

*Full Length Research Paper*

## Involvement of pro-inflammatory cytokines and nociceptive pathways on the pharmacological activity of hydantoin derivative 5-(4-isopropylphenyl)-3-phenyl-imidazolidine-2,4-dione

Fabiola Lélis de Carvalho<sup>1</sup>, Diogo Vilar da Fonsêca<sup>1</sup>, Antônia Rosangela Soares Penha<sup>1</sup>, Mirian Graciela da Silva Stiebbe Salvadori<sup>1</sup>, Paula Regina Rodrigues Salgado<sup>1</sup>, Charlane Kelly Souto Pereira<sup>1</sup>, Fagner Carvalho Leite<sup>2</sup>, Márcia Regina Piuvezam<sup>1,2</sup>, Severino Araújo de Sousa<sup>3</sup>, Petrônio Filgueiras de Athayde-Filho<sup>3</sup>, Liana Clébia de Moraes Pordeus<sup>1</sup> and Reinaldo Nóbrega de Almeida<sup>1\*</sup>

<sup>1</sup>Laboratório de Psicofarmacologia, Centro de Ciências da Saúde, Universidade Federal da Paraíba (UFPB), João Pessoa, PB, 58051-900, Brazil.

<sup>2</sup>Laboratório de Imunologia, Centro de Ciências da Saúde, UFPB, João Pessoa, PB, 58051-900, Brazil.

<sup>3</sup>Laboratório de Química Orgânica, Departamento de Química, UFPB, João Pessoa, PB, 58051-900, Brazil.

Received 8 September, 2016; Accepted 19 September, 2016

Hydantoin is often reported as potent anticonvulsant drug; however, recent studies have highlighted their antinociceptive potential. Based on these reports, this study investigated the antinociceptive and anti-inflammatory activities of the hydantoin derivative 5-(4-isopropylphenyl)-3-phenyl-imidazolidine-2,4-dione (IM-7) using animal models. Treated mice submitted to the acetic acid-induced writhing test showed increase ( $p < 0.01$  or  $p < 0.001$ ) in the latency to the first writhing and reduction in the number of abdominal writhing ( $p < 0.001$ ). Furthermore, all doses reduced the nociceptive response in the first ( $p < 0.05$  or  $p < 0.001$ ) and second ( $p < 0.001$ ) phases of the formalin test. This effect was inhibited by pretreatment with the antagonists naloxone, sulphuride and caffeine. Additionally, IM-7 (75, 150 and 300 mg/kg, i.p.) reduced ( $p < 0.05$  or  $p < 0.001$ ) the glutamate-induced nociceptive response. Carrageenan-induced paw edema was strongly reduced following treatment with all doses of IM-7 ( $p < 0.05$ ,  $p < 0.01$  or  $p < 0.001$ ), and so were leukocyte migration and levels of interleukin- $1\beta$  (300 mg/kg:  $p < 0.001$ ) and tumor necrosis factor- $\alpha$  (300 mg/kg:  $p < 0.01$ ) in carrageenan-induced peritonitis. Therefore, IM-7 decreased the nociceptive response via a mechanism involving the opioid, dopaminergic, and adenosinergic receptors. Its anti-inflammatory action also contributed by decreasing the release of pro-inflammatory cytokines.

**Key words:** Pain, inflammation, IM-7, nociception.

### INTRODUCTION

Pain is the symptom that most often leads patients to seek medical care (Rief et al., 2012). It currently affects about 100 million Americans, generating an estimated

economic impact of 560 to 635 billion dollars per year (Dubois and Follett, 2014). Although there is an extensive therapeutic arsenal available for the treatment of pain, it

is accompanied by a range of undesirable side effects. Some examples include the gastric irritation that can ensue as a result of non-steroidal anti-inflammatory drug (NSAID) usage or opioid tolerance. This issue is, therefore, one of the factors that motivates research into the development of new drugs for pain relief.

It has been reported previously that anticonvulsants may be useful in some painful conditions such as trigeminal neuralgia and diabetic neuropathy (Bhattacharya et al., 2009). Phenytoin, for example, blocks presynaptic voltage-gated sodium channels, thereby inhibiting the pathological release of excitatory neurotransmitters, such as glutamate, which are directly involved in nociceptive pathways (Sakaue et al., 2004; Webb and Kamali, 1998). This drug belongs to the class of drugs known as the hydantoins, which are compounds with a chemical structure containing a derivative of the imidazolidine ring. These substances have been extensively studied, both in regards to their chemical structure and their pharmacological properties. Some of these properties include acting as a muscle relaxant, antiarrhythmic, antibacterial, antitumor and, particularly, anticonvulsant (Oliveira et al., 2008).

While studies investigating the anticonvulsant activity of hydantoins are common in the scientific literature, especially because of the established efficacy of phenytoin against partial and generalized tonic-clonic seizures, increasing efforts have been undertaken to characterize the antinociceptive profile of new hydantoins. Behavioral studies have demonstrated that hydantoins are able to reduce nociceptive behavior and inflammation in animal models (Guerra et al., 2011; Sudo et al., 2010; Sztanke et al., 2005; Walls et al., 2012). These results were similar to those observed by our research group in mice subjected to the acetic acid-induced writhing test, formalin and hot plate tests, and carrageenan-induced peritonitis after treatment with the hydantoin derivatives IM-3 and HPA-05 (Maia, 2013; Queiroz et al., 2015; Salgado, 2011).

Studies concerning the antinociceptive effects of hydantoins are still scarce; however, there is a strong indicator of the potential of these substances. Thus, in this study the antinociceptive and anti-inflammatory properties of the hydantoin derivative 5-(4-isopropylphenyl)-3-phenyl-imidazolidine-2,4-dione (IM-7) were investigated in mice.

## MATERIALS AND METHODS

### Hydantoin derivative IM-7

The synthetic compound IM-7 was provided by Prof. Dr. Petrônio Filgueiras de Athayde Filho (Organic Synthesis Laboratory, Federal

University of Paraíba, Brazil). Synthesis was performed as previously described (de Sousa et al., 2009).

### Animals

Male Swiss mice (25 to 35 g, approximately 3 months old) were obtained from the vivarium of Prof. Dr. Thomas George (UFPB), where they were monitored and kept at a constant temperature ( $21 \pm 1^\circ\text{C}$ ), exposed to light/dark cycles of 12 h each (light phase from 0600 to 1800), and given water and food ad libitum. Animals were used only once and euthanized at the end of each experiment. All procedures were approved by the Animal Experimentation Ethics Committee (CEPA/UFPB/BRAZIL, protocol number 0601/11).

### Drugs and reagents

The following materials were used: Acetic acid (Reagen, Brazil); cytokines assay kits (e-Bioscience, USA); atropine, bicuculline, caffeine, carrageenan, dexamethasone, glibenclamide, glutamate, sulpiride and yohimbine (Sigma, USA); formaldehyde P.A. (Vetec, Brasil); morphine hydrochloride (Merck, USA) and naloxone hydrochloride (Research Biochemical, USA). The substances were solubilized in distilled water (LTF/UFPB, Brazil) and administered intraperitoneally (i.p.) at 0.1 mL per 10 g of body weight (except formaline, glutamate and carrageenan: 20  $\mu\text{L}$ , via intraplantar; and naloxone and dexamethasone used subcutaneously). IM-7, glibenclamide and sulpiride were dissolved in Tween 80 (Vetec, Brazil) and distilled water.

### Evaluation of the antinociceptive activity of IM-7

#### Acetic acid-induced writhing test

The injection of 0.85% acetic acid into the peritoneal cavity of mice triggers a nociceptive response characterized by abdominal contortions followed by stretching of the hind limbs (Koster et al., 1959). Mice were distributed into groups ( $n=8$ ), and treated with a vehicle (3.4% v/v Tween 80), morphine (10 mg/kg), or IM-7 at doses of 75, 150, and 300 mg/kg. After 30 min (morphine treatment group) or 60 min (vehicle and IM-7 treatment groups), the animals were administered an intraperitoneal (i.p.) injection of 0.85% acetic acid (0.1 mg/mL) and were observed for 15 min. The latency to the first writhing and the number of abdominal contortions that occurred in the interval of 5 to 15 min after the administration of acetic acid were recorded. A decrease in the number of contortions was considered indicative of an antinociceptive effect.

#### Formalin test

This methodology, described by Dubuisson and Dennis (1977) and adapted by Hunskaar et al. (1985) for mice, involves inducing nociception by the subcutaneous (s.c.) injection of formalin 2.5% (20  $\mu\text{L}$ ) into the hind paws of mice. Animals respond to this stimulus by licking the injected paw, and the time spent in this activity is recorded in 2 phases. The first phase (0 to 5 min) occurs due to the direct stimulation of nociceptors, while the second phase (15 to 30 min) involves the participation of inflammatory mediators. Mice were distributed into groups ( $n=8$ ), and injected with a vehicle (3.4% v/v

\*Corresponding author. E-mail: reinaldoan@uol.com.br. Tel: +55 83 32167175. Fax: +55 83 32167804.

Author(s) agree that this article remain permanently open access under the terms of the [Creative Commons Attribution License 4.0 International License](https://creativecommons.org/licenses/by/4.0/)

solution of Tween 80), morphine (10 mg/kg) or IM-7 (75, 150, and 300 mg/kg). An intraplantar (i.pl.) injection of formalin 2.5% (20  $\mu$ L) was administered into the right hind paw of the mice 30 min (morphine group) or 60 min (vehicle and IM-7 groups) after treatment. The times spent in the 2 phases were recorded.

### Glutamate-induced nociception

Glutamate is the major excitatory neurotransmitter involved in nociceptive signal transmission (Beirith et al., 2002). In order to evaluate the influence of IM-7 on the glutamatergic system, mice were treated with an i.p. injection of a vehicle (3.4% v/v solution of Tween 80) or IM-7 (75, 150, or 300 mg/kg). After 60 min, 20  $\mu$ L of

glutamate solution (20  $\mu$ mol/paw) was injected into the right hind paw of the mice. Animals were immediately placed into individual observation boxes and the nociceptive response (paw licking time) was recorded for 15 min.

### Mechanism of action

To demonstrate the possible mechanism of action involved in the IM-7-induced antinociceptive effect we used pharmacological tools and formalin. Briefly, 15 min prior to treatment with IM-7 (75 mg/kg, i.p.), pattern drugs were administered to the mice. These included naloxone (5 mg/kg, s.c.), an opioid receptor antagonist; glibenclamide (10 mg/kg, i.p.), an ATP-sensitive potassium channel ( $K_{ATP}$ ) blocker; sulpiride (20 mg/kg, i.p.), a D2 dopamine receptor antagonist; yohimbine (0.15 mg/kg, i.p.), an  $\alpha_2$ -adrenergic receptor antagonist; atropine (5 mg/kg, i.p.) a muscarinic receptor antagonist; bicuculline (1 mg/kg, i.p.) a gamma amino butyric acid-A receptor ( $GABA_A$ ) antagonist; and caffeine (10 mg/kg, i.p.), an adenosine receptor antagonist.

### Evaluation of the anti-inflammatory activity of IM-7

#### Carrageenan-induced paw edema

The injection of carrageenan elicits an inflammatory reaction characterized by edema, erythema, and hyperalgesia, which can be reversed by steroid and non-steroid anti-inflammatory agents (Winter et al., 1962). Mice were distributed into groups (n=8) and treated as follows: negative control group, 3.4% v/v Tween 80, i.p.; positive control group 3.4% v/v Tween 80, i.p. and IM-7 (75 mg/kg, i.p.); IM-7 (150 mg/kg, i.p.); IM-7 (300 mg/kg, i.p.); and dexamethasone (2 mg/kg, s.c.). Mice received 20  $\mu$ L of a 1% solution of carrageenan in the subplantar region of the right hind paw and 20  $\mu$ L of saline into the left hind paw 60 min after treatment. The negative control group received saline in both paws (data not shown). Hind paw thickness was recorded for each mouse with a digital micrometer just before carrageenan injection (baseline measurement, data not shown), and then after 1, 2, 3, 4, 5, 24, 48, and 72 h. Edema was calculated as the difference between right and left hind paw thickness.

#### Carrageenan-induced peritonitis

Carrageenan-induced peritonitis was used to determine if IM-7 could inhibit cell migration that results from the animal's exposure to an acute inflammatory stimulus. Mice (n=6) were distributed into groups and treated with a vehicle (3.4% v/v Tween 80); IM-7 at 75, 150, or 300 mg/kg i.p.; or dexamethasone (2 mg/g, s.c.). After 60 min, the inflammatory process was induced by an i.p. injection of 1% carrageenan. Four hours later, animals were euthanized and

the peritoneal exudate was removed after washing the peritoneal cavity with 3 mL of phosphate buffered saline (PBS). The peritoneal exudate was centrifuged at 1500 rpm for 5 min at 4°C, and the supernatant was stored at -20°C for further measurement of the pro-inflammatory cytokines. Precipitated cells were diluted with Turk's solution (1:10) to determine the total cell count in a Neubauer chamber under an optical microscope.

### Differential cell counts in the peritoneal exudate

For each animal, the peritoneal exudate was diluted 1:10 in PBS and 100  $\mu$ L of this cell suspension was cytocentrifuged (Citopin, Bio Research) at 1500 rpm for 15 min to prepare slides. The slides were then stained with May-Grunwald Giemsa to allow differential cell counts of polymorphonuclear and mononuclear leukocytes using an optical microscope.

### Pro-inflammatory cytokine assay

Tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-1 $\beta$  levels in the peritoneal fluid were assessed using specific cytokine ELISA assay kits and a microplate reader set at 450 nm (VERSAmix Tunable, Molecular Devices). The protocol was performed according to the manufacturer's instructions. The levels of cytokines were calculated from the standard curves and expressed as the total amount per milliliter (pg/ml).

### Statistical analysis

Differences between the means of the groups were evaluated by one-way analysis of variance (ANOVA), followed by Dunnett's test or Tukey's multiple comparisons test depending on the case. Results were expressed as the mean  $\pm$  standard error of mean (SEM) and considered significant when  $p < 0.05$ .

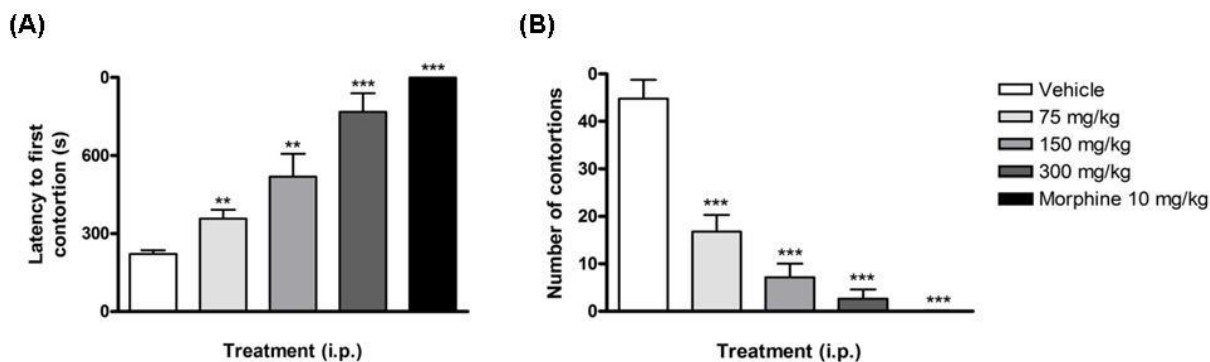
## RESULTS

### Effects of IM-7 on the acetic acid-induced writhing test

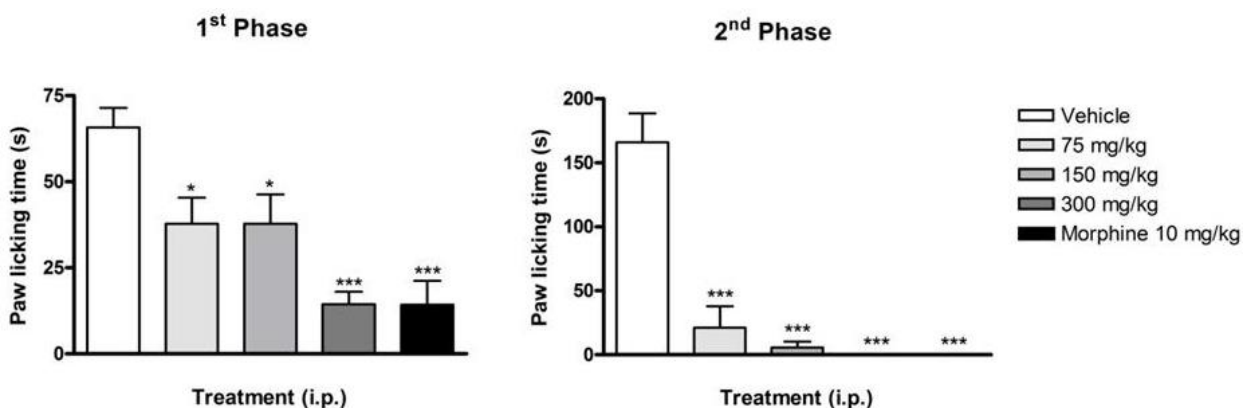
The latency to the first writhing was significantly increased in mice treated with doses of 75 mg/kg (357.4  $\pm$  34.6 s;  $p < 0.05$ ), 150 mg/kg (518.5  $\pm$  87.9 s;  $p < 0.01$ ) and 300 mg/kg (767.9  $\pm$  71.2 s;  $p < 0.001$ ) of IM-7 when compared to the control group (221.3  $\pm$  15.0 s) (Figure 1A). The number of contortions decreased in animals treated with IM-7. While the vehicle-treated group showed an average of 44.8  $\pm$  4.0 contortions, the groups treated with doses of 75, 150, and 300 mg/kg IM-7 exhibited 16.8  $\pm$  3.5, 7.1  $\pm$  2.9, and 2.6  $\pm$  2.0 contortions during the 10 min of observation, respectively (Figure 1B). Animals treated with morphine (10 mg/kg, i.p.) exhibited no abdominal contortions.

### Effects of IM-7 on formalin-induced nociception

According to data shown in Figure 2A, in the first phase of this test all doses of IM-7 (75, 150, and 300 mg/kg, i.p.)



**Figure 1.** Figure (A): the effect of IM-7 on the latency to the first contortion in acetic acid-induced writhing test. Figure (B): the effect of IM-7 on the number of contortions in acetic acid-induced writhing test. Each column represents mean  $\pm$  S.E.M. ANOVA followed by Dunnett's test. \*\* $p < 0.01$ , \*\*\* $p < 0.001$  versus control group.



**Figure 2.** Effect of IM-7 on the licking response induced by intraplantar injection of formalin in the first (neurogenic) and in the second (inflammatory) phases of formalin test. Each column represents mean  $\pm$  S.E.M. ANOVA followed by Dunnett's test. \* $p < 0.05$ , \*\*\* $p < 0.001$  versus control group.

were able to reduce the paw licking time ( $37.8 \pm 7.6$  s,  $37.8 \pm 8.6$  s, and  $14.4 \pm 3.6$  s, respectively) when compared to the control group ( $65.8 \pm 5.7$  s). A similar reduction was observed with morphine ( $14.3 \pm 6.9$  s). In the second phase, the paw licking time also decreased significantly ( $p < 0.001$ ) at all tested doses of IM-7 when compared to the control group ( $165.9 \pm 22.7$  s). The obtained values for mice treated with doses of 75 and 150 mg/kg were  $21.1 \pm 16.8$  and  $5.5 \pm 4.7$  s, respectively. Animals that received morphine or IM-7 at the dose of 300 mg/kg did not lick their paws in this phase (Figure 2B).

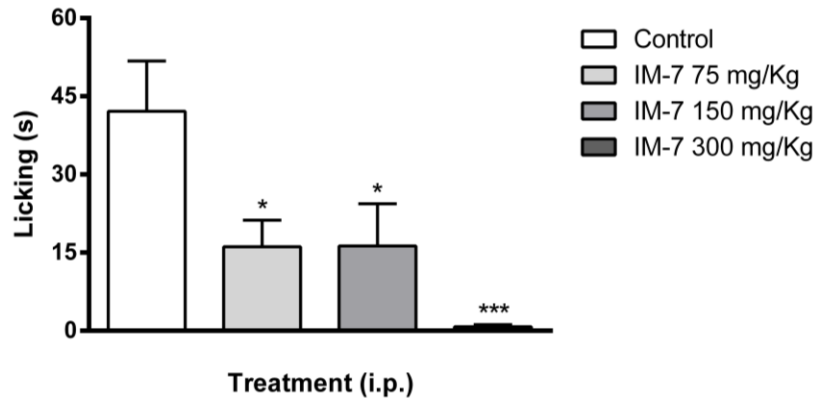
#### Effect of IM-7 on glutamate-induced nociception

At all tested doses, IM-7 (75 mg/kg,  $16.1 \pm 5.1$  s; 150 mg/kg,  $16.3 \pm 8.1$  s; 300 mg/kg,  $0.75 \pm 0.4$  s) significantly inhibited nociceptive behavior induced following

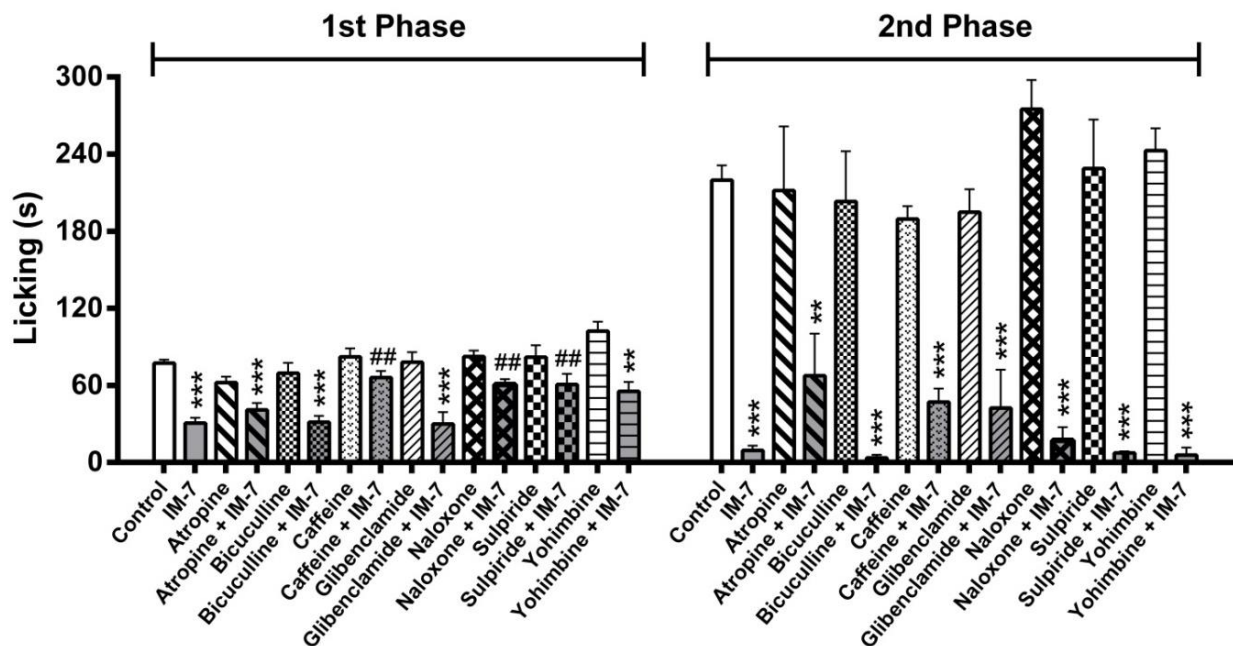
intraplantar injection of glutamate in mice, when compared to the vehicle-treated group ( $42.1 \pm 9.7$  s,  $p < 0.05$ , Figure 3).

#### Investigation of the mechanism of action of IM-7

The pretreatment of mice with naloxone (5 mg/kg, s.c., opioid receptor antagonist), sulpiride (20 mg/kg, i.p., D2 dopamine receptor antagonist) or caffeine (10 mg/kg, i.p., adenosine receptor antagonist) 15 min prior to the administration of IM-7 (75 mg/kg, i.p.) significantly inhibited the antinociceptive response induced by IM-7 in the first phase of the formalin test (Figure 4). However, the pretreatment with glibenclamide (10 mg/kg, i.p.,  $K_{ATP}$  channel blocker), atropine (5 mg/kg, i.p., muscarinic receptors antagonist), yohimbine (0.15 mg/kg, i.p.,  $\alpha_2$ -adrenergic receptor antagonist) or bicuculline (1 mg/kg, i.p., GABA<sub>A</sub> receptor antagonist) did not alter the effect



**Figure 3.** Effect of IM-7 on the licking response induced by intraplantar injection of glutamate in mice. Each column represents mean  $\pm$  S.E.M. ANOVA followed by Dunnett's test. \* $p < 0.05$ , \*\*\* $p < 0.001$  versus control group.



**Figure 4.** Effect of pretreatment of mice with atropine (5 mg/kg, i.p.), bicuculline (1 mg/kg, i.p.), caffeine (10 mg/kg, i.p.), glibenclamide (10 mg/kg, i.p.), naloxone (5 mg/kg, s.c.), sulpiride (20 mg/kg, i.p.) and yohimbine (0.15 mg/kg, i.p.) on the antinociceptive behavior induced by IM-7 in the formalin test. Animals were pretreated with antagonists 15 min prior treatment with IM-7. Each column represents mean  $\pm$  S.E.M. ANOVA followed by Dunnett's test. \*\* $p < 0.01$ , \*\*\* $p < 0.001$  versus vehicle-treated group; ### $p < 0.01$  when comparing antagonist+IM-7 group with IM-7 group.

of IM-7 in this test (Figure 4).

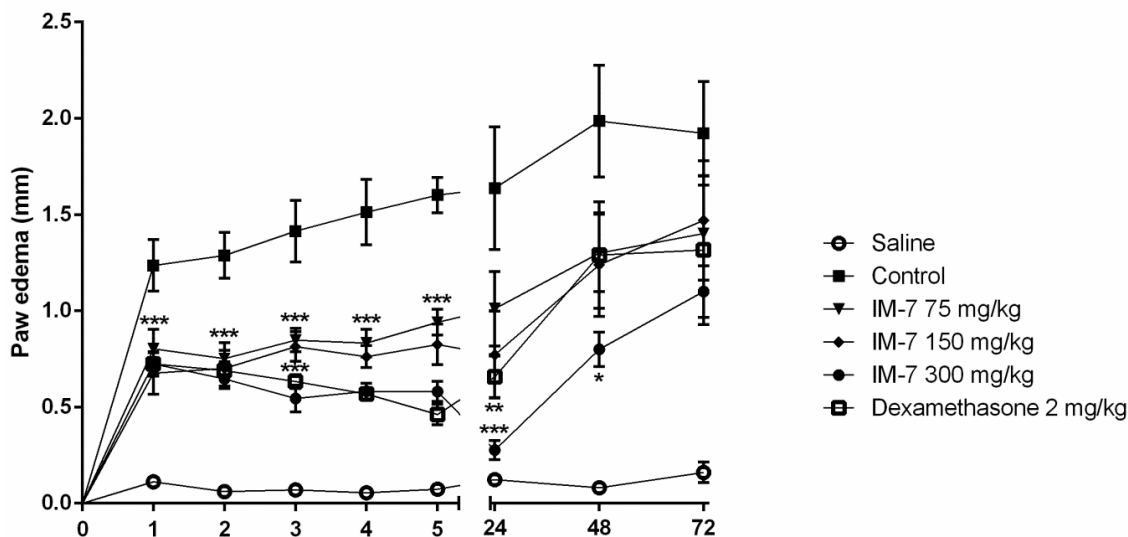
#### Effect of IM-7 on carrageenan-induced paw edema

As shown in Figure 5, the administration of IM-7 (i.p.) significantly inhibited carrageenan-induced paw edema from the first hour after carrageenan injection until the fifth hour of observation at all tested doses ( $p < 0.05$ ). This

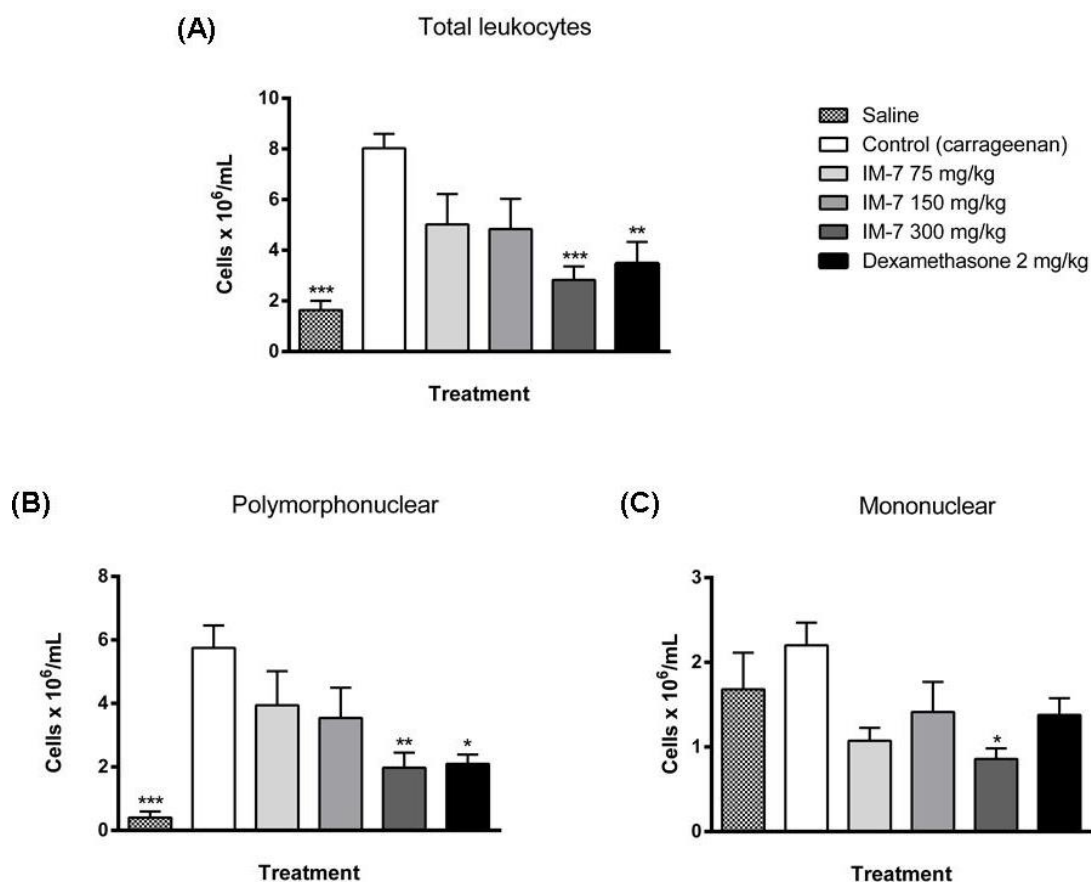
effect persisted until 24 h at a dose of 150 mg/kg, and until 48 h in mice treated with 300 mg/kg.

#### Effect of IM-7 on carrageenan-induced cellular influx into the peritoneal cavity

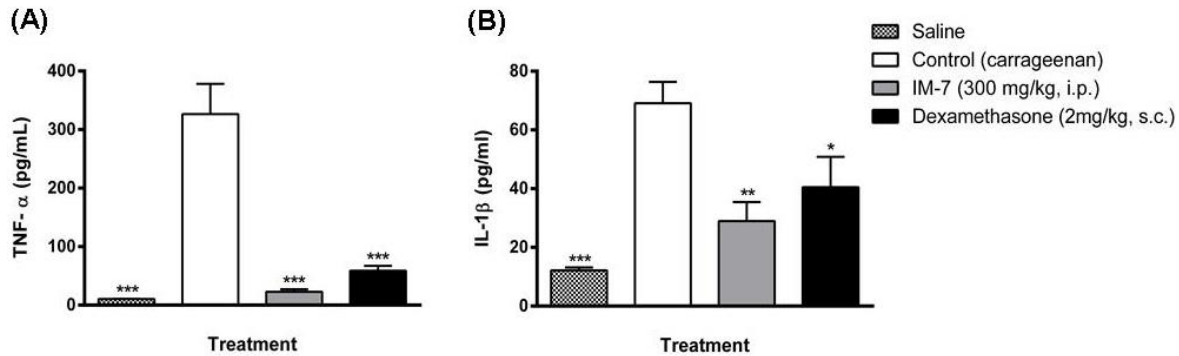
As shown in Figure 6, leukocyte migration induced by intraperitoneal carrageenan administration ( $8.0 \pm 0.6 \times$



**Figure 5.** Effect of IM-7 on carrageenan-induced paw edema in mice. Results are shown as mean  $\pm$  S.E.M., and the % of inhibition when compared to control group is shown in parenthesis ANOVA one-way followed by Tukey's multiple comparisons test. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  versus vehicle-treated group.



**Figure 6.** Effect of intraperitoneal treatment of mice with IM-7 on carrageenan-induced inflammatory cells influx into peritoneal cavity. (A) Total leukocyte, (B) Polymorphonuclear cells, (C) Mononuclear cells. Each column represents mean  $\pm$  S.E.M. ANOVA followed by Dunnett's test. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  versus control group.



**Figure 7.** Effect of IM-7 on the levels of the pro-inflammatory cytokines TNF- $\alpha$  (A) and IL-1 $\beta$  (B) in the carrageenan-induced peritonitis model in mice. Each column represents mean  $\pm$  S.E.M. ANOVA followed by Dunnett's test. \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001 control group.

$10^6$  cells/mL) was significantly reduced following treatment with 300 mg/kg of IM-7 ( $2.8 \pm 0.5 \times 10^6$  cells/mL;  $p < 0.001$ ). This effect seems to be due to the reduction of the influx of both polymorphonuclear (control,  $5.8 \pm 0.7 \times 10^6$  cells/mL; IM-7 300 mg/kg,  $2.0 \pm 0.5 \times 10^6$  cells/mL;  $p < 0.01$ ) and mononuclear (control,  $2.2 \pm 0.3 \times 10^6$  cells/mL; IM-7 300 mg/kg,  $0.9 \pm 0.1 \times 10^6$  cells/mL;  $p < 0.05$ ) cells into the peritoneal cavity. Treatment with doses of 75 and 150 mg/kg of IM-7 did not show significant decreases in cellular migration in this model.

#### Effect of IM-7 on pro-inflammatory cytokine levels in the peritoneal fluid

Figure 7 shows that intraperitoneal injections of carrageenan significantly increased the amount of the pro-inflammatory cytokines TNF- $\alpha$  ( $326.4 \pm 51.7$  pg/mL) and IL-1 $\beta$  ( $69.1 \pm 7.2$  pg/mL) in peritoneal exudate when compared to the saline group ( $10.6 \pm 0.3$  pg/mL and  $12.1 \pm 1.0$  pg/mL, respectively). Treatment with 300 mg/kg of IM-7 significantly reduced the TNF- $\alpha$  level (93% decrease;  $p < 0.001$ ) when compared to the control group. At the same dose, IM-7 also reduced the IL-1 $\beta$  level (58% decrease;  $p < 0.01$ ) in the peritoneal exudate. Dexamethasone (2 mg/kg), a well-known anti-inflammatory drug, reduced both TNF- $\alpha$  (82% decrease;  $p < 0.001$ ) and IL-1 $\beta$  (41% decrease;  $p < 0.05$ ) levels.

#### DISCUSSION

In this study, the antinociceptive and anti-inflammatory effects of the hydantoin derivative, IM-7 in mice were investigated. It was observed that i.p. treatment of mice with IM-7 to decrease the number of abdominal contortions induced by the intraperitoneal injection of 0.85% acetic acid. The injection of i.p. acetic acid enhances the release of several pro-inflammatory mediators such as prostaglandin (PG) $E_2$ , PGF $_{2\alpha}$ , TNF- $\alpha$ ,

IL-1 $\beta$ , and IL-8 (Deraedt et al., 1980; Ribeiro et al., 2000). Additionally, it also enhances the release of the excitatory neurotransmitters, aspartate and glutamate, in the cerebrospinal fluid by sensitizing and stimulating primary afferent neurons (Gu et al., 2013).

Despite its high sensitivity to centrally and peripherally acting drugs, the acetic acid-induced writhing test has poor specificity. This is due to substances lacking analgesic effects, like sedatives, antidepressants, antihistamines, muscle relaxants, and neuroleptic agents, being able to inhibit abdominal writhing and, therefore, producing false-positive results (Le Bars et al., 2001). On the other hand, the formalin test, a widely used model of persistent pain, is more selective and permits the differentiation between 2 types of antinociceptive mechanisms: neurogenic and anti-inflammatory (Hunskar et al., 1985, 1987).

The antinociceptive effect of IM-7 was also detected by the formalin test, since all tested doses significantly reduced the paw licking response in both phases, in similarity to morphine. Centrally acting drugs such as opioids inhibit both phases of the formalin test, while peripherally acting drugs, such as NSAIDs and corticosteroids, only inhibit the second phase (Hunskar et al., 1987). Thus, these results demonstrated that IM-7 exerts effects both at the central and peripheral level, by interfering with direct nociceptive and inflammatory mediators or receptors. Glutamate is the major excitatory neurotransmitter involved in nociceptive signal transmission. Beirith et al. (2002) demonstrated that the intraplantar injection of glutamate into the mouse paw produces a nociceptive response (licking) and paw edema. These effects seem to be mediated by both ionotropic and metabotropic glutamate receptors and involve peripheral, spinal, and supraspinal sites. When given intraperitoneally, IM-7 significantly inhibits the nociceptive response induced by the peripheral injection of glutamate. To further characterize the underlying mechanisms of the antinociception induced by IM-7, a series of antagonists/blockers of known pathways

involved in pain regulation were employed. The antinociceptive response induced by IM-7 in the formalin test was significantly inhibited by the pretreatment of mice with naloxone, caffeine, or sulphuric acid. This supports the proposal that opioid, adenosine, and D2 dopamine receptors play a role in the mechanism of action of IM-7, since their activation reduce the transmission of nociceptive impulse. Pretreatment with atropine, yohimbine, bicuculline, or glibenclamide did not modify the antinociceptive effect induced by IM-7, therefore, eliminating the participation of muscarinic,  $\alpha_2$ -adrenergic, and GABA<sub>A</sub> receptors, as well as K<sub>ATP</sub> channels. However, Sudo et al., (2010) demonstrated the involvement of  $\alpha_{2A}$ -adrenergic receptors in the antinociceptive response evoked by the hydantoin derivative PT-31 in mice. In their study, pretreatment with yohimbine or BRL 44408 (selective  $\alpha_{2A}$ -adrenergic receptor antagonists) significantly reduced the effect of PT-31. These results explain only the central antinociceptive mechanism of IM-7, since all antagonists/blockers failed to inhibit the effect of this hydantoin on the second phase of the formalin test. This could be explained by the involvement of anti-inflammatory mechanisms on the antinociceptive activity of IM-7.

Edema formation resulting from the increase in vascular permeability and vasodilatation of the arterioles (Szolcsányi, 1988) allows sensory neurons to release active mediators in the inflammatory process. These substances released by stimulated neurons are considered the neurogenic component of inflammation mediation (Fantini et al., 1995; Maggi and Meli, 1988). This means that the formation of edema is the major consequence of neurogenic inflammation (Bon et al., 2013). The obtained results demonstrated the significant inhibition of paw edema post-carrageenan injection by IM-7 at different times. One of the mechanisms by which this can happen is through the direct blocking of a receptor that contributes to the edema formation, such as transient receptor potential ankyrin 1 (Moilanen et al., 2012), a non-selective cation channel whose activation contributes to the increased excitability of nociceptors and neurogenic inflammation (Story et al., 2003). It is noteworthy that the inflammatory response induced by carrageenan in this model is biphasic. The first phase lasts approximately 6 h and is mediated by the release of histamine, serotonin, and kinins. The late phase involves the release of prostaglandins and is characterized by more pronounced edema that starts approximately 24 h after administration of carrageenan, with peak at 72 h (Henriques et al., 1987; Shibata et al., 1989). All the doses of IM-7 inhibited paw edema in the first 5 h of observation. Furthermore, treatment with doses of 150 mg/kg and 300 mg/kg of IM-7 inhibited paw edema by 53 and 83%, respectively, 24 h after the administration of carrageenan. After 48 h, the inhibitory effect of the 300 mg/kg dose could still be detected, with an inhibition of

60%. However, after 72 h, there was no significant inhibition resulting from IM-7 treatment at the tested doses. According to these results, it seems that IM-7 can inhibit the inflammatory process by reducing the release of mediators involved in both phases of carrageenan-induced paw edema.

Guerra et al. (2011) observed that some hydantoins reduce leukocyte migration and inhibit TNF- $\alpha$  and IL-1 $\beta$  release in air pouch and peritonitis models. At least in part, these anti-inflammatory effects may be attributed to the selective and potent inhibition of the TNF- $\alpha$  converting enzyme (TACE), responsible for the activation of TNF- $\alpha$  (Sheppeck et al., 2007; Yu et al., 2010) or inhibition of the nuclear factor (NF)-kappaB pathway (Wang et al., 2015).

In this study, IM-7 also reduced leukocyte migration and inhibited TNF- $\alpha$  and IL-1 $\beta$  release induced by carrageenan in a peritonitis model; therefore, reinforcing the anti-inflammatory activity of this hydantoin.

## Conclusion

The results of this study strongly suggest that the acute intraperitoneal injection of the hydantoin derivative IM-7 in mice induces a dose-dependent antinociceptive response. This response is probably mediated by its interaction with opioid, dopaminergic, and adenosinergic receptors, along with its anti-inflammatory activity resulting in reduced leukocyte migration and TNF- $\alpha$  and IL1- $\beta$  release.

## Conflicts of Interests

The authors have not declared any conflict of interestS.

## ACKNOWLEDGMENTS

The authors wish to thank Mr. José Crispim Duarte for his technical assistance. This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

## REFERENCES

- Bhattacharya A, Wickenden AD, Chaplan SR (2009). Sodium channel blockers for the treatment of neuropathic pain. *Neurother.* 6(4):663-678.
- Beirith A, Santos AR, Calixto JB (2002). Mechanisms underlying the nociception and paw oedema caused by injection of glutamate into the mouse paw. *Brain Res.* 924(2):219-228.
- Deraedt R, Jouquey S, Delevallée F, Flahaut M (1980). Release of prostaglandins E and F in an allogenic reaction and its inhibition. *Eur. J. Pharmacol.* 61(1):17-24.
- Dubois MY, Follett KA (2014). Pain medicine: The case for an independent medical specialty and training programs. *Acad. Med.* 89(6):863-868.
- Dubuisson D, Dennis SG (1977). The formalin test: A quantitative study



- of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. *Pain*. 4(2):161-174.
- Fantini F, Magnoni C, Pincelli C, Giannetti A (1995). Neurogenic inflammation and the skin: Neural modulation of cutaneous inflammatory reactions. *Eur. J. Dermatol*. 5:349-357.
- Gu WW, Ao GZ, Zhu YM, Sun SC, Zhou Q, Fan JH, Nobuhiko K, Ishidoh K, Zhang HL, Gao XM (2013). Autophagy and cathepsin L are involved in the antinociceptive effect of DMBC in a mouse acetic acid-writhing model. *Acta Pharmacol. Sin*. 34(8):1007-1012.
- Guerra AS, Malta DJ, Laranjeira LP, Maia MB, Colaço NC, de Lima Mdo C, Galdino SL, Pitta Ida R, Gonçalves-Silva T (2011). Anti-inflammatory and antinociceptive activities of indole-imidazolidine derivatives. *Int. Immunopharmacol*. 11(11):1816-1822.
- Henriques MG, Silva PM, Martins MA, Flores CA, Cunha FQ, Assreuy-Filho J, Cordeiro RS (1987). Mouse paw edema. A new model for inflammation? *Braz. J. Med. Biol. Res*. 20(2):243-249.
- Hunskar S, Fasmer OB, Hole K (1985). Formalin test in mice, a useful technique for evaluating mild analgesics. *J. Neurosci. Methods*. 14(1):69-76.
- Hunskar S, Hole K (1987). The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. *Pain*. 30(1):103-114.
- Koster R, Anderson M, De Beer EJ (1959). Acetic acid for analgesic screening. *Fed. Proc*. 18:412-418.
- Le Bars D, Gozariu M, Cadden SW (2001). Animal models of nociception. *Pharmacol. Rev*. 53(4):597-652.
- Maggi CA, Meli A (1988). The sensory-efferent function of capsaicin-sensitive sensory neurons. *Gen. Pharmacol*. 19(1):1-43.
- Maia AKHL (2013). Estudos psicofarmacológicos pré-clínicos do 3-fenil-5-(4-metilfenil)-imidazolidin-2,4-diona (HPA-05) em camundongos. Tese (Pós-Graduação em Produtos Naturais e Sintéticos Bioativos). Universidade Federal da Paraíba, João Pessoa.
- Moilanen LJ, Laavola M, Kukkonen M, Korhonen R, Leppänen T, Högestätt ED, Zygmunt PM, Nieminen RM, Moilanen E (2012). TRPA1 contributes to the acute inflammatory response and mediates carrageenan-induced paw edema in the mouse. *Sci. Rep*. 2:380.
- Oliveira SM, Silva JBP, Hernandez MZ, Alves de Lima MC, Galdino, SL, Pitta IR (2008). Structure, reactivity, and biological properties of hydantoins. *Quim. Nova*. 31(3):614-622.
- Queiroz RB, de Carvalho FL, Fonsêca DV, Barbosa-Filho JM, Salgado PR, Paulo LL, de Queiroz AB, Pordeus LC, Souza SA, Souza HD, Lira BF, de Athayde-Filho PF (2015). Antinociceptive effect of hydantoin 3-phenyl-5-(4-ethylphenyl)-imidazolidine-2,4-dione in mice. *Molecules*. 20:974-986.
- Ribeiro RA, Vale ML, Thomazzi SM, Paschoalato AB, Poole S, Ferreira SH, Cunha FQ (2000). Involvement of resident macrophages and mast cells in the writhing nociceptive response induced by zymosan and acetic acid in mice. *Eur. J. Pharmacol*. 387(1):111-118.
- Rief W, Kaasa S, Jensen R, Perrot S, Vlaeyen JW, Treede RD, Vissers KC (2012). New proposals for the International Classification of Diseases-11 revision of pain diagnoses. *J. Pain*. 13(4):305-316.
- Salgado PRR (2011). Avaliação da atividade antinociceptiva central de HPA-05: um derivado imidazolidínico. Dissertação (Pós-Graduação em Produtos Naturais e Sintéticos Bioativos). Universidade Federal da Paraíba, João Pessoa.
- Sakaue A, Honda M, Tanabe M, Ono H (2004). Antinociceptive effects of sodium channel-blocking agents on acute pain in mice. *J. Pharmacol. Sci*. 95(2):181-188.
- Sheppeck JE, Gilmore JL, Tebben A, Xue CB, Liu RQ, Decicco CP, Duan JJ (2007). Hydantoins, triazolones, and imidazolones as selective non-hydroxamate inhibitors of tumor necrosis factor-alpha converting enzyme (TACE). *Bioorg Med Chem Lett*. 17(10):2769-2774.
- Sheppeck JE, Gilmore JL, Yang A, Chen XT, Xue CB, Roderick J, Liu RQ, Covington MB, Decicco CP, Duan JJ (2007). Discovery of novel hydantoins as selective non-hydroxamate inhibitors of tumor necrosis factor-alpha converting enzyme (TACE). *Bioorg. Med. Chem. Lett*. 17(5):1413-1417.
- Shibata M, Ohkubo T, Takahashi H, Inoki R (1989). Modified formalin test: characteristic biphasic pain response. *Pain* 38(3):347-352.
- de Sousa Luis JA, Barbosa Filho JM, Freitas Lira B, Almeida Medeiros I, Soares Lima de Moraes LC, Dos Santos AF, Soares de Oliveira C, De Athayde-Filho PF (2009). Synthesis of new imidazolidin-2,4-dione and 2-thioxoimidazolidin-4-ones via C-phenylglycine derivatives. *Molecules*. 15(1):128-137.
- Story GM, Peier AM, Reeve AJ, Eid SR, Mosbacher J, Hricik TR, Earley TJ, Hergarden AC, Andersson DA, Hwang SW, McIntyre P, Jegla T, Bevan S, Patapoutian A (2003). ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. *Cell*. 112(6):819-829.
- Sudo RT, Calasans-Maia JA, Galdino SL, Lima MC, Zapata-Sudo G, Hernandez MZ, Pitta IR (2010). Interaction of morphine with a new alpha 2-adrenoceptor agonist in mice. *J. Pain* 11(1):71-78.
- Szolcsányi J (1988). Antidromic vasodilatation and neurogenic inflammation. *Agents Actions*. 23(1-2):4-11.
- Szatanke K, Fidecka S, Kedzierska E, Karczmarzyk Z, Pihlaja K, Matosiuk D (2005). Antinociceptive activity of new imidazolidine carbonyl derivatives. Part 4. Synthesis and pharmacological activity of 8-aryl-3,4-dioxo-2H,8H-6,7-dihydroimidazo[2,1-c] [1,2,4]triazines. *Eur. J. Med. Chem*. 40(2):127-134.
- Walls TH, Grindrod SC, Beraud D, Zhang L, Baheti AR, Dakshanamurthy S, Patel MK, Brown ML, MacArthur LH (2012). Synthesis and biological evaluation of a fluorescent analog of phenytoin as a potential inhibitor of neuropathic pain and imaging agent. *Bioorg. Med. Chem*. 20(17):5269-5276.
- Wang Y, Qin Z, Shen S, Xiang N, Liu J, Lin X, Bai Z, Wu Z (2015). A novel fibrinogenase from *Agkistrodon acutus* venom protects against LPS-induced endotoxemia via regulating NF-κB pathway. *Immunopharmacol. Immunotoxicol*. 37(5):413-420.
- Webb J, Kamali F (1998). Analgesic effects of lamotrigine and phenytoin on cold-induced pain: a crossover placebo-controlled study in healthy volunteers. *Pain* 76(3):357-363.
- Winter CA, Risley EA, Nuss GW (1962). Carrageenin-induced edema in hind paw of the rat as an assay for antiinflammatory drugs. *Proc. Soc. Exp. Biol. Med*. 111:544-547.
- Yu W, Guo Z, Orth P, Madison V, Chen L, Dai C, Feltz RJ, Girijavallabhan VM, Kim SH, Kozlowski JA, Lavey BJ, Li D, Lundell D, Niu X, Piwinski JJ, Popovici-Muller J, Rizvi R, Rosner KE, Shankar BB, Shih NY, Siddiqui MA, Sun J, Tong L, Umland S, Wong MK, Yang DY, Zhou G (2010). Discovery and SAR of hydantoin TACE inhibitors. *Bioorg. Med. Chem. Lett*. 20(6):1877-1880.