Trypsin inhibitor isolated from the seeds of Inga laurina (sw.) Willd decreased inflammatory and nociceptive response in mice

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Abstract

Serine proteases (SP) are a defense mechanism for neutrophil, induce recruitment and activation of immune cell, cytokine production and degradation of the extracellular matrix. However, the deregulated activity of SP or their inhibitors can lead to the development of inflammatory diseases. Natural inhibitors of serine proteases (iSP) modulate inflammation and can be found in the Fabaceae family. *Inga laurina*, for exemple, is a species that has an iSP (ILTi) in its seeds.

To evaluate the anti-inflammatory and antinociceptive response of a protease inhibitor purified of *Inga laurina* seeds (ILTi). Leukocyte infiltrate, mast cell degranulation, paw edema, writhing abdominal, formalin and determination of cytokines levels were the assays realized. Male Swiss mice, 18-25g, were distributed in the groups: Saline (10 mL/kg); Dexamethasone (0.5 mg/kg); ILTi (0.3; 3 or 30 mg/kg); Dipyrone (500 mg/kg), in the writhing abdominal assay and Morphine (5 mg/kg), in the formalin test. Cytokine levels were determined by flow cytometry. The results showed that ILTi (0.3 mg/kg) reduced the total leukocytes (64.5%) and polymorphonuclear cell infiltrate (74%), mast cell degranulation (30.2 ± 3.2%) and inhibit paw edema in all times at the doses 3 and 30 mg/kg. In addition, ILTi reduced IL-6 (35.6%) and TNF (79.2%) levels. In antinociceptive evaluation ILTi reduced abdominal writhing (56.4%) and paw licking time at the both phases (neurogenic, 43.2%; inflammatory, 23. 4%). ILTi has anti-inflammatory and antinociceptive effects probably due to inhibition of inflammatory proteases or blocking the activation of protease-activated receptors.

Introduction

Serine proteases (SP) are enzymes that regulate various pathophysiological processes (Soualmia and el Amri 2018). Their proteolytic action is as a defense mechanism for neutrophils. SP are involved in the formation of extracellular traps (NETs) utilized for pathogen capture (Huang and Li 2020). However, an imbalance between SP and their natural inhibitors can lead to the development of inflammatory diseases (Soualmia and el Amri 2018).

Natural serine protease inhibitors (iSP) have been targeted for the development of drugs with potential therapeutic applications in treatment cardiovascular disorders, chronic inflammation, Alzheimer's disease and cancer (Burster et al. 2021; Coppinger et al. 2024). Inhibitors from the Kunitz family act by inhibiting the active site of the enzyme through non-covalent binding, thus regulating protease activity (Ranasinghe and McManus, 2013).

These inhibitors are found in various plants, particularly in legumes belonging to the Fabaceae family (Shamsi; Parveen; Fatima, 2016; Rodríguez-Sifuentes et al. 2020). Some of these compounds have demonstrated the ability to modulate inflammation by inhibiting the activity of proteases involved in this process (Shigetomi et al. 2010; Wittenauer et al. 2015; Bacha et al. 2017). Species from the genus *Inga*, representatives of the Fabaceae family, exhibit anti-inflammatory (Machado 2013; Shamsi 2018), antioxidant (Luís et al. 2010; Maziero et al. 2020), antiparasitic (Lima et al. 2020), fungicide (Pereira et al. 2024), microbicide (Macedo et al. 2016; Jin et al. 2024), anticancer (Carneiro et al. 2018; Kakali 2024), and insecticide (Machado et al. 2017) activities.

Among these species, *Inga laurina*, commonly known as white inga, is found in the cerrado and is primarily recognized for its insecticidal effect, attributed to the presence of an iSP in its seeds, classified as a trypsin inhibitor known as ILTi (Inga laurina trypsin inhibitor) (Macedo et al. 2007). Therefore, this study aimed to evaluate the anti-inflammatory and antinociceptive responses of the trypsin inhibitor purified from *I. laurina* seeds (ILTi) in mice.

Material and methods

Plant material

The Inga laurina trypsin inhibitor (ILTi) was obtained from *Inga laurina* seeds in the city of Campo Grande, Mato Grosso do Sul, Brazil (20°29'49"S 54°36'47"W) and purified at the Laboratory for the Purification of Proteins and their Biological Functions-LPPFB/ UFMS. Access to the genetic heritage was registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SISGEN), under number A315278.

Inga laurina Trypsin inhibitor (ILTi) purification

ILTi was purified as previously described (Macedo et al. 2007). A crude extract of *I. laurina* (ILCE) was obtained by extraction with 100 mM phosphate buffer (pH 7.6). After, ILCE has been centrifuged at 7500g for 30 min, followed by ammonium sulphate precipitation into three fractions: 30%, 60% and 80% saturation. The fraction with the highest inhibitory activity (30–60%) was selected for purification by ion-exchange chromatography on HiTrap Q Sepharose column equilibrated with Tris–HCl (20 mM, pH 8.0) and eluted with bugger containing NaCl (0–1M).

Animals

Male swiss mice (18–25g) were used in the experiments. There animals were supplied by the Federal University of Mato Grosso do Sul (UFMS), maintained in a room under standard conditions, at temperature of 22 ± 2ºC, light cycle (12/12 h light/dark) and controlled humidity, with access to food and water. Experiments were approved by UFMS Animal Ethics Committee (protocol number 1225/2022) and were according with National Institutes of Health (NIH, 2024) regulations on the use animals for scientific assays.

Experimental design

Animals were pretreated with sterile saline (0.9% NaCl), ILTi (0.3, 3 or 30 mg/kg) for the leukocyte infiltrate and 0.3 mg/kg for the others assays, or the standard drug, subcutaneously (s.c.; 200 µL) 30 min before the injection of the inflammatory stimuli. Dexamethasone (1 mg/kg; Ache®, 4mg/mL), dipyrone (500 mg/kg; Farmace®, 500 mg/mL) or morphine (2 mg/kg; Cristália®, 10 mg/mL) were used as the standard drug (anti-inflammatory or analgesic) diluted in sterile saline. The saline group was pretreated with sterile 0.9% NaCl (s.c.; 200 µL) and 30 min later the animals received sterile 0.9% NaCl as stimuli. For euthanasia, anesthetic overdose (Xylazine 2%, Syntec®; Ketamyne 10%, Syntec®; Xilazine:Ketamyne, 1:2) was used.

Anti-inflammatory evaluation

Leukocyte infiltrate into the peritoneal cavity

Inflammation was induced via intraperitoneal (i.p.) injection of 1% carrageenan (500 µL; Sigma Aldrich®). Four hours later, the cell from the euthanized animals were harvested with 3 mL of phosphate-buffered saline (PBS) and heparin (10 U/mL; Basel Switzerland). Total leukocyte counts were determined using a Neubauer chamber with Turk's solution (0.2% crystal violet in 30% acetic acid). For differential counts, exudates were centrifuged (1000 rpm/8 min), supernatants stored at − 20°C for cytokine analysis and cell pellets resuspended in 3% bovine serum albumin (200 µL; Prothermo®). Cells were stained with Hema3® (NewProv®) and classified as polymorphonuclear (PMN) or mononuclear (Souza and Ferreira 1985). Data were expressed as cells/mm³.

Mast cell degranulation

Mast cell degranulation in mice was induced by i.p. administration of compound 48/80 (1 µg/mL, 500 µL; Sigma Aldrich®), while control group received saline solution. After 30 minutes, mesenteries were removed, washed with PBS and fixed in 4% paraformaldehyde for 2 hours. Slides were mounted with the tissues, stained with Hema3®. Degranulation was determined by granule dispersion, with mast cells exhibiting ≥ 10 extruded granules classified as degranulated and examined under light microscopy (Galvão-Nascimento et al. 2010). Results were expressed as the percentage of degranulated cells per 100 counted.

Paw edema

Paw edema was induced via intraplantar injection of 1% carrageenan (40 µL) into the right hind paw, while the contralateral paw was injected with saline solution (40 µL) as a control. Edema volume was measured at 30, 60, 120, and 240 minutes after injection using a digital plethysmometer (Insight®). Edema was calculated as the difference between the volumes of the control and stimulated paws (Winter et al. 1962). Data were presented as mL and percentage inhibition relative to controls.

Determination of cytokines levels in peritoneal cavity

Levels of interleukin-6 (IL-6), interleukin-10 (IL-10), monocyte chemoattractant protein-1 (MCP-1), and tumor necrosis factor (TNF) were quantified in peritoneal exudates collected 4 hours after carrageenan injection. Supernatants from centrifuged samples were analyzed using a flow cytometry (CBA; BD Biosciences®). Cytokines concentrations were expressed as pg/mL.

Antinociceptive evaluation

Abdominal writhing

Abdominal writhing was performed via i.p. injection of 0.6% acetic acid (500 µL, Vetec®) in according Koster et al., 1959. The total number of writhings was recorded over 30 minutes after injection.

Formalin test

Nociception was evaluated by intraplantar injection of 1.2% formalin (Neon®, 40 µL) into the right hind paw. Paw licking duration was recorded in two phases: 0–5 minutes (neurogenic pain) and 15–30 minutes

(inflammatory pain) (Hunskaar et al. 1985). Data were expressed as seconds of licking.

Statistical analysis

The results were expressed as mean ± standard error of the mean (SEM). Statistical significance was determined using one-way or two-away ANOVA followed by Bonferroni's post test (GraphPad Prism 5, USA). A p-value < 0.05 was considered significant.

Results

Anti-inflammatory activities of ILTi

Effect of ILTi on leukocyte infiltrate into the peritoneal cavity

The infiltrate of leukocytes induced by carrageenan (4863.0 ± 995.3 cells/mm^3) increased when compared to saline (1425.0 ± 222.2 cell/mm^3). ILTi pretreatment inhibited carrageenan induced leukocyte infiltrate into mice peritoneal cavity (Fig. 1a) at 0.3 mg/kg in 64.5% (1725.0 ± 92.4 cell/mm^3), at 3 mg/kg in 34.3% (3188.0 ± 629.9 cell/mm^3) and at 30 mg/kg in 36.8% (3075.0 ± 385.4 cell/mm^3). However, there was not difference as doses. Therefore, dexamethasone (0.5 mg/kg) reduced leukocyte influx in 50.4% (2413.0 ± 611.1 cell/mm^3). ILTi pretreatment (0.3 mg/kg) decreased the polymorphonuclear cells infiltrate (Fig. 1b) in 74.0% (394.8 ± 128.0 PMN/mm^3) when compared to control (1517.0 ± 182.0 cell/mm^3). But at 3 mg/kg (664.8 ± 70.45 PMN/mm^3) and at 30 mg/ kg (409.3 ± 43.7 PMN/mm^3) this effect was not observed. Dexamethasone reduced PMN recruitment in 42.1% (877.8 ± 277.6 PMN/mm^3). Regarding mononuclear cells, the treatment with ILiT in all doses (0.3 mg/kg, 1330.0 ± 140.0 MN/mm^3; 3 mg/kg, 2523.0 ± 581.2 MN/mm^3; 30 mg/kg, 2666.0 ± 348.8 MN/mm^3) and dexamethasone (1535.0 ± 437.4 MN/mm^3) were not inhibited their recruitment.

Effect of ILTi on peritoneal mast cell degranulation

Fig. 2 shows the mast cell degranulation. Compound 48/80 induced degranulation (42.6 ± 4.0%) when compared to the negative control group (19.3 ± 0.7%). The treatment with dexamethasone reduced mast cell degranulation in 21 ± 2.3 %. ILTi inhibit this effect at all doses (0.3 mg/kg, 30.2 ± 3.2%; 3 mg/kg, 23.0 ± 2.7%; 30 mg/kg, 20.6 ± 3.2%).

Effect of ILTi on paw edema

ILTi pretreatment inhibited paw edema induced by carrageenan in mice (Table 1). ILTi (0.3 mg/kg) dit not reduced paw edema at all times observed. This effect was observed at doses 3 mg/kg at all times and 30 mg/kg until 120 minutes.

Table 1

Effects of the inhibitor from *Inga laurina* seeds (ILTi) on carrageenan induced mice paw edema.

Group	Dose	Paw edema (mL) and % inhibition									
	(mg/Kg)	-	%	30 min	%	60 min	%	120 min	%	240 min	%
Saline	-	-	-	0.045 ± 0.015	-	0.047 ± 0.007	-	0.047 ± 0.007	-	0.045 ± 0.004	-
Dexamethasone	0.5	-	-	0.025 ± 0.002**	44	0.025 ± 0.002**	47	0.020 ± 0.003***	57	0.022 ± 0.004**	51
ILTi	0.3	-	-	0.045 ± 0.008	-	0.047 ± 0.006	-	0.045 ± 0.002	4	0.038 ± 0.005	16
ILTi	3	-	-	0.027 ± 0.007*	40	0.027 ± 0.007**	43	0.018 ± 0.004***	62	0.022 ± 0.003**	51
ILTi	30	-	-	0.022 ± 0.005**	51	0.025 ± 0.006**	47	0.020 ± 0.004***	57	0.040 ± 0.003	11

Animals were pretreated with saline (0.9% NaCl, vehicle), dexamethasone or ILTi, subcutaneously (s.c.; 200 µL), 30 min before intraplantar injection of carrageenan (1%, 40 µL) in the right hind paw. Sterile saline (0.9% NaCl) was injected into the left hind paw. Values represent the mean ± SEM (n = 4) of edema (mL) difference between right and left paws. Two-way ANOVA followed by Bonferroni test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to the negative control group (saline).

Effect of ILTi on cytokines concentration

ILTi reduced IL-6 and TNF concentration into mice peritoneal cavity (Fig. 3) in 35.6% (7228.0 ± 887.1 pg/mL) and 79.2% (229.2 ± 25.9 pg/mL), respectively, compared to control (11224.0 ± 701.9 pg/mL, IL-6; 1104.0 ± 164.2 pg/mL, TNF). For IL-10 and MCP this effect was not observed. The treatment with dexamethasone also reduced IL-6 (76.2%; 2672.0 ± 809.7 pg/mL) and TNF (91.5%; 93.3 ± 27.1 pg/mL) concentration.

Antinociceptive activities of ILTi

Effect of ILTi on abdominal writhing

Fig. 4 shows the number of writhings induced by acetic acid. ILTi (0.3 mg/kg) inhibited writhing response in 56.4% (52.8 ± 10.5 writhings) compared to control (121.0 ± 11.1 writhings). Dipyrone pretreatment (500 mg/kg) decreased the number of writhings by 95.9% (5.0 ± 0.0 writhings).

Effect of ILTi on the formalin induced paw licking

The ILTi (0.3 mg/kg) reduced the paw licking response (43.2%, 40.5 ± 5.7 s) when compared to the group without treatment (71.3 ± 1.3 s) at first phase (Fig. 5a). Morphine (65.4%, 24.7 ± 5.4 s) and dipyrone (100%) reduced licking response. At the second phase (Fig. 5b), morphine (77.1%, 55.7 ± 16.8 s) and dipyrone (100%) reduced paw licking time. ILTi (23.4%, 187.0 ± 6.7 s) was effective when compared to the group without treatment (244.0 ± 10.8 s).

Discussion

SPs are enzymes that act in the acute inflammatory response by recruitment leukocytes, production of cytokines and formation of NETs (Soualmia and El Amri 2018; Huang and Li 2020). However, it can cause tissue damage and prolonged inflammation when in excess (Hidalgo et al. 2022). This has driven the search for natural inhibitors of SPs (Sananes et al. 2023; Kontoh-Twumasi et al. 2024), such as those found in species of the Fabaceae family, known for their anti-inflammatory and analgesic effects (Carvalho et al. 2023; Paulo-Pereira et al. 2012; Ferreira et al. 2019).

Our results showed that ILTi reduced the leukocyte infiltrate into the peritoneal cavity of mice, as well as the number of PMN, predominant cells in the acute inflammatory response. In accordance with this results, in another study conducted by our research group, an inhibitor obtained from *Libidibia Ferrea* (LfTI) reduced carrageenan-induced total leukocyte and PMN influx (Carvalho et al. 2023). Furthermore, another Kunitz-type inhibitor, from *Schistosoma mansoni* (Sm KI-1) decreased neutrophil migration into the pleural cavity of mice in response to carrageenan (Morais et al. 2018). PMN are immune response cells that play a defense role in cases of infection or tissue damage by recognizing the inflammatory stimulus through toll-like receptors (TLR) (Jain; Pandey; Shukla 2015; Oishi and Manabe 2018). For this, they release the contents of their granules, such as proteases, enzymes that remodel the extracellular matrix (ECM). One example is neutrophil elastase, which at high levels degrades ECM components such as collagen and the intercellular junctions of the endothelium, facilitating cell migration and exudate in the inflamed tissue (Zhu et al. 2021; Morales-Primo et al. 2022). In acute inflammation, the ECM fragments generated by the action of elastase act as damage-associated molecular patterns (DAMPs), are recognized by immune cells via TLR receptors and activate the synthesis of cytokines, which amplify the inflammatory response (Zhu et al. 2021).

A possible explanation for the results found regarding leukocyte infiltrate is the binding of ILTi to neutrophil proteases, especially elastase, an enzyme involved in the degradation of ECM, the migration of cells to the focus of inflammation and the production of pro-inflammatory cytokines.

Following to mast cell degranulation, we observed that ILTi reduced this effect. These are granular and resident cells that initiate allergic and inflammatory responses by inducing vasodilation, increased vascular permeability and cell infiltrate (Elieh Ali Komi et al. 2020).

Mast cells express various receptors, which are activated by specific substances as FcεR and FcγR that are stimulated by immunoglobulins; TLR bind to antigens, CR to complement factors, HR to histamine and CXCR to chemokines; ILR, TGFβR and INFγR respond to interleukins, TGFβ and INFγ, respectively; while P2X and EP2 are activated by ATP and prostaglandin E_2 (PGE_2) during degranulation (Sabato et al. 2021; Haque and Frischmeyer-Guerrerio 2022; Schulman et al. 2023).

The interaction between these receptors and their ligands triggers the phosphorylation of intracellular protein residues, which ends in degranulation, leading to the secretion of proteases such as chymase, tryptase and metalloprotease and vasoactive substances, for example, serotonin and histamine (Haque e Frischmeyer-Guerrerio, 2022). Furthermore, mast cells release newly formed lipid mediators, including platelet aggregation factor (PAF), prostaglandin (PG), like PGE_2 and leukotrienes (LT), mainly LTB_4 and LTD_4, which positively modulate the inflammatory response (Elieh Ali Komi; Rambasek; Wöhrl 2018; Elieh Ali Komi et al. 2020).

In addition, mast cells, neutrophils and endothelial cells have protease-activated receptors on their surface, called PARs. These can be cleaved by endogenous proteases, such as thrombin and trypsin, or exogenous proteases, such as proteases from microorganisms. Thus, they trigger G protein-mediated intracellular signaling and are irreversibly activated (Heuberger and Schuepbach 2019). The PAR_1 receptor is present on platelets and endothelial cells. Its cleavage by thrombin results in platelet aggregation and PAR_1 activation on endothelial cells results in secretion of IL-1, IL-6, TNF and cell adhesion molecules, like intracellular adhesion molecule 1 (ICAM-1) and adhesion molecule vascular cell adhesion molecule 1 (VCAM-1) (Peach et al. 2023).The PAR_2 receptor is expressed in the stomach, small intestine, gallbladder and inflammation, is mainly activated by trypsin and is related to nociceptor sensitization (Heuberger and Schuepbach 2019; Peach et al. 2023). Finally, receptor 3 is a regulator of the other PARs and PAR_4 is cleaved by thrombin and trypsin, mediating platelet responses (Peach et al. 2023).

The SP promotes leukocyte migration by degrading the ECM (Zhu et al. 2021; Morales-Primo et al. 2022) or by activating PAR receptor (Heuberger; Schuepbach 2019), suggesting that ILTi may inhibit proteases released from mast cells and neutrophils, which are responsible for the formation of DAMPs and the degranulation of more leukocyte granules. Furthermore, ILTi can block PAR receptors, like PAR_1 and PAR_2, present in mast cell and neutrophil. This receptors, when activated, lead to degranulation of these cells and secretion of IL-6 and TNF (Ramachandran et al. 2012; Peach et al. 2023), effects whose reduction was observed in our study.

Therefore, the serine proteases released by mast cell degranulation not only degrades ECM components, generating DAMPs, which are a stimulus for leukocyte recruitment and inflammatory response amplification (Zhu et al. 2021), but also cleave PAR_2 receptors in inflammatory cells and induce nociception (Peach et al. 2023). Physiological serine protease inhibitors, such as the leukocyte protease inhibitor present in secretions, are capable of blocking the action of proteases whose degranulation is mediated by IgE, via FcεR (Kwiecinska et al. 2023). The inhibition of leukocyte degranulation by natural serine protease inhibitors has been previously described (Carvalho et al. 2023; Buster et al. 2021). A protease inhibitor isolated from tick saliva attenuates inflammation by inhibiting mast cell chymases and neutrophil cathepsin G, enzymes released by degranulation, which modulate the influx of leukocytes (Fu et al. 2021). The activation of PAR_2 and PAR_4 by trypsin or tryptase increases leukocyte degranulation, leading to the release of pro-inflammatory mediators, like PGE_2 and cytokines (Heuberger; Schuepbach 2019).

In edematogenic activity of ILTi, the results showed that the inhibitor was effective in reducing edema at superior doses. A Kunitz-type inhibitor isolated from the seeds of *Enterolobium Contortisiliquum* reduced paw edema induced by *Crotalus durissus* terrificus venom (Costa et al. 2023). Similarly, Carvalho et al. (2023) evaluated the effect of a protease inhibitor obtained from the seeds of *Libidibia ferrea* and confirmed this activity.

Cg-induced paw edema consists of two phases, the first is mediated mainly by histamine, serotonin and bradykinin from mast cells (30–60 minutes) and the second involves the participation of PGE_2 and NO (after 60 minutes) (Di Rosa; Giroud; Willoughby 1971; Mehrzadi et al. 2021), whose increase derives from STAT3 and NF-κB activation by carrageenan (Mehrzadi et al. 2021). Histamine, serotonin and bradykinin are

released from pre-formed mast cell granules, promoting vasodilation in initial phase, which favors the migration of neutrophils to the inflamed site (Elieh Ali Komi; Rambasek; Wöhrl 2018). At the site of inflammation, the leukocytes initiate the synthesis of PGE_2, a majority product of COX-2, after the first hour (Claudino et al. 2006).

ILTi was effective in reducing edema in both phases. Confirming this result, the inhibitor reduced mast cell degranulation, which involves the release of vasoactive mediators in the first phase, such as histamine, serotonin and bradykinin (Elieh Ali Komi; Rambasek; Wöhrl 2018). The explanation for these results is that ILTi may be blocking intracellular serine proteases, such as mast cell tyrptase, or signaling pathways that lead to the release of granules, thus reducing the secretion of vasoactive mediators. This effect can be confirmed by the lower mast cell degranulation in treated animals with ILTi. Moreover, edema in the second phase (after 60 minutes) was also reduced, suggesting that the inhibitor acts on the PG pathway. Possible mechanisms of action include blocking the COX-2 enzyme or some stage of STAT3 signaling pathway, which results in the production of this mediator (Mehrzadi et al. 2021).

In addition, edema in rodents can be modulated by administration of PAR receptor agonists and antagonists, demonstrating the participation of these receptors in this event (Suen et al. 2012; Peach et al. 2023). The cleavage of PAR_2 receptors on inflammatory cells leads to the release of PGE_2 and cytokines (Heuberger; Schuepbach, 2019). PGE_2 is a substance produced by the COX-2 enzyme from arachidonic acid, whose function is to increase the vasodilation, induce fever and sensitize nociceptors to the stimulation of algesic substances, such as bradykinin (Calder 2020).

SPs released during the inflammatory response can stimulate the production of cytokines such as IL-6, interleukin 1 beta (IL-1β) and TNF (Caughey 2016; Heuberger; Schuepbach 2019). This occurs through the cleavage of G-protein-coupled PAR receptors, which activates the NFκB and MAPKs (JNK/p38) signaling pathways, regulating the transcription of pro-inflammatory genes (Nennig and Schank 2017; Ren et al. 2023). In the present study, ILTi inhibited the concentration of IL-6 and TNF in the peritoneal exudate of mice injected with carrageenan. Corroborating our results, Silva et al. (2023) evaluated the anti-inflammatory efficacy of a kallikrein inhibitor isolated from *Bauhinia bauhinioides* in an experimental asthma model and observed a reduction inflammatory mediators in the alveolar fluid and in the leukocyte infiltrate, including IL-6 and TNF. Rajegowda and SnehaRani (2023) observed the decrease in the release of inflammatory mediators (NO, IL-6, TNF) and on the activity of elastase (NE) in macrophage cell of a protease inhibitor isolated from the seeds of Caesalpinia decapetala (CdTI), a species belonging to the same family as ILTi. Therefore, lower levels of nuclear factor kappa B (NFkB) and inducible nitric oxide synthase (iNOS) were observed in animals treated with this inhibitor.

NFkB is a transcription factor for the production of cytokines production (Nennig and Schank 2017). DAMPs from ECM degradation by SPs are detected by macrophage toll-like receptors and activate the NFkB phosphorylation, leading to the synthesis of proinflammatory cytokines (Mitchell, Vargas and Hoffmann 2016). In addition, activation of PAR_2 by proteases induces TNF/IL-6 synthesis by this same pathway (Ramachandran et al. 2012). Thus, ILTi may have prevented NFkB activation by binding to the SP and reducing the formation of DAMPs or may have impeded PAR_2 activation, resulting in lower cytokine production.

However, ILTi did not reduce IL-10 and MCP-1 and levels. IL-10 is a cytokine secreted by activated macrophages that regulates the expression of pro-inflammatory cytokines, preventing tissue damage as a result of an exacerbated defense cell response (Ouyang et al. 2011). MCP-1 is regulated via PPAR-γ and activated by IL-10 (via STAT3) in response to TLR3/TLR4 activation in macrophages. Its function to stimulate the migration and differentiation of monocytes (Zhang et al. 2018; Singh, Anshita and Ravichandiran 2021). Corroborating, ILTi also did not change MN migration (data not shown).

Thus, it is possible that ILTi acts on NF-κB/MAPK dependent pathways that increase TNF and IL-6 and does not interfere with compensatory (MCP-1) or anti-inflammatory (IL-10) mechanisms. This is positive for a therapeutic profile, as maintaining the synthesis of IL-10 and MCP-1 can prevent excessive immunosuppression.

The results of the nociception showed that ILTi has antinociceptive andantihyperalgesic properties. The induction of nociception by acetic acid promotes an increase in the concentration of prostaglandins in the peritoneal cavity of mice and the activation of mast cells by trypsin, via PAR_2 receptors, contribute to the production of PGE_2 (Ribeiro et al. 2000; Heuberger and Schuepbach 2019). Moreover, the elastase secreted by neutrophils activates PAR_2 receptors, which results in the conversion of AA into prostaglandins (Muley et al. 2016). This effect can be reversed with the administration of PAR_2 antagonists as demonstrated by Zhao et al. (2015). The cleavage of PAR_2 by elastase activates transient receptor potentials (TPRs) in sensory neurons, which process the effects of prostaglandins, histamine, serotonin and bradykinin and lead to the detection of pain (Sun et al. 2020).

Therefore, ILTi might interacted with neutrophil elastase, preventing the activation or the cleavage of PAR_2 resulting in less activation of nociceptors through TPR and decreased pain signaling. In addition, the decrease in degranulation of mast cells and neutrophils by ILTi may have contributed to the reduced release of algesic mediators such as histamine, serotonin and bradykinin.

In the formalin test, a reduction in paw licking time was observed in both phases of the experiment. Carvalho et al. (2023) reported similar results when evaluating the antinociceptive potential of LfTI. The explanation given for these results was the possible blockade of PAR_3 receptors or a decrease in substance P, a neuropeptide that activates nociceptors and is produced via activation of PARs.Thus, Clark et al. (2007) described a cathepsin S inhibitor with anti-hyperalgesic and antinociceptive effects in neuropathic pain models. Zhao et al. (2014) observed that this same protease exerts pro-inflammatory and nociceptive effects via PAR_2 activation.

In our study, ILTi showed efficacy in phase I of formalin, which can be attributed to the blocking of proteases. This prevents the action of SP on the PAR receptors of pain fibres, since these receptors, mainly PAR_1, PAR_2 and PAR_4, increase nociceptor sensitization when activated by proteases (Ribeiro et al. 2000; Heuberger and Schuepbach 2019). This can occur in the presence of inflammatory mediators, such as PGE_2 (Calder, 2020) or pro-nociceptive peptides, such as substance P and CGRP, released from nerve terminals (Merighi 2024). Thus, the possible blockade of PAR receptors by ILTi may have contributed to the reduction in pain

perception induced by formalin. Similarly, blocking the PAR3 of nociceptive fibres can reduce the release of neurotransmitters, and decrease the sensitization of nociceptive fibres (Bunnet 2006).

In the inflammatory phase of the formalin test (phase II), the results corroborate those obtained in the abdominal writhing model, given that the mediators involved are similar (Carvalho et al., 2023). In this context, it is possible that ILTi mechanism of action is related to reducing leukocyte degranulation, considering that mediators present in mast cell and neutrophil granules, such as, histamine, serotonin and bradykinin, are responsible for activating nociceptors, while PG, pre-formed or synthesized by COX during inflammation, sensitive the nerve fibers (Caughey 2016).

Nociceptors are constituted of type Aσ or C fibers, myelinated or unmyelinated, respectively. Type Aσ fibers conduct acute and rapid pain, while C fibers conduct moderate and persistent pain (Feng 2025). These fibers respond immediately to any irritation or local stimulus and after this first interpretation, there is the central signaling that will lead to the inflammatory response and the release of neurotransmitters.

In addition, formalin activates TRP in sensory neurons, causing pain (Pereira 2014) and the cleavage of PAR receptors by SP causes a similar effect (Heuberger and Schuepbach 2019). Therefore, ILTi may have prevented the cleavage of PARs by SP, decreasing the degranulation of mast cells and neutrophils and the release of algesic substances from their granules. This effect, particularly on PAR_2 and PAR_4 receptors, may decrease signaling for the release of cytokines and prostaglandins, as well as reducing leukocyte degranulation (Heuberger and Schuepbach 2019). Thus, the involvement of substances responsible for the direct activation of nociceptors or for reducing the pain threshold is reduced.

Conclusion

This study showed that ILTi has anti-inflammatory and antinociceptive effects. Our results suggest that these effects may be associated with the inhibition of inflammatory proteases by prevention of the cleavage of PAR receptors, whose activation leads to the degranulation of mast cells and neutrophils, the production of pro-inflammatory cytokines and PGE_2 and nociception. This possible mechanism is promising and because it is different from the inhibition of PGE_2 synthesis promoted by conventional anti-inflammatory drugs, which block COX-2 leading to the traditional side effects known.

Abbreviations

ILTi
Inga laurina trypsin inhibitor
SP
Serine proteases
iSP
Inhibitors of serine proteases
NETs
Neutrophil extracellular traps
PMN

Polymorphonuclear cells
MN
Mononuclear cells
ECM
Extracellular matrix
DAMPs
Damage associated molecular patterns
TLR
Toll–like receptors
PARs
Protease activated receptors
PGE_2
Prostaglandin E_2
LT
Leukotrienes
PAF
Platelet aggregation factor
ICAM
1–Intracellular adhesion molecule 1
VCAM
1–Vascular cell adhesion molecule 1
COX
2–Cyclooxygenase–2
NF
κB–Nuclear factor kappa B
MAPKs
Mitogen activated protein kinases
JNK
c–Jun N–terminal kinase
iNOS
Inducible nitric oxide synthase
STAT3
Signal transducer and activator of transcription 3
PPAR
γ–Peroxisome proliferator activated receptor gamma
TRP
Transient receptor potential
CGRP
Calcitonin gene related peptide
CBA
Cytometric bead array
PBS

Phosphate buffered saline
IL
6–Interleukin–6
IL
10–Interleukin–10
TNF
Tumor necrosis factor
MCP
1–Monocyte chemoattractant protein–1
UFMS
Federal University of Mato Grosso do Sul
SISGEN
National System for the Management of Genetic Heritage and Associated Traditional Knowledge
NIH
National Institutes of Health
SEM
Standard error of the mean
ANOVA
Analysis of variance

Declarations

Author contributions

AS, WM and SA performed the experimental work. WY and Alamgeer designed the study, MNHM helped for qPCR study. MZH and JU helped in molecular docking studies. AMU and UHS write the initial draft that was further fnalized by KSL. ANH, FARL and AGJ writing, reviewing and editing the original draft. All authors read and approved the fnal manuscript.

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Declarations conflict of interest

The authors declare that they have no financial interest or non-fnancial interests to disclose.

Ethical approval

Experiments were approved by UFMS Animal Ethics Committee (protocol number 1225/2022) and were according with National Institutes of Health regulations on the use animals for scientific assays.

Data availability statement

All data are available in this publication.

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Figures

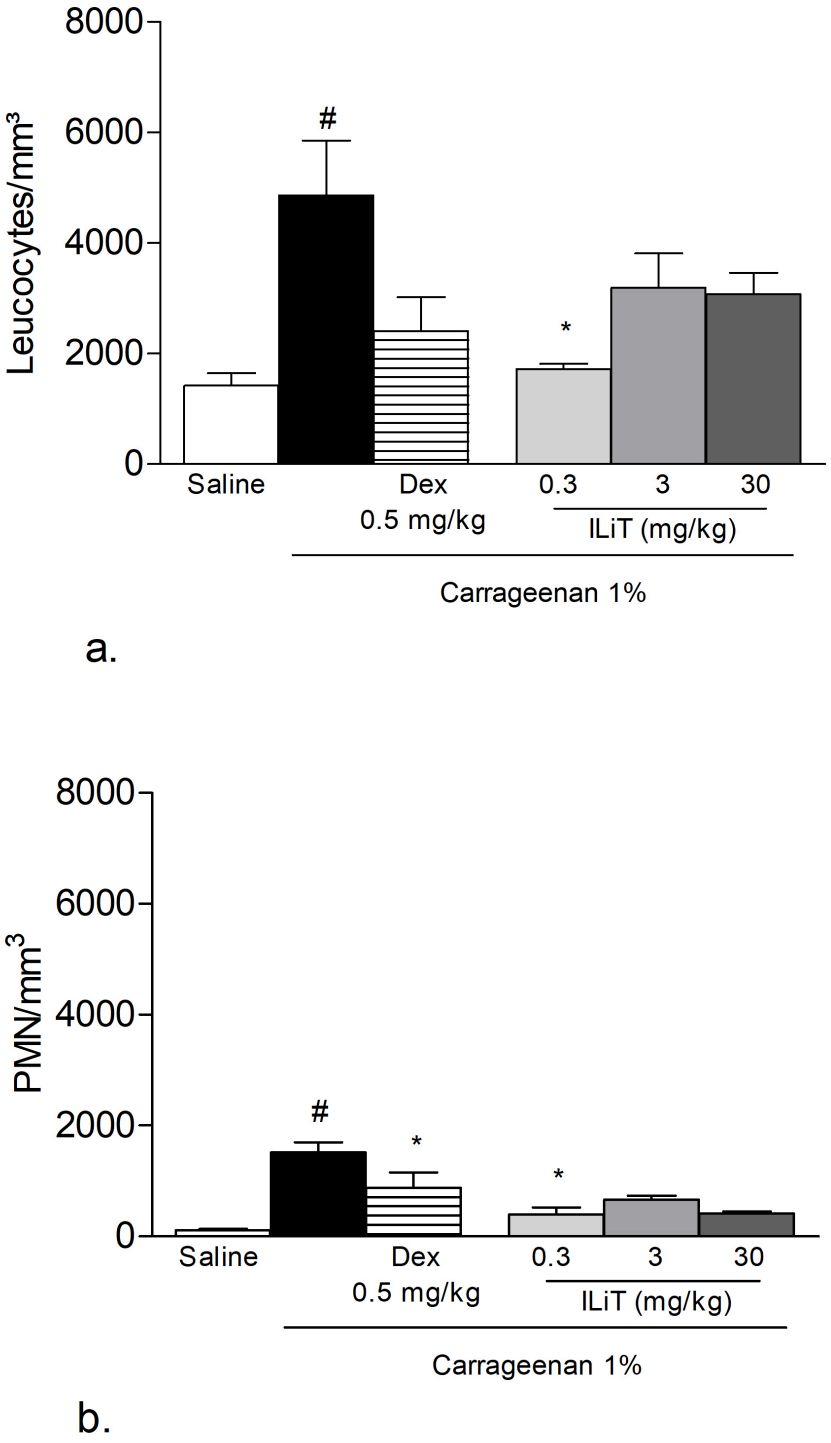

Figure 1

Leukocyte infiltrate into mice peritoneal cavity after treatment with *Inga laurina* trypsin inhibitor (ILTi). Animals were pretreated (s.c.) with saline (0.9% NaCl, vehicle), dexamethasone (Dex, 0.5 mg/kg) or ILTi (0.3, 3 and 30 mg/kg), subcutaneously (s.c.; 200 µL), 30 min before injection of carrageenan (1%, i.p.). Four hours later, exudates were collected for cell counting. Columns represent the mean ± SEM (n = 5) of cell

numbers/mm^3. ANOVA was performed followed by the Bonferroni test. # $p < 0.05$, compared to the saline group. * $p < 0.05$, compared to the groups without treatment.

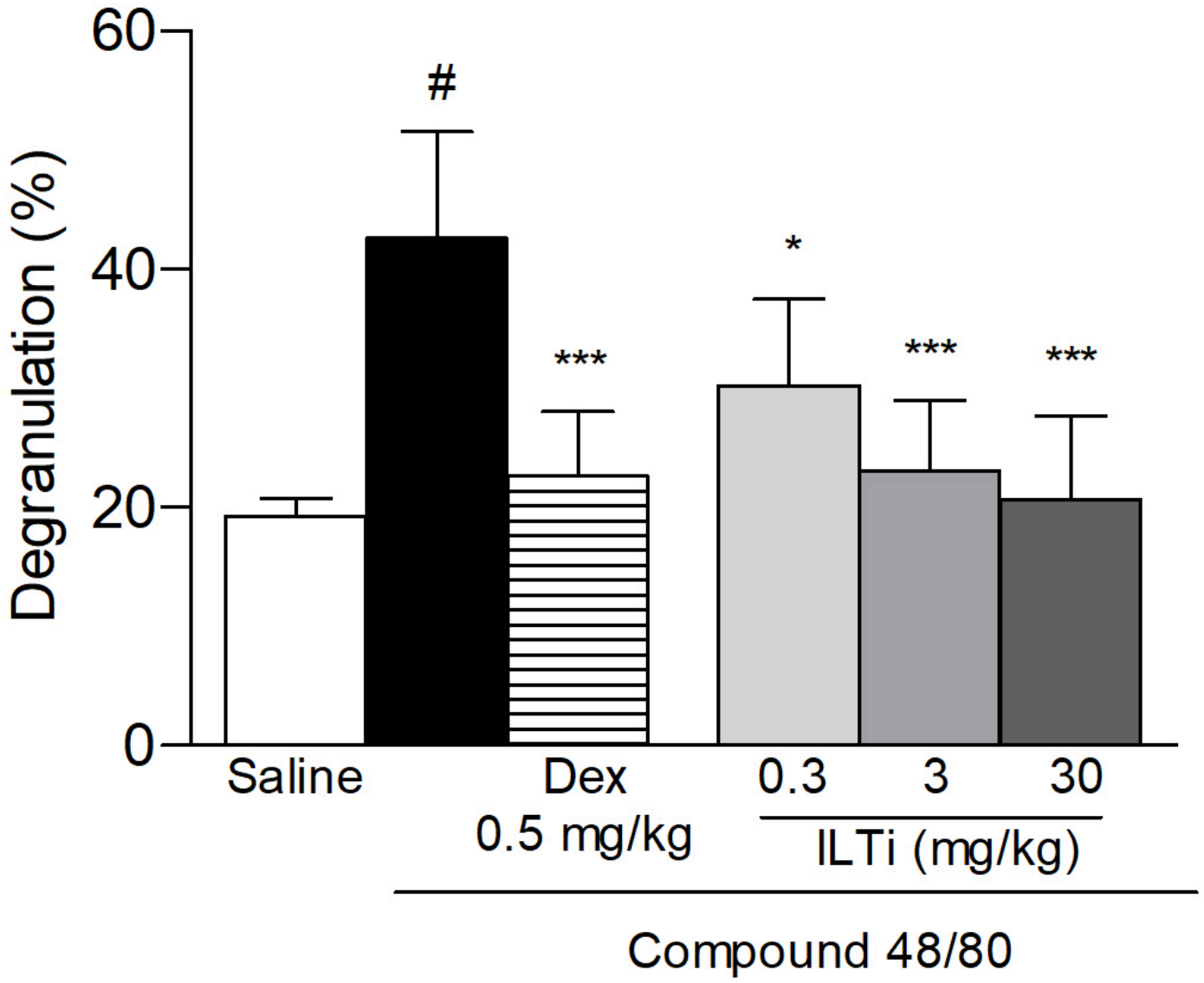

Figure 2

Mast cell degranulation writhing after treatment with *Inga laurina* trypsin inhibitor (ILTi) in mice. Animals were pre-treated (s.c.) with saline (0.9% NaCl, vehicle), dexamethasone (Dex, 0.5 mg/kg) or ILTi (0.3 mg/kg), subcutaneously (s.c.; 200 µL), 30 min before intraperitoneal injection of compound 48/80 (1 µg/mL). The saline group pretreated and stimulated with sterile saline (0.9% NaCl). The mesenteries were removed and fixed with 4% paraformaldehyde for cell counting. Columns represent the mean ± SEM (n = 5) of % of degranulated mast cells. Values were subjected to one-way ANOVA followed by by the Bonferroni test. # $p < 0.001$, compared to the saline group. * $p < 0.05$, *** $p < 0.001$, compared to the groups without treatment.

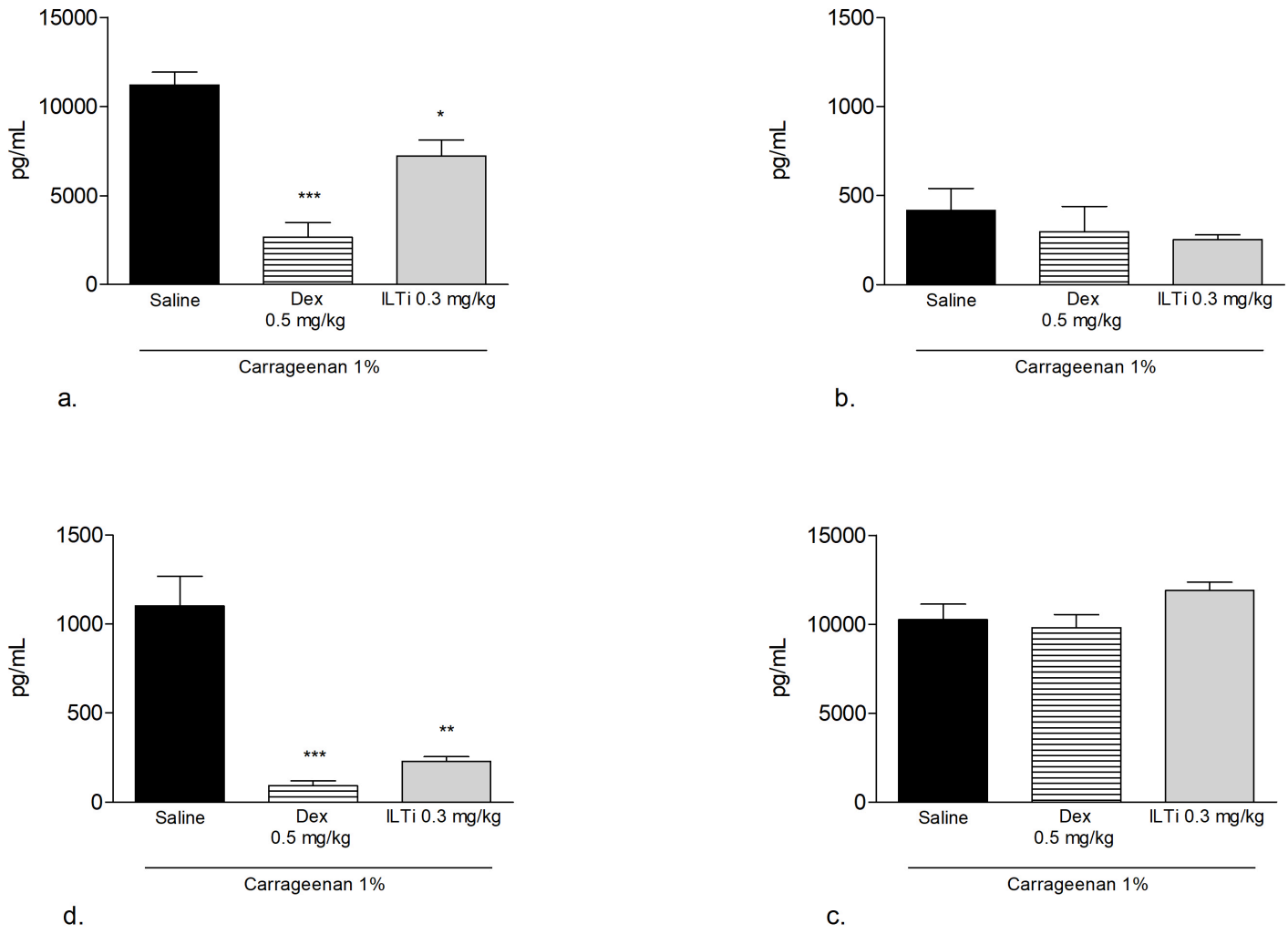

Figure 3

Cytokines concentration after treatment with *Inga laurina* trypsin inhibitor (ILTi) in mice. Animals were pretreated (200 µL, s.c.) with saline (0.9% NaCl, vehicle), dexamethasone (Dex, 0.5 mg/kg) or ILTi (0.3 mg/kg). Four hours after injection of carrageenan (1%, i.p.) the exudate was collected and stored for cytokines **a** IL-6, **b** IL-10, **c** MCP and **d**TNF quantified by flow cytometry. Columns represent the mean ± SEM (n = 3). ANOVA was performed followed by the Bonferroni test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to the saline group.

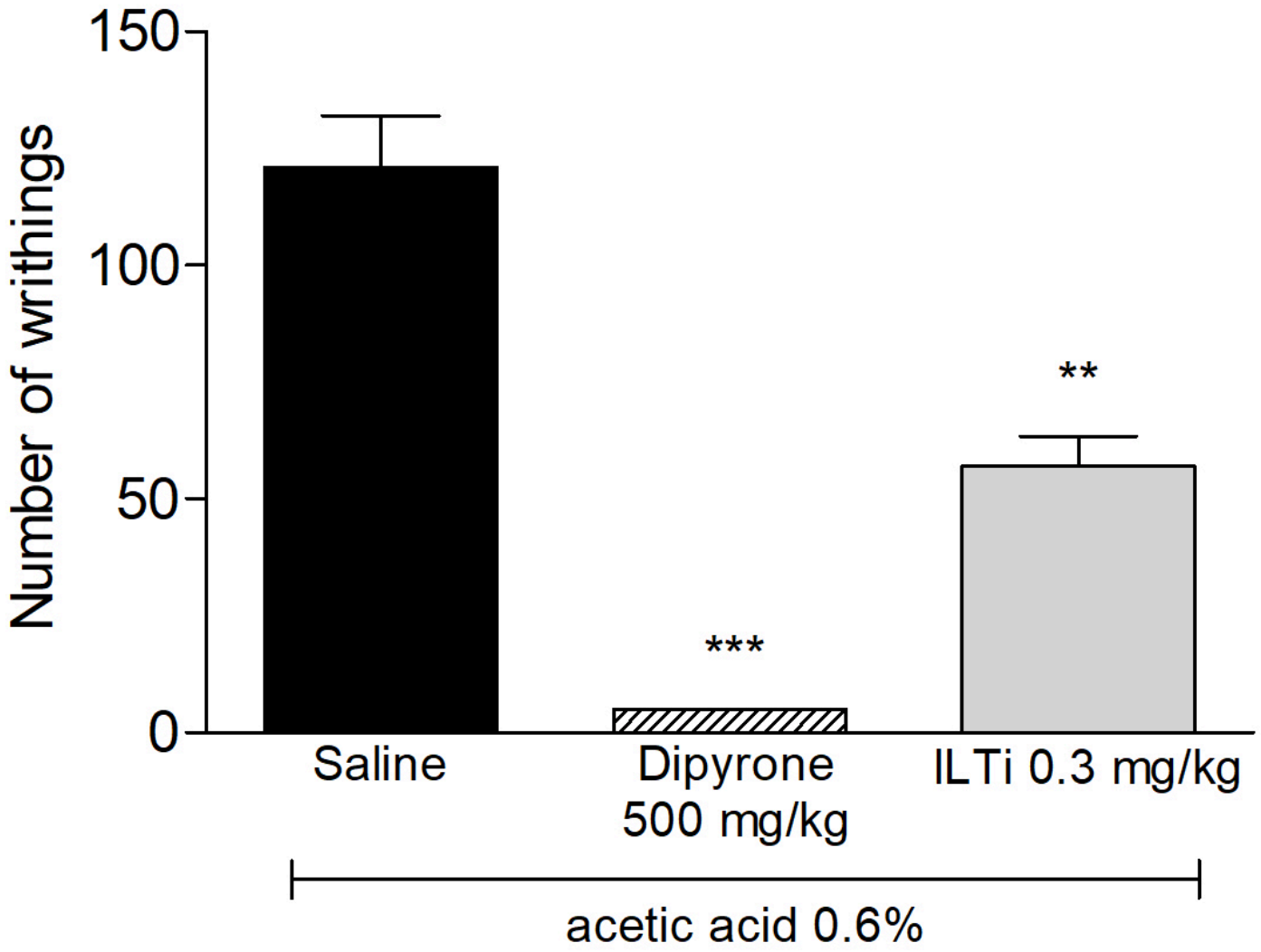

Figure 4

Abdominal writhing after treatment with *Inga laurina* trypsin inhibitor (ILTi) in mice. Animals were pretreated (200 µL, s.c.) with saline (0.9% NaCl, vehicle), dipyrone, or ILTi (0.3 mg/kg), 30 min before acetic acid injection (0.6%, 500 µL, i.p.). Columns represent the mean ± SEM (n = 5) of writhings. ANOVA was performed followed by the Bonferroni test. ** $p < 0.01$, *** $p < 0.001$, compared to the saline group.

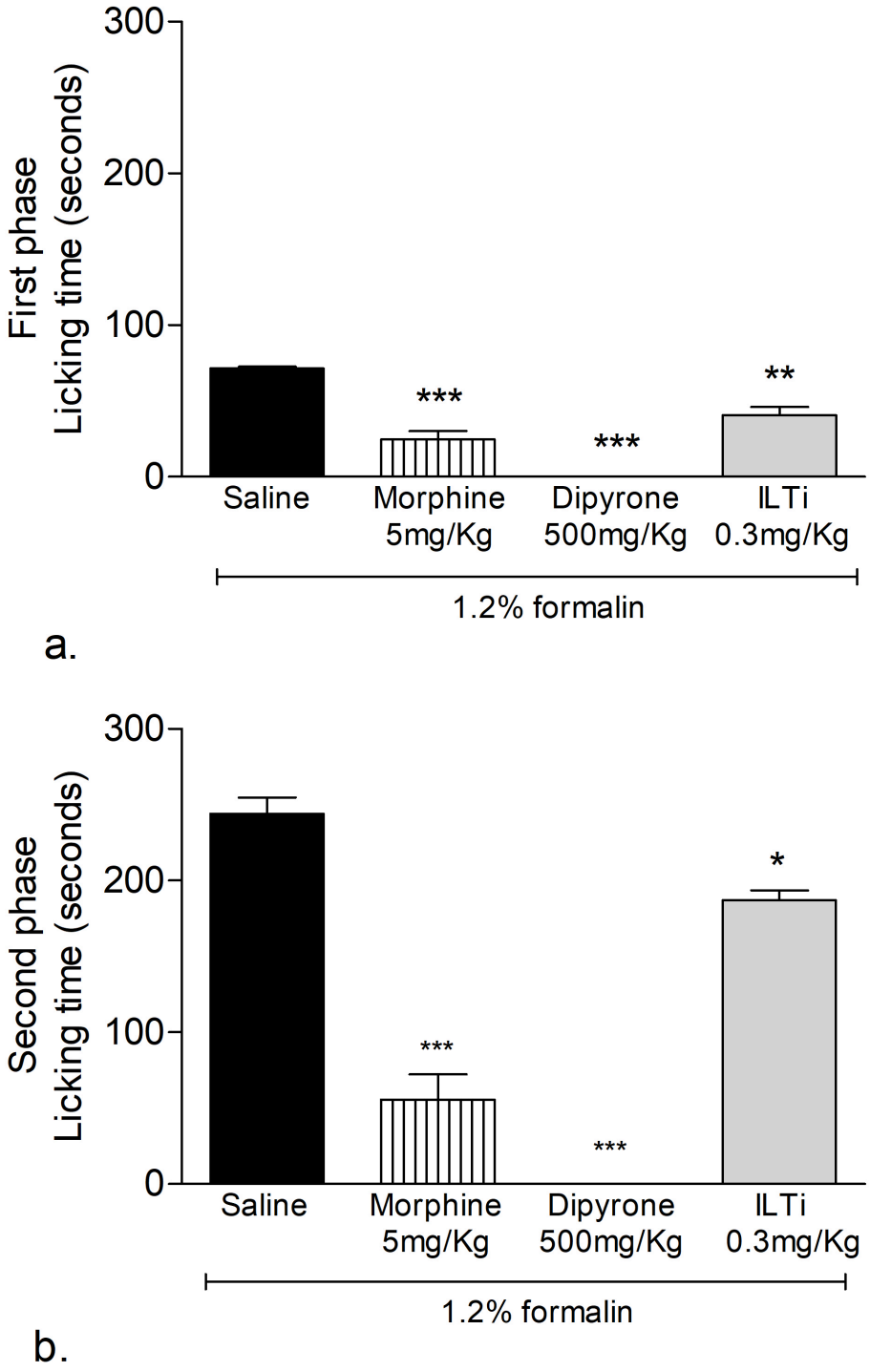

Figure 5

Formalin induced paw licking after treatment with *Inga laurina* trypsin inhibitor (ILTi) in mice. Animals were tested in the first phase (0 - 5 minutes, a) and second phase (15 - 30 minutes, b). Animals were pretreated (200 µL, s.c.) with saline (0.9% NaCl, vehicle), morphine (5 mg/kg), dipyrone (500 mg/kg) or ILTi (0.3 mg/kg) 30 min before intraplantar injection of formalin (1.2%, 40 µL). ANOVA was performed followed by the Bonferroni test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the saline group.