the invagination between adjacent villi. An average of 5 to 10 different intestinal crypt/villus measurements per experimental group was taken in the histological section, and the villus-to-crypt length ratios were calculated.

***In vitro* contractility of duodenum.** Duodenum segments (0.6 cm long) were dissected and processed to investigate *in vitro* contractility according to the method of Araújo *et al*. (2005). A dose–response curve to the cholinergic agonist acetylcholine was constructed, using increasing and cumulative concentrations ranging from 10^{-10} to 10^{-4} M. The data obtained from the cholinergic dose–response curve were analyzed as the percentage contractile response in comparison with the mean of two contractions initially observed using the 60 mM KCl standard.

Myeloperoxidase assay (MPO). The intensity of neutrophil accumulation in intestinal samples was indirectly estimated by the measurement of MPO activity through colorimetric analysis (Bradley *et al*., 1982). The changes in absorbance were recorded and plotted on a standard curve of neutrophil density. Data were expressed as myeloperoxidase activity (neutrophils/mg of tissue).

Detection of cytokines (TNF-α, IL-1β) in the duodenum. The tissue was homogenized and processed as previously described (Safieh-Garabedian *et al*., 1995). The measurement of TNF-α and IL-1β concentrations was performed using murine TNF-α and IL-1β DuoSet® ELISA Kits (R&D Systems, Inc., Minneapolis, USA). The steps were carried out according to the manufacturer's instructions.

Immunohistochemistry. Detection of TNF-α, IL-1β, iNOS, COX-2, and NF-κB was developed using the streptavidin–biotin–peroxidase method in formalin-fixed, paraffin-embedded tissue sections (4 μm thick), mounted on poly-L-lysine-coated microscope slides (Hsu *et al*., 1981). Primary antibodies against TNF-α (Sigma), IL-1β, iNOS, COX-2, or nuclear localization sequence (NLS) of the NF-kB p50 subunit (Santa Cruz) were used at a 1:200 dilution in PBS–BSA. The avidin–biotin–horseradish peroxidase conjugate (Strep ABC complex; Santa Cruz Biotechnology) was used according to the manufacturer's protocol. The reaction was visualized with the chromogen 3,3 diaminobenzidine (DAB; liquid DAB + substrate chromogen system; Dako). Slides were counterstained with Mayer's hematoxylin (Invision Flex-Dako) or methyl green (for NF-κB). A qualitative evaluation using light microscopy was performed as previously described (Yeoh *et al*., 2005).

Data analyses. Data are expressed as mean ± standard error of the mean (SEM) or median (non-parametric data) of at least three independent observations. Analysis of variance (ANOVA), followed by Bonferroni's test, was used to compare the means, and the Kruskal–Wallis test, followed by Dunn's test, was used to compare medians. Statistical significance was accepted when $P < 0.05$. All data were analyzed using GraphPad Prism software version 5.0 (Graph-Pad Software, Inc., La Jolla, CA, USA).

RESULTS

LP improves the clinical parameters of intestinal mucositis in mice

Fig. 2A shows increasing mortality over time in animals given CPT-11. The survival of animals in this group reached a minimum (41.67%) at day 12. As expected, the survival rate in untreated animals (normal control) was 100%. Therefore, the vehicle used to dissolve the latex did not affect the clinical parameters. The performance of animals given CPT-11 with LP at 1 mg/kg was quite similar to that documented in the CPT-11 group. However, the groups given CPT-11 as a preventative treatment with LP at 5 or 50 mg/kg statistically differed from the CPT-11 group; the survival rate in these groups was above 70%. Animals in the normal group progressively gained body weight until day 12. No significant differences were observed between the groups given CPT-11 and CPT-11 followed by LP treatment in terms of body weight (data not shown). Table 1 presents the scores according to the occurrence and intensity of diarrhea in animals. As observed, the data are coherent with those shown in Fig. 2A. While animals receiving LP at the lower concentration almost replicated the performance of animals given CPT-11 ($p < 0.05$), exhibiting strong and persistent diarrhea, this condition was mitigated in animals given CPT-11 but treated with LP at the higher concentrations, differing from CTP-11 group ($p < 0.05$) and being similar to the normal control group.

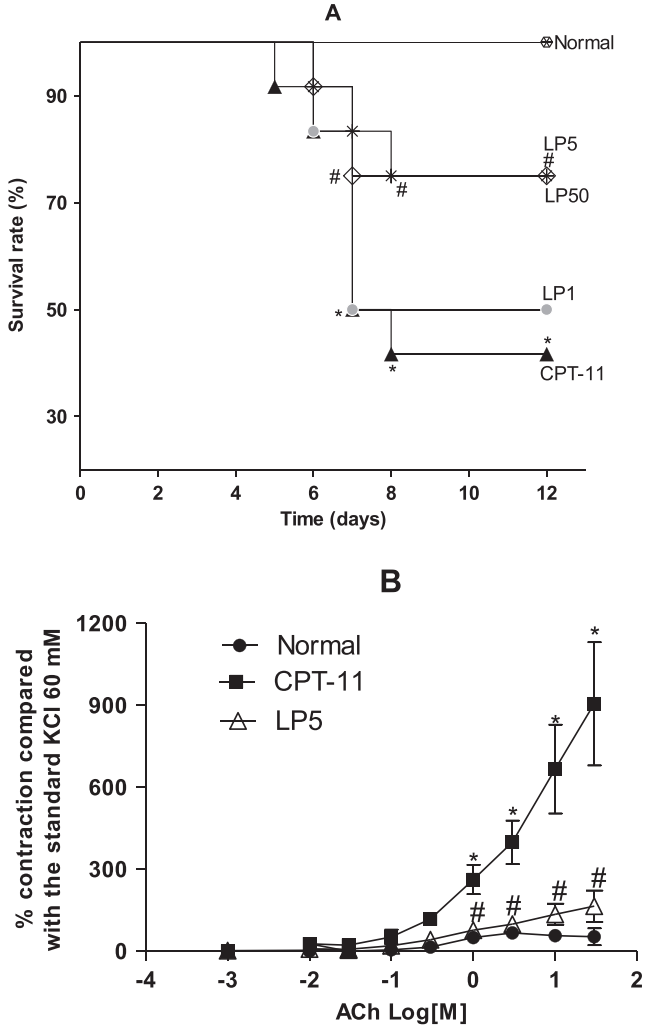

Figure 2. Treatment with LP improved the survival rate of mice with intestinal mucositis (A). Values are expressed as percentage of the median from three independent observations. $*p < 0.05$ versus normal group and $\#p < 0.05$ versus CPT-11 group; ($n = 8$ animals per group; Mantel–Cox log rank test). Inhibitory effect of LP on mice duodenal contractility *in vitro* (B). Values are expressed as mean ± standard error mean (S.E.M.) from three independent determinations. $*p < 0.05$ versus normal group and $\#p < 0.05$ versus CPT-11 group ($n = 8$ animals per group; ANOVA—Bonferroni test).

Table 1. The severity of irinotecan-induced diarrhea is alleviated in LP-treated mice. Values are expressed as medians

Group	Diarrhea assessment scores
Normal	0 (0–0)
CPT-11	3 (2–3)*
LP—1 mg/kg	2.5 (1–3)*
LP—5 mg/kg	1 (0–2)#
LP—50 mg/kg	1 (0–2)#

*$p < 0.05$ versus the normal group.
#$p < 0.05$ versus the CPT-11 group.
$n = 8$ animals per group; Kruskal–Wallis and Dunn's test.

Damage to the intestinal mucosa induced by CPT-11 is prevented by LP treatment

As shown in Table 2 and Fig. 3, the use of CPT-11 induced severe injury to the intestinal mucosa of animals. The intense diarrhea observed in the animals of this group is thought to be, at least in part, a consequence of this histological damage. The full integrity of the intestinal mucosa observed in animals in the control group was confirmed. The parameters used to assess the intestinal mucosa of animals treated with LP at 1 mg/kg group were statistically similar to those in the group given CPT-11 alone. Of note, the histological integrity of the intestinal mucosa of animals given CPT-11 and treated with LP at 5 or 50 mg/kg was preserved and comparable to that of animals given only sterile saline. Further, the histological integrity of the mucosa in animals given LP was probably the main factor driving the observed homeostasis in the *in vitro* contractility assays. The data in Fig. 2B show that the responsiveness of the intestinal mucosa of animals from the normal control and the group given CPT-11 followed by LP treatment were both statistically different to that of animals receiving only CPT-11. These findings also corroborate the important reduction in diarrhea in these groups.

LP modulates pro-inflammatory mediators in CTP-11-induced mucositis

Fig. 4A shows the differences in MPO activity observed in the duodenal tissue of animals. The inflammatory process resulting from irinotecan delivery was indicated by increasing levels of myeloperoxidase measured in the duodenum (Fig. 4A). MPO activity indicates the presence of activated neutrophils, found only in inflamed tissues. The inflammatory response stimulated by CPT-11 was reduced in animals given CPT-11 and treated with LP at 5 or 50 mg/kg, that is, by 83% and 77.21%, respectively. This is the first experimental evidence that the beneficial effects of LP treatment on animals given CPT-11 may be because of its well-documented ability to modulate inflammatory processes. This observation was supported by reductions in the levels of pro-inflammatory mediators, that is, TNF-α (Fig. 4B) and IL-1β (Fig. 4C), as well as the decrease in immunostaining for both markers (Fig. 4D) in intestinal tissues of animals given CPT-11 and treated with LP.

Reduced immunohistochemical detection of iNOS and COX-2 in LP-treated animals

Fig. 5A provides images of the intestinal tissues of animals given CPT-11 and animals given CPT-11 followed by treatment with LP. Table 3 presents the histological scores in each group. Immunohistochemical detection of both iNOS and COX-2 were statistically comparable to the control and different to that in the group given CPT-11 alone. The increase in iNOS and COX-2 detected in the duodenal tissues of animals given only CPT-11 indicates an inflammatory process in progress. The reduced detection of iNOS accompanied by previous observation that fewer activated neutrophils were found in animals given CPT-11 followed LP treatment (western blot analysis also suggested this profile—data not shown). However, the reduced detection of COX-2 in animals given CPT-11 followed by LP treatment as compared to the level of COX-2 in samples from

Table 2. Histopathological damage to the intestinal mucosa in mice is prevented by LP treatment in irinotecan-induced mucositis

Groups	Histological grading	Villus/crypt ratio	Histological description
Normal	0 (0–0)	$2.7 \pm 0.15^{\#}$	Healthy intestinal mucosa
CPT-11	4 (1–4)*	1.6 ± 0.17*	CPT-11 promoted intestinal crypt destruction, shortened villi (vertical arrows), and led to intense infiltration of inflammatory cells in the lamina propria (slanted arrows) and vacuolization of the epithelial cell lining (horizontal arrow).
LP 1 mg/kg	3.5 (1–4)*	1.8 ± 0.21*	
LP 5 mg/kg	1 (0–2)$^{\#}$	$2.8 \pm 0.17^{\#}$	LP (5 and 50 mg/kg) prevented the histopathological damage induced by irinotecan (CPT-11) on the intestinal mucosa.
LP 50 mg/kg	1 (0–3)$^{\#}$	$2.7 \pm 0.09^{\#}$	

*$p < 0.05$ versus the normal group.
#$p < 0.05$ versus the CPT-11 group.
$n = 8$ animals per group; Kruskal–Wallis test followed by Dunn's test or ANOVA followed by the Bonferroni test.
The values of histological grading are expressed as medians. The values of the villi/crypt ratio are expressed as mean ± standard error of the mean (SEM). Magnification (× 100).

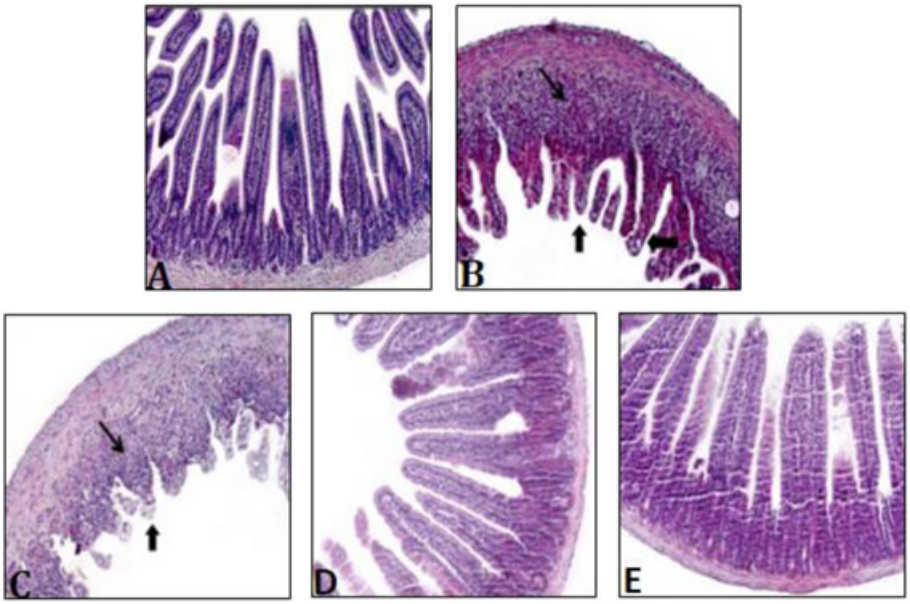

Figure 3. Photomicrographs of histological sections show intestinal mucosa of Normal (A), CPT-11 (B), LP 1 (C), LP 5 (D), and LP 50 mg/kg (E) groups (magnification × 100). [Colour figure can be viewed at wileyonlinelibrary.com]

animals given CPT-11 only provides evidence of the ability of LP to robustly modulate CPT-11-induced IM.

NF-κB expression is stimulated in animals given CPT-11 but down-regulated by LP treatment

Immunostaining for NF-κB in tissues collected from animals given only CPT-11 was increased by almost 10-fold. The NLS of the NF-kB p50 subunit is markedly increased in CPT-11-injected mice and attenuated in LP5-treated animals. This increase was statistically reduced by half in animals given CPT-11 and treated with LP (Fig. 5A, B). The variation in the immunoexpression for each inflammatory marker is to be mainly observed in the submucosal layer. In the study of Collet *et al.* (2008) early epithelial changes associated with altered responsiveness to bacteria precede increased permeability and mucosal inflammation in a model of colitis. Normal and damaged epithelial cells in the intestine express the studied markers, because the epithelial surface is continuously exposed to antigens from the intestinal lumen (Collett *et al.*, 2008). However, during mucositis the submucosa is exposed to translocating bacteria and resident cells become activated by pathogen-associated molecular patterns and damage associated molecular patterns, which signal to induce pro-inflammatory cytokine expression (Wong *et al.*, 2015; Lima-Júnior *et al.*, 2014).

DISCUSSION

Persistent episodes of diarrhea constitute a central adverse concern of almost all patients undergoing chemotherapy for malignancy. It must be kept in mind that the diarrhea resulting from chemotherapy protocols is clinically different of other etiological causes, such as infections (Gibson and Stringer, 2009). Therefore, the rationality of its clinical management is also different.

Late diarrhea provoked by use of CPT-11 is secretory, because of the reduced intestinal absorption of fluids, the presence of exudate components, and also because of the lack of specificity of the drug and its metabolically active derivative, SN-38 (Ikuno *et al.*, 1995). The

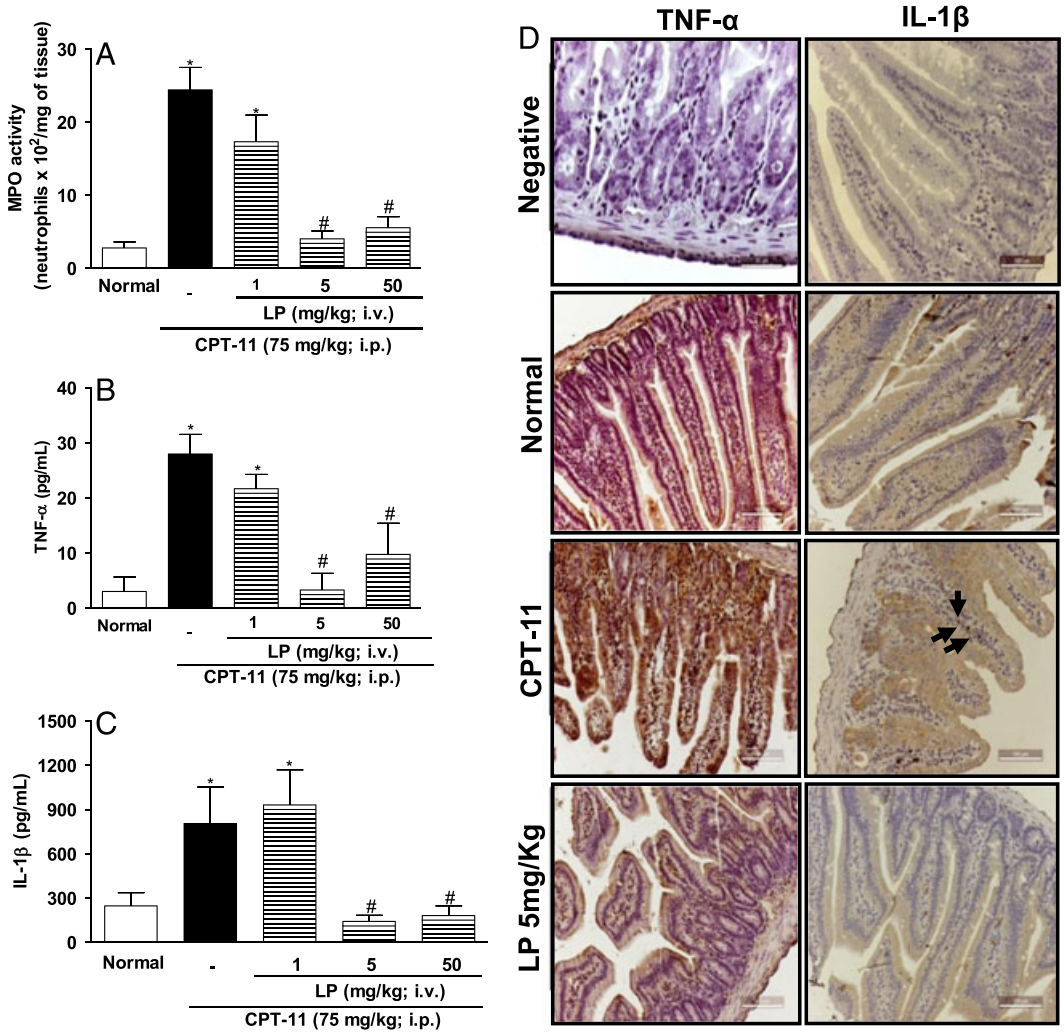

Figure 4. The myeloperoxidase activity and pro-inflammatory cytokines are down-modulated in LP-treated mice subjected to irinotecan-induced intestinal mucositis (A). Values are expressed as mean ± standard error mean (S.E.M.) of neutrophils number × 10^2/mg of tissue. The concentration of TNF-α (B) or IL-1β (C) is expressed as mean ± standard error mean (S.E.M.) in pg/mL from three independent determinations. Photomicrographs of sections show immunoexpression of TNF-α and IL-1β (D). The negative control represents a sample of the duodenum where the first antibody was replaced with PBS-BSA 5%, and no immunostaining was detected. Arrows indicate labeled cells (magnification × 200). $^*p < 0.05$ versus normal group and $^\#p < 0.05$ versus CPT-11 group ($n = 8$ animals per group; ANOVA—Bonferroni test). [Colour figure can be viewed at wileyonlinelibrary.com]

unspecific activity of currently used drugs, such as CPT-11, not only severely impairs the natural rate of cellular replacement, but also disrupts any regenerative process. This explains mucositis and anemia as the first impairments faced by patients treated with anticancer drugs. Bearing in mind this current scenario, even aggravated by the poor perspective regarding the immediate availability of new and more specific medicinal drugs for the management of neoplasia and other malignancies, the search for complementary and alternative supporting protocols to current therapies is appreciated.

For more than a decade, we have dedicated efforts to scientifically validate the folk use of the latex of *C. procera*. In the beginning, we decided to clean the latex of organic soluble compounds in order to study the soluble protein fraction, because all available literature at that time reported data on the whole dried latex or organic extracts obtained after extraction of the whole dried latex, including toxicology (Kumar and Shivkar, 2004).

In our initial studies, LP, the laticifer proteins, were shown to inhibit inflammation artificially induced by phlogistic agents such as carrageenan and dextran, reducing peritonitis and paw edema (Alencar *et al.*, 2004). Later, we showed that LP could suppress tumor growth (*in vivo*), prevent septic shock in lethally infected animals (Oliveira *et al.*, 2010), and treat arthritic animals (Kumar *et al.*, 2011). These activities were accompanied by down-regulation of pro-inflammatory cytokines and other inflammatory mediators, the preservation of tissue architecture, and the maintenance of oxidative homeostasis (Chaudhary *et al.*, 2015). Even though LP has demonstrated important antiinflammatory features, it was still surprising to document the ability of LP to abolish oral mucositis in animals subjected to 5-fluorouracil treatment (Freitas *et al.*, 2012). It was found that LP inhibited the expression of iNOS, COX-2, and the pro-inflammatory mediators IL-1β and TNF-α. These pharmacological characteristics of LP and the later findings on oral mucositis were therefore the justification to investigate the presumed protective effect of LP on IM.

In previous studies, Indian researchers evaluated the effect of the dried latex (500 mg/kg) of *C. procera* in rats orally treated with castor oil (Kumar *et al.*, 2001). The main findings included a decrease in the frequency of

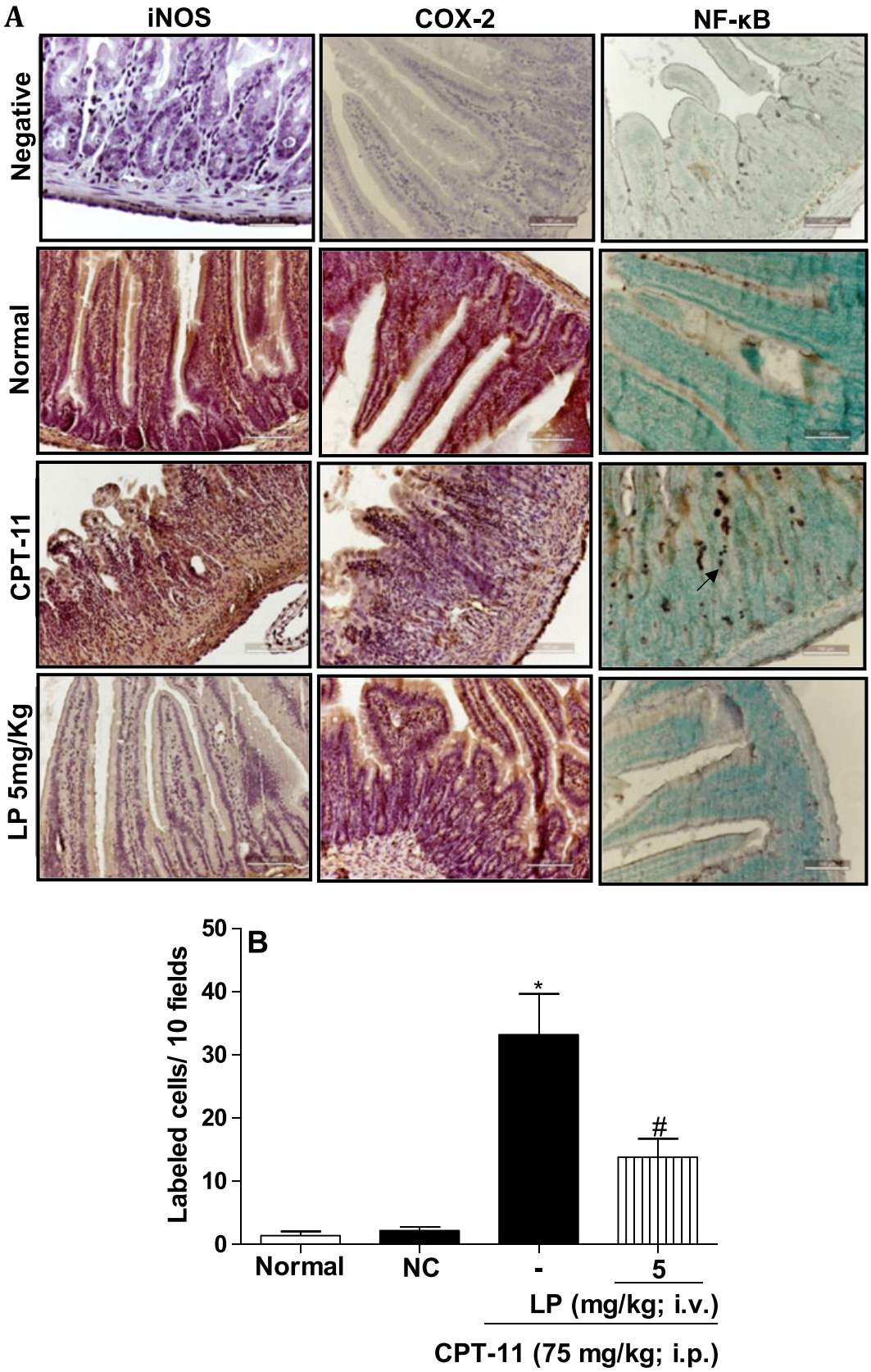

Figure 5. Photomicrographs of sections show immunoexpression of iNOS, COX-2, and NF-κB on the irinotecan-induced intestinal mucositis (A). The negative control represents a sample of the duodenum where the first antibody was replaced with PBS-BSA 5%, and no immunostaining was detected. Arrow shows labeled cells. Magnification (200×). Values are expressed as mean ± standard error mean (S.E.M.) of the number of labeled cells to NF-κB in 10 fields/slide from three independent observations. (B). $^{*}p < 0.05$ versus normal group and $^{\#}p < 0.05$ versus CPT-11 group ($n = 8$ animals per group; ANOVA—Bonferroni test). [Colour figure can be viewed at wileyonlinelibrary.com]

Table 3. The immunoexpression of iNOS and COX-2 is attenuated in LP-treated mice subjected to irinotecan-induced intestinal mucositis

Enzymes	Negative control	Normal	CPT-11	LP 5 mg/kg
iNOS	0 (0–0)	1 (1–2)	4 (3–4)*	1 (1–2)#
COX-2	0 (0–0)	1 (1–2)	4 (2–4)*	1 (1–2)#

*$p < 0.05$ versus the normal group.
#$p < 0.05$ versus the CPT-11 group.
Kruskal–Wallis test followed by Dunn's test.

defecation and the prevention of castor oil-induced enteropooling. Thus, the present data, showing a reduction in diarrhea and the reduced responsiveness of the duodenum to stimulation with acetylcholine, corroborate the study reported by our Indian colleagues. It is also indicative that LP may have multiple independent activities, because it was able to retard defecation independently of IM, because castor oil does not induce inflammation. In the clinical setting, physicians use diarrhea as a clinical sign of IM. However, we have some evidence that the modulation of diarrhea with loperamide does not interfere with underlying inflammatory response associated with IM (Lima-Júnior *et al*., 2012). In addition, the modulation of inflammatory parameters not always prevents diarrheic events (Wong *et al*., 2015). Those findings suggest that the underlying mechanisms of diarrhea and IM might be partially different, although diarrhea is sometimes consequent to intestinal injury (Murray and Rubio-Tapia, 2012). Interestingly, LP prevented both diarrhea and intestinal injury. Thus, in the present study, LP may have played a dual role by down-regulating key pro-inflammatory mediators and inhibiting overexcited duodenal contractility driven by the effects of CPT-11.

The failure of LP to prevent body weight loss, a characteristic observed in animals given CPT-11, needs to be approached in a different way. In the present study, the duodenum was chosen for analyses because of the length of villus, which would allow us to quantify the extension of injury more easily, as also reported in previous studies (Lima-Júnior *et al*., 2012). Even though LP contributed to the preservation of intestinal tissue structure and down-regulated pro-inflammatory mediators, the body weight progress in the LP groups was as bad as that in animals give. Even though LP contributed to the preservation of intestinal tissue structure and down-regulated pro-inflammatory mediators, the body weight progress in the LP groups was as bad as that in animals given CPT-11 alone. In other studies, the treatment of animals with glutamine and alanyl glutamine, aimed at protecting animals against IM induced by 5-fluorouracil, preserved tissue architecture but did not prevent body weight loss (Carneiro-Filho *et al*., 2004). These findings indicate the multifactorial negative effects associated with CPT-11 administration and are in line with the lack of specificity in the mechanism of action of this drug. Even if LP or other compounds can overcome IM, the broad effects of CPT-11 on general metabolism probably play a decisive role on the negative performance of animals in terms of weight gain. As a whole, IM and diarrhea seem to be only part of the harmful effects observed in animals given CPT-11, and thus the loss of body weight observed in animals given CPT-11 even when treated with IM regenerative drugs suggests the high degree of metabolic catabolism promoted by CPT-11 and its toxic metabolites. For instance, the treatment of animals with thalidomide or pentoxifylline reduces the histopathological damage induced by CPT-11 in the intestinal mucosa. However, only pentoxifylline reduces diarrhea (Melo *et al*., 2008).

In the present study, we used the expression of pro-inflammatory cytokines and enzymes as putative markers of intestinal inflammation associated with irinotecan injection. It is expected that an antiinflammatory drug is capable of attenuating the expression of most of these inflammatory markers, because the inflammatory reaction is orchestrated in a sequence of events as a cascade (Lima-Júnior *et al*., 2012). As described here, LP significantly attenuated the immunoexpression of these markers. The precise mechanism through which LP controls the inflammatory reaction is still a matter of debate. However, one possible explanation might involve the direct reduction of neutrophil influx to the inflammatory foci (Alencar *et al*., 2004).

LP has been characterized in terms of its protein content and related activities. Although it is currently impossible to determine the role played by any latex protein (LP) on inflammation, potential proteins involved in the modulation of inflammatory processes have emerged. Chitinases, proteases, and osmotins are now recognized as the major proteins found in LP (Ramos *et al*., 2010). To a lesser extent, anti-oxidant enzymes are also found (Freitas *et al*., 2007). The fractionation of chitinases and proteases *plus* osmotins suggests that the antiinflammatory activity persists in both fractions (Ramos *et al*., 2009). As far as we are concerned, there are no data on chitinases having antiinflammatory effects. Also, the data associating proteases and the modulation of inflammatory processes are sparse. Recent literature, however, proposes an unexpected effect of osmotins on inflammatory events (Freitas *et al*., 2011). We have recently purified the latex osmotin of *C. procera* and characterized the protein in some detail (Ramos *et al*., 2015). It is now our primary goal to determine whether this protein plays any role on the mitigation of the side-effects induced by CPT-11 treatment.

During IM, inflammatory cytokines induce tissue injury and mediate complications, such as diarrhea. The severity and maintenance of injuries are associated with high levels of cytokines such as IL-1β, IL-6, TNF-α, and IL-2 (Williams, 2001). Overall, the mechanistic events supporting the beneficial effects of LP observed in animals exposed to IM are indicated in the present study. Accordingly, the inhibition of TNF-α and IL-1β release was characteristic of LP treated animals. According to Melo *et al*., 2008 on day 7 after irinotecan-induced mucositis, high levels of TNF-α, IL-1β, and KC are involved with the pathogenesis and the inflammatory response. A study by Freitas *et al*. (2012) also demonstrated in 5-fluorouracil-induced oral mucositis that LP (5 mg/kg) was able to reduce the immunoreactivity for TNF-α, IL-1β, iNOS, and COX-2 in the oral mucosa of hamsters. In addition to the immunohistochemistry results, a suggestive reduction in iNOS expression by Western blot confirmed the involvement of nitric oxide in the antiinflammatory effects of LP in irinotecan-induced mucositis (data not shown).

In conclusion, our results demonstrate that the inflammatory response in irinotecan-induced mucositis was modulated by the laticifer proteins of *C. procera* (LP) in mice. The morphological alterations as well the functional and inflammatory disorders associated with mucositis were suppressed by treatment with LP. This study shows that proteins endogenously expressed in the latex of the medicinal plant *C. procera* are able to impair the establishment of IM in animals subjected to irinotecan treatment. The results of this study and the literature reporting the pharmacological properties of LP strongly support it as a reliable and promising material for the development of new phytotherapeutics.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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