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Edema and nociception induced by *Philodryas patagoniensis* venom in mice: a pharmacological evaluation with implications for the accident treatment

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Running Title: *Philodryas patagoniensis* venom-induced edema and nociception

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Abbreviations: *Philodryas patagoniensis* venom, PpV; minimum edematogenic dose, MED; EDTA, Ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonylfluoride; FRET, Free Ressonance Energy Transfer; Abz, *o*-aminobenzoic acid; EDDnp, *N*-(2,4-dinitrophenyl)ethylenediamine; standard error of the mean, SEM.

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ABSTRACT

We have investigated the mechanisms involved in the genesis of edema and nociception induced by the *Philodryas patagoniensis* venom (PpV) injected into mice footpad. The PpV induced dose-related edema and nociceptive effects. Pre-treatment of mice with cyclooxygenase inhibitor (indomethacin), but not with cyclooxygenase 2 inhibitor (celecoxib) markedly inhibited both effects. Pre-treatments with H_1 receptor antagonist (promethazine) or with dual histamine-serotonin inhibitor (cyproheptadine) failed in inhibiting both effects. In groups pre-treated with captopril (angiotensin-converting enzyme inhibitor) the edema was unaltered, but nociception was clearly increased, suggesting the participation of kinins in the pathophysiology of the nociception but not of the edema-forming effect of PpV. When PpV was treated with EDTA, the nociception was similar to the one induced by untreated venom, but edema was markedly reduced. We concluded that PpV-induced edema and nociception have cyclooxygenase eicosanoids as main mediators and no participation of vasoactive amines. Kinins seem to participate in nociception but not in edema induced by PpV. Results also suggest that metalloproteinases are the main compounds responsible for the edema, but not for the nociception induced by this venom.

INTRODUCTION

In Brazil, accidents induced by venomous snakes of Viperidae and Elapidae families have significant human and veterinary importance. They are responsible for a high number of accidents in humans, but from 20 to 40% of the snakebite cases are caused by the so-called non-venomous snakes, which belong to the families Boidae and Colubridae (Salomão *et al.*, 2003). The latter is the largest group of snakes with approximately 300 genera and 1850 species (Vidal, 2002). This group was recently divided into five new families, among which is the Dipsadidae Family (Zaher *et al.*, 2009).

Among the colubrid snakes belonging to the Dipsadidae family, the genus *Philodryas* is widespread in all South America, and they are considered to be of medical interest. These snakes produce venom (Duvernoy's gland secretion) with sufficient toxicity to elicit serious lesions at the site of the bite. Although most accidents caused by these snakes do not result in serious consequences, some reports emphasize the importance of their toxins in causing local manifestations such as pain, edema, ecchymosis, hemorrhage, and in some cases necrosis, but no coagulation disturbances (Ribeiro *et al.*, 1999; Prado-Franceschi and Hyslop, 2002 Medeiros *et al.*, 2010)).

Similarly, *Philodryas patagoniensis* venom (PpV) causes local tissue damage as hemorrhage, edema, myonecrosis and dermonecrosis, besides pain (Acosta *et al.*, 2003; Peichoto *et al.*, 2004; Rocha and Furtado, 2007; Medeiros *et al.*, 2010).

The complexity of rear-fanged snake venom composition is reflected in the clinical symptoms of envenomation (Kuch and Mebs, 2002; Peichoto *et al.*, 2012; Weinstein *et al.*, 2014). Metalloproteinases play a critical role in the pathophysiology of colubrid envenomation since they are responsible for the hemorrhagic activity exhibited by many rear-fanged snake venoms (Peichoto *et al.*, 2007; Weldon and Mackessy, 2012). Recent studies have described the purification and characterization of various components of colubrid venoms. Peichoto *et al.* have isolated and characterized a metalloproteinase from the *P. patagoniensis* venom (Peichoto *et al.*, 2007, 2009, 2010). This toxin, named Patagonfibrase, presents alpha-fibrinolytic and

hemorrhagic activities, and can induce hemorrhage and inflammation when injected in mice (Peichoto *et al.*, 2011). These observations and proteomic studies (Weldon and Mackessy, 2010, 2012; Peichoto *et al.*, 2012) have demonstrated that colubrid snake venoms have many proteins in common with the Viperidae venoms, and that severity of envenomation depends on the nature of the venom components (Peichoto *et al.*, 2012).

Similarities between the pathophysiological manifestations of *Bothrops* spp. and *P. patagoniensis* may result in a misidentification of accidents by *Philodryas* as *Bothrops* envenomation (Cardoso and Fan, 1995; França and Málaque, 2003). In many instances, *Bothrops* antivenom was administered (Nishioka and Silveira, 1994; de Araújo and Santos, 1997; Ribeiro *et al.*, 1999), potentially causing adverse effects (Nishioka and Silveira, 1994).

As edema and pain are the main local signs in accidents caused by *Philodryas* snakes, in some cases conservative treatments with antihistamine and anti-inflammatory drugs are employed (Medeiros *et al.*, 2010), but there is no information on the inflammatory mediators involved in these symptoms.

Here, we studied the mechanisms involved in the genesis of the edema and the nociceptive effect induced by the venom of *Philodryas paragoniensis* in mice.

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MATERIAL AND METHODS

Philodryas patagoniensis Venom

Venom from adult specimens of *Philodryas patagoniensis* (90 to 120 cm body length) was obtained after the previous injection of pilocarpine (10 mg/kg) to induce secretion in Duvernoy's glands. The venom was collected with the aid of capillary tubes, lyophilized, and stored at -20 °C (Ferlan *et al.*, 1983). The venom solutions were prepared with sterile physiological saline (0.15 M NaCl) at the moment of use, and the protein content was evaluated by the method of Markwell et al. (Markwell *et al.*, 1978) using bovine serum albumin as standard.

Animals

Male Swiss mice weighing 18 to 22g, supplied by the Central Animal House of the Butantan Institute were used. Animals were maintained and used under strict ethical conditions according to the International Animal Welfare Organization. This study was submitted to the Institutional Animal Care Committee at the Butantan Institute (CEUAIB) and was approved by protocol number 295/06.

Evaluation of edema

Mice (n= 6/ group) were injected into the footpad of one of the hind paws with 50 µL of sterile saline solution (0.15 M NaCl) containing different doses of PpV, and the contralateral paw receives the same volume of saline as control. The paw volumes were evaluated with the aid of a plethysmometer (Letica, Spain) at 15 and 30 minutes and 1h, 2h, 3h, 4h, 6h and 24 hour after the paw injections. The edema was expressed as the difference (%) between the volumes of venom and saline-injected paws. The minimum edematogenic dose (MED) was defined as the

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venom dose able to induce 30% of paw increase (Yamakawa *et al.*, 1976). In experiments of pharmacological modulation, the edema was evaluated only at 30 minutes after the injection of 02 MED of PpV.

Evaluation of nociception

Nociception was assessed after subcutaneous injection of 50 µL of solutions containing saline, 0.5, 1.0, 3.0 or 5.0 µg of PpV into the footpad of the right hind paw of mice (n= 9/ group). The animals were placed in a glass chamber on a reflector surface for the observation of the nociceptive behavior. During 30 minutes of observation, we recorded the time (in seconds) that animals spent in a nociceptive behavior (licking, shaking or lifting the injected paw), according to described by Hunskaar et al. (Hunskaar *et al.*, 1985). In experiments of pharmacological modulation, the nociception was induced with 2.0 µg of PpV.

Treatment of animals

To evaluate the pharmacological modulation of edema and nociception, non-blinded groups from 6 to 9 mice were injected into a hind paw with PpV after the following pre-treatments: (1) dexamethasone (corticosteroid, phospholipase A_2 inhibitor: Hypofarma, Brazil), 1 mg/kg, i.p., 1 h before; (2) indomethacin (cyclooxygenase inhibitor: Fluka Chemie AG, Switzerland), 2 mg/kg, s.c., 30 minutes before; (3) celecoxib (cyclooxygenase 2 inhibitor, Pfizer, Brazil), 5 mg/kg, i.p., 30 minutes before; (4) promethazine (H_1 receptor antagonist: EMS Laboratories, Brazil), 5 mg/kg, i.p., 15 minutes before; (5) cyproheptadine (histamine and serotonin receptors antagonist: Sigma, USA), 2 mg /kg, s.c., 30 minutes before, or (6) captopril (angiotensin-converting enzyme inhibitor, Medley, Brazil), 10 mg/kg, i.p., 2h before. Control groups received only PpV into the paw.

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Effect of EDTA on PpV-induced edema and nociception

The effectiveness of EDTA treatment as a metalloprotease inhibitor was evaluated using a FRET-peptide Abz-RPPGFSPFRQ-EDDnp according to described by Kuniyoshi et al.(Kuniyoshi *et al.*, 2012). Briefly, the PpV activity assay was conducted in a 7.4 pH PBS buffer (final volume 100 μL) containing 50 mM phosphate and 20 mM NaCl , using Corning® 96 well plates, and the peptide substrate in a final concentration of 5 μM. The reactions occurred at 37ºC and were initiated by the addition of 0.5 μg of PpV. There was an incubation period of 30 minutes at room temperature when phenylmethanesulfonylfluoride (1 mM, PMSF), 1,10-phenantroline (5 mM) and EDTA (5 mM) where tested. When necessary, control samples were made in the presence of the same volume of ethanol used in the preparation of inhibitors stock solutions (PMSF and 1,10-phenantroline). The experiments were made in triplicate.

After that, PpV venom was treated with 5 mM of EDTA for 30 minutes at room temperature to procedure *in vivo* experiments. EDTA-treated venom was injected into the hind paw of mice at doses used to evaluate edema or nociception as described above. Control groups were injected into the hind paws with crude venom or with the same concentration of EDTA.

Statistical analysis

Results were presented as mean ± standard error. They were analyzed by one-way ANOVA followed by Bonferroni test or, when appropriated, by Student's *t*-test using the software GraphPad Prism 5.01. Results were considered significant when $p<0.05$.

RESULTS

Evaluation of edema and nociception induced by PpV

The PpV induced an edema of rapid onset, with peak 30 minutes after the venom injection, decreasing after that, and disappearing 24h after the experimental envenomation (Fig. 1A). The edematogenic activity was intense and dose-dependent (Fig. 1B) and the MED was 0.82 µg. A dose of 1.64 µg (2 MED) was used for the study of pharmacological modulation.

This venom also induced a dose-dependent nociception (Fig. 2). A dose of 2.0 µg was used for studying pharmacological modulation.

Pharmacological evaluation of PpV-induced edema and nociception

The edema induced by PpV was significantly inhibited in groups pre-treated with dexamethasone and indomethacin (Fig. 3). In contrast, nociception was significantly inhibited in group pre-treated with indomethacin. In the group treated with captopril the nociception was significantly increased (Fig. 4). In groups pre-treated with celecoxib, promethazine or cyproheptadine edema and nociception were not affected (Fig. 3 and Fig. 4).

Effect of EDTA on PpV-induced edema and nociception

Figure 5 shows that Abz-RPPGFSPFRQ-EDDnp hydrolysis was totally inhibited by both EDTA and 1,10-phenantroline and, thus, indicating a complete inhibition of metalloproteinases presents in the venom. This was unlike the results obtained with PMSF, a serine protease inhibitor. Treatment of PpV with EDTA significantly inhibited its edema-forming activity (Fig. 6A) but did not affect the venom-induced nociception (Fig. 6B). Groups injected only with EDTA did not present significant edema or nociception (not shown).

DISCUSSION

In accidents caused by colubrid snakes, it is frequent the presence of prominent edema, and hyperalgesia (Prado-Franceschi and Hyslop, 2002), as characteristic signs of acute inflammation.

The venom of *Philodryas patagoniensis* causes a rapid inflammatory response, with marked edema and hyperalgesia. These local effects are intense, as those observed in *Bothrops*-induced envenomation, which can cause misinterpretation in the clinical diagnostic, despite the lack of blood coagulation disturbances (Puorto and França, 2003; Medeiros *et al.*, 2010).

Regarding the time-course and intensity of the edematogenic response, our results are in agreement with studies that have shown a peak of activity 30 minutes after the venom injection, with a dose-effect response (Rocha and Furtado, 2007). In fact, the edema induced by PpV reached the maximal intensity faster than in edema induced by some viperid venoms (Chaves *et al.*, 1995; Gonçalves and Mariano, 2000; Barbosa *et al.*, 2003; Al-Asmari and Abdo, 2006).

We also found that the *P. patagoniensis* venom elicits a marked dose-dependent nociceptive response, which is more intense than the described to *P. olfersii* venom (Rocha and Furtado, 2007).

Apart from case reports, epidemiologic studies, and studies on characterization of biological activities of PpV, to the best of our knowledge, this is the first experimental study on the pharmacological evaluation of inflammatory effects induced by this venom. Our results show that derivatives of arachidonic acid are main mediators of edema induced by PpV since treatment with dexamethasone significantly inhibited this effect. Results of treatment with indomethacin and with celecoxib indicate that eicosanoids from the cyclooxygenase pathway, but not from cyclooxygenase 2, participate in this mediation. Arachidonic acid derivatives are major players in the modulation of edema induced by some viperid snake venoms (Perales *et al.*, 1992; Chaves *et al.*, 1995; Gonçalves and Mariano, 2000; Barbosa *et al.*, 2003)

In contrast, results obtained in groups treated with promethazine (H_1 receptor antagonist), cyproheptadine (histamine and serotonin inhibitor), or captopril (angiotensin-

converting enzyme inhibitor) suggest that vasoactive amines and kinins do not play a role in the edema induced by PpV. Other studies have shown that histamine does not participate in the edema induced by *Bothrops jararaca* or *B. asper* venom in mice (Perales *et al.*, 1992; Chaves *et al.*, 1995). However, this mediator can participate of the edema induced by other animal venoms, such as viperid venoms (Al-Asmari and Abdo, 2006; Nascimento *et al.*, 2010; Sebia-Amrane and Laraba-Djebari, 2013), *Lonomia* caterpillars venom (De Castro Bastos *et al.*, 2004), *Potamotrygon motoro* stingray venom (Kimura *et al.*, 2015) or *Echinometra lucunter* sea urchin coelomic fluid (Sciani *et al.*, 2014).

The nociceptive effect of PpV has a pharmacologic modulation distinct from the edema induced by this venom. Besides eicosanoids, the increase of the nociceptive behavior after the inhibition of the angiotensin-converting enzyme by captopril is an indicative of the kinins participation on the modulation of the nociception induced by PpV.

Bradykinin participates in nociception induced by some *Bothrops* venoms (Chacur *et al.*, 2001, 2002), but not in the edema induced by these venoms (Trebien and Calixto, 1989; Chacur *et al.*, 2001). Nevertheless, this mediator participates of edema and nociception induced by *Thalassophryne nattereri* fish venom (Lopes-Ferreira *et al.*, 2004).

The inhibition of FRET substrate hydrolysis by EDTA corroborates that metalloproteinases have a central role in the edematogenic effect, as a significant reduction of this effect in the EDTA-treated PpV was found. This fact agrees with previous studies with an isolated toxin and with proteomic studies showing metalloproteinases as the main class of toxins present in the PpV (Peichoto *et al.*, 2011, 2012).

In contrast, treatment with EDTA did not affect the nociceptive action of the PpV, suggesting the participation of other class of toxins. It is well known that bradykinin is a major mediator of the pain induced by *Bothrops jararaca* venom (Chacur *et al.*, 2002) and that serine proteinases in this venom are the class of toxins responsible for the liberation of this nonapeptide (Serrano and Maroun, 2005; Serrano, 2013). Serine proteinases are present in colubrid venoms (Ching *et al.*, 2006; Peichoto *et al.*, 2012), but until now, there are no

information on kinin-releasing enzymes from colubrid venoms. This issue is under investigation by our group.

In *Bothrops jararaca* venom, metalloproteinases are the main compounds responsible for the inflammatory effect, and serine proteinases do not have consequences in its local inflammatory effect, despite having kininogenase activity (Zychar *et al.*, 2010).

As cited above, some accidents caused by *Philodryas* snakes have had wrong diagnostics of *Bothrops* envenomation, including the use of antivenom (Medeiros *et al.*, 2010). The use of *Bothrops* antivenom to treat cases of *Philodryas* envenomation do not have clinical basis, despite the fact that some of toxins of *Philodryas* venom are immunologically recognized by the *Bothrops* antivenom (Rocha *et al.*, 2006). As specific antivenoms are not available for treatment of colubrid-induced envenomation in Brazil, the knowledge of the pharmacological mediation of the edema and pain induced by PpV can allow a therapeutic strategy. Clinical studies describe the use of antihistamine drugs as supportive treatment of this envenomation (Medeiros *et al.*, 2010), but present results indicate that histamine does not participate in the mediation of edema and nociception induced by PpV. In contrast, our results suggest that treatment with cyclooxygenase inhibitors, such as indomethacin, may be beneficial in the treatment of edema and pain induced by *Philodryas patagoniensis* venom, but further experimental and clinical studies need to be carried out to confirm this observation. Experimental studies have shown that in envenomation-induced by *Bothrops* spp., the use of anti-inflammatory drugs, such as dexamethasone, associated with serum therapy has a beneficial effect, reducing faster the inflammatory edema and avoiding muscle damage (Araújo *et al.*, 2007; Patrão-Neto *et al.*, 2013).

In conclusion, our results show that eicosanoids are the primary mediators of edema and nociception induced by *Philodryas patagoniensis* venom, and suggest that nociception may also be mediated of kinins.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Lopes; Rocha; Portaro; and Gonçalves

Conducted experiments: Lopes; Rocha; and Kunyioshi

Performed data analysis: Lopes; Portaro; and Gonçalves.

Wrote or contributed to the writing of the manuscript: Lopes; Rocha; Kunyioshi; Portaro; and Gonçalves.

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Footnote

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Figure Legends

Fig. 1. Edema-forming activity of *Philodryas patagoniensis* venom. Time course of edema induced by 1.0 µg/50 µL of PpV in mice footpad (A) and effect of different doses of PpV on edema evaluated at 30 min after the injection (B). Edema (%) was expressed as mean ± SEM of six animals, analyzed by One-way ANOVA and Bonferroni post-test. (*) Statistically different from saline induced edema.

Fig. 2. Nociceptive effect of the *Philodryas patagoniensis* venom. Effect of different doses of PpV in 50 µL injected in footpad of mice. Nociceptive behavior, represented as the licking, shaking or lifting time in seconds, was expressed as mean ± SEM of nine animals for each dose, analyzed by One-way ANOVA and Bonferroni post-test. (*) Statistically different from saline induced nociception.

Fig. 3. Effect of the pre-treatment with different drugs on the paw edema induced by *Philodryas patagoniensis* venom. The edema was evaluated 30 min after the injection of 1.64 µg/50 µL of PpV in the footpad of mice. Edema (%) was expressed as mean ± SEM of six animals, analyzed by One-way ANOVA and Bonferroni post-test. (*) Result statistically lower ($p<0.05$) than the control untreated PpV injected group.

Fig. 4. Effect of the pre-treatment with different drugs on the nociceptive behavior induced by *Philodryas patagoniensis* venom. The nociceptive behavior induced by the injection of 2.0 µg/50 µL of PpV was expressed as mean ± SEM of the licking time (seconds) of nine animals, analyzed by One-way ANOVA and Bonferroni post-test. (*) Results statistically lower or (#) result statistically higher ($p<0.05$) than the control untreated PpV injected group.

Fig. 5. Hydrolysis of FRET substrate by *Philodryas patagoniensis* venom and the effect of classical inhibitors of metalloproteases and serine proteases. Inhibition effect of EDTA (5 mM),

1,10-phenantroline (5 mM) and PMSF (1 mM) upon the hydrolysis of Abz-RPPGFSPFRQ-EDDnp by *Philodryas patagoniensis* venom (0.5 µg). The results were expressed as mean ± SEM of triplicates.

Fig. 6. Effect of treatment of the *Philodryas patagoniensis* venom with EDTA on its edematogenic and nociceptive effect. Venom (1.64 and 2.0 µg/ 50 µL) treated with 5 mM of EDTA were injected into the footpad of mice for evaluation of edema (A) and nociception (B). Results were expressed as mean ± SEM, analyzed by Student's t-test. (*) Statistically different ($p<0.05$).

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Fig 1.

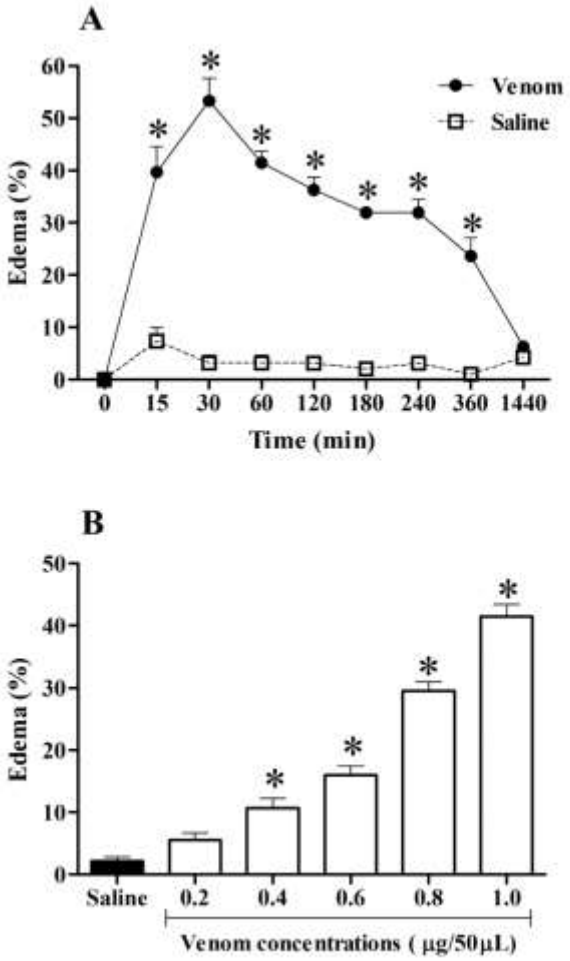

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Fig 2.

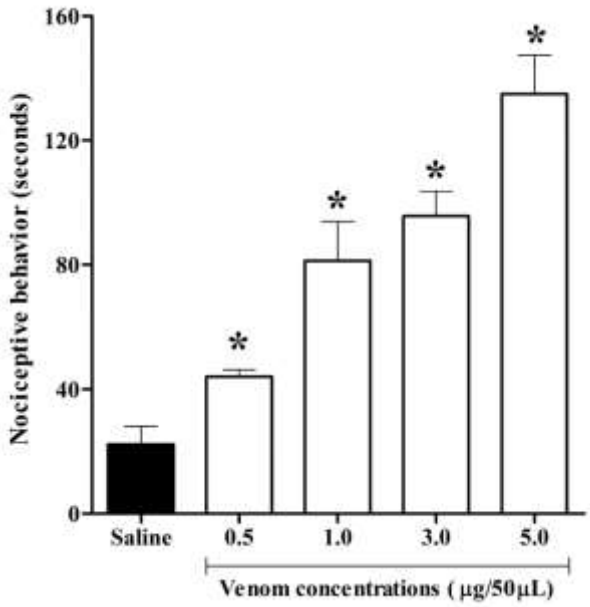

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Fig 3.

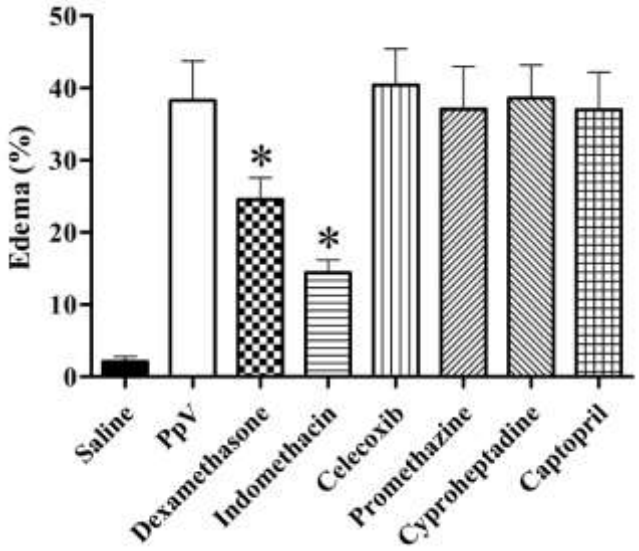

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Fig 4.

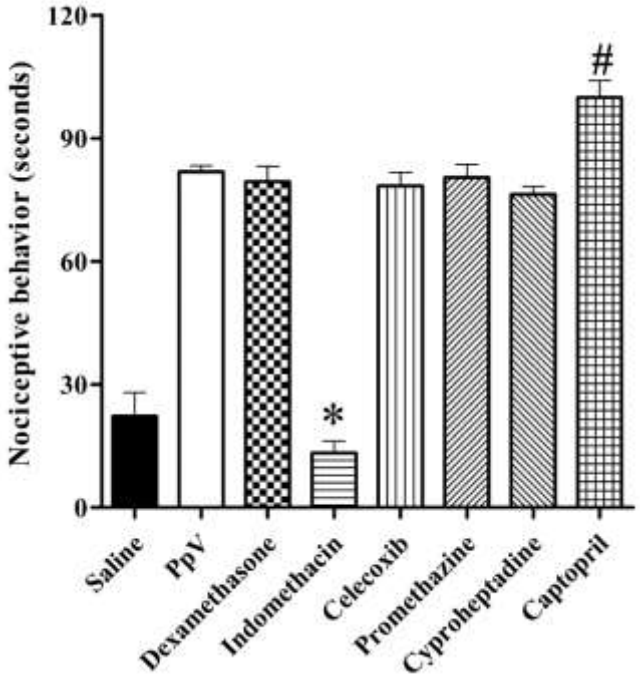

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Fig 5.

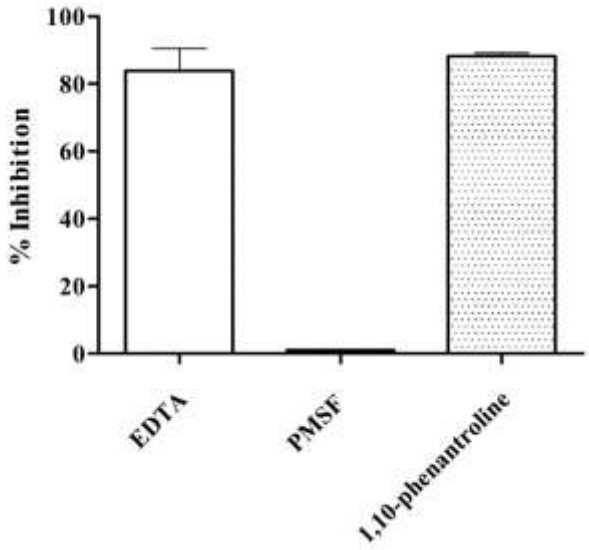

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Fig 6.