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MARIA ALICE MIRANDA BEZERRA MEDEIROS

AVALIAÇÃO DOS EFEITOS ANTINOCICEPTIVO E ANTI-INFLAMATÓRIO DE HIDRAZONAS OBTIDAS POR SÍNTESE

Feira de Santana, BA
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Tese apresentada ao Programa de Pós-graduação em Biotecnologia, da Universidade Estadual de Feira de Santana como requisito parcial para obtenção do título de Doutor em Biotecnologia.

Orientador: Prof. Dr. Jackson Roberto Guedes da Silva Almeida

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RESUMO

A presente tese de doutorado está dividida em dois capítulos. O primeiro capítulo trata-se da avaliação dos efeitos antinociceptivo e anti-inflamatório de derivados hidrazonas e seu possível mecanismo de ação. Foram realizados vários testes, dentre eles o de contorções abdominais induzidas por ácido acético e o da formalina (utilizando bloqueadores farmacológicos para investigação do mecanismo de ação). Foi observado que todas as hidrazonas possuem efeito antinociceptivo e atuam perifericamente. H5 apresentou melhor potencial antinociceptivo, inibiu 78,92% (20 mg/Kg) e 100% (40 mg/Kg) da nocicepção na segunda fase do teste da formalina. A via de sinalização de H5 tem envolvimento dos sistemas opioide e nitrérgico, além do efeito anti-inflamatório nos testes de migração de leucócitos na cavidade peritoneal, edema de pata induzido por carragenina e edema de pata induzido por histamina. O docking demonstrou que H5 inibe COX-2 de maneira semelhante ao meloxicam. Além disso, H5 apresentou um perfil farmacocinético adequado. Portanto, é um forte candidato a fármaco antinociceptivo e anti-inflamatório. O segundo capítulo envolve o efeito antinociceptivo de LASSBio-2012. A atividade antinociceptiva foi avaliada por meio dos testes de contorções abdominais induzidas por ácido acético e da formalina (utilizando bloqueadores farmacológicos). LASSBio-2012 exibiu potencial antinociceptvo perifericamente, na segunda fase do teste da formalina inibiu 50,38% e 65% (20 mg/Kg e 40 mg/Kg). Seu mecanismo de ação demonstrou possível envolvimento do sistema opioide. No docking foi observada uma interação com os receptores delta e mu opioides. Seu perfil farmacocinético foi moderado. Portanto, LASSBio-2012 é um candidato a novo fármaco antinociceptivo.

Palavras-chave: Efeito antinociceptivo, efeito anti-inflamatório, hidrazona, *N*-acilhidrazona, dor, nocicepção.

ABSTRACT

This doctoral thesis is divided into two chapters. The first chapter deals with the evaluation of the antinociceptive and anti-inflammatory effects of hydrazone derivatives and their possible mechanism of action. Several tests were carried out, among them the abdominal contortions induced by acetic acid and the formalin test (using pharmacological blockers to investigate the mechanism of action). It was observed that all hydrazones have an antinociceptive effect and act peripherally. H5 showed better antinociceptive potential, inhibiting 78.92% (20 mg/kg) and 100% (40 mg/kg) of nociception in the second phase of the formalin test. The H5 signaling pathway involves the opioid and nitrergic systems, in addition to the anti-inflammatory effect in leukocyte migration tests in the peritoneal cavity, carrageenan-induced paw edema, and histamine-induced paw edema. Docking demonstrated that H5 inhibits COX-2 in a similar way to meloxicam. In addition, H5 had an adequate pharmacokinetic profile. Therefore, it is a strong candidate asan antinociceptive and anti-inflammatory drug. The second chapter involves the antinociceptive effect of LASSBio-2012. Antinociceptive activity was assessedthrough abdominal contortion tests induced by acetic acid and formalin (using pharmacological blockers). LASSBio-2012 exhibited antinociceptive potential peripherally.In the second phase of the formalin test, it inhibited 50.38% and 65.00% (20 mg/kg and 40 mg/kg, respectively). Its mechanism of action demonstrated a possible involvement of the opioid system. After docking, interactions with delta and mu-opioid receptors were observed and its pharmacokinetic profile was moderate. Therefore, LASSBio-2012 is a candidate for a new antinociceptive drug.

Keywords: antinociceptive effect, anti-inflammatory effect, hydrazones, *N*-acylhydrazone, pain, nociception.

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INTRODUÇÃO GERAL

A dor é uma experiência sensitiva e emocional desagradável associada a uma lesão tecidual real ou potencial, ou descrita nos termos de tal lesão, de acordo com a IASP (RAJA, 2020). A dor não ocorre apenas por uma estimulação sensorial anormal, que promove uma sensação indesejável, mas sim por um conjunto do reconhecimento do desconforto somático acompanhado de uma resposta emocional. A percepção da dor modifica-se de acordo com a experiência anterior, antecedentes culturais e fatores situacionais. Normalmente, a dor aguda ocorre devido a uma lesão tecidual, enquanto a dor crônica ocorre por uma lesão irreversível ou doença, como a fibromialgia (WANG; MULLALLY, 2019; MAXWELL; FRAGA; MALAVOLTA, 2019).

Dor não é equivalente a nocicepção, são conceitos distintos. A sensação dolorosa é decorrente da interação entre acontecimentos biológicos, psicológicos e sociais, cada fenômeno interferindo um ao outro. Já nocicepção, possui apenas o componente biológico, disponibiliza informações ao cérebro através de elementos sensoriais e cognitivos. A nocicepção é composta pelas seguintes etapas: transdução (transformação do sinal sensorial em nervoso), condução (encaminhamento da informação ao sistema nervoso central - SNC), transmissão (deslocamento da informação via sinapse), percepção (fenômeno cerebral da sensação dolorosa) e modulação (regulação da nocicepção e percepção) (RISCH; POINTEAU; POQUET, 2017).

A nocicepção é amplamente estudada há mais de cinquenta anos, entretanto, permanece como um elemento bastante complexo, não linear. A percepção é influenciada pela nocicepção devido suas dispersas projeções cerebrais e seus diversos elementos sensitivo-discriminatórios, afetivomotivacionais e cognitivos. Contudo, a nocicepção também pode ser influenciada pelas vias modulatórias (RISCH; POINTEAU; POQUET, 2017).

A nocicepção é um mecanismo protetor e sensorial que, consente aos animais, perceber e impedir a ocorrência de danos teciduais graves. Este mecanismo necessita dos nociceptores, que são neurônios especializados em detectar e responder esses sinais. Os nociceptores sintetizam moléculas com o intuito de identificar e informar a presença de lesão. Estes neurônios são instigados por temperaturas intensas (frio ou calor), estímulos mecânicos ríspidos e diversos estímulos químicos (TRACEY, 2017).

A dor inflamatória se dá pela sensibilização dos neurônios nociceptivos sensoriais primários (CUNHA *et* al., 2005). O processo inflamatório é um mecanismo fisiopatológico

que ocorre devido uma infecção ou lesão, representado por diversas reações locais e sistêmicas do organismo (SILVA, 2009).

A inflamação é uma defesa do organismo em relação a estímulos endógenos ou exógenos. Este evento é composto por diversos episódios nos vasos sanguíneos, como dilatação vascular, o que gera vermelhidão na região afetada, migração leucocitária, edema, sensibilização de nociceptores terminais, estímulo da síntese e liberação de fatores pró-inflamatórios, tais como cininas, óxido nítrico (NO), fator de necrose tumoral (TNF-α), e interleucinas (por exemplo, IL-1β e IL-6) (MEDZHITOV, 2010; KUMAR *et* al., 2013; SÁ *et* al., 2014; OLIVEIRA-TINTINO *et* al., 2018).

O tratamento da dor é realizado com analgésicos, que podem atuar centralmente ou perifericamente. A ação analgésica central pode ser mediada através da inibição dos receptores da dor central, enquanto o efeito analgésico periférico pode ser mediado através da inibição da cicloxigenase (COX) e/ou lipoxigenase (LOX), além de outros mediadores inflamatórios (CHAVAN *et* al., 2010). Os analgésicos de ação central, opioides, como morfina e codeína, incluem fármacos capazes de aliviar a dor por atuarem em receptores de superfície celular denominados de receptores opioides (QUINTÃO *et* al., 2011). Entretanto, o uso destes é limitado devido seus efeitos secundários, potencial de dependência e desenvolvimento de tolerância (MORGAN; CHRISTIE, 2011; SILVA, 2013).

O tratamento da dor e da inflamação também pode ser realizado através dos anti-inflamatórios, que podem ser classificados como anti-inflamatórios esteroidais (AIEs) e anti-inflamatórios não esteroidais (AINEs) (ABDELLATIF *et* al., 2015). Os AIEs são medicamentos bastante utilizados pelas suas conhecidas propriedades anti-inflamatórias e imunossupressoras, no tratamento de várias doenças (PATRICIO et al., 2006). Entretanto, seus efeitos terapêuticos como anti-inflamatório e imunossupressor são, constantemente, acompanhados por graves efeitos colaterais, como diabetes mellitus, úlcera péptica, síndrome de Cushing com supressão do eixo hipotálamo-hipófise-adrenal, osteoporose, atrofia cutânea, psicose, glaucoma, entre outras, ficando seu uso limitado por estes efeitos colaterais (SILVA, 2013).

Já os AINEs possuem seu efeito devido à inibição da enzima COX (1,2 ou 3), portanto, a administração a longo prazo, mesmo em doses profiláticas, pode levar a efeitos secundários gastrintestinais (desde gastrite, úlceras até perfurações e sangramentos) (ABDELLATIF *et* al., 2015). Determinados AINEs possuem especificidade parcial para a COX-2, por exemplo, o meloxicam. Enquanto outros são totalmente específicos e inibem apenas a COX-2, sem intervenção na atividade da COX-1, que são denominados de coxibes,

por exemplo, o celecoxibe (SILVA, 2013). Alguns coxibes apresentaram efeitos colaterais e adversos no sistema cardiovascular, entretanto, o celecoxibe não foi associado ao aumento da incidência de eventos cardiovasculares quando comparado ao placebo e aos AINEs não seletivos, então ainda é um medicamento altamente comercializado (ABDELLATIF et al., 2015).

Os AINEs são bem aceitos por alguns pacientes, entretanto, os fármacos não seletivos podem promover efeitos colaterias e adversos graves, como citado anteriormente (OLIVEIRA-TINTINO *et* al., 2018). Portanto, é necessária a síntese de fármacos antinociceptivos e anti-inflamatórios, que possuem o mesmo efeito dos AINEs, entretanto, com uma probabilidade muito pequena de efeitos colaterais indesejáveis no trato gastrintestinal (ABDELLATIF *et* al., 2015).

A estratégia de modificação estrutural de fármacos já existentes para a descoberta de novos compostos é amplamente utilizada na indústria farmacêutica. Isto proporciona, muitas vezes, a obtenção mais acelerada de fármacos com propriedades farmacocinéticas e farmacodinâmicas talvez mais desejáveis que a molécula protótipo. Em certos casos, com este tipo de alteração química também é possível obter derivados com atividades farmacológicas distintas dos fármacos utilizados como modelo (BARREIRO, 2009).

As hidrazonas constituem uma classe versátil de compostos orgânicos, sendo usadas como intermediários sintéticos, na química de heterociclos, na identificação de compostos carbonílicos e como complexantes de cátions metálicos (AL-THIB, 2016). Os derivados hidrazônicos são utilizados na agricultura, indústria e, principalmente, no setor farmacêutico. Em 1883, Emil Fischer descobriu esta classe quando sintetizava um derivado indólico a partir da reação entre fenil-hidrazina com propanona (CAIXEIRO, 2007). Desde então, essas substâncias foram sintetizadas em larga escala devido à sua ação no tratamento de diversas doenças. Os derivados hidrazônicos possuem um amplo espectro de propriedades farmacológicas (HUSSAIN, 2017), o que proporciona a síntese destes compostos e enorme interesse farmacêutico (SILVA, 2015). Desde 1980, pesquisas científicas relatam que compostos com a porção hidrazona em sua estrutura podem atuar como analgésico e anti-inflamatório (AZIZIAN, 2016; BARREIRO *et* al, 2002; BISPO-JÚNIOR *et* al., 2011; SILVA, 2015), antioxidante e citoprotetor (BELKHEIRI, 2010; VANUCCI-BACQUÉ 2014), anti-hipertensivo (BAKALE, 2014), leishmanicida (CHARRETE, 2011), antitumoral (JAGER, GUDE, ARIAS-PÉREZ, 2018), antiviral (TIAN et al., 2011), antibacteriano (GU et al, 2012; JAGER, GUDE, ARIAS-PÉREZ, 2018), e antifúngico (CACHIBA et al., 2012; JAGER, GUDE, ARIAS-PÉREZ, 2018).

Do ponto de vista químico, as hidrazonas compõem uma classe de compostos com estrutura geral: R1R2C=N–NH–R3, sendo consideradas bases de Schiff (AL-THIB, 2016). São substâncias obtidas normalmente pela condensação de hidrazinas com cetonas ou aldeídos, sendo produtos de derivatização clássica de compostos carbonilados (BARREIRO, 2002; GUIMARÃES, 2017).

Por tais razões, o desenvolvimento de novas estruturas moleculares contendo este grupo funcional e a subsequente avaliação de atividades biológicas de interesse constituem etapas iniciais e que consideramos importantes para o desenvolvimento de novos protótipos candidatos a fármacos.

OBJETIVOS

OBJETIVO GERAL

Avaliar o efeito antinociceptivo de 06 hidrazonas obtidas por síntese, bem como o possível mecanismo de ação e o efeito anti-inflamatório do composto mais promissor em camundongos, caracterizando ainda propriedades físico-químicas, ADMET e análise de docking molecular através de métodos *in silico*.

OBJETIVOS ESPECÍFICOS

- ✓ Avaliar o efeito antinociceptivo das hidrazonas H1, H2, H3, H4, H5, e da *N*-acilhidrazona LASSBio-2012 através do teste de contorções abdominais induzidas por ácido acético e do teste da formalina.
- ✓ Investigar o possível mecanismo de ação antinociceptivo da hidrazona H5 e da *N*-acilhidrazona LASSBio-2012 através do teste da formalina utilizando os seguintes bloqueadores farmacológicos: naloxona, Nω-nitro-L-arginina (L-NAME), ondansetrona, atropina, glibenclamida e vermelho de rutênio.
- ✓ Investigar o efeito anti-inflamatório da hidrazona H5 através dos testes de migração de leucócitos na cavidade peritoneal, edema de pata induzido por carragenina, e edema de pata induzido por histamina.
- ✓ Investigar a influência da hidrazona H5 sobre a coordenação motora através do teste Rota-Rod.
- ✓ Investigar a toxicidade da hidrazona H5 através do teste de toxicidade com *Artemia salina*.
- ✓ Investigar as propriedades físico-químicas e o perfil ADMET da hidrazona H5 e da *N*-acilhidrazona LASSBio-2012, *in silico*, em comparação com os anti-inflamatórios indometacina e meloxicam, através do programa ACD/Percepta.
- ✓ Realizar a análise molecular através do docking da Hidrazona H5 e da *N*-acilhidrazona LASSBio-2012.

CAPÍTULO 1

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Antinociceptive and anti-inflammatory effects of hydrazone derivatives and their possible mechanism of action in mice

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Abstract

Pain and inflammation are an unpleasant experience usually resulting from actual or imminent tissue damage. Despite the number of existing analgesic drugs, side-adverse effects limit their use, stimulating the search for new therapeutic alternatives. Therefore, hydrazones derivatives are a class of organic compounds with the general structure $R_1R_2C=NNR_3R_4$. Five hydrazone derivatives (H1, H2, H3, H4, and H5) were synthesized with molecular modification strategies. In this paper, we describe the ability of hydrazone derivatives to attenuate nociceptive behavior and the inflammatory response in mice. Antinociceptive activity was evaluated through acetic acid-induced writhing and formalin-induced nociception tests. In these experimental models, the hydrazone with the greatest potential (H5) significantly ($p<0.05$) reduced nociceptive behavior. Several methods of acute and chronic inflammation induced by different chemical (carrageenan and histamine) to evaluate the anti-inflammatory effect of H5. In addition, our group performed molecular docking studies, indicating that H5 can block the COX-2 enzyme, reducing arachidonic acid metabolism and consequently decreasing the production of prostaglandins, which are important inflammatory mediators. H5 also changes locomotor activity. In summary, H5 exhibited relevant antinociceptive and anti-inflammatory potential and acted on several targets, making it a candidate for a new multi-target oral anti-inflammatory drug.

Keywords: Hydrazone; antinociceptive activity; anti-inflammatory activity; pain; inflammation.

Introduction

Pain is an unpleasant sensory and emotional experience thatusually occurs as a result of actual or imminent tissue damage. This important public health problem causes disability, suffering, and is associated with increased anxiety [1]. Pharmacological treatment of pain initially includes non-opioids, followed by opioids, and finally, if necessary, adjuvants like anticonvulsants and antidepressants. However, despite the number of existing analgesic drugs, side-adverse effects limit their use, stimulating the search for new therapeutic alternatives [2].

Hydrazones derivatives are a class of organic compounds with the general structure $R_1R_2C=NNR_3R_4$ [3], being considered Schiff bases. They are substances normally obtained by the condensation of hydrazines with ketones or aldehydes, being products of classic derivatization of carbonyl compounds [4,5].

Therefore, seeking to obtain structurally novel compounds, the organic hydrazine used in this work for the synthesis of the desired hydrazones was hydralazine hydrochloride. The use of this drug for the synthesis of new organic compounds and studies of medicinal chemistry has been increasing in scientific publications year by year. They indicate that hydrazine has a privileged chemical structure for the coordination with metallic cations [5], and for its biological activity, mainly due to the presence of the pyridazine heterocycle in its structure [6].

Given this, five hydrazone derivatives (compounds H1, H2, H3, H4, and H5) were prepared based on the combination of hydralazine with α,β-unsaturated carbonyl compounds previously synthesized. Studies have shown that the combination of hydrazones with other functional groups improve its biological properties and provide pharmacologically active molecules [7]. Besides that, a variety of hydrazone derivatives have been developed to minimize gastrointestinal discomfort and toxicity, especially when it comes to analgesic drugs [8].

Thus, this work evaluates the antinociceptive activity of the hydrazone derivatives in different experimental models as well its possible mechanism of action in mice. In addition, we performed docking studies with some of the main targets of the nociceptive and inflammatory processes to better understandtheir interactions on the molecular level.

Materials and methods

Synthetic intermediates i1-i5 (α,β-unsaturated ketones) were prepared using aldolic condensation reaction between aldehydes (a1-a5) and propanone, and using the methodologies described by Murtinho and coworkers [9]. Hydrazone derivatives (H1-H5) were prepared using condensation reaction between carbonyl compounds (i1-i5) and inorganic hydrazine (hydralazine drug), and using the methodologies described by Ananthnag and coworkers [10]. Fig 1 shows the synthetic routeused for the preparation of these hydrazone derivatives.

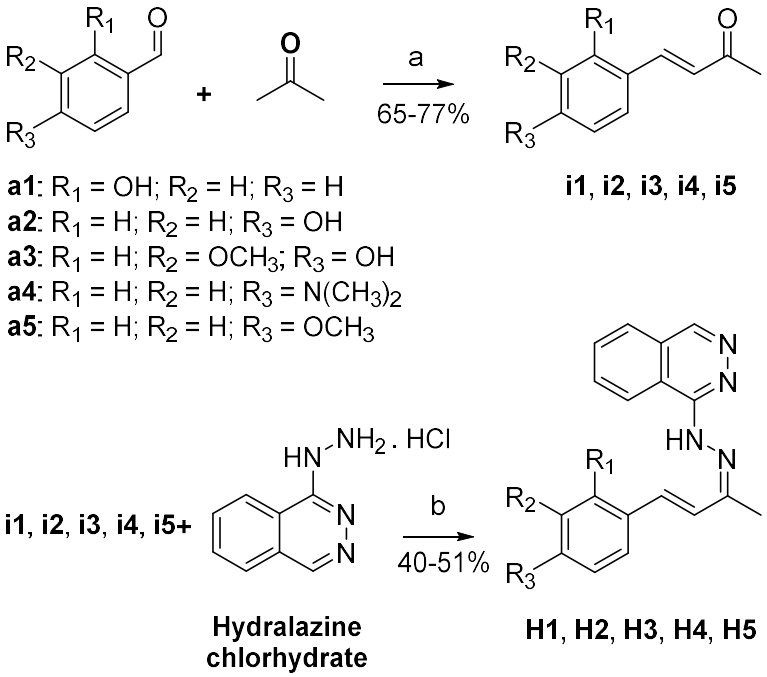


Fig 1. Synthetic route for hydrazone derivatives H1-H5. *a (first stage), *b (second stage); *a1-a5 (aldehydes); i1-i5 (intermediates).

Animals and ethics statement

All experiments were conducted using 8-week-old male Swiss mice (*Mus musculus*) (30 ± 40 g). The total number of animals involved in this study was 378, and each individual was involved in a single pain full procedure (not reused). The animals were kept in groups of six animals (n = 6) in polypropylene cages at a temperature of 22 ± 1°C with arelative humidity of 60 ± 80%, light/dark cycle of 12 h (start 06:00 and end 18:00), and free access to food (Purina Labina) and water. This study was performed in accordance with the Conselho Nacional para o Controle de Experimentação Animal (CONCEA, Brazil) and complied with the recommendations of the International Association for the Study of Pain [11,12]. The protocols were approved by the Comitê de Ética no Uso de Animais of the Universidade Federal do Vale do São Francisco (CEUA-UNIVASF, Brazil) with authorization number 0004/241017. All efforts were made to minimize animal suffering. At the end of the experiments, the animals were anesthetized with 60 mg/kg of ketamine plus 7.5 mg/kg of xylazine intraperitoneally and euthanized by cervical dislocation. For intraperitoneal administrations, syringes of 1 ml with aneedle of 13 x 0.45 mm were used. For the oral route, a gavage needle was used [12].

Acetic acid-induced writhing test

The writhing test was chosen as a classic model to assess the analgesic or anti-inflammatory properties of a new agent [13,12]. This test was performed using the method described by Collier and collaborators [14,12] with modifications. Mice received 10 ml/kg of a 0.9% acetic acid solution intraperitoneally (i.p.) [15,12]. The number of abdominal writhing was recorded for 10 min, beginning 5 min after administration of the acetic acid solution [16,12]. Writhing reflexes were defined as contractions of the abdominal muscles and pelvic rotation, followed by hind limb extension. Animals were divided into thirteen groups of six animals each (n = 78) and were treated orally (p.o.) with H1, H2, H3, H4, H5 (20 and 40 mg/kg, p.o.) and saline (negative control, p.o.) 1 h before the nociceptive agent. Indomethacin (20 mg/kg, i.p.) and morphine (10 mg/kg, i.p.) were used as reference drugs and were administered 30 min before the acetic acid solution [12].

Formalin-induced nociception test

The formalin test was performed as described by Hunskaar and Hole [17,12]. Animals were divided into thirteen groups of six animals each (n = 78) and were treated with saline (p.o.), H1, H2, H3, H4, H5 (20 and 40 mg/kg, p.o) given 1 h prior to the formalin injection, while indomethacin (20 mg/kg, i.p.) and morphine (10 mg/kg, i.p.) were given 30 min before the formalin injection. A formalin solution (2.5% in 0.9% sterile saline; 20 µl/animal) was injected into the right hind paw of mice [18,12]. Immediately after the formalin injection, animals were placed back in the chambers with a mirror and were observed for 30 min. The amount of time (in seconds) spent licking and biting the injected paw was measured as an indicator of pain. The formalin injection produced a biphasic nociceptive response: (I) an acute phase, 5 min after formalin injection, followed by a quiescent period of approximately 10 min and (II) a longer-lasting tonic phase 15 to 30 min after this period [19,12]. To verify the possible involvement of the nitrergic, serotonergic, ATP-sensitive potassium channel, vanilloid, muscarinic, and opioid systems in the pharmacological effect of H5 (hydrazone with more promising effect), animals were divided into twenty-one groups of six animals each (n = 126) and were pretreated with the respective blockers: *N*(*G*)-Nitro-*L*-arginine methyl ester (*L*-NAME, 20 mg/kg, i.p), *L*-arginine (60 mg/Kg, i.p.), ondansetron (0.5 mg/kg, i.p), glibenclamide (2 mg/Kg, i.p.), ruthenium red (3 mg/Kg, i.p.), atropine (0.1 mg/Kg, i.p.) and naloxone (1.5 mg/Kg, i.p.) 30 min before treatment with H5 (20 mg/kg, p.o) [20,12].

Leukocyte migration to the peritoneal cavity induced by carrageenan

Leukocyte migration was induced with 250 µl of 1% carrageenan (i.p.) into the peritoneal cavity of mice 1 h after administration of saline (p.o.) and H5 (20 and 40 mg/kg, p.o) and 0.5 h after injection of dexamethasone (2 mg/kg, i.p.). Animals (n = 24) were euthanized, as described above, 4 h later, and the peritoneal cavity was washed with 3 ml of a saline solution containing 1mM EDTA [21]. The collected fluid was centrifuged (3000 rpm for 6 min) at room temperature. Subsequently, 10 µl of this suspension was dissolved in 200 µl of Turk solution, and a total cell count was performed using a Neubauer chamber. The results are expressed as the number of leukocytes/ml [22,12].

Carrageenan-induced hind paw edema

Mice were divided into five groups of six animals each (n = 30) and were pretreated with H5 (20 and 40 mg/kg, p.o), saline (p.o.) or indomethacin (20 mg/kg, i.p.) 1 h before subcutaneous injection of carrageenan (2.0% ƛ-carrageenan) or saline (0.9%) into the right hind paw of animals at a volume of 20 µl/animal [23,24,12]. The mice pedal volume up to the ankle joint was measured using a plethysmometer (PanLab LE 7500, Spain) 0, 1, 2, 3, and 4 h after administration of carrageenan as described previously [25,12]. Inhibition of the paw edema was calculated by edema = (foot volume at measurement time - foot volume at time zero)/foot volume at time zero.

Histamine-induced hind paw edema

To evaluate the involvement of histaminic receptors, the mice were divided into three groups of six animals each (n = 18) mice were pretreated with H5 (20 mg/kg, p.o), saline (p.o.), or indomethacin (20 mg/kg, i.p.) 1 h before the subcutaneous injection of histamine (100 µg/paw) or saline (0.9%) into their right hind paw at a volume of 20 µl/animal [26,12]. The volume was measured 0, 30, 60, 90, 120, and 150 min after theinjection of histamine or saline [27,12].

Rota-Rod test

A rota-rod apparatus (Insight, Brazil) was used for the assessment of motor coordination. Initially, animals capable of remaining on the rotarod apparatus for 60 s (7 rpm) were selected 24 h before the test. The mice were divided into four groups of six animals each (n = 24) and were pretreated with H5 (20 and 40 mg/kg, p.o), saline, or diazepam (2.5 mg/kg). Each animal

was individually evaluated on the rota-rod apparatus at 30, 60, 90, and 120 min after treatments, and the time (s) spent on top of the bar was recorded for up to 180 s [28,29].

Artemia salina toxicity test

The methodology described by Meyer et al. (1982) with some modifications. *Artemia salina* cysts were placed in an aquarium with water salted under aeration and temperature control (20-30 °C) and after 48 h of incubation, the larvae were removed for the test [30].

Physicochemical properties and ADMET profile

The physicochemical properties and ADMET profile of hydrazones derivatives were predicted *in silico* in comparison with the anti-inflammatory drugs indomethacin and meloxicam, using the ACD/Percepta Program.

Molecular docking analysis

Molecules were constructed on Spartan'16 (Wavefunction Inc.) software and conformational analysis by molecular mechanic method (MMFF - Merck molecular force field) was performed. Starting from minor energy conformer, equilibrium geometry was calculated by PM6 semi empiric method [31].

The X-ray crystallographic structure of murine COX-2 enzyme, complexed with meloxicam (MXM), was obtained from the RCSB Protein Data Bank (PDB ID: 4M11) [32,33].

Molecular docking studies were performed in triplicate with GOLD 5.4 with all scoring functions available (ChemPLP, GoldScore, ChemScore, and ASP) [34], with the default parameter. The binding site was determined within 8Å around the MXM as a reference. 10 poses were generated for each compound and the best scoring complexes for each ligand were selected. Firstly, for validation purposes, meloxicam (MXM) was redocked in the binding site to evaluate the accuracy of the docking procedure with the 4 function, in this system, evaluating the RMSD (Root Mean Square Deviation) between the native and post-redocking conformation of MXM. The same step was conducted for H5 to view its possible interaction modes and score value.

GOLD uses a genetic algorithm for docking compounds into protein (3D crystallographic structure or 3D model) binding sites [35]. GOLD presents high accuracy and reliability and considers the full ligand flexibility and partial protein flexibility.

Statistical analysis

The results are presented as the mean ± standard error of the mean (SEM), and statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's test. Values of $p<0.05$ were considered statistically significant. All analyses were performed using GraphPad Prism 5.0 (Graph Pad Prism Software, Inc., San Diego, CA, USA).

Results and discussion

Initially, the antinociceptive effect of hydrazones derivatives (H1-H5) was evaluated using the acetic acid-induced nociception test. In this model, H1 attenuated the nociceptive activity, reducing the number of writhings by 83.87% and 78.78% at the highest doses tested (20 mg/kg and 40 mg/kg), as shown in Fig 2. H2 attenuated the nociceptive activity, reducing the number of writhings by 96.00% and 89.93% at the highest doses tested (20 mg/kg and 40 mg/kg), as shown in Fig 3. H3 also attenuated the nociceptive activity, reducing the number of writhings by 77.81% and 80.84% at the highest doses tested (20 mg/kg and 40 mg/kg), as shown in Fig 4. H4 attenuated the nociceptive activity, reducing the number of writhings by 87.87% and 96.00% at the highest doses tested (20 mg/kg and 40 mg/kg), as shown in Fig 5. Finally, H5 attenuated the nociceptive activity, reducing the number of writhings by 86.90% at the highest doses tested (20 mg/kg and 40 mg/kg), as shown in Fig 6.

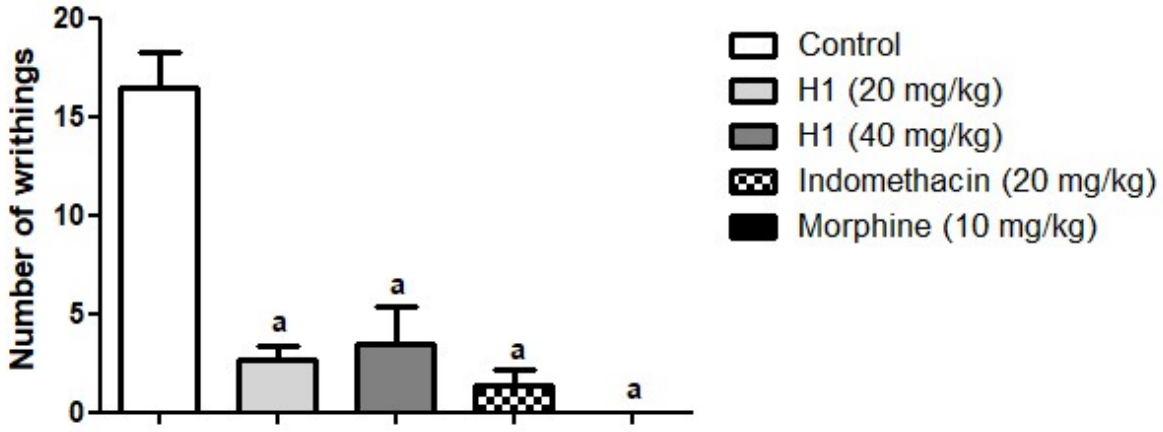


Fig 2. Effect of H1 (20 and 40 mg/kg, p.o.), morphine (10 mg/kg, i.p.) and indomethacin (20 mg/kg, i.p.) in the acetic acid-induced writhing test in mice (n = 6, per group). Values are expressed as the mean ± SEM, where *a* indicates $p<0.05$, significantly different from the control group, according to ANOVA, followed by Tukey's test.

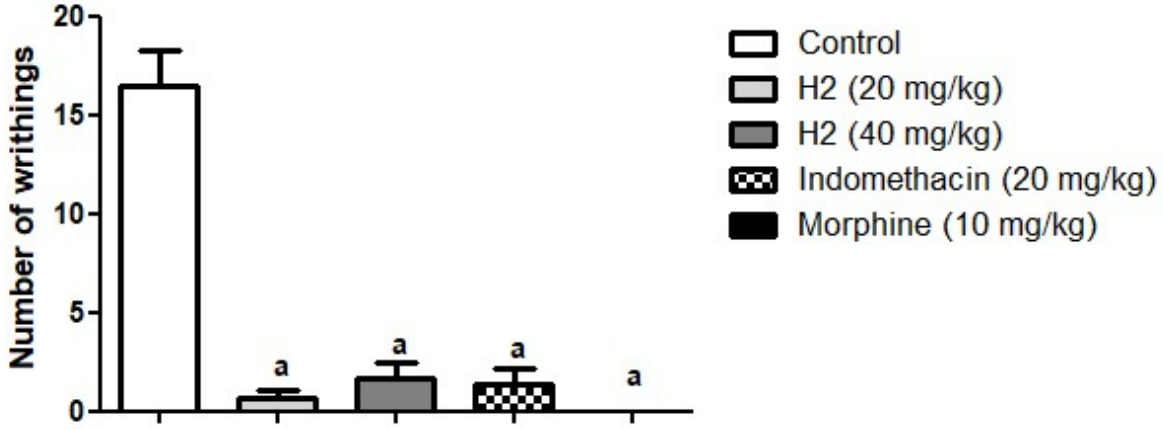


Fig 3. Effect of H2 (20 and 40 mg/kg, p.o.), morphine (10 mg/kg, i.p.), and indomethacin (20 mg/kg, i.p.) in the acetic acid-induced writhing test in mice (n = 6, per group). Values are expressed as the mean ± SEM, where *a* indicates $p<0.05$, significantly different from the control group, according to ANOVA, followed by Tukey's test.

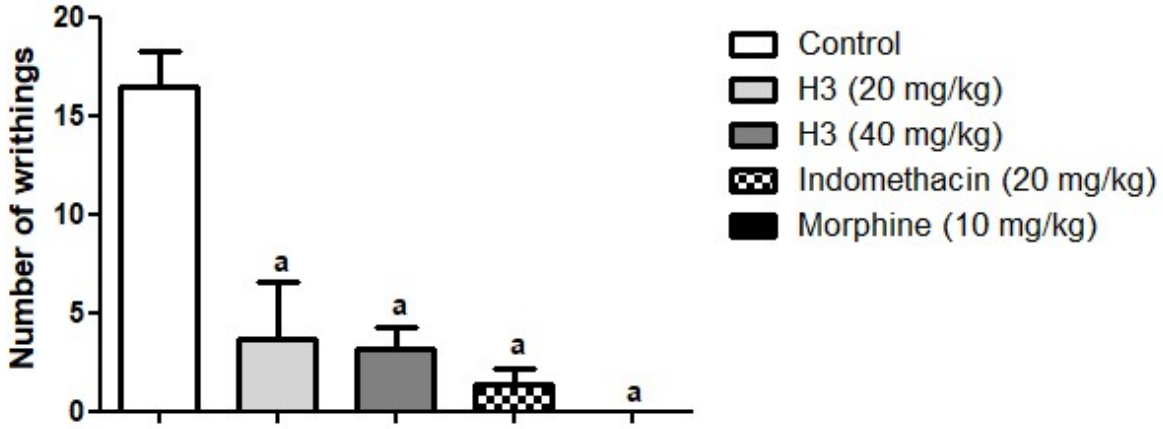


Fig 4. Effect of H3 (20 and 40 mg/kg, p.o.), morphine (10 mg/kg, i.p.) and indomethacin (20 mg/kg, i.p.) in the acetic acid-induced writhing test in mice (n = 6, per group). Values are expressed as the mean ± SEM, where *a* indicates $p<0.05$, significantly different from the control group, according to ANOVA, followed by Tukey's test.

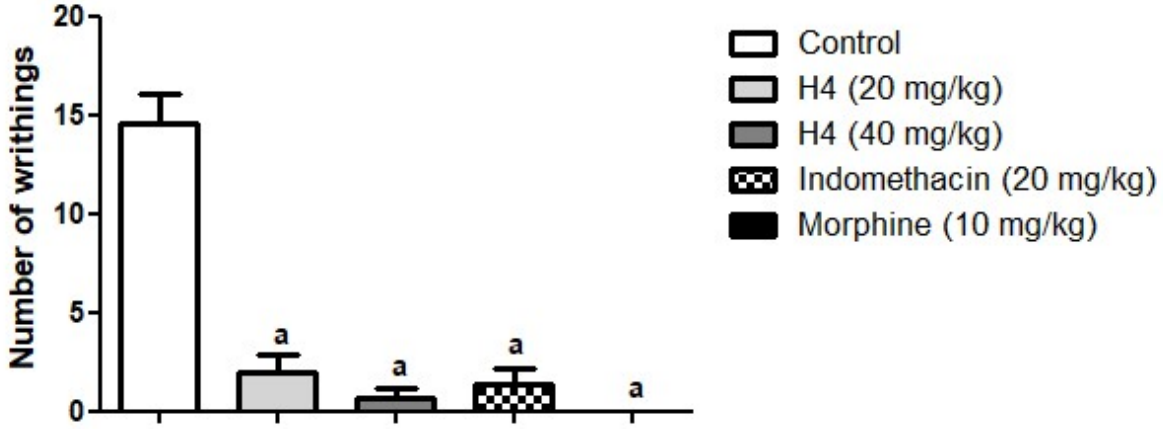


Fig 5. Effect of H4 (20 and 40 mg/kg, p.o.), morphine (10 mg/kg, i.p.) and indomethacin (20 mg/kg, i.p.) in the acetic acid-induced writhing test in mice (n = 6, per group). Values are expressed as the mean ± SEM, where *a* indicates $p<0.05$, significantly different from the control group, according to ANOVA, followed by Tukey's test.

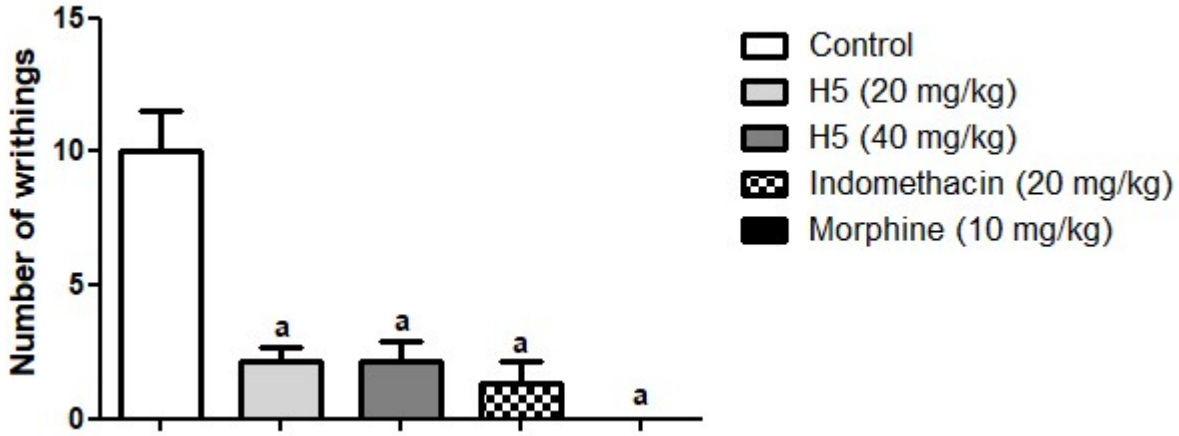


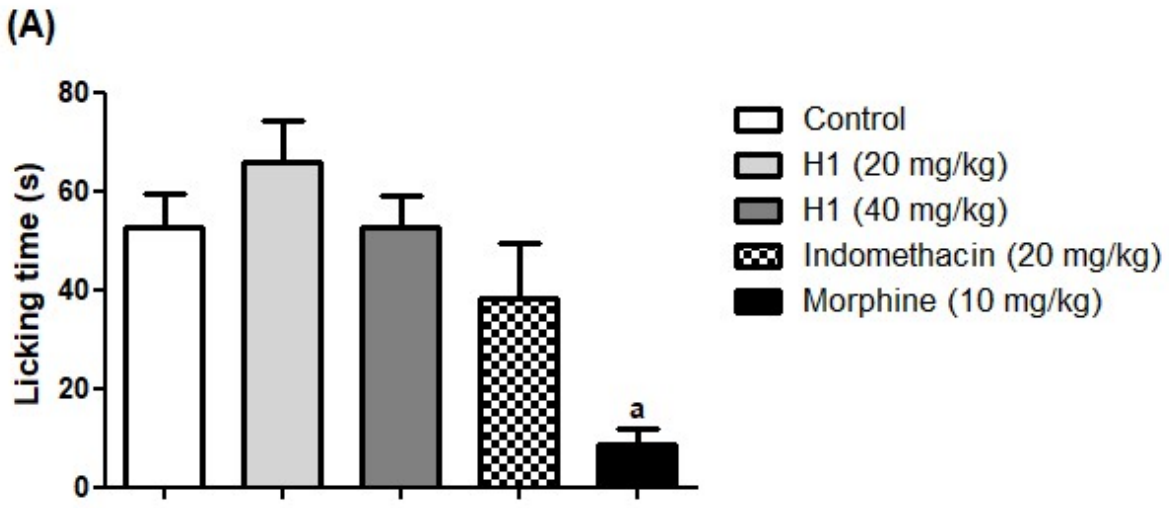
Fig 6. Effect of H5 (20 and 40 mg/kg, p.o.), morphine (10 mg/kg, i.p.) and indomethacin (20 mg/kg, i.p.) in the acetic acid-induced writhing test in mice (n = 6, per group). Values are expressed as the mean ± SEM, where *a* indicates $p<0.05$, significantly different from the control group, according to ANOVA, followed by Tukey's test.

According to the statistical analysis, all hydrazones tested had similar effects compared to indomethacin, which inhibited 91.93% of nociception. These results were also similar to *N*-acylhydrazone LASSBio-1586, which presented 88.97% of inhibition at the highest dose of 40 mg/kg [12].

The acetic acid-induced writhing test is quite unspecific. Intraperitoneal administration of the chemical agent induces activation of nociceptors and stimulates the release of a diversity of painful and inflammatory mediators, including histamine, bradykinin, serotonin, glutamate, noradrenaline, substance P, nitric oxide, and prostaglandins [14,12]. For this reason, it is not possible to specify the nociceptive pathways in which the molecule acts. From this perspective, the formalin-induced nociception test was performed.

The formalin test is an experimental model that can corroborate the two different phases of a nociceptive incident. The first phase comprises a nociceptive response elicited by central-acting mediators, including those that activate serotonergic, muscarinic, vanilloid, and glutamatergic receptors. In the second phase, there is a prevalence of inflammatory mediators, mainly histamine, bradykinin, and prostaglandins [17,12].

In his test, H1 reduced nociceptive behavior only in the second phase of nociception, being the doses of 20 and 40 mg/kg responsible for 59.61% and 39.64% of normal antinociceptive effect respectively (Fig 7B). H2 reduced nociceptive behavior only in the second phase of nociception, being the dose of 20 mg/kg responsible for 51.67% of normal antinociceptive effect (Fig 8B). H3 reduced nociceptive behavior only in the second phase of nociception, being the dose of 40 mg/kg responsible for 64.04% of normal antinociceptive effect (Fig 9B). H4 reduced nociceptive behavior only in the second phase of nociception, being the doses of 20 and 40 mg/kg responsible for 96.41% and 78.16% of normal antinociceptive effect respectively (Fig 10B). And H5 reduced nociceptive behavior only in the second phase of nociception, being the doses of 20 and 40 mg/kg responsible for 78.92% and 100% of normal antinociceptive effect respectively (Fig 11B).



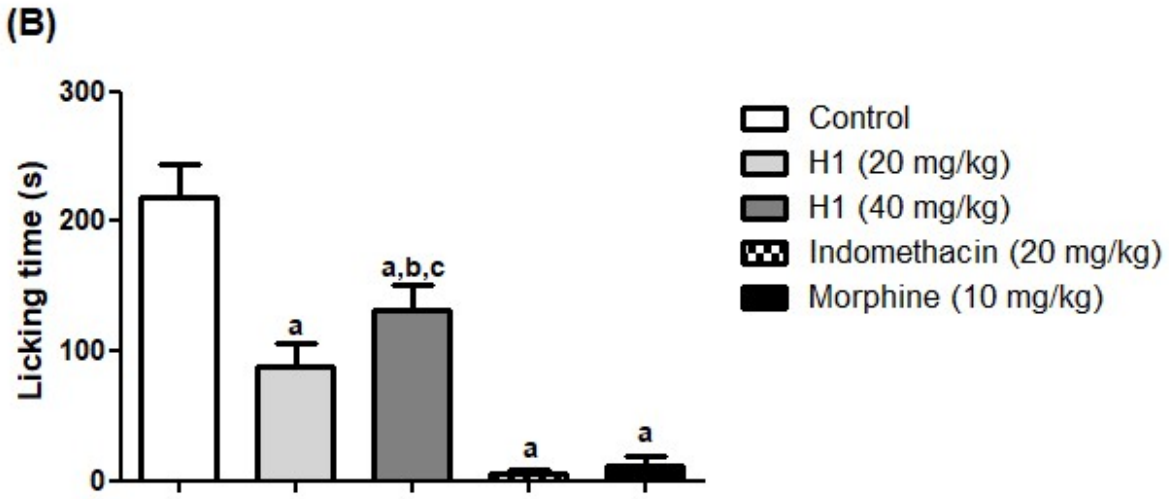
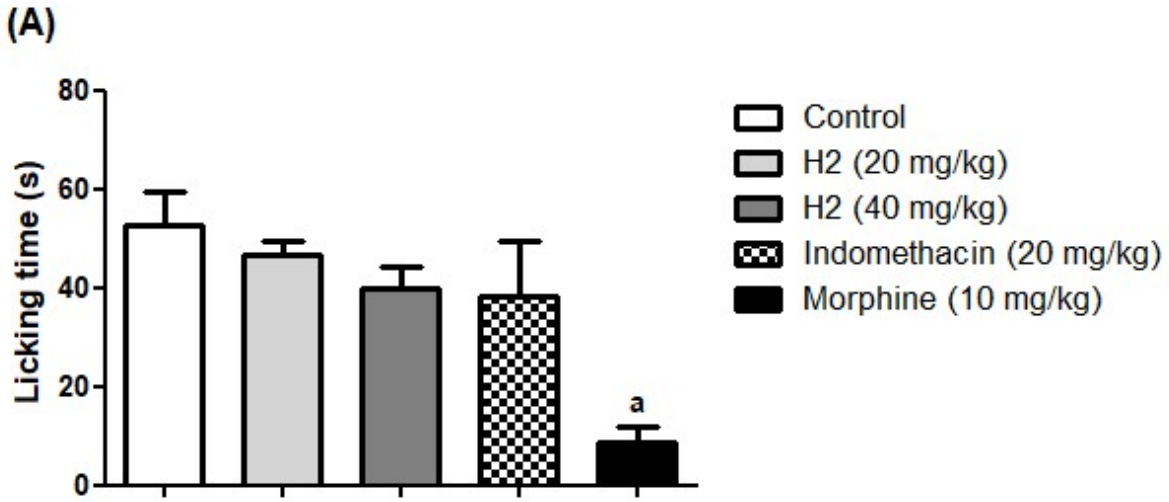


Fig 7. Effect of H1 (20 and 40 mg/kg, p.o.), morphine (10 mg/kg, i.p.) and indomethacin (20 mg/kg, i.p.) in the first (A) and second (B) phases of the formalin-induced nociception test in mice (n = 6, per group). Values are expressed as the mean ± SEM, where *a* indicates $p<0.05$, significantly different from the control group, *b* indicates $p<0.05$ in comparison with indomethacin group and *c* indicates $p<0.05$ in comparison with morphine group, according to ANOVA, followed by Tukey's test.



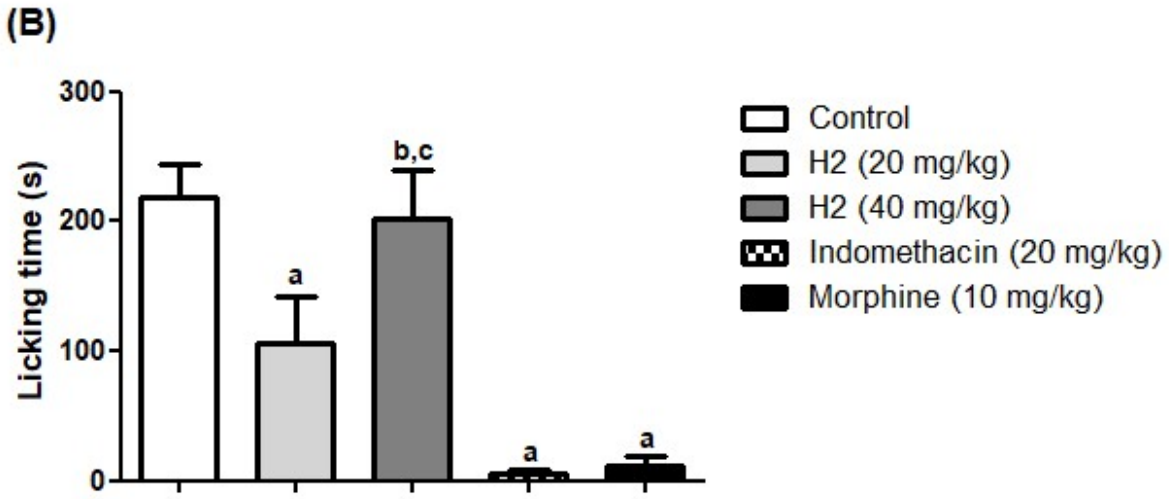
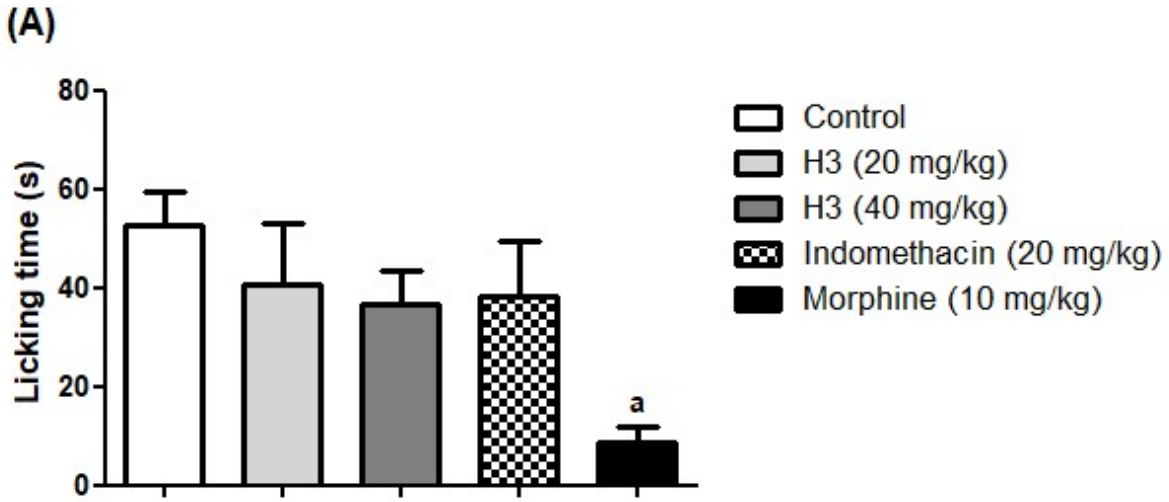


Fig 8. Effect of H2 (20 and 40 mg/kg, p.o.), morphine (10 mg/kg, i.p.) and indomethacin (20 mg/kg, i.p.) in the first (A) and second (B) phases of the formalin-induced nociception test in mice (n = 6, per group). Values are expressed as the mean ± SEM, where *a* indicates $p<0.05$, significantly different from the control group, *b* indicates $p<0.05$ in comparison with indomethacin group and *c* indicates $p<0.05$ in comparison with morphine group, according to ANOVA, followed by Tukey's test.



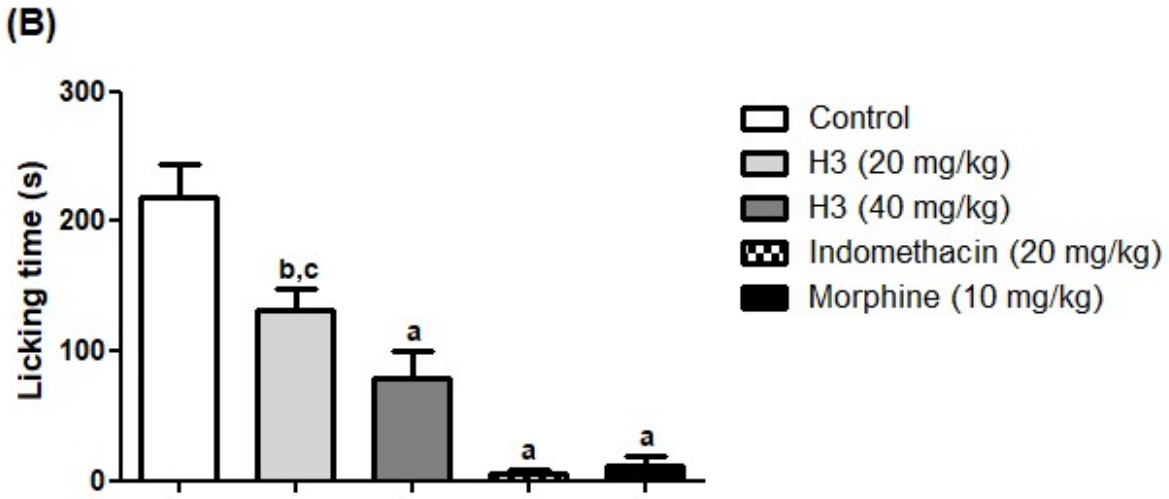


Fig 9. Effect of H3 (20 and 40 mg/kg, p.o.), morphine (10 mg/kg, i.p.) and indomethacin (20 mg/kg, i.p.) in the first (A) and second (B) phases of the formalin-induced nociception test in mice (n = 6, per group). Values are expressed as the mean ± SEM, where *a* indicates $p<0.05$, significantly different from the control group, *b* indicates $p<0.05$ in comparison with indomethacin group and *c* indicates $p<0.05$ in comparison with morphine group, according to ANOVA, followed by Tukey's test.

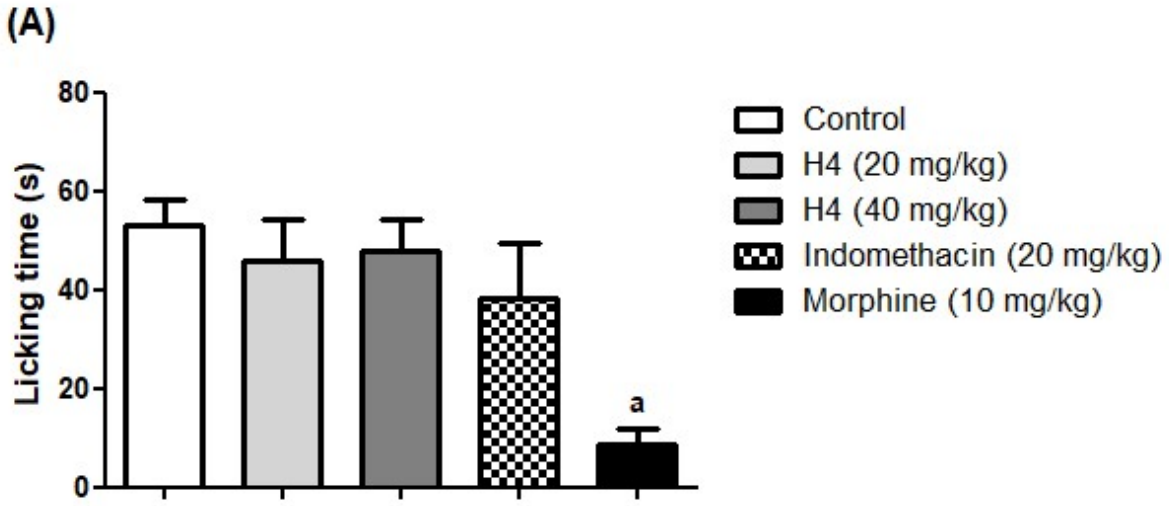
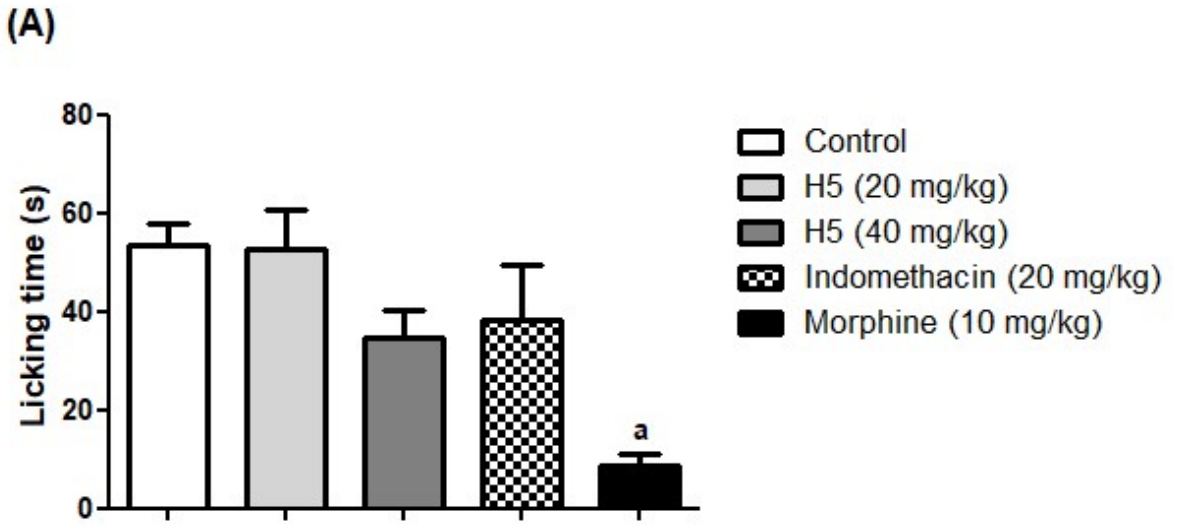


Fig 10. Effect of H4 (20 and 40 mg/kg, p.o.), morphine (10 mg/kg, i.p.) and indomethacin (20 mg/kg, i.p.) in the first (A) and second (B) phases of the formalin-induced nociception test in mice (n = 6, per group). Values are expressed as the mean ± SEM, where *a* indicates $p<0.05$, significantly different from the control group, according to ANOVA, followed by Tukey's test.



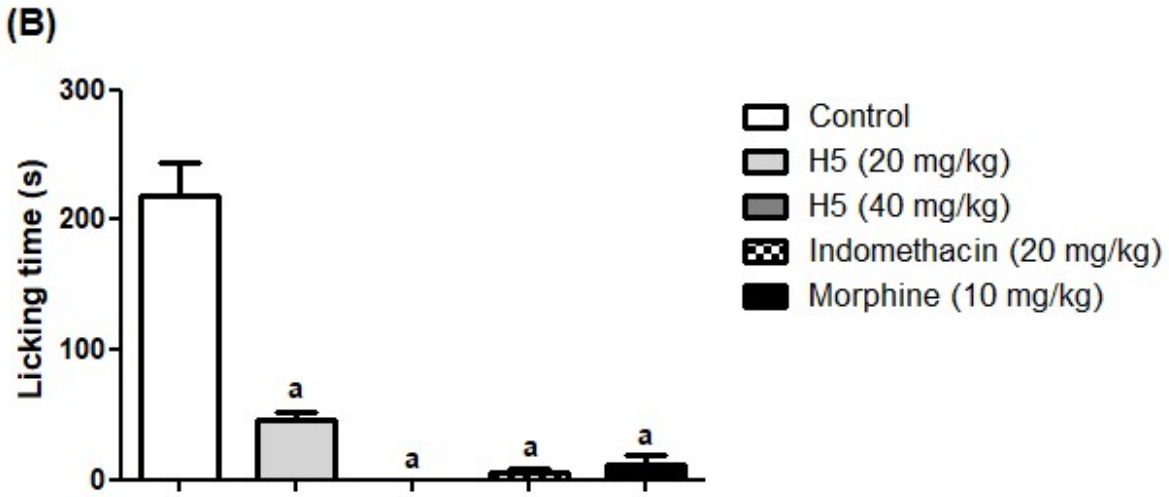


Fig 11. Effect of H5 (20 and 40 mg/kg, p.o.), morphine (10 mg/kg, i.p.) and indomethacin (20 mg/kg, i.p.) in the first (A) and second (B) phases of the formalin-induced nociception test in mice (n = 6, per group). Values are expressed as the mean ± SEM, where *a* indicates $p<0.05$, significantly different from the control group, according to ANOVA, followed by Tukey's test.

Therefore, the hydrazones H4 and H5 showed greater potential to become an antinociceptive and anti-inflammatory drug, however, H5 is more chemically stable. Regarding the statistical analysis, there was no statistical difference between the two doses. Similar result were observed for indomethacin (97.25%) and LASSBio-1586 (96.74%) at a dose of 20 mg/kg [14]. According to these results, H5 does not affect the first phase of the test, suggesting that this chemical has a peripheral (not central) effect that reduces nociception only in the second phase of the formalin test, similar to indomethacin.

Similarly to our results with H5, Meymandi *et* al. (2019) showed that celecoxib (10-30 mg/kg), a specific COX-2 inhibitor, had antinociceptive and anti-inflammatory activity in mice submitted to the formalin test, being this effect visible only in the second phase of the test [36,37,38].

The effect of H5 was related to a reduction in nociception and inflammation. In this context, we conducted several tests to explore the antinociceptive and anti-inflammatory potential of H5.

When animals were pretreated with naloxone (1.5 mg/kg, i.p.), the pharmacological effect of H5 (20 mg/kg, p.o.) was completely reversed in the second phase of the test (Fig12), suggesting that its peripheral antinociceptive response was involved at least in part with the opioid system.

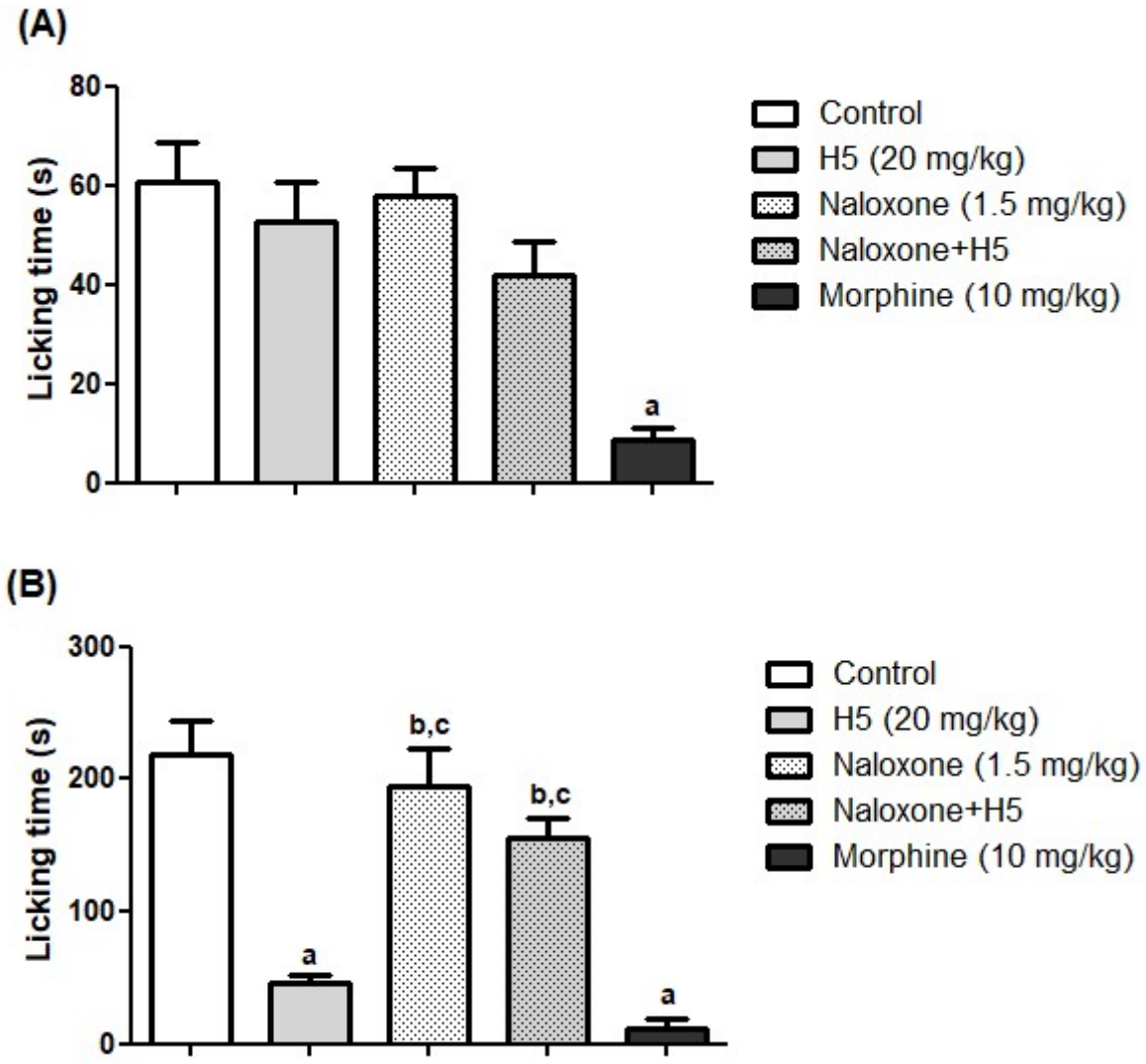


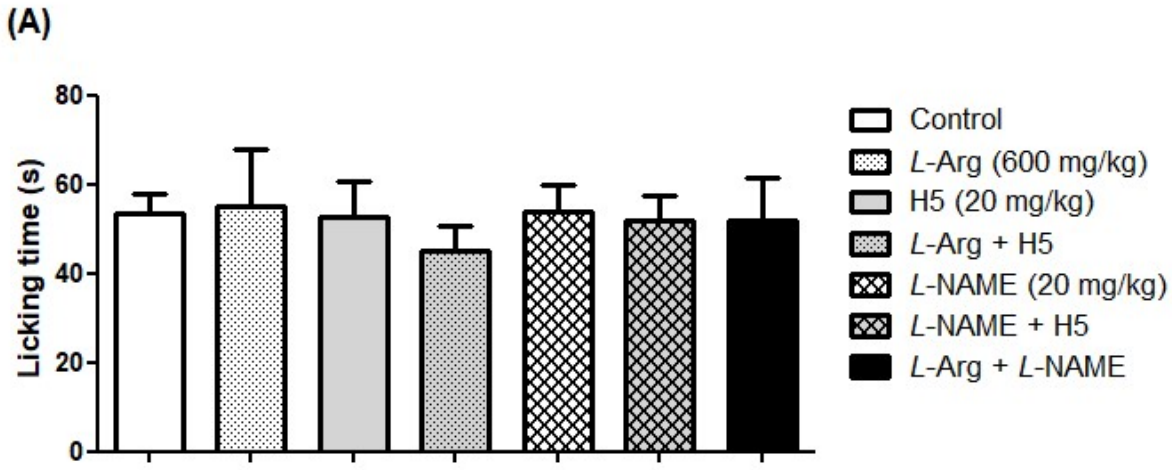
Fig 12. Effect of H5 (20 mg/kg, p.o.), naloxone (1.5 mg/kg, i.p.), naloxone + H5 and morphine (10 mg/kg, i.p.) in the first (A) and second (B) phases of the formalin-induced nociception test in mice (n = 6, per group). Values are expressed as the mean ± S.E.M., where *a* indicates $p<0.05$ in comparison with control group, *b* indicates $p<0.05$ in comparison with morphine group, and *c* indicates $p<0.05$ in comparison with H5 group, according to ANOVA, followed by Tukey's post-test.

When animals were pretreated with naloxone, the pharmacological effect of H5 was completely reversed in the second phase of the formalin test, suggesting that its peripheral antinociceptive response was involved at least in part in the opioid system. Naloxone is an opioid antagonist and for this reason, it significantly blocks the activity of morphine in both phases of the test. The results of Mehanna *et* al. (2018) [39] demonstrated that naloxone

completely reversed the effect of tadalafil in the first phase of the same test and partially in the second phase, suggesting that this drug have a peripheral antinociceptive effect that activates the opioid receptors, which was also demonstrated by Florentino *et* al. (2015) with pyrazole compounds [39,40,41,42].

We also found in the scientific literature that the bergamot essential oil (BEO)-induced antinociception possibly occurred through μ-opioid and κ-opioid receptors in the periphery [43]. Beyond that, the capacity of flavonoids to decrease hyperalgesia can also involve μ and δ-opioid receptors [44,45,46]. In addition, several studies have shown that activation of peripheral opioid receptors inhibits inflammatory pain and activates *L*-arginine/NO/cGMP pathway [39,41,42].

When animals were pretreated with *L*-arginine (600 mg/kg, i.p.), the pharmacological effect of H5 (20 mg/kg, p.o.) was completely reversed, and when were pretreated with *L*-NAME (20 mg/kg, i.p.) the pharmacological effect of H5 (20 mg/kg, p.o.) was not reversed in the second phase of the test (Fig13), suggesting that its peripheral antinociceptive response was involved, at least in part, with the nitrergic system.



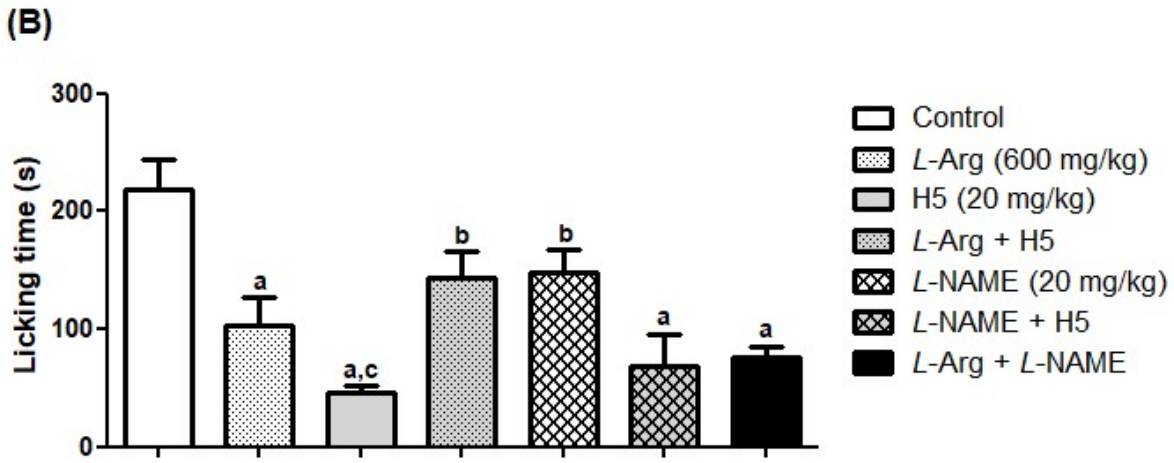


Fig13. Effect of *L*-arginine (600 mg/kg, i.p.), H5 (20 mg/kg, p.o.), *L*-arginine + H5, *L*-NAME (20 mg/kg, i.p.), *L*-NAME + H5, and *L*-arginine + *L*-NAME in the first (A) and second (B) phases of the formalin-induced nociception test in mice (n = 6, per group). Values are expressed as the mean ± S.E.M., where *a* indicates $p<0.05$ in comparison with control group, *b* indicates $p<0.05$ in comparison with H5 group, and *c* indicates $p<0.05$ in comparison with *L*-arginine + H5 group, according to ANOVA, followed by Tukey's post-test.

When nociceptors are activated, intracellular signaling cascades lead to an increase in the production of a variety of neuromodulators such as NO and cGMP. Thus, a sufficient increase of NO concentration boosts the cGMP production and leads to the activation of glutamatergic receptors, which make the NO concentrations directly associated with nociception [47,48,49,50,51]. Our results showed that H5 antinociceptive effect involves the *L*-arginine-nitric oxide pathway since *L*-arginine reversed this effect, similar to the result of isopulegol (ISO), studied by Próspero et al. (2018) [51].

The cGMP acts directly or through the stimulation of protein kinases that phosphorylate ion channels favoring the firing of action potentials that culminate in the production of nociception [51,52,53]. Systemically, the *L*-arginine/NO/cGMP pathway blockade causes a decrease in nociception [51,54]. Our results showed that the H5 effect is involved with the nitrergic system, similar that ISO [51]. In the second phase of the formalin test, in the presence of *L*-NAME, H5 showed no reversibility of its antinociceptive effect. This result corroborates the one presented by Silva et al. 2018 about the LASSBio-1586 [12].

When animals were pretreated with ondansetron (0.5 mg/kg, i.p.), the pharmacological effect of H5 (20 mg/kg, p.o.) was not reversed in the second phase of the test (Fig14),

suggesting that its peripheral antinociceptive response was not involved in the serotonergic system.

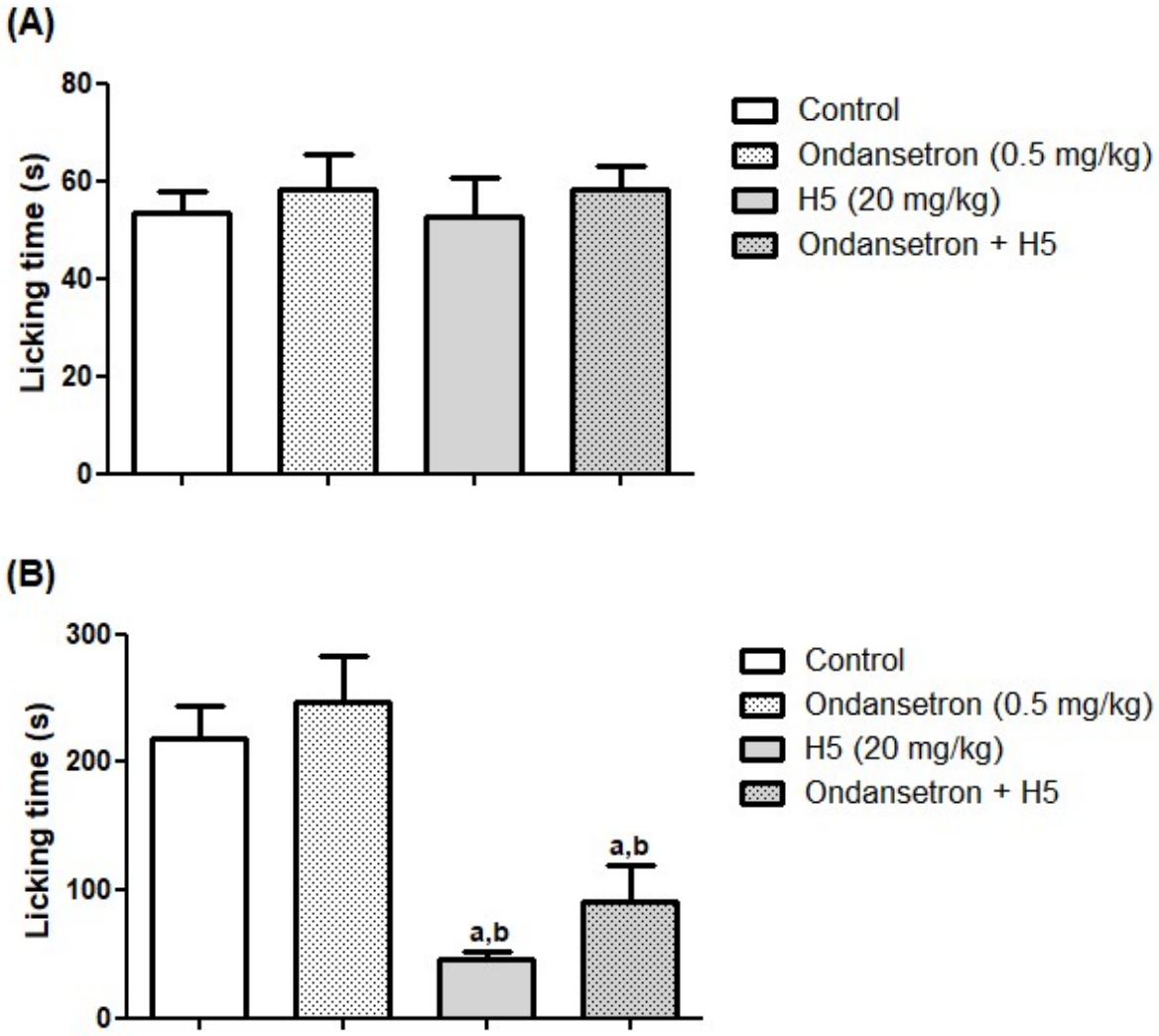


Fig 14. Effect of ondansetron (0.5 mg/kg, i.p.), H5 (20 mg/kg, p.o.), ondansetron + H5 in the first (A) and second (B) phases of the formalin-induced nociception test in mice (n = 6, per group). Values are expressed as the mean ± S.E.M., where *a* indicates $p<0.05$ in comparison with control group and *b* indicates$p<0.05$ in comparison with ondansetron group, according to ANOVA, followed by Tukey's post-test.

Ondansetron did not alter the antinociceptive effect of H5. Therefore, its effect has no involvement with the serotonergic system. Similar to LASSBio-1586, in the second phase H5 also showed no reversibility of its antinociceptive effect [12]. Diverse serotonin (5-HT) receptors are present in the central and peripheral nervous systems [55,56]. Studies have

shown that 5-HT1 receptors are implicated in the process of antinociception, whereas 5-HT2 receptors have pronociceptive effects [56,57,58].

When animals were pretreated with atropine (0.1 mg/kg, i.p.), the pharmacological effect of H5 (20 mg/kg, p.o.) was not reversed in the second phase of the test (Fig 15), suggesting that its peripheral antinociceptive response was not involved in the muscarinic system.

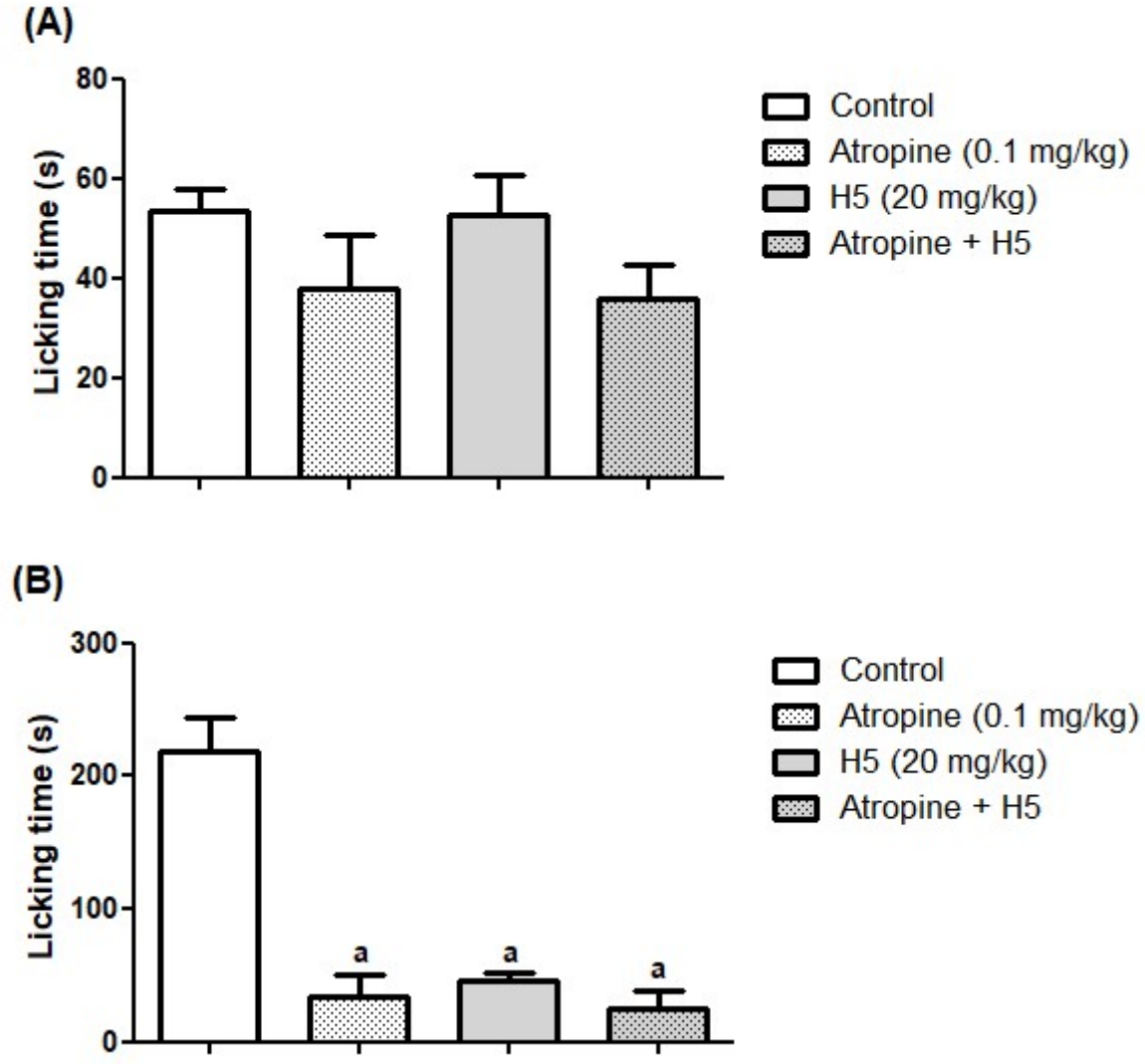


Fig15. Effect of atropine (0.1 mg/kg, i.p.), H5 (20 mg/kg, p.o.), atropine + H5 in the first (A) and second (B) phases of the formalin-induced nociception test in mice (n = 6, per group). Values are expressed as the mean ± S.E.M., where *a* indicates $p<0.05$ in comparison with control group, according to ANOVA, followed by Tukey's post-test.

When animals were pretreated with glibenclamide (2 mg/kg, i.p.), the pharmacological effect of H5 (20 mg/kg, p.o.) was not reversed in the second phase of the test (Fig16), suggesting that its peripheral antinociceptive response was not involved with the ATP sensitive potassium channels.

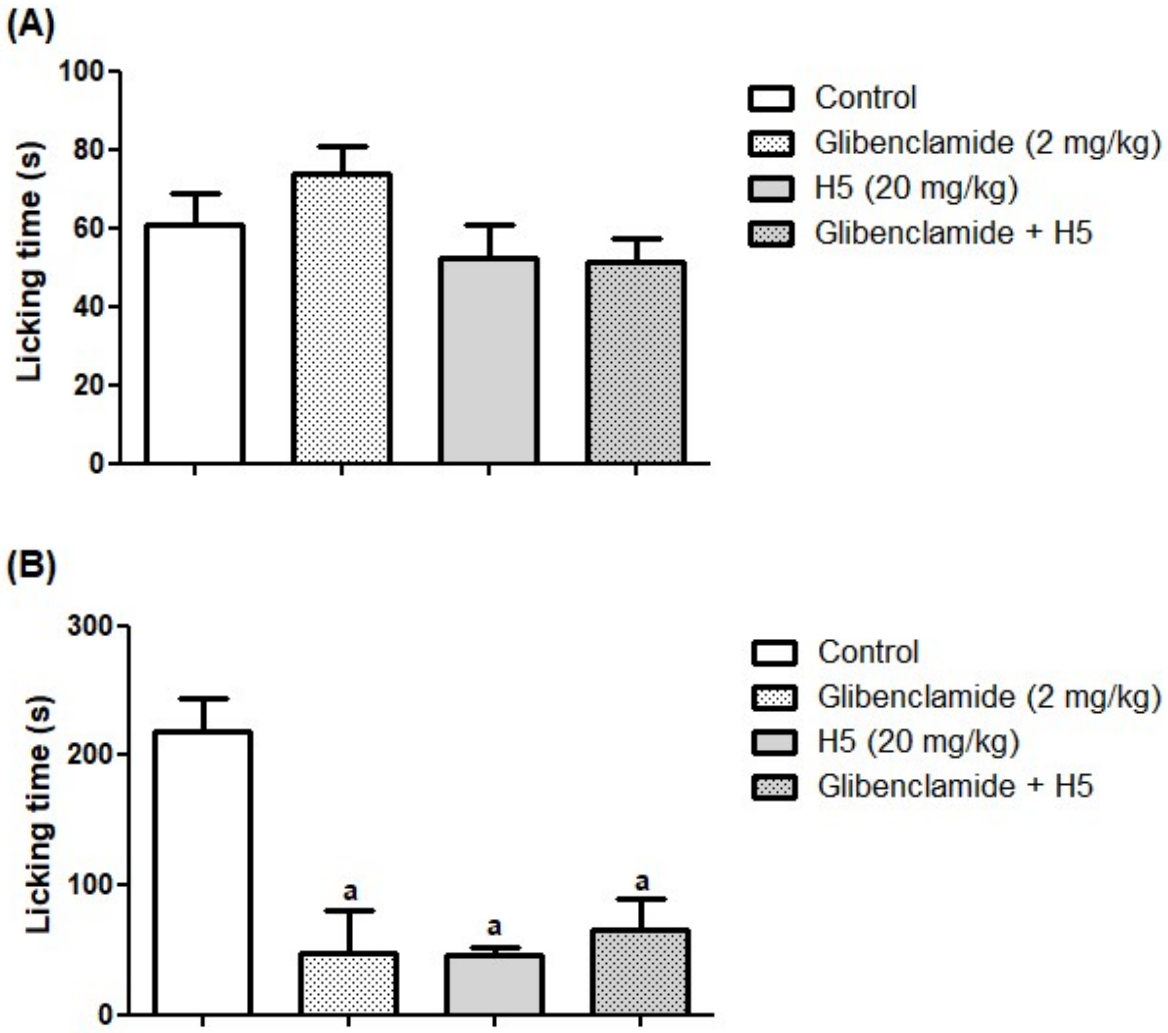


Fig16. Effect of glibenclamide (2 mg/kg, i.p.), H5 (20 mg/kg, p.o.), glibenclamide + H5 in the first (A) and second (B) phases of the formalin-induced nociception test in mice (n = 6, per group). Values are expressed as the mean ± S.E.M., where *a* indicates $p<0.05$ in comparison with control group, according to ANOVA, followed by Tukey's post-test.

When animals were pretreated with ruthenium red (3 mg/kg, i.p.), the pharmacological effect of H5 (20 mg/kg, p.o.) was not reversed in the second phase of the test (Fig17), suggesting that its peripheral antinociceptive response was not involved in the vanilloid

system. Our results with H5 also showed no interference of the muscarínico and vanilloid system nor the K_{ATP} ion channels.

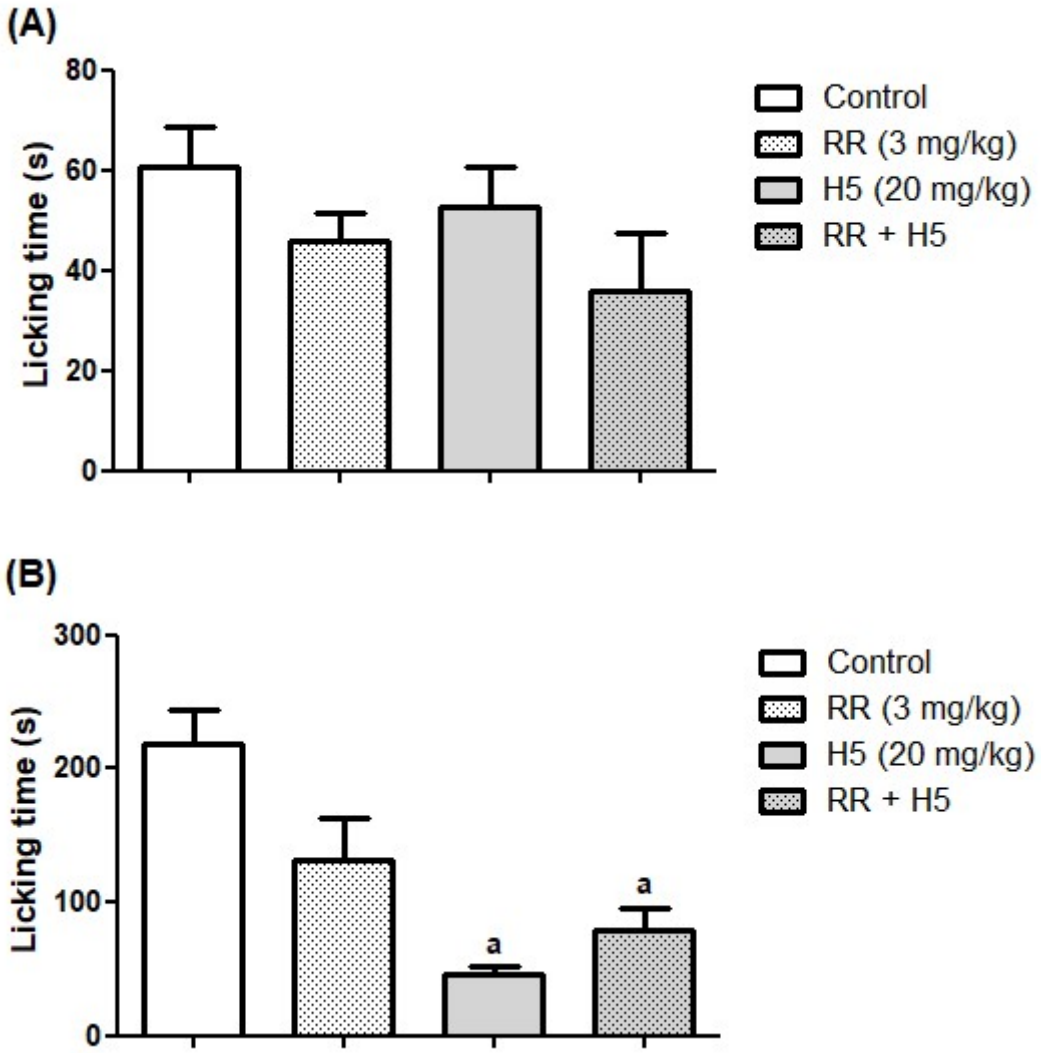


Fig17. Effect of ruthenium red (3 mg/kg, i.p.), H5 (20 mg/kg, p.o.), ruthenium red + H5 in the first (A) and second (B) phases of the formalin-induced nociception test in mice (n = 6, per group). Values are expressed as the mean ± S.E.M., where *a* indicates $p<0.05$ in comparison with control group, according to ANOVA, followed by Tukey's post-test.

In this context, we assessed the anti-inflammatory potential of H5. Firstly, the anti-inflammatory effect of H5 was analyzed in tests of acute inflammation, such as the leukocyte migration in the carrageenan-induced peritoneal cavity test. In this model, H5 reduced leukocyte migration independent of dose (Fig 18). The anti-inflammatory effect of H5

(20mg/kg - 37.17% and 40 mg/kg - 47.42%) was equivalent to that observed for dexamethasone (2 mg/kg - 57.34%).

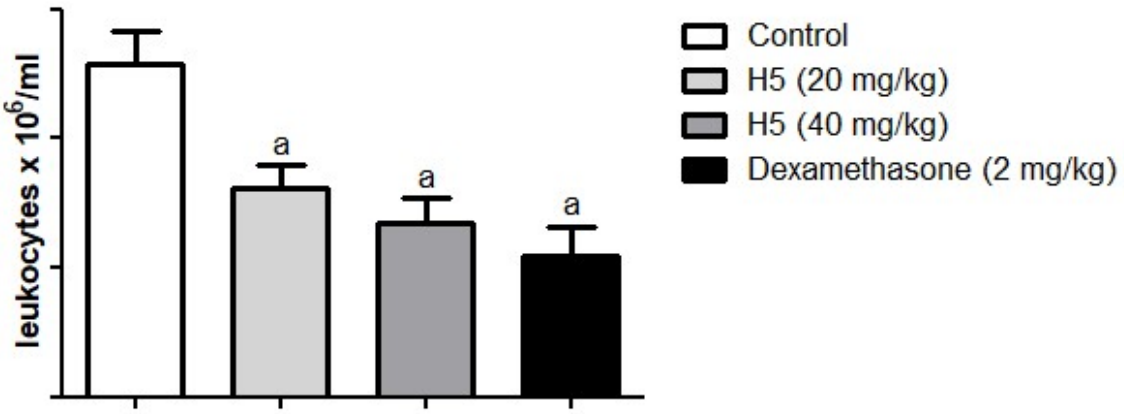


Fig 18. Effect of H5 (20 and 40 mg/kg, p.o.) and dexamethasone (2 mg/kg, i.p.) on leukocyte migration into the peritoneal cavity induced by carrageenan in mice. Values are expressed as the mean ± S.E.M. (n = 6, per group), where *a* indicates $p<0.05$, significantly different from the control group, according to ANOVA, followed by Tukey's test.

Another methodology used was the carrageenan-induced hind paw edema model. In this test, H5 significantly decreased ($p<0.05$) paw edema at all doses tested, especially at 1, 2, 3, and 4 hours after hydrazone treatment, suggesting a high anti-inflammatory, effect as shown in Fig 19.

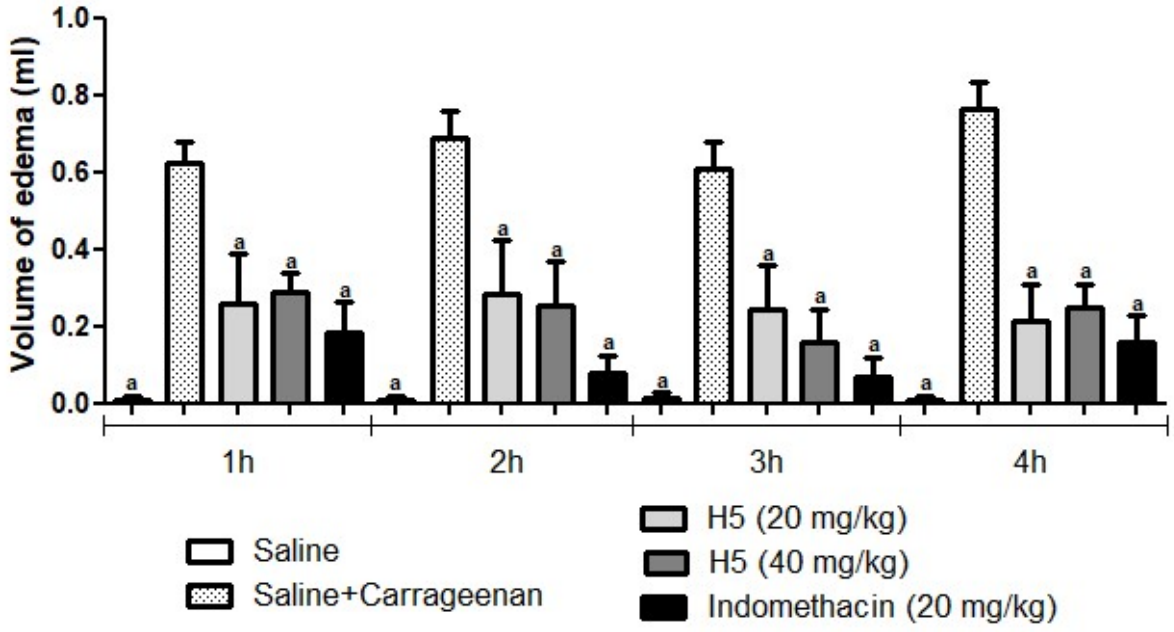


Fig 19. Effect of H5 (20 and 40 mg/kg, p.o.) and indomethacin (20 mg/kg, i.p.) on paw edema induced by carrageenan in mice. The sham group was treated only with saline, whereas the control group received saline and carrageenan. Values are expressed as the mean ± S.E.M. (n = 6, per group), where *a* indicates $p<0.05$, significantly different from the control group, according to ANOVA, followed by Tukey's post-test.

In the carrageenan-induced paw edema model, stimulated inflammation promotes the release of inflammatory mediators in two phases. The first phase occurs one hour after administration of carrageenan, and histamine, serotonin, and cytokines are released. Meanwhile, the second phase is characterized by the release of bradykinins, proteases, and prostaglandins, for example. Therefore, the second phase is more sensitive to anti-inflammatory drugs (ex: diclofenac) used clinically, which promote the inhibition of cycloxygenases (COX-1 and COX-2), inhibiting the synthesis of prostaglandins [59].

As histamine is one of the first mediators produced (first phase), its vasodilator action makes it essential for the formation of edema [60,12]. Therefore, a similar protocol was performed using histamine to induce paw edema to assess the involvement of histaminergic receptors. Fig 20 shows that H5 (20 mg/kg, p.o.) significantly reduced ($p<0.05$) histamine-induced paw edema at 30, 60, 90, 120, and 150 minutes, suggesting the involvement of histamine receptors in its anti-inflammatory effect.

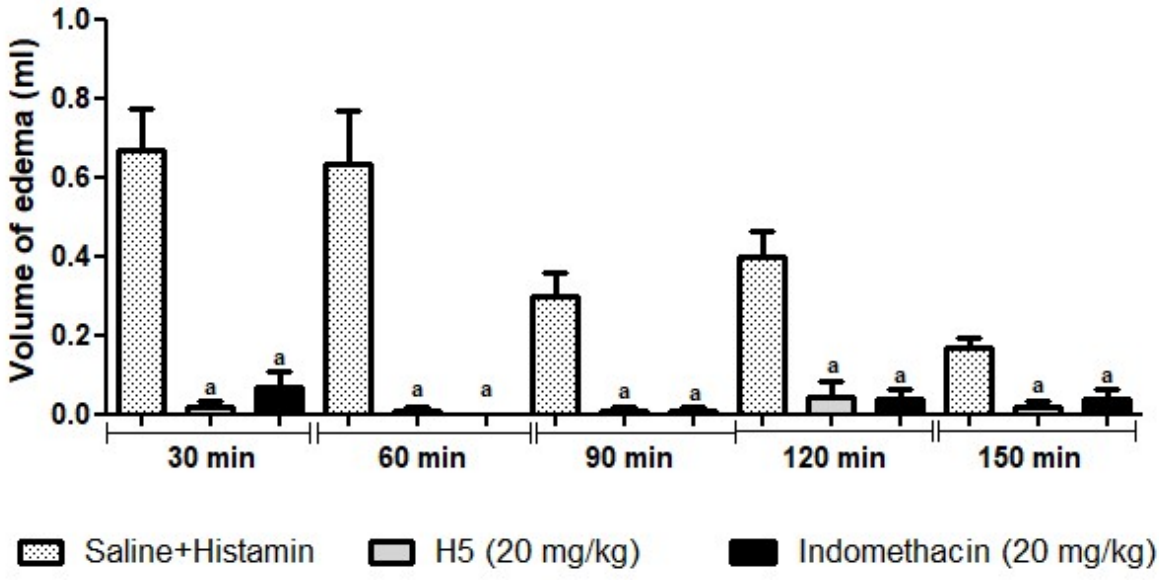


Fig 20. Effect of H5 (20 mg/kg, p.o.) on paw edema induced by histamine in mice. The sham group was treated only with saline, whereas the control group received saline and histamine. Values are expressed as the mean ± S.E.M. (n = 6, per group), where *a* indicates $p<0.05$, significantly different from the control group, according to ANOVA, followed by Tukey's post-test.

In addition, the rota-rod test was performed to assess the influence on motor coordination. Rota-rod test showed that there was achange in motor coordination of animals treated with H5 at all doses after 1, 1.5, and 2 h after administration of H5 (Fig 21). Similarly, diazepam significantly decreased the permanence time on the bar when compared to the negative control.

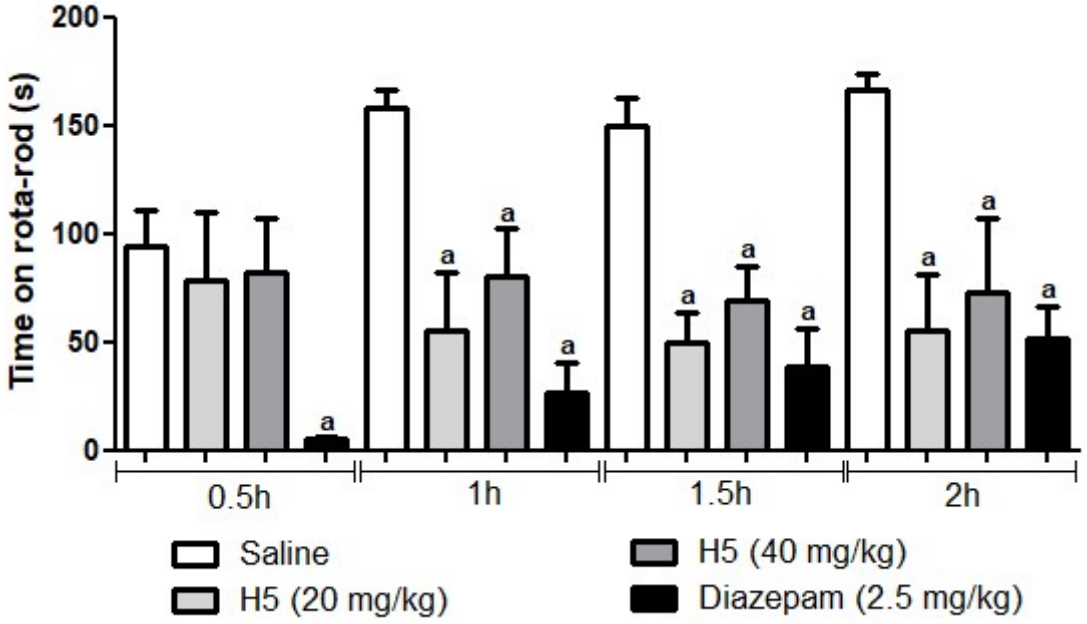


Fig 21. Effects of H5 (20 and 40 mg/kg, p.o.), and diazepam (2.5 mg/kg, i.p.) in the Rota-rod test in mice. Values are expressed as mean ± S.E.M. (n = 6, per group), where *a* indicates $p<0.05$, significantly different from the control group, according to ANOVA, followed by Tukey's post-test.

H5 toxicity was assessed using the *Artemia salina* test. The *Artemia salina* assay is a simple, economical, and efficient method for determining acute toxicity. Table 1 describes the lethality rates for H5, positive control, and negative control. It was observed that the positive control showed 40% lethality in the first 24 hours and after 48 hours it resulted in 100%. In the negative control, only one larva of the triplicates did not survive, which showed a lethality rate of 3.4% after 48 hours. For H5 in the first 24 hours, a lethality rate of 54% was observed at a concentration of 250 µg/ml, and after 48 hours a higher lethality rate was observed from the concentration of 100 µg/ml.

Meyer et al. (1982) [30] considers toxic the substance that has an LC_{50} value less than 1000 µg/ml compared to *Artemia salina*. In this sense, Table 2 shows the results of H5 toxicity through LC_{50} values. The analysis of the results indicated that H5 presented toxicity after 24 and 48 hours, since it presented LC_{50} of 210.6 µg/ml after 24 hours, and 81.95 µg/ml after 48 hours. However, according to Dolabela (1997) [61], toxicity is considered moderate with LC50 values between 80 µg/ml and 250 µg/ml. Therefore, H5 showed moderate toxicity.

Table 1. Lethality rate of *Artemia salina* nauplius to hydrazone (H5).

Sample	Concentration (µg/ml)	Lethality rate – 24h (%)	Lethality rate – 48h (%)
H5	1	0	4
	50	7	34
	100	17	57
	250	54	84
	500	100	100
	1000	100	100
CP	800	40	100
CN	0.0038	0	3.4

Table 2. *Artemia salina* toxicity test of hydrazone H5.

Sample	CE_{50}± SD of H5 (µg/ml)
24 hours	210.6 ± 68.38
48 hours	81.95 ± 11.10

Considering the docking results, the redocking studies were performed and we get the best RMSD value of 0.28 Å (Figure 22) for ChemPLP function with score value of 65.25. We observed that compound H5 has a higher score of 70.54 than meloxicam (MXM) with its interaction modes shown in figure 23.

H5 fit in a similar way in the binding site like, with his phthalazine moiety occupying a larger space than the methylthiazole moiety of MXM. We can observe a π-π interaction between methoxyphenyl group and Tyr355; also phthalazine and Trp387. It seem that the most important interaction are the hydrogen bond that phthalazine has with Tyr355 and Ser530 (Figure 23). All this interaction through docking studies may explain why H5 have a high antinociceptive and anti-inflamatory effects.

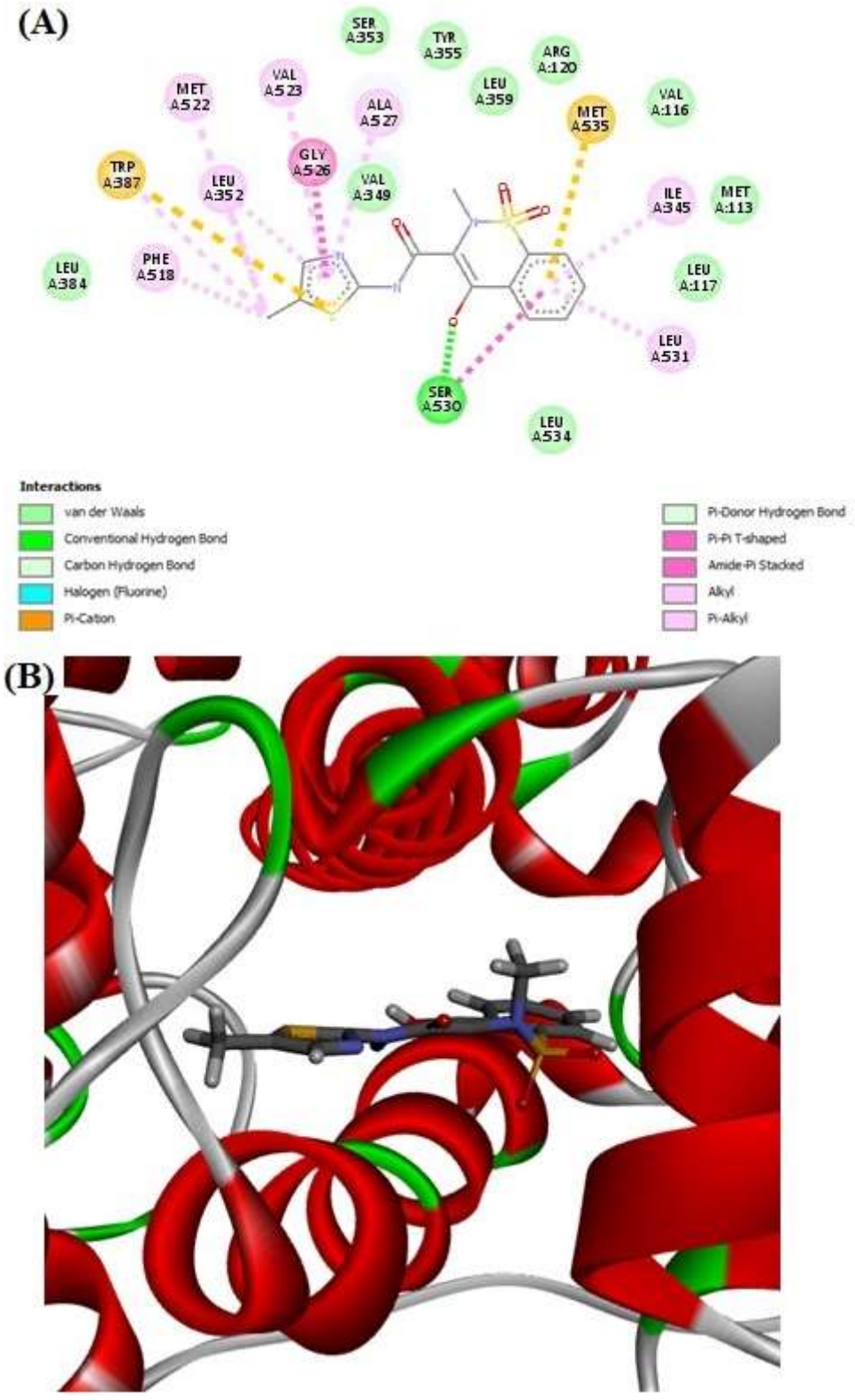


Fig 22. Interaction profile of meloxicam (MXM) in the murine COX-2 enzyme binding site after the redocking process.

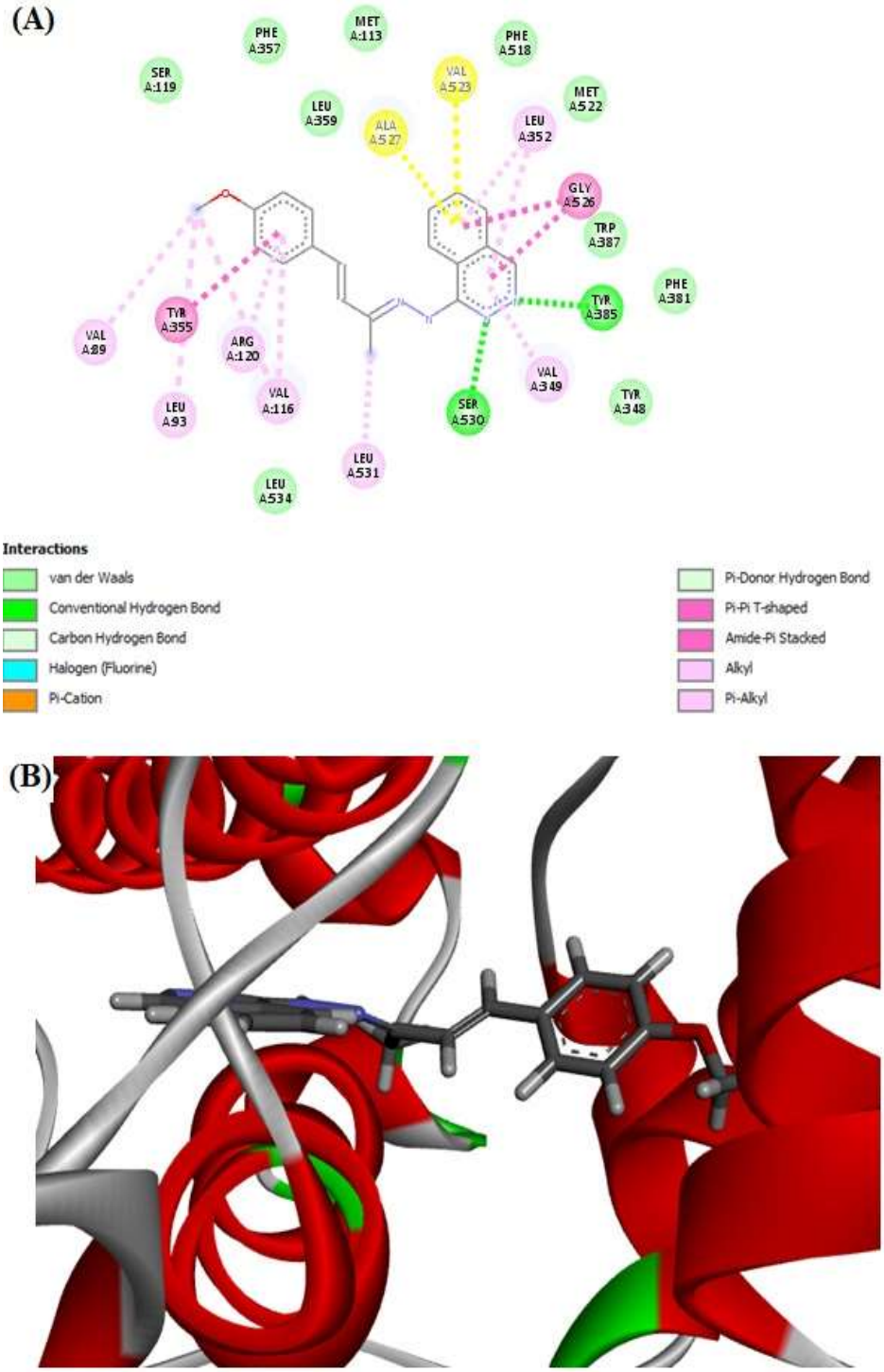


Fig 23. Interaction profile of H5 in the murine COX-2 enzyme binding site after the docking process.

According to the physicochemical properties and ADMET profile presented in Table 3, no violation of Lipinsky rule of five (Ro5) was found for H5 pointing out that this compound has properties that would make it a likely orally active drug in humans [62,12]. However, H5 was predicted to have a lower solubility once compared to drug like indomethacin and meloxicam. Concerning their comparative ADMET profile, they were predicted to be highly absorbed (HIA = 100%), highly permeable (Pe $> 7 \times 10 - 6$ cm/s) and extensively bound to plasma protein (PPB > 90%). All compounds have indicated a great oral bioavailability *in silico* (F = 80 to 99%). The principal differences amongst H5, indomethacin and meloxicam rely on their metabolic stability in human liver microsomes (HLM) and their ability to penetrate in CNS. As described in Table 3, indomethacin and meloxicam were predicted as stable in HLM (scores of 0.26 and 0.32, respectively), while an undefined result was found for H5 (Score = 0.54). Both anti-inflammatory drugs have been predicted as non-penetrant to CNS (scores of -4.32 and -5.24, respectively) whereas H5 was defined as able to penetrate CNS. To have some index about the toxicity of H5, the ability of this compound to inhibit hERG (the human Ether-à-go-go-Related Gene), and its mutagenic profile (i.e. probability of a positive Ames test) were predicted. The results were converted into classification scores, and an undefined hERG and mutagenic activities (score > 0.33 and ≤ 0.67) were predicted for H5. Taken together, those *in silico* results suggest an adequate pharmacokinetic profile for H5, meanwhile the *in silico* approach was not able predicted its toxicological profile [12].

Table 3. Comparative *in silico* physicochemical properties and ADMET profile of the anti-inflammatory drugs indomethacin and meloxicam and new hydrazone serie H1 to H5.

Predicted Properties*	Compounds						
	H1	**H2**	**H3**	**H4**	**H5**	**Indomethacin**	**Meloxicam**
MW (g/mol)	304.35	334.37	331.41	304.35	318.37	357.79	351.40
H-Donors	2	2	1	2	1	1	2
H-Acceptors	5	6	5	5	5	5	7
Rotable Bonds	4	5	5	4	5	4	2
TPSA	70.4	79.63	53.41	70.4	59.4	68.53	136.22
LogP	3.7	2.92	4.19	3.15	3.63	4.02	2.38
Solubility (mg/ml)	0.01	0.03	0.004	0.03	0.008	2.5	5.96
Caco-2 (cm/s)	Pe= $215x10^{-6}$	Pe= $194x10^{-6}$	Pe= $232x10^{-6}$	Pe= $205x10^{-6}$	Pe= $235x10^{-6}$	Pe = 129×10^{-6}	Pe = 233×10^{-6}
HIA (%)	100	100	100	100	100	100	100
F (oral) (%)	90	93	80	97	92	99	96
PPB (%)	99	99	99	99	99	99	99
CNS Score	-3.19	-3.4	-3.36	-3.33	-3.19	-4.32	-5.24
HLM	0.51	0.53	0.54	0.56	0.54	0.26	0.32
hERG	0.48	0.47	0.49	0.48	0.48	0.23	0.41
AMES	0.52	0.53	0.53	0.52	0.52	0.27	0.21

*Determined *in silico* using the ACD/Percepta Program. MW = molecular weight; H-Donors = hydrogen bond-donors; H-Acceptors = hydrogen bond-acceptors; TPSA = topological polar surface area; LogP = the logarithm of the drug partition coefficient between n-octanol and water; Caco-2 = human epithelial cell line Caco-2; HIA= human intestinal absorption; F = Bioavailability; CNS = central nervous system; HLM = human liver microsomes; hERG = the human Ether-à-go-go-Related Gene; AMES = Ames test = *Salmonella typhimurium* reverse mutation assay.

Conclusion

Based on the results presented here, H1, H2, H3, H4, and H5 showed a significant antinociceptive effect in both experimental models, especially H5. This later hydrazone showed significant antinociceptive and anti-inflammatory activities in all of the experimental models. Its mechanism of action appears to be peripheral, with the involvement of opioid and nitrergic signaling pathways, in addition to inhibition of COX-2, as demonstrated by the molecular docking study. In summary, H5 has emerged as a strong candidate for a multi-target antinociceptive and anti-inflammatory drug.

Supporting information

S1 Dataset. Raw data.
(RAR)

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Attachment

Preparation of the intermediate carbonyl compounds

In a reaction flask, 2 mmol of each aldehyde (a1-a5), 10 ml of propanone PA (higher excess) and 10 ml of anaqueous solution of KOH at 56 g/l were mixed. The reaction mixture was closed and left to reacta troom temperature for 24 h (protected from light). The resulting mixture was poured into a beaker containing 50 ml of a solution of H_3CCOOH 0.20 mol/l and left in refrigerator for another 24 h for crystallization. The solid formed was filtered, washed with distilled water and dried a troom temperature (protected from light). These synthetic intermediates (i1-i5) were characterized only by melting point determination since their syntheses and properties are already reported in scientific papers.

Intermediate properties

*Intermediate*i 1: 4-(2-(hydroxyphenyl)but-3-en-2-one
yellow solid, 65% yield; mp134-136 ºC.

*Intermediate*i 2: 4-(4-(hydroxyphenyl)but-3-en-2-one
yellow solid, 70% yield; mp 99-102 ºC

*Intermediate*i 3: 4-(4-(hydroxy-3-methoxyphenyl)but-3-en-2-one
yellow solid, 69% yield; mp 125-128 ºC

*Intermediate*i 4: 4-(4-(dimethylamino)phenyl)but-3-en-2-one
yellow solid, 77% yield; mp 123-125ºC

*Intermediate*i 5: 4-(4-methoxyphenyl)but-3-en-2-one
yellow solid, 74% yield; mp 68-70ºC

Preparation of the hydrazone derivatives

In a reaction flask, 0.50 mmol of each intermediate (i1-i5), 0.471 g of hydralazine drug (previously pulverizated with using mortar and pestle and containing 0.50 mmol drug equivalent), 5.0 ml of absolute ethanol, 4.0 ml of distilled water, 3.0 ml of glacial H_3CCOOH and 3 drops of concentrated H_2SO_4 were mixed. The reaction mixture was closed and left to reacta troom temperature for 6 h with Constant magnetics tirring. The resulting mixture was filtered and the filtrate collected in a beaker. The residuereta in edo the paper was discarded (excipients). To the filtrate was added 20 ml of an ice cold aqueous solution of 5.0% (w/v)

$NaHCO_3$. The solid formed was filtered, washed with distilled water and dried at room temperature (protected from light). The products were purified by chromatographic column, using silica gel as stationary phase, and the mixture of hexane:ethylacetate (8:2 v/v) as eluent. The hydrazone derivatives (H1-H5) were characterized by melting point determination, FTIR, ^{1}H-NMR and ^{13}C-NMR. These purified products were used *in vivo* tests to assessanti nociceptive and anti-inflammatory activities.

Hydrazone derivatives properties

Hydrazone H1: 2-(3-(2-(phthalazin-1-yl)hydrazono)but-1-en-1-yl)phenol: orange solid, 46% yield, mp 263-265 ºC. FTIR (KBr, cm^{-1}): 3107 (υN–H), 1605 (υC=N azomethine), 1584 and 1458 (υC=C). ^{1}H-NMR (400 Mhz, DMSO-d_6, δppm): 2.30 (s, 3H, CH_3); 6.85 (m, 2H, Ar-H); 7.12 (m, 2H, =CH + Ar-H); 7.27 (dd, 1 H, =CH, J = 16.63 Hz); 7.48 (d, 1H, Ar-H); 7.70 (m, 3H, Ar-H); 8.02 (s, 1H, Ar-H); 8.30 (d, 1H, Ar-H); 9.88 (s, 1H, OH) e 11.72 (s, 1H, NH). ^{13}C-NMR (100 Mhz, DMSO-d_6, δ ppm): 12.47; 116.37; 119.85; 123.84; 124.17; 126.78; 126.82; 127.15; 127.42; 128.27; 129.62; 130.68; 132.14; 132.51; 137.90; 146.77; 155.61 e 159.87 (C=N, azomethine).

Hydrazone H2: 4-(3-(2-(phthalazin-1-yl)hydrazono)but-1-en-1-yl)phenol: yellow solid, 44% yield, mp 240-242 ºC. FTIR (KBr, cm^{-1}): 3243 (υN–H), 1605 (υC=N azomethine), 1584 and 1512 (υC=C). ^{1}H-NMR (400 Mhz, DMSO-d_6, δ ppm): 2.28 (s, 3H, CH_3); 6.78 (d, 2H, Ar-H); 6.93 (d, 1H, =CH, J = 16.50 Hz); 7.03 (d, 1H, =CH, J = 16.50 Hz); 7.40 (d, 2H, Ar-H); 7.70 (m, 3H, Ar-H); 8.02 (s, 1H, Ar-H); 8.30 (d, 1H, Ar-H); 9.73 (s, 1H, OH) e 11.68 (s, 1H, NH). ^{13}C-NMR (100 Mhz, DMSO-d_6, δ ppm): 12.28; 115.78 (2C); 123.76; 126.41; 126.84; 127.04; 127.64; 127.85; 128.24 (2C); 131.74; 132.08; 132.95; 137.44; 146.24; 157.86 e 159.30 (C=N, azomethine).

Hydrazone H3: 2-methoxy-4-(3-(2-(phthalazin-1-yl)hydrazono)but-1-en-1-yl)phenol: yellow solid, 40% yield, mp 241-243ºC. FTIR (KBr, cm^{-1}): 3264 (υN–H), 1604 (υC=N azomethine), 1584 and 1511 (υC=C). ^{1}H-NMR (400 Mhz, DMSO-d_6, δ ppm): 2.31 (s, 3H, CH_3); 3.84 (s, 3H, OCH_3); 6.80 (d, 1H, =CH, J = 8.04 Hz); 7.00 (m, 3H, =CH + Ar-H); 7.17 (d, 1H, Ar-H); 7.73 (m, 3H, Ar-H); 8.04 (s, 1H, Ar-H); 8.32 (m, 1H, Ar-H); 9.31 (s, 1H, OH) e 11.66 (s, 1H, NH). ^{13}C-NMR (100 Mhz, DMSO-d_6, δ ppm): 12.17; 55.44; 109.64; 120.44; 123.58; 126.25; 126.67; 126.87; 127.70; 128.23; 131.58; 131.94; 133.10; 137.31; 146.12; 147.11; 147.75 e 159.08 (C=N, azomethine).

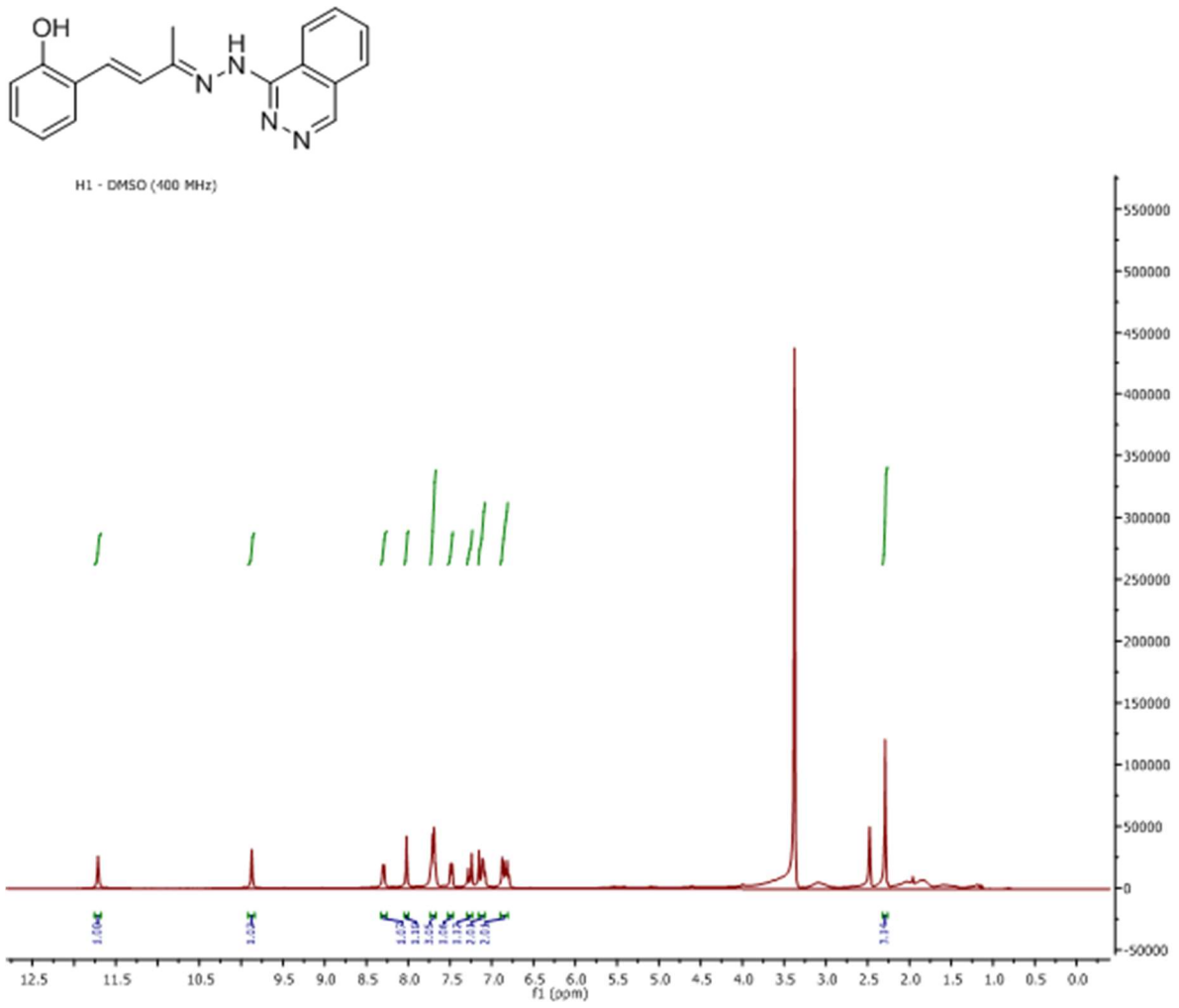
Hydrazone H4: N,N-dimethyl-4-(3-(2-(phthalazin-1-yl)hydrazono)but-1-en-1-yl)aniline: orange solid, 51% yield, mp 176-178 ºC. FTIR (KBr, cm^{-1}): 3242 (υN–H), 1601 (υC=N

azomethine), 1585 and 1519 (υC=C). ^{1}H-NMR (400 Mhz, DMSO-d_6, δ ppm): 2.30 (s, 3H, CH_3); 2.95 (s, 6H, $N(CH_3)_2$); 6.74 (d, 2H, Ar-H); 6.90 (d, 1H, =CH, J = 16.56 Hz); 7.02 (d, 1H, =CH, J = 16.56 Hz); 7.41 (d, 2H, Ar-H); 7.72 (m, 3H, Ar-H); 8.02 (s, 1H, Ar-H); 8.30 (m, 1 H, Ar-H) e 11.62 (s, 1H, NH). ^{13}C-NMR (100 Mhz, DMSO-d_6, δ ppm): 12.09; 39.77; 112.11; 123.53; 124.37; 125.84; 126.21; 126.78; 126.85; 127.73; 131.54; 131.83; 133.19; 137.15; 145.81; 150.16; 155.40 e 159.32 (C=N, azomethine).

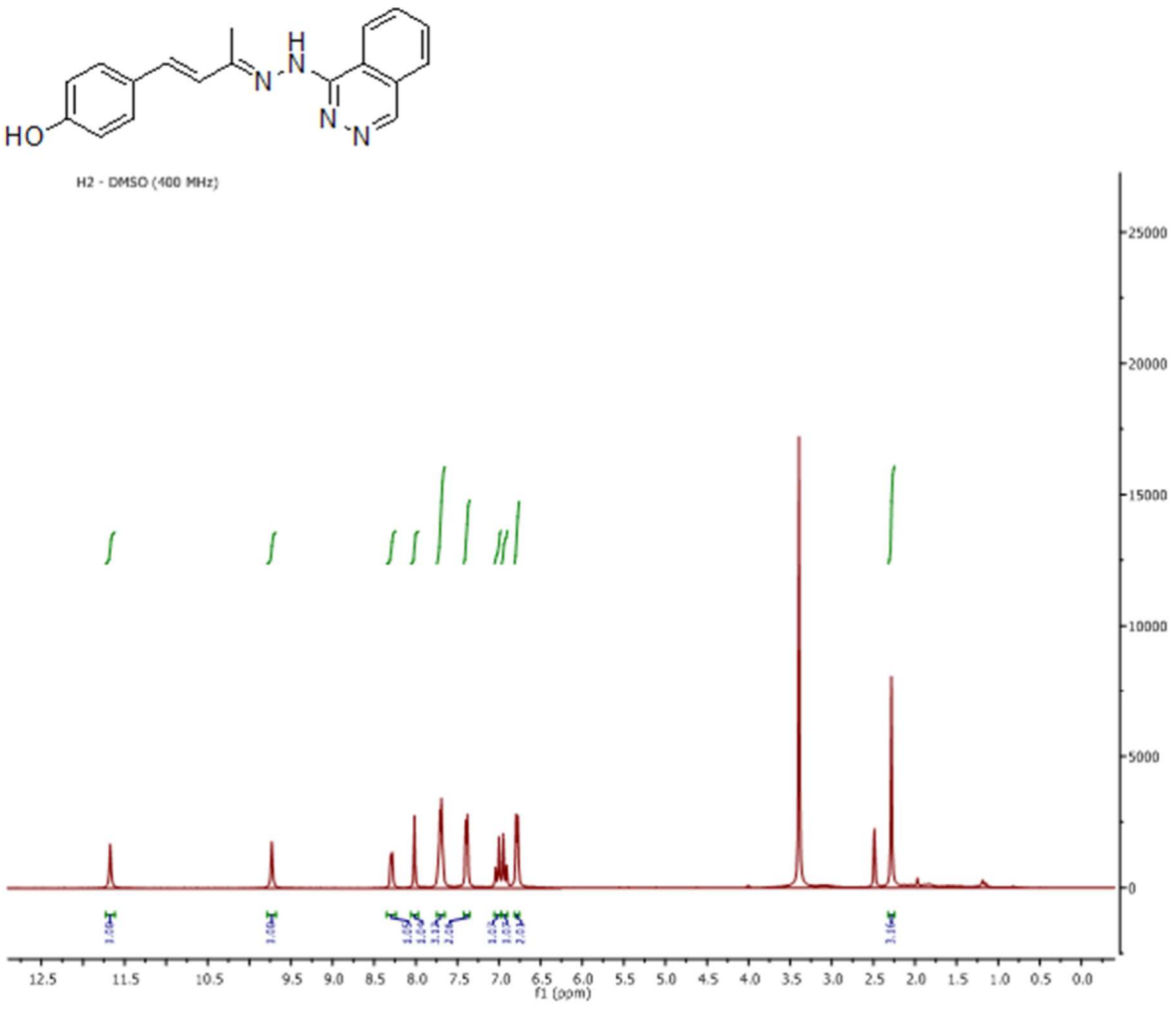
Hydrazone H5: 1-(2-(4-(4-methoxyphenyl)but-3-en-2-ylidene)hydrazinyl)phthalazine: yellow solid, 48% yield, mp165-168 ºC. FTIR (KBr, cm^{-1}): 3254 (υN–H), 1606 (υC=N azomethine), 1588 and 1508 (υC=C). ^{1}H-NMR (400 Mhz, DMSO-d_6, δ ppm): 2.30 (s, 3H, CH_3); 2.95 (s, 6H, $N(CH_3)_2$); 6.74 (d, 2H, Ar-H); 6.90 (d, 1H, =CH, J = 16.56 Hz); 7.02 (d, 1H, =CH, J = 16.56 Hz); 7.41 (d, 2H, Ar-H); 7.72 (m, 3H, Ar-H); 8.02 (s, 1H, Ar-H); 8.30 (m, 1 H, Ar-H) e 11.62 (s, 1H, NH). ^{13}C-NMR (100 Mhz, DMSO-d_6, δ ppm): 12.09; 39.77; 112.11; 123.53; 124.37; 125.84; 126.21; 126.78; 126.85; 127.73; 131.54; 131.83; 133.19; 137.15; 145.81; 150.16; 155.40 e 159.32 (C=N, azomethine).

^{1}H-NMR of the hydrazone derivatives

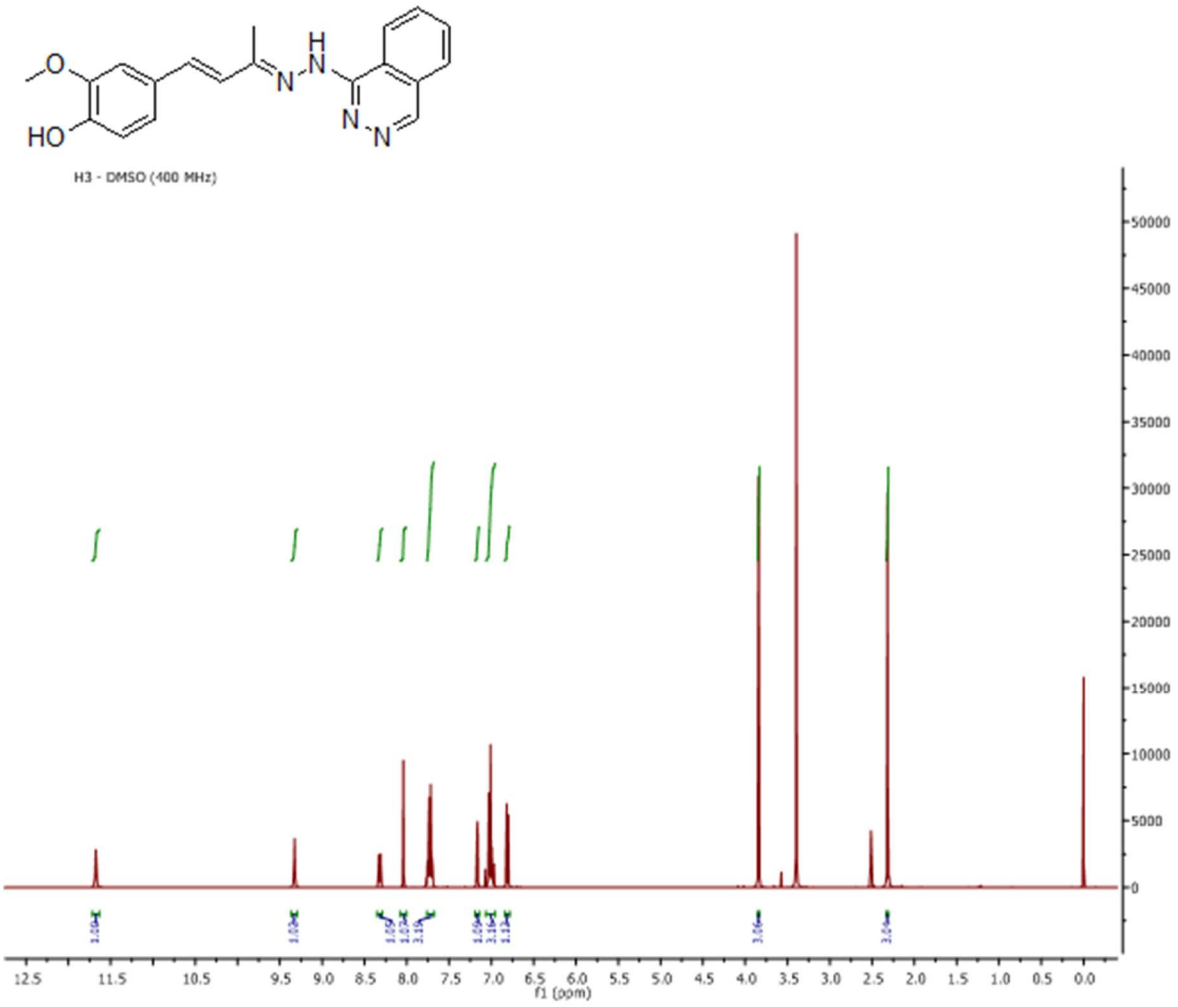
^{1}H-NMR spectra (400 MHz) in DMSO-d6 of hydrazone H1



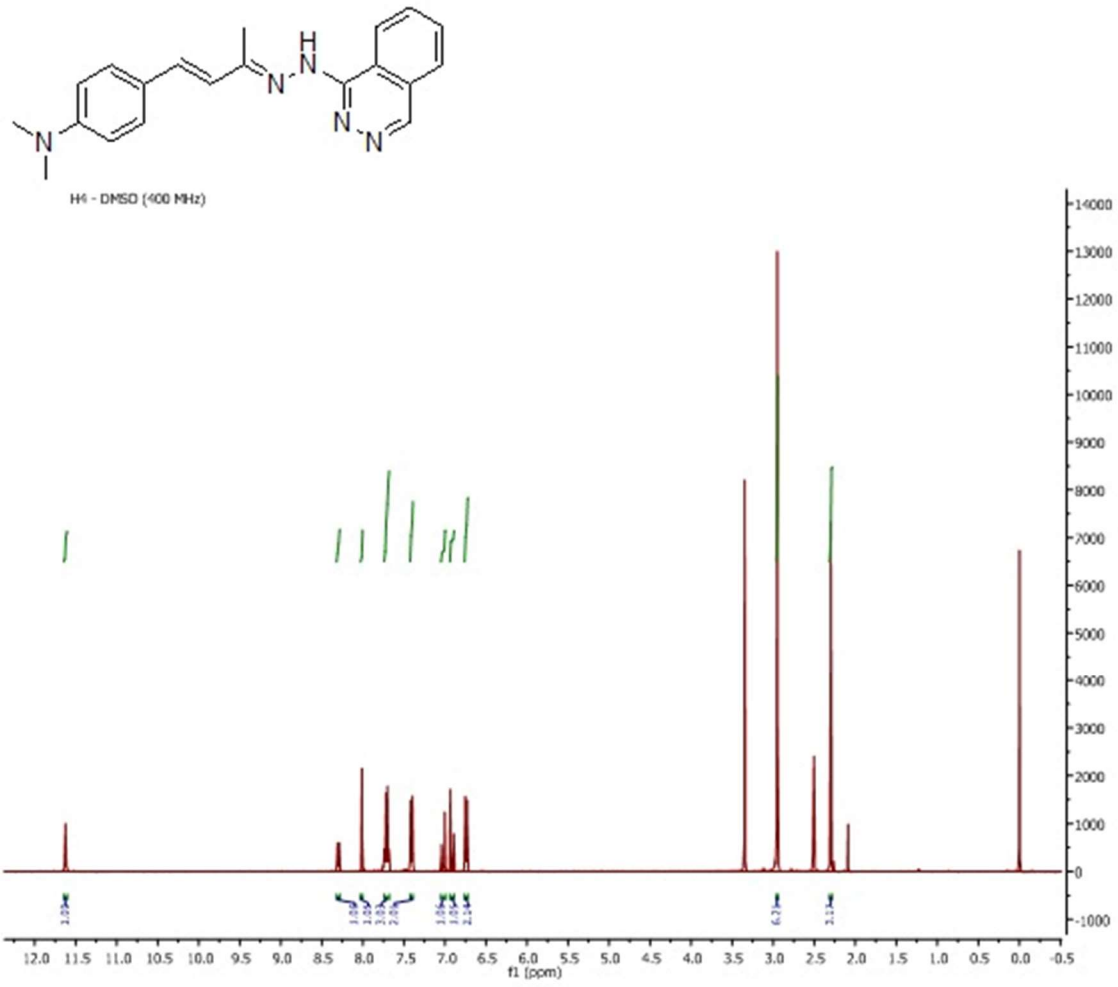
[1]H-NMR spectra (400 MHz) in DMSO-d6 of hydrazone H2



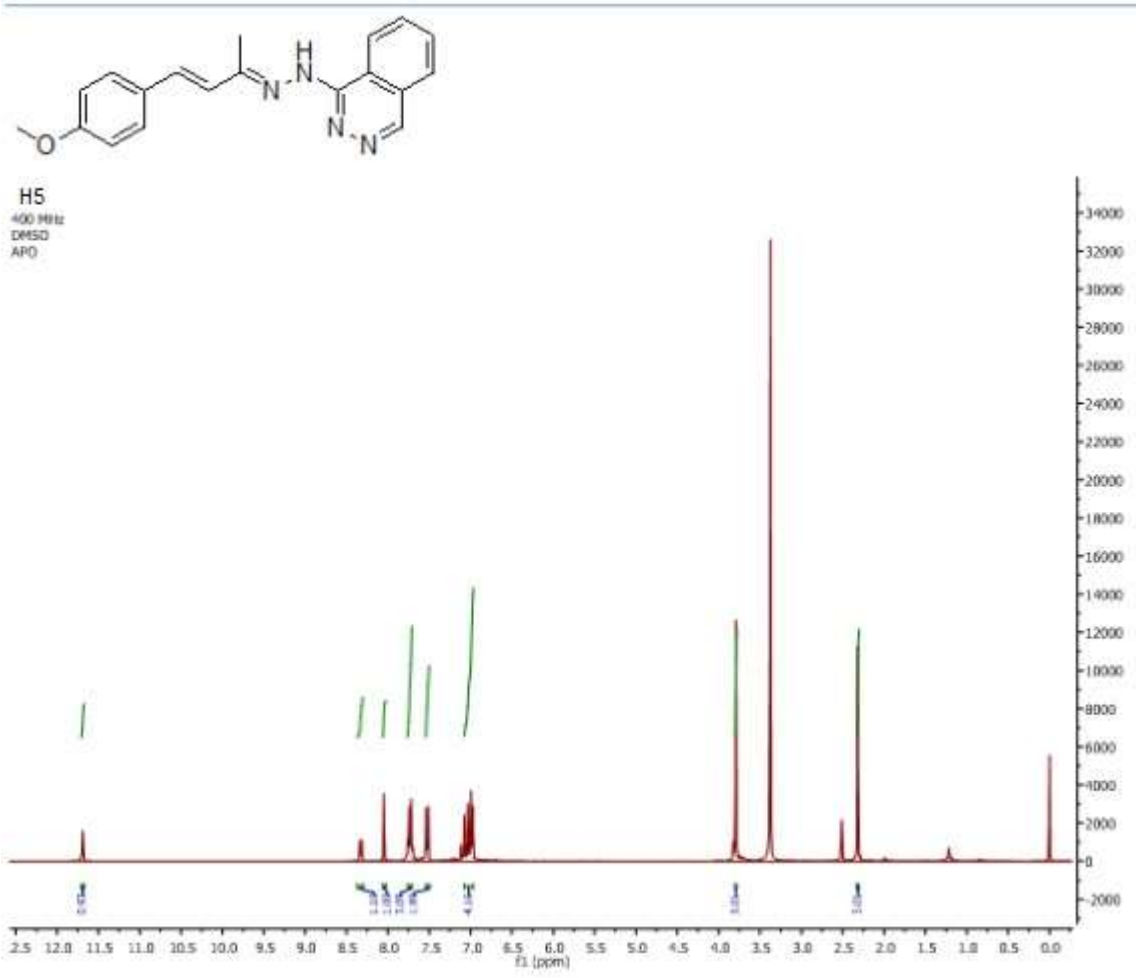
[1]H-NMR spectra (400 MHz) in DMSO-d6 of hydrazone H3



[1]H-NMR spectra (400 MHz) in DMSO-d6 of hydrazone H4

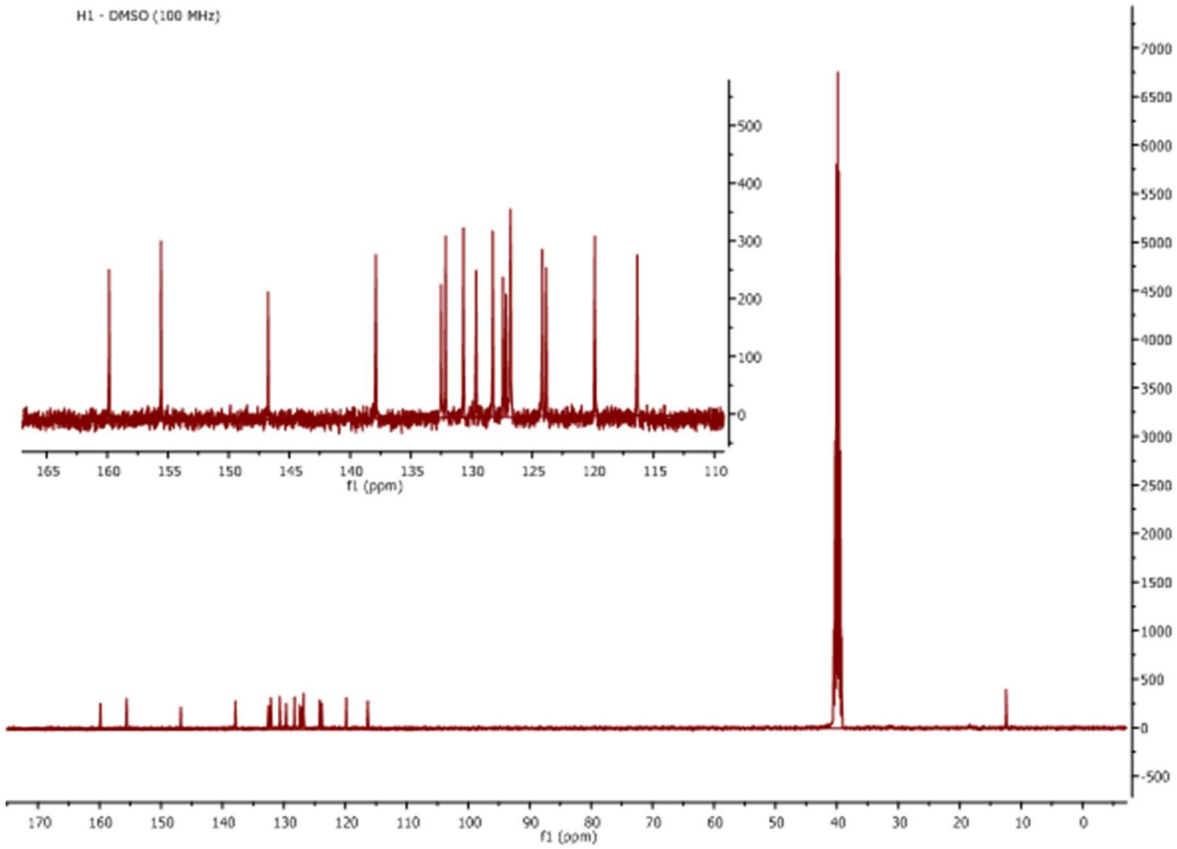


[1]H-NMR spectra (400 MHz) in DMSO-d6 of hydrazone H5

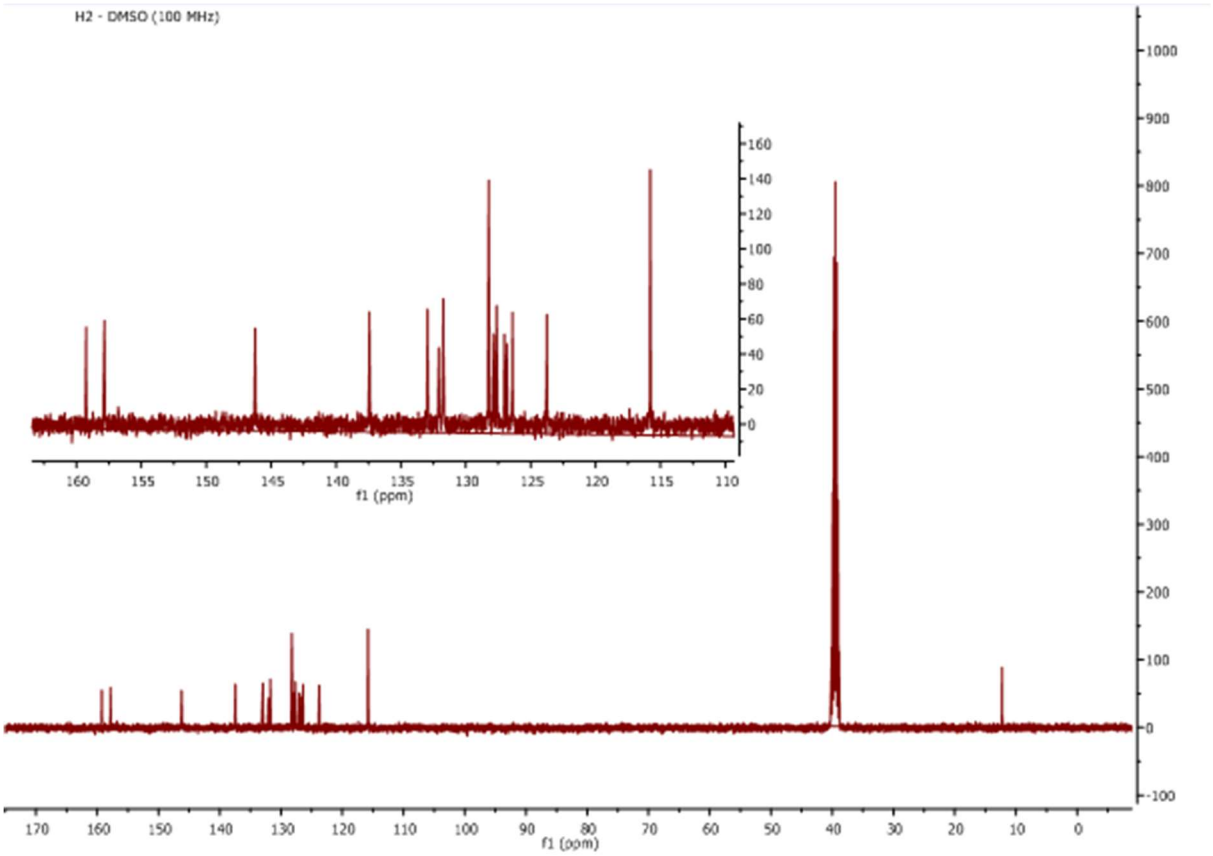


^{13}C-NMR of the hydrazone derivatives

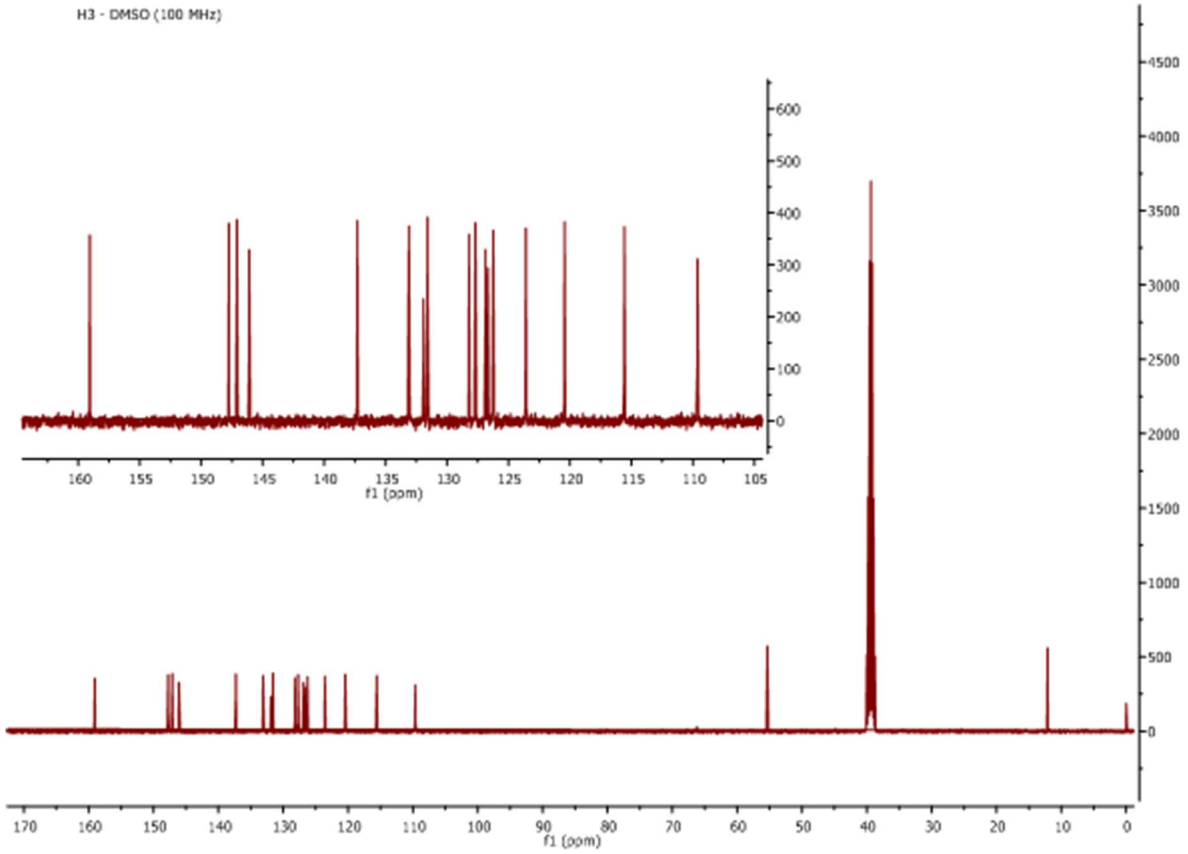
^{13}C-NMR spectra (400 MHz) in DMSO-d6 of hydrazone H1



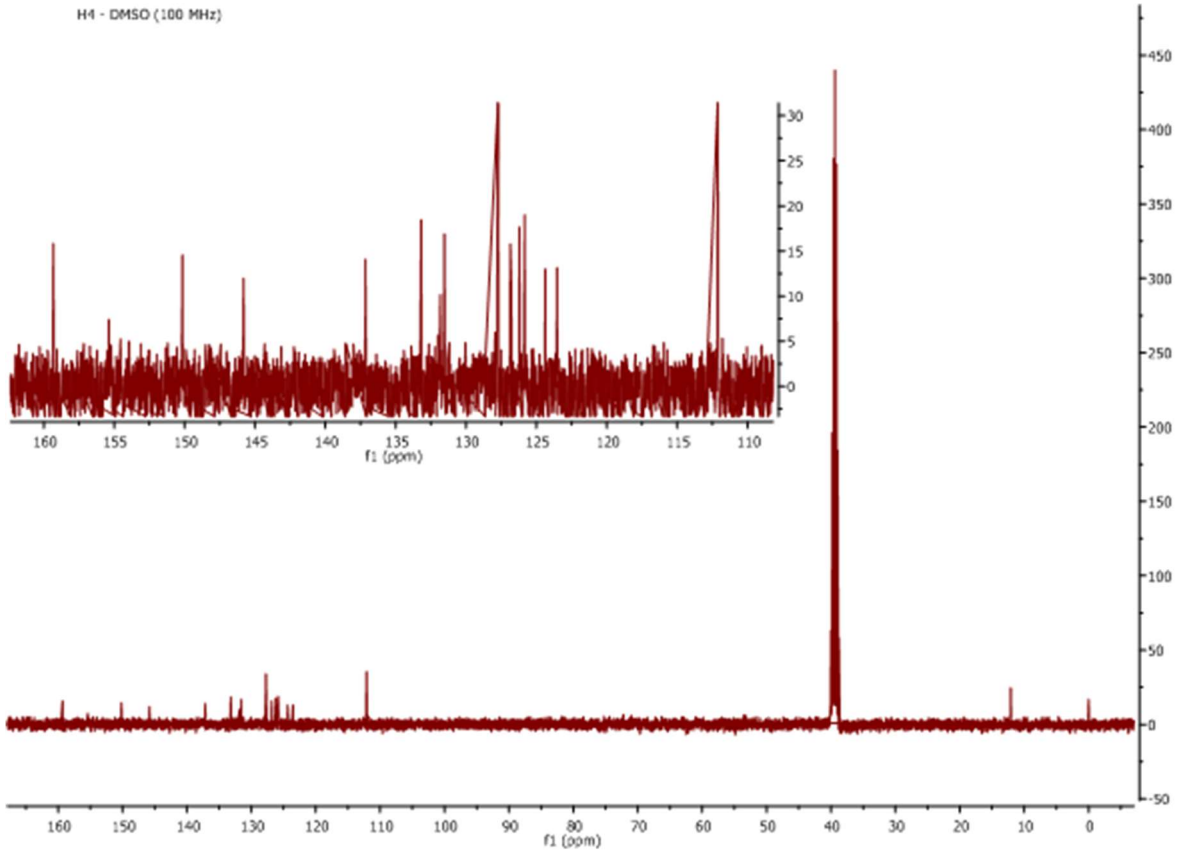
^{13}C-NMR spectra (400 MHz) in DMSO-d6 of hydrazone H2



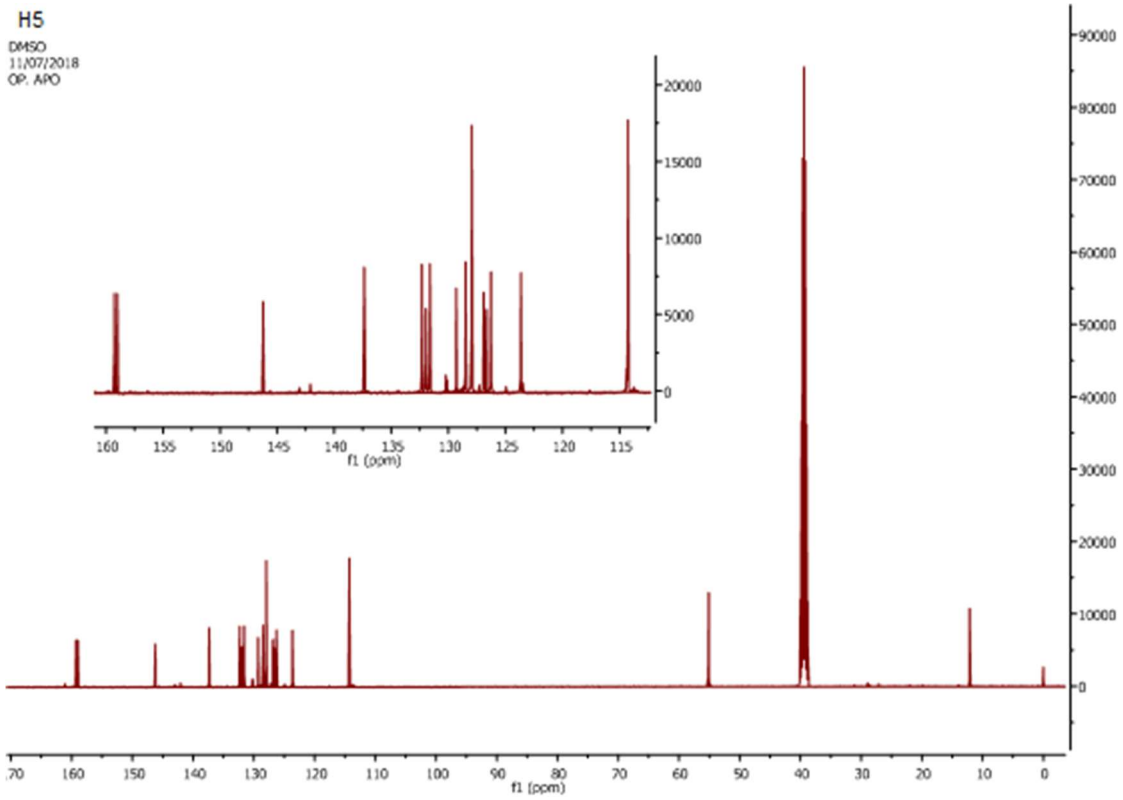
^{13}C-NMR spectra (400 MHz) in DMSO-d6 of hydrazone H3



^{13}C-NMR spectra (400 MHz) in DMSO-d6 of hydrazone H4



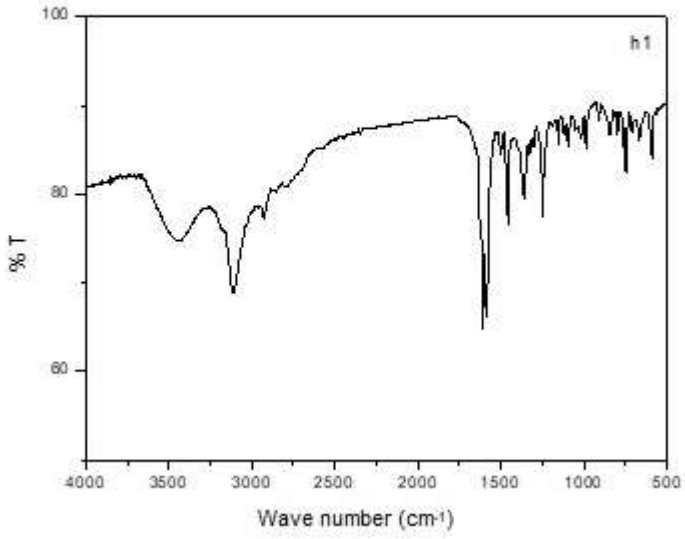
^{13}C-NMR spectra (400 MHz) in DMSO-d6 of hydrazone H5



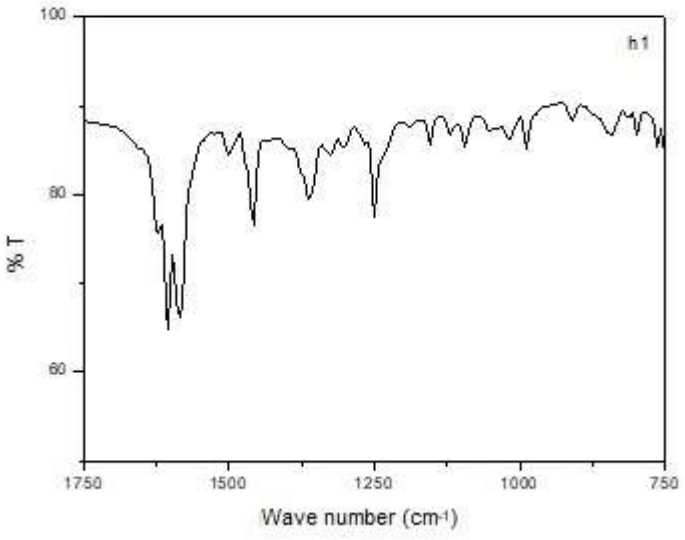
FTIR of the hydrazone derivatives

FTIR spectra (KBr) of hydrazone H1

(A)



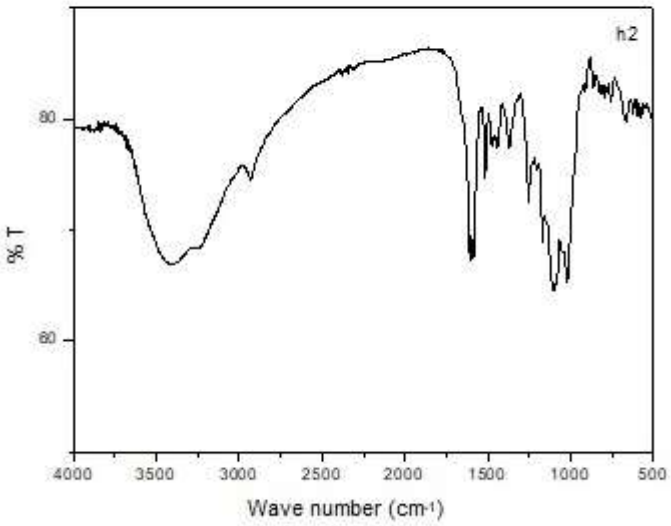
(B)



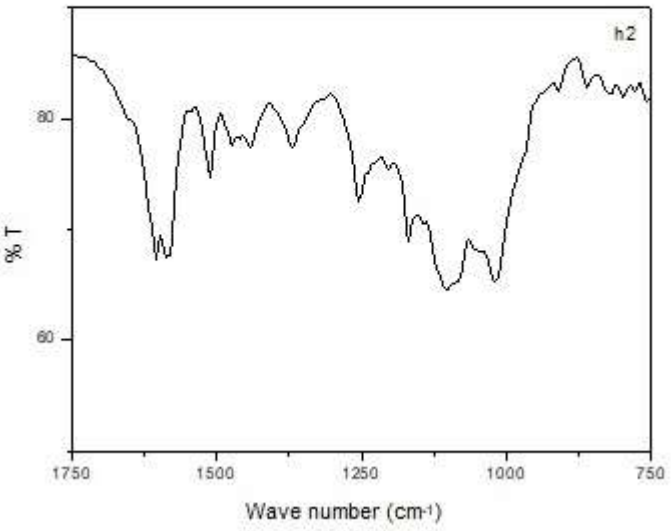
Peaks (cm^{-1}):3447 (υO–H), 3107 (υN–H), 1605 (υC=N azomethine), 1584 e 1458 (υC=C), 1362 (δO–H), 1250, 1153 e 1093 (υC–O) e 1015 (υC–N).

FTIR spectra (KBr) of hydrazone H2

(A)



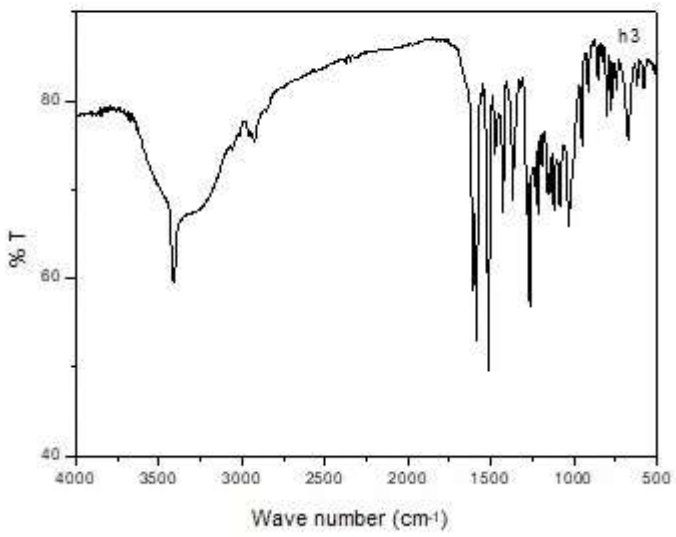
(B)



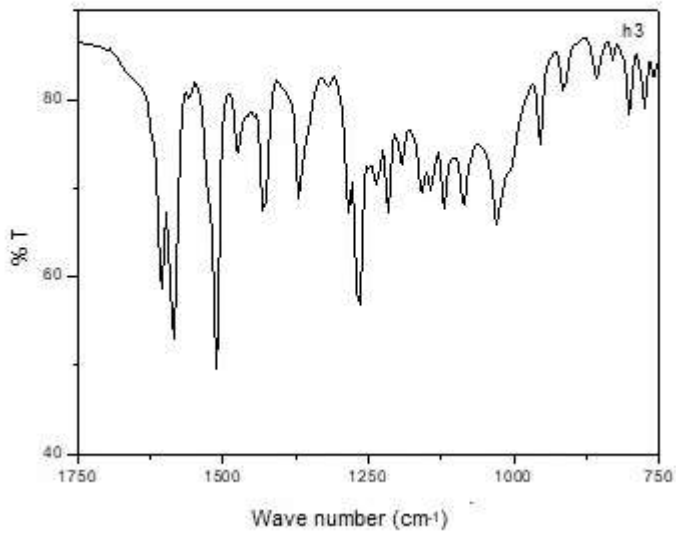
Peaks(cm^{-1}):3408 (νO–H), 3243 (νN–H), 1605 (νC=N azomethine), 1584 e 1512 (νC=C), 1368 (δO–H), 1254, 1168, 1102 (νC–O) e 1018 (νC–N).

FTIR spectra (KBr) of hydrazone H3

(A)



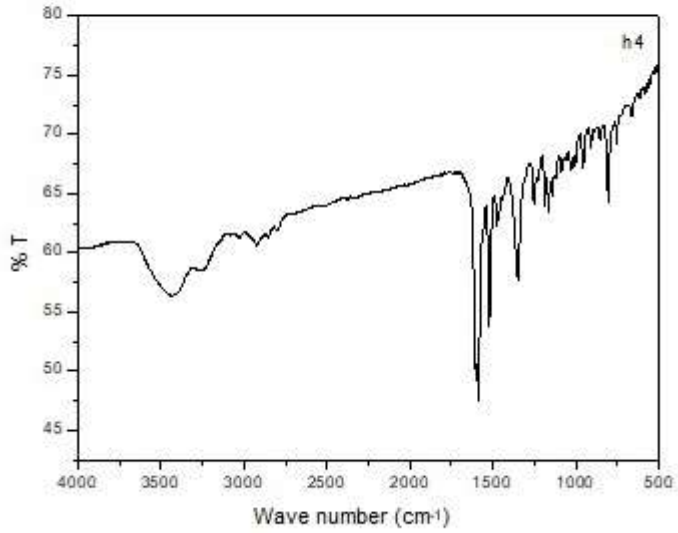
(B)



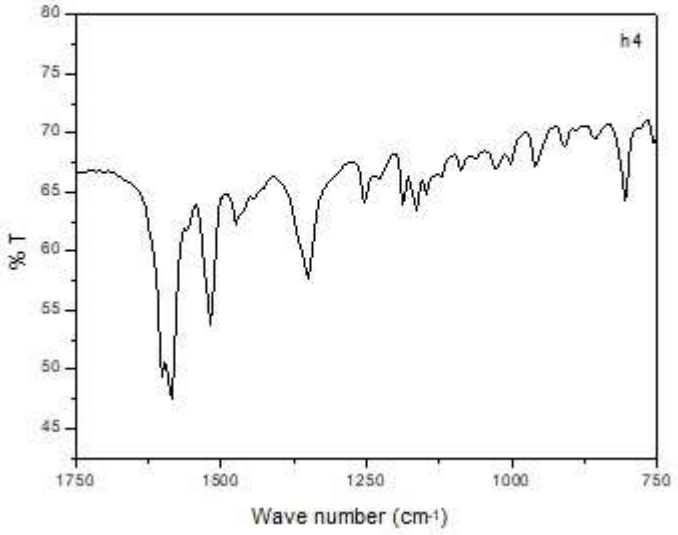
Peaks (cm^{-1}):3408 (νO–H), 3264 (νN–H), 2924 (νCH₃eter), 1605 (νC=N azomethine), 1584 e 1511 (νC=C), 1431 (δCH₃eter), 1370 (δO–H), 1265, 1215 e 1120 (νC–O–C), 1086 e 955 (νC–O) e 1029 (νC–N).

FTIR spectra (KBr) of hydrazone H4

(A)



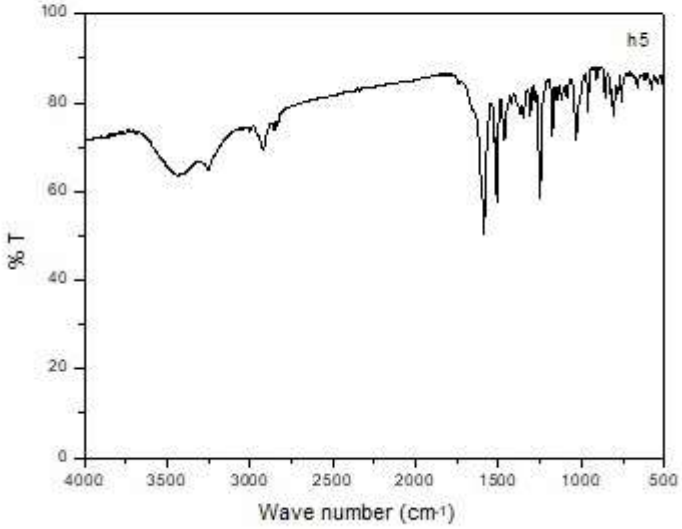
(B)



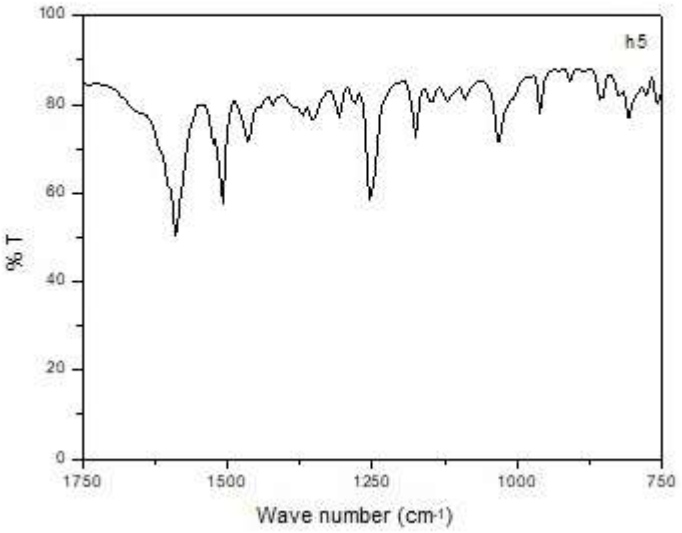
Peaks (cm^{-1}): 3242 (νN–H), 1601 (νC=N azomethine), 1585 e 1519 (νC=C), 1350 (νC–N) e 804 (δC–H).

FTIR spectra (KBr) of hydrazone H5

(A)



(B)



Peaks (cm^{-1}):3254 (υN–H), 2921 (υCH_3eter), 1605 (υC=N azomethine, "shoulder peak"), 1588, 1508 (υC=C), 1465 (δCH_3eter), 1252 e 1177 (υC–O–C) e 1032 e 961 (υC–O).

CAPÍTULO 2

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Antinociceptive effect of LASSBio-2012, an *N*-acylhydrazone derivative, in mice

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Abstract

Objectives The objective of this work was to evaluate the antinociceptive effect of the *N*-acilhydrazone LASSBio-2012 in different experimental models as well as its possible antinociceptive mechanism of action in mice.

Methods Antinociceptive activity was evaluated through acetic acid-induced writhing and formalin tests. The antinociceptive mechanism of action was investigated using pharmacological blockers (naloxone, *L*-NAME, *L*-arginine, ondansetron, atropine, red ruthenium, and glibenclamide) in the formalin test. Additional *in silico* procedures were carried out to evaluate the physicochemical properties, ADMET profile, and molecular docking analysis of LASSBio-2012.

Results In these two experimental models, LASSBio-2012 significantly ($p<0.05$) reduced nociceptive behavior. LASSBio-2012 attenuated the nociceptive behavior induced by acetic, reducing the number of writhings by 79.66% and 85.22% at the doses tested (20 mg/kg and 40 mg/kg, respectively). In the formalin test, LASSBio-2012 reduced nociceptive behavior only in the second phase being the doses of 20 and 40 mg/kg responsible for 50.38% and 65.00% of thenormal antinociceptive effect, respectively. The study of the mechanism of action demonstrated the possible involvement of the opioid system.

Conclusion In summary, LASSBio-2012 exhibited relevant antinociceptive potential acted on several targets, making it a candidate for a new oral antinociceptive drug.

Keywords: *N*-acylhydrazone; antinociceptive effect; pain

Introduction

Nociception is the transmission of painful signals by nociceptors through afferent nerve fibers in response to harmful stimuli is defined.[1] Nociceptors are neurons capable of identifying and signaling likely injuries. Neural signals reach the brain, stimulate protective events that can be identified as painful. These neurons can be triggered by thermic, chemical, and mechanical stimuli. According to the literature, they can be classified according to stimuli response, myelination, diameter, and speed of conduction. According to electrophysiology, the transduction of the nociceptive impulse occurs in the form of action potentials that result in the release of neurotransmitters in the dorsal horn.[2]

Pain is defined as an unpleasant sensation, both sensory and emotional, related to tissue damage, according to the International Association for the Study of Pain. Because of emotional involvement, pain is considered subjective, so it is immeasurable. However, nociception, unlike pain, is not subjective and can be monitored. Pathophysiological and acute pain management studies are constantly growing.[3,4]

In the induction of pain, several chemical components, such as serotonin, bradykinin, prostaglandins, nitric oxide, and histamine, in the process of tissue damage can stimulate nociceptive fibers. Given this, pain can be characterized as a basic defense of the body in cases of injury, invasion by microorganisms, and other harmful stimuli.[5,6,7,8]

The pain is a symptom that can arise due to various disorders, representing an urgent medical problem.[1] Pain restricts productivity and decreases quality of life, causing high social and economic impacts.[8]

Every year millions of people around the world deal with pain and do not treat it adequately.[9] Pharmacological treatment of pain initially includes non-opioids drugs, followed by opioids, and finally, if necessary, adjuvants like anticonvulsants and antidepressants. However, despite the high number of existing analgesic drugs, side-adverse effects limit their use, such as gastrointestinal disorders, stimulating the search for new therapeutic alternatives. Therefore, pain management remains a huge challenge for current medicine.[10,8]

Given this, several studies seek new pharmacological alternatives for the treatment of pain. Receptors, neurotransmitters, and pharmacological targets are the focus of research for drug discovery. In order to discover new analgesics, *N*-acylhydrazones derivatives have been researched in several pain models.[8] Hydrazones are widely used in medicinal chemistry, due

to the ease of synthesis and high chemical stability.[11] This is a class of organic compounds with the general structure $R_1R_2C=NNR_3R_4$.[12] LASSBio-2012 is an *N*-acylhydrazone derivative that was developed through molecular modification strategies.

They present a variety of biological properties, such as analgesic, anti-inflammatory, anticonvulsant, antidepressant, antiplatelet, antimalarial, antimicrobial, antimycobacterial, anticancer, vasodilating, antiviral, anti-schistosomiasis, anti-HIV, anthelmintic, antidiabetic, and trypanocidal.[13,14]

Studies have shown that the combination of hydrazones with other functional groups improve its biological properties and provide pharmacologically active molecules.[15] Besides that, a variety of hydrazone derivatives have been developed to minimize gastrointestinal discomfort and toxicity, especially when it comes to analgesic drugs.[16]

Therefore, this study aimed to evaluate the antinociceptive activity of *N*-acylhydrazone LASSBio-2012 in different experimental models, as well as its possible mechanism of action in mice, in order to contribute to the development of new antinociceptive drugs.

Materials and Methods

The *N*-acilhydrazone LASSBio-2012 was characterized, and its purity was determined using the methodologies described by Amaral and coworkers.[17]

Animals and ethics statement

All experiments were conducted using 8-week-old male Swiss mice (*Mus musculus*) (30 ± 40 g). The total number of animals used in this work was 186 animals, and each individual was involved injust one painful procedure (not reused). The animals were kept in groups of six (n = 6) in polypropylene cages at a temperature of 22 ± 1°C with a relative humidity of 60 ± 80%, light/dark cycle of 12 h (start 06:00 and end 18:00), and with free access to food (Purina Labina) and water. This study was performed following the Conselho Nacional para o Controle de Experimentação Animal (CONCEA, Brazil) and complied with the recommendations of the International Association for the Study of Pain.[18,8] The experimental procedures were approved by the Comitê de Ética no Uso de Animais of the Universidade Federal do Vale do São Francisco (CEUA-UNIVASF, Brazil) with authorization number 0004/241017. We made all sorts of efforts to minimize animal suffering. At the end of the

experiments, the animals were anesthetized with 60 mg/kg ketamine associated with 7.5 mg/kg xylazine intraperitoneally and euthanized by cervical dislocation. To administrate all substances intraperitoneally, we used syringes of 1 ml with a needle of 13 x 0.45 mm. For the oral route, we used a gavage needle.[8]

Acetic acid-induced writhing test

We choose the acetic acid-induced writhing test as a classic model to assess the analgesic or anti-inflammatory properties of a new agent.[19,8] This test was performed according to the method described by Collier and collaborators[20,8] with few modifications. Mice received 10 ml/kg of a 0.9% acetic acid solution intraperitoneally (i.p.).[21,8] The number of abdominal writhing was recorded for 10 min, starting 5 min after administration of the acetic acid solution.[22,8] We defined the writhing reflexes as contractions of the abdominal muscles and pelvic rotation, followed by hind limb elongation. Mice were divided into five groups of six animals each (n = 30) and were treated orally (p.o.) with LASSBio-2012 (20 and 40 mg/kg, p.o.) and saline (negative control, p.o.) 1 h prior to the nociceptive agent. Indomethacin (20 mg/kg, i.p.) and morphine (10 mg/kg, i.p.) were used as reference drugs, being administered 30 min prior to the acetic acid solution.[8]

Formalin-induced nociception test

We performed the formalin test as described by Hunskaar and Hole.[23,8] Animals were divided into five groups of six animals each (n = 30) and were treated with saline (p.o.), LASSBio-2012 (20 and 40 mg/kg, p.o) given 1 h prior to the formalin injection. Indomethacin (20 mg/kg, i.p.) and morphine (10 mg/kg, i.p.) were given 30 min prior to the formalin injection. The formalin solution (2.5% in 0.9% sterile saline; 20 µl/animal) was injected into the right hind paw of mice.[24,8] Right after the formalin injection, animals were returned to the chambers with mirrors and were observed for 30 min. The total time (in seconds) that the animal spent licking and biting the treated paw was measured as an indicator of pain. A biphasic nociceptive response was produced by formalin injection: (I) the acute phase, 5 min after formalin injection, followed by a quiescent period of 10 min, and (II) the tonic phase, longer-lasting, 15 to 30 min after this period.[25,8] In order to investigate the involvement of the nitrergic, serotonergic, ATP-sensitive potassium channel, vanilloid, muscarinic and opioid systems in the pharmacological activity of LASSBio-2012, mice were divided into twenty-one groups of six animals each (n = 126) and were pretreated with the following blockers in their respective doses: *N*(*G*)-Nitro-*L*-arginine methyl ester (*L*-NAME, 20 mg/kg, i.p), *L*-

arginine (60 mg/kg, i.p.), ondansetron (0.5 mg/kg, i.p), glibenclamide (2 mg/kg, i.p.), ruthenium red (3 mg/kg, i.p.), atropine (0.1 mg/kg, i.p.) and naloxone (1.5 mg/kg, i.p.) 30 min before administration of LASSBio-2012 (20 mg/kg, p.o).[26,8]

Physicochemical properties and ADMET profile

The physicochemical properties and ADMET profile of *N*-acilhydrazone LASSBio-2012 were predicted *in silico* in comparison with the anti-inflammatory drugs indomethacin and meloxicam, using the ACD/Percepta Program.

Molecular docking analysis

The structure of delta-opioid receptor in complex with naltrindole (PDB ID 4N6H)[27] and mu-opioid receptor in complex with methyl 4-{[(5beta,6alpha)-17-(cyclopropylmethyl)-3,14-dihydroxy-4,5-epoxymorphinan-6yl]amino}-4-oxobutanoate (PDB ID 4DKL)[28] were downloaded from the protein data bank (http://www.rcsb.org/pdb/home/home.do). The structure of compound LASSBio-2012 was submitted to molecular docking using the Molegro Virtual Docker, v. 6.0.1 (MVD).[29] All structures were prepared using the same default parameter settings in the same software package (score function: MolDock Score; Ligand evaluation: Internal ES, Internal HBond, Sp2-Sp2 Torsions, all checked; Number of runs: 10 runs; Algorithm: MolDock SE; Maximum Interactions: 1500; Max. population size: 50; Max. steps: 300; Neighbour distance factor: 1.00; Max. number of poses returned: 5). The redocking procedure was performed using a GRID of 15 Å in radius and 0.30 in resolution to cover the ligand-biding site of the receptors. The moldock score [GRID] algorithm was used as the score function, and the moldock search algorithm was used.[29,30]

Statistical analysis

The results are presented as the mean ± standard error of the mean (SEM), and statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's as post-test. Values of $p<0.05$ were considered statistically significant. All analyses were performed using GraphPad Prism 5.0 (Graph Pad Prism Software, Inc., San Diego, CA, USA).

Results

Acetic acid-induced writhing test

Firstly, the antinociceptive effect of LASSBio-2012 was evaluated using the acetic acid-induced writhing test. In this model, LASSBio-2012 attenuated the nociceptive activity, reducing the number of contortions by 79.66% and 85.22% at the highest doses tested (20 mg/kg and 40 mg/kg), as shown in Figure 1. This preliminary test indicated that LASSBio-2012 could be a promising antinociceptive agent. However, the acetic acid-induced writhing test is quite unspecific. From this perspective, the formalin-induced nociception test was performed.

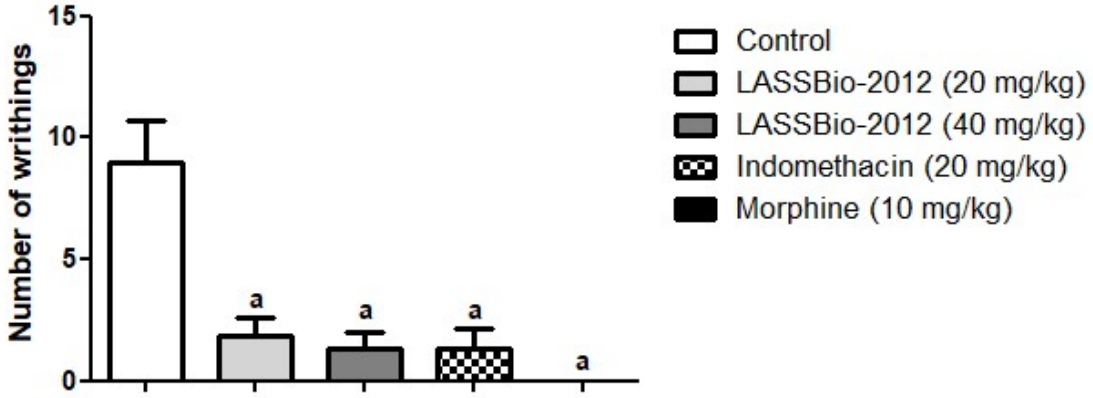

Figure 1 Antinociceptive effect of LASSBio-2012 using the acetic acid-induced nociception test. Effect of LASSBio-2012 (20 and 40 mg/kg, p.o.), morphine (10 mg/kg, i.p.) and indomethacin (20 mg/kg, i.p.) in the acetic acid-induced writhing test in mice (n = 6, per group). Values are expressed as the mean ± SEM, where a indicates $^{a}p<0.05$ in comparison with the control group, according to ANOVA, followed by Tukey's post-test.

Formalin

In the formalin test, LASSBio-2012 reduced nociceptive behavior only in the second phase of nociception, being the doses of 20 and 40 mg/kg responsible for 50.38% and 65.00% of normal antinociceptive effect respectively (Figure 2B).

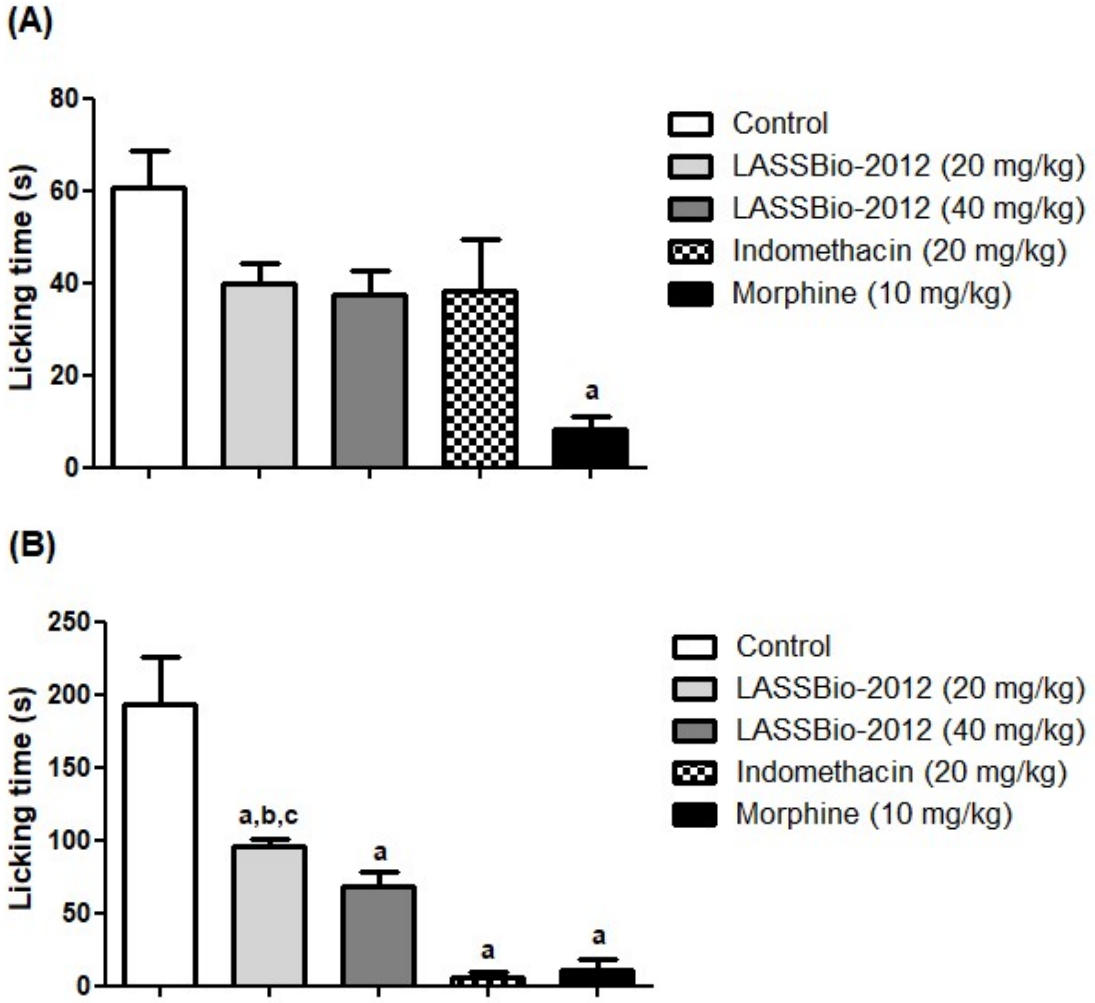

Figure 2 Antinociceptive effect of LASSBio-2012 using the formalin test. Effect of LASSBio-2012 (20 and 40 mg/kg, p.o.), morphine (10 mg/kg, i.p.) and indomethacin (20 mg/kg, i.p.) in the first (A) and second (B) phases of the formalin-induced nociception test in mice (n = 6, per group). Values are expressed as the mean ± SEM, where a indicates $^{a}p<0.05$ in comparison with the control group, b indicates $^{b}p<0.05$ in comparison with indomethacin group, and c indicates $^{c}p<0.05$ in comparison with morphine group according to ANOVA, followed by Tukey's post-test.

LASSBio-2012 had an antinociceptive effect. In this context, we conducted numerous tests to explore the antinociceptive potential of LASSBio-2012. First, when animals were pretreated with naloxone (1.5 mg/kg, i.p.), the pharmacological effect of LASSBio-2012 (20 mg/kg, p.o.) was completely reversed in the second phase of the test (Figure 3), suggesting that its peripheral antinociceptive response was involved at least in part with the opioid system.

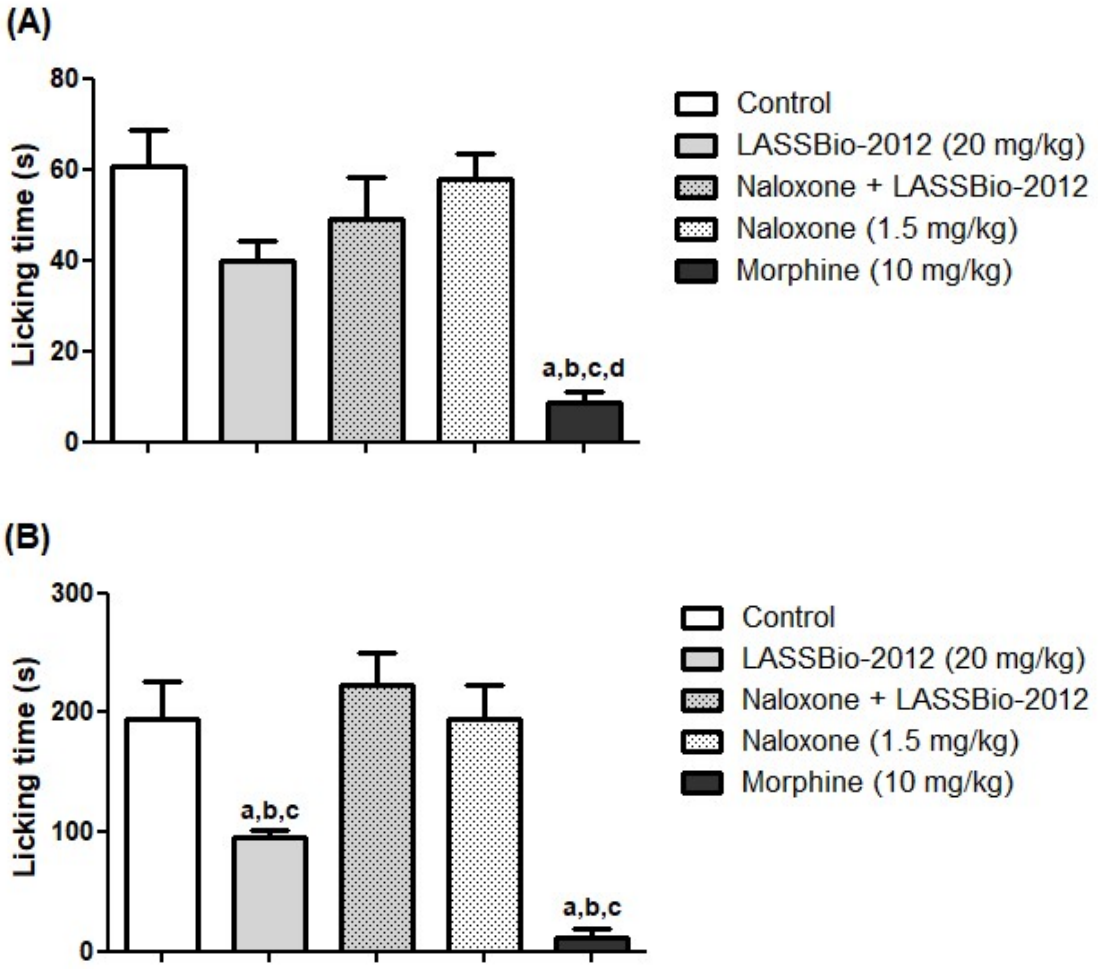

Figure 3 Involvement of opioid receptors in the antinociceptive effect of LASSBio-2012 using the formalin test. Effect of LASSBio-2012 (20 mg/kg, p.o.), naloxone (1.5 mg/kg, i.p.), naloxone + LASSBio-2012 and morphine (10 mg/kg, i.p.) in the first (A) and second (B) phases of the formalin-induced nociception test in mice (n = 6, per group). Values are expressed as the mean ± S.E.M., where a indicates $^{a}p<0.05$ in comparison with control group, b indicates $^{b}p<0.05$ in comparison with naloxone group, c indicates $^{c}p<0.05$ in comparison with Naloxone + LASSBio-2012 group, and d indicates $^{d}p<0.05$ in comparison with LASSBio-2012 group according to ANOVA, followed by Tukey's post-test.

When animals were pretreated with *L*-arginine (600 mg/kg, i.p.) and *L*-NAME (20 mg/kg, i.p.) the pharmacological effect of LASSBio-2012 (20 mg/kg, p.o.) was not reversed in the second phase of the test (Figure 4), suggesting that its peripheral antinociceptive response was not involved in the nitrergic system.

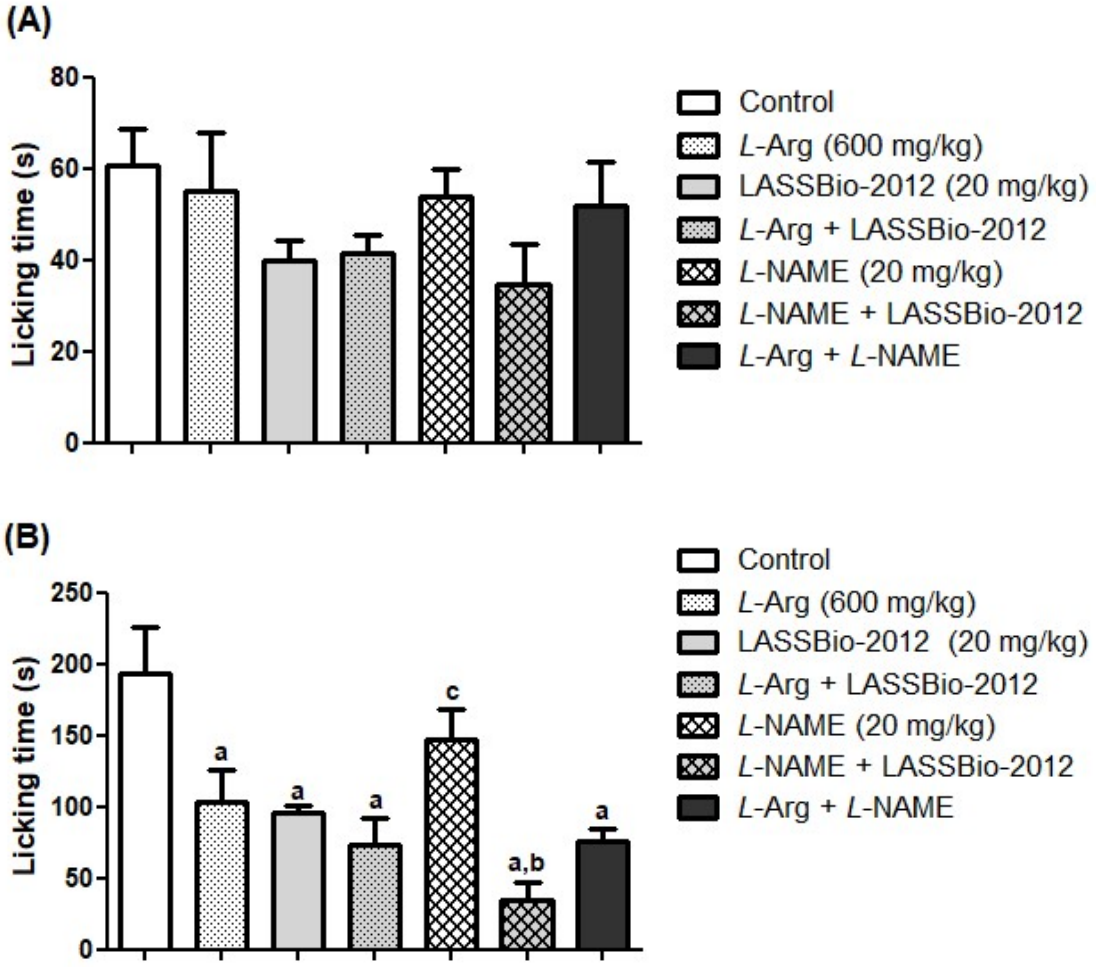

Figure 4 Involvement of nitrergic receptors in the antinociceptive effect of LASSBio-2012 using the formalin test. Effect of *L*-arginine (600 mg/kg, i.p.), LASSBio-2012 (20 mg/kg, p.o.), *L*-arginine + LASSBio-2012, *L*-NAME (20 mg/kg, i.p.), *L*-NAME + LASSBio-2012, and *L*-arginine + *L*-NAME in the first (A) and second (B) phases of the formalin-induced nociception test in mice (n = 6, per group). Values are expressed as the mean ± S.E.M., where a indicates $^{a}p<0.05$ in comparison with the control group, b indicates $^{b}p<0.05$ in comparison with the *L*-NAME group, c indicates $^{c}p<0.05$ in comparison with *L*-NAME + LASSBio-2012 group according to ANOVA, followed by Tukey's post-test.

When animals were pretreated with ondansetron (0.5 mg/kg, i.p.), the pharmacological effect of LASSBio-2012 (20 mg/kg, p.o.) was not reversed in the second phase of the test (Figure 5), suggesting that its peripheral antinociceptive response was not involved in the serotonergic system.

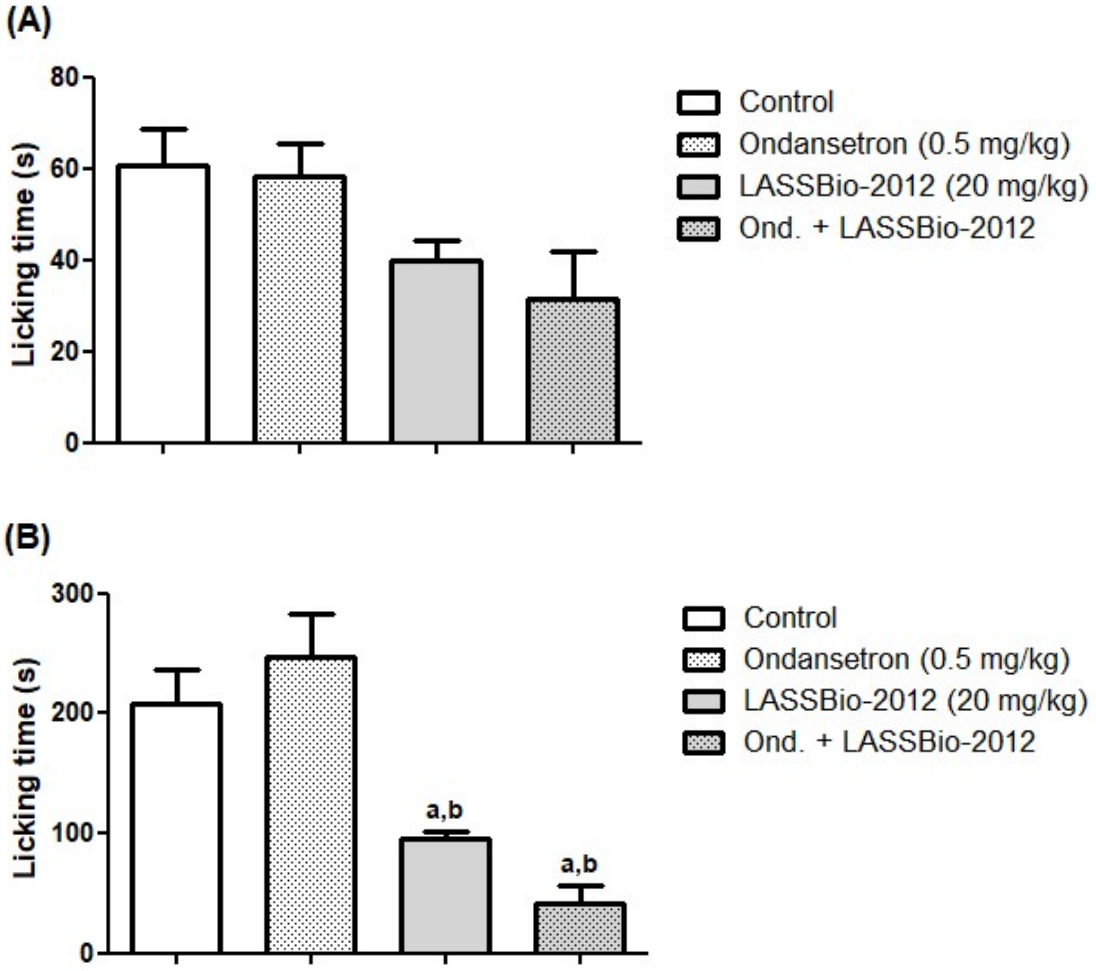

Figure 5 Involvement of serotonergic receptors in the antinociceptive effect of LASSBio-2012 using the formalin test. Effect of ondansetron (0.5 mg/kg, i.p.), LASSBio-2012 (20 mg/kg, p.o.), ondansetron + LASSBio-2012 in the first (A) and second (B) phases of the formalin-induced nociception test in mice (n = 6, per group). Values are expressed as the mean ± S.E.M., where a indicates $^{a}p<0.05$ in comparison with control group and b indicates $^{b}p<0.05$ in comparison with the ondansetron group, according to ANOVA, followed by Tukey's post-test.

When animals were pretreated with atropine (0.1 mg/kg, i.p.), the pharmacological effect of LASSBio-2012 (20 mg/kg, p.o.) was potentiated in the second phase of the test (Figure 6), suggesting that its peripheral antinociceptive response was not involved in the muscarinic system.

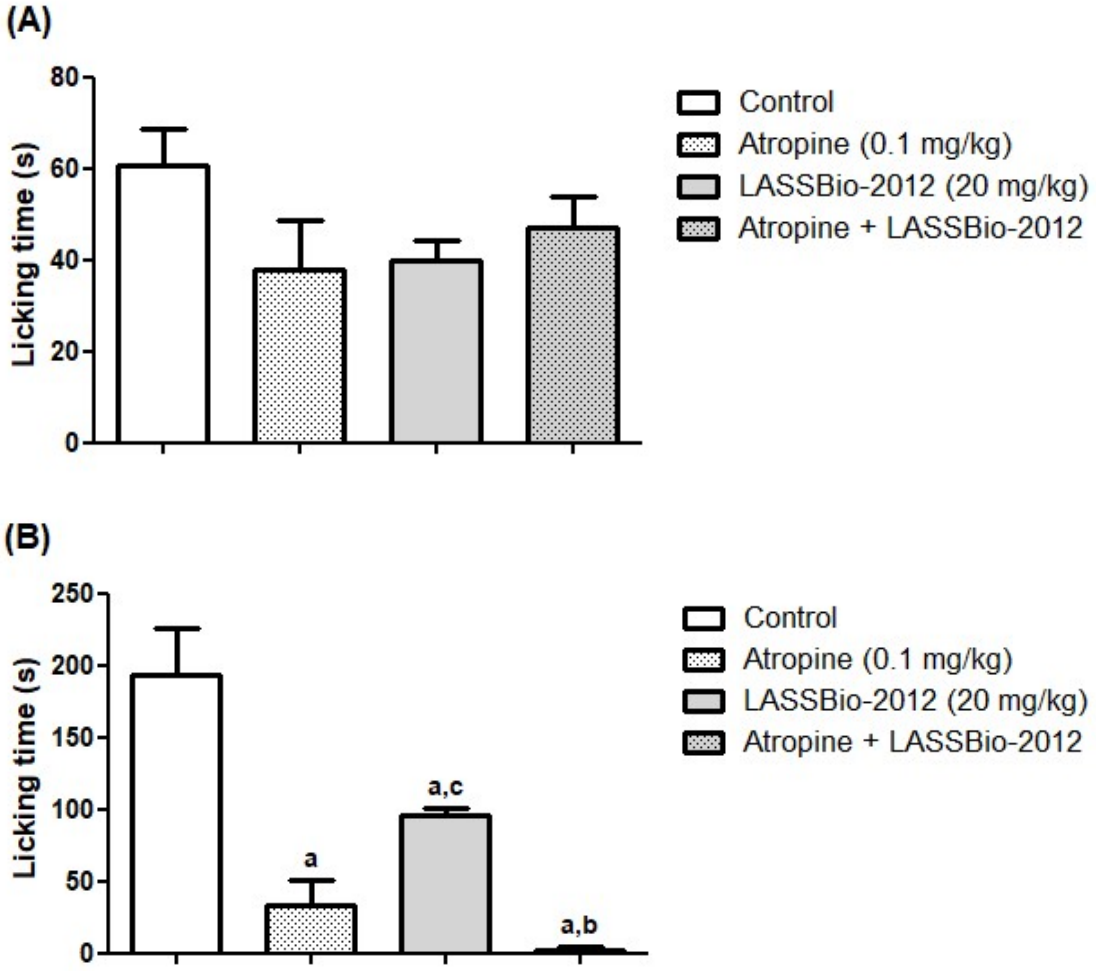

Figure 6 Involvement of muscarinic receptors in the antinociceptive effect of LASSBio-2012 using the formalin test. Effect of atropine (0.1 mg/kg, i.p.), LASSBio-2012 (20 mg/kg, p.o.), atropine + LASSBio-2012 in the first (A) and second (B) phases of the formalin-induced nociception test in mice (n = 6, per group). Values are expressed as the mean ± S.E.M., where a indicates $^{a}p<0.05$ in comparison with control group, b indicates $^{b}p<0.05$ in comparison with the LASSBio-2012 group, and c indicates $^{c}p<0.05$ in comparison with Atropine + LASSBio-2012 group according to ANOVA, followed by Tukey's post-test.

When animals were pretreated with glibenclamide (2 mg/kg, i.p.), the pharmacological effect of LASSBio-2012 (20 mg/kg, p.o.) was not reversed in the second phase of the test (Figure 7), suggesting that its peripheral antinociceptive response was not involved with the ATP sensitive potassium channels.

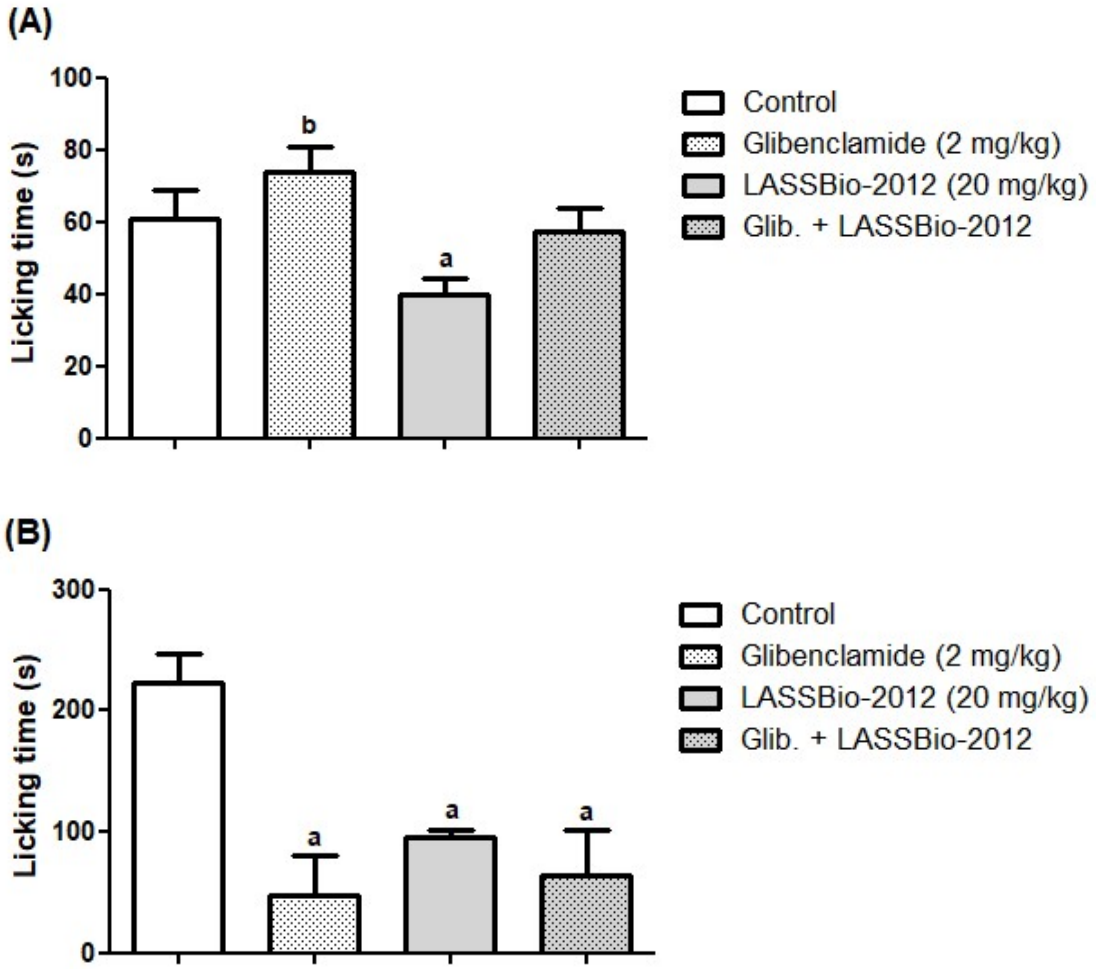

Figure 7 Involvement of K_{ATP} in the antinociceptive effect of LASSBio-2012 using the formalin test. Effect of glibenclamide (2 mg/kg, i.p.), LASSBio-2012 (20 mg/kg,p.o.), glibenclamide + LASSBio-2012 in the first (A) and second (B) phases of the formalin-induced nociception test in mice (n = 6, per group). Values are expressed as the mean ± S.E.M., where a indicates $^{a}p<0.05$ in comparison with control group, and b indicates $^{b}p<0.05$ in comparison with the LASSBio-2012 group according to ANOVA, followed by Tukey's post-test.

When animals were pretreated with ruthenium red (3 mg/kg, i.p.), the pharmacological effect of LASSBio-2012 (20 mg/kg, p.o.) was not reversed in the second phase of the test (Figure 8), suggesting that its peripheral antinociceptive response was not involved in the vanilloid system.

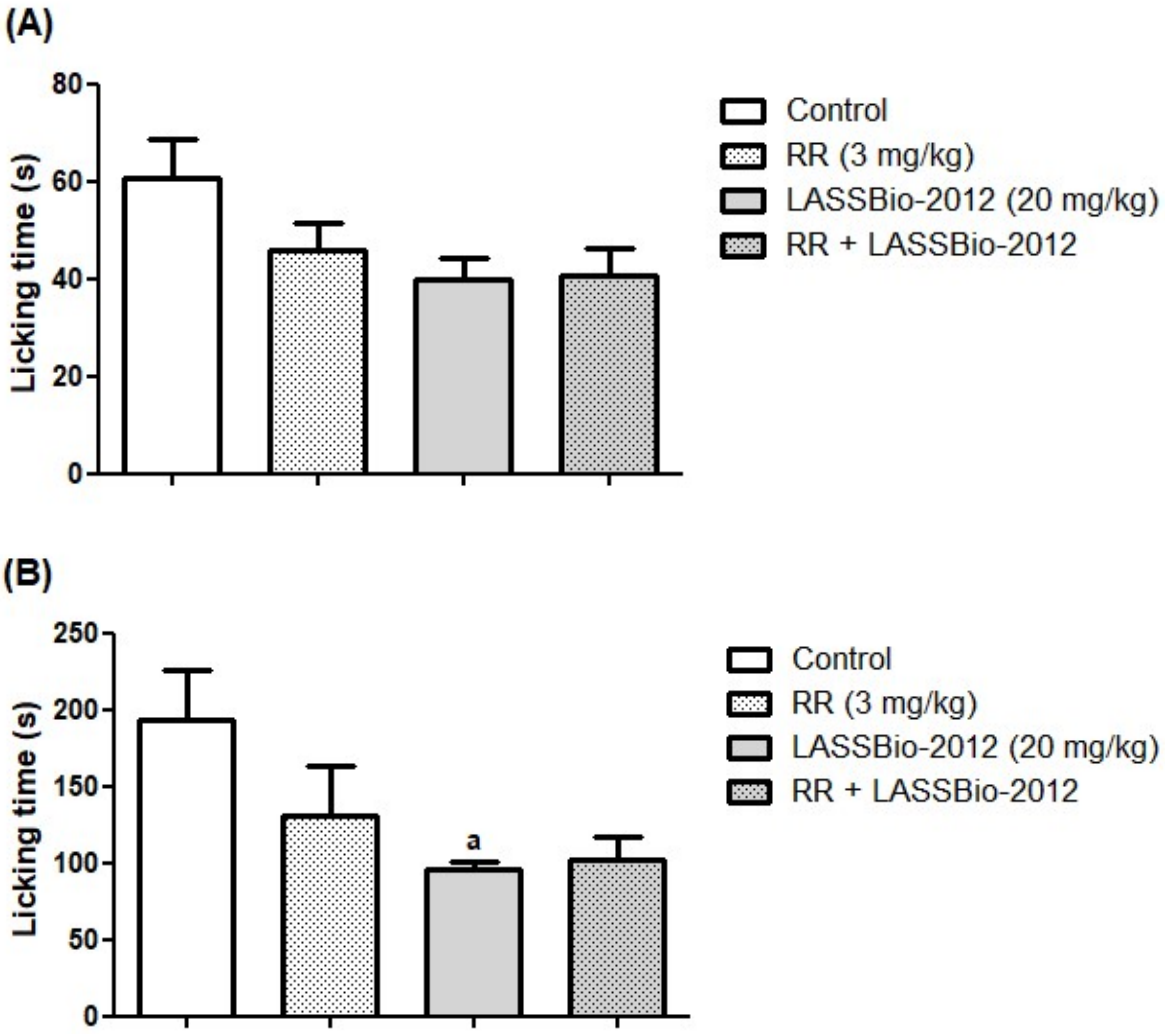

Figure 8 Involvement of vanilloid receptors in the antinociceptive effect of LASSBio-2012 using the formalin test. Effect of ruthenium red (3 mg/kg, i.p.), LASSBio-2012 (20 mg/kg, p.o.), ruthenium red (VR) + LASSBio-2012 in the first (A) and second (B) phases of the formalin-induced nociception test in mice (n = 6, per group). Values are expressed as the mean ± S.E.M., where a indicates $^{a}p<0.05$ in comparison with control group, according to ANOVA, followed by Tukey's post-test.

Physicochemical properties and ADMET profile determined *in silico* using the ACD/Percepta Program

In Table 1 the *in silico* physicochemical properties and ADMET profile LASSBio-2012 and of the anti-inflammatory drugs indomethacin and meloxicam are compared.

Table 1 Comparative *in silico* physicochemical properties and ADMET profile of LASSBio-2012 and the anti-inflammatory drugs indomethacin and meloxicam.

PredictedProperties*	**LASSBio-2012**	**Indomethacin**	**Meloxicam**
MW (g/mol)	543.57	357.79	351.40
H-Donors	1	1	2
H-Acceptors	10	5	7
RotableBonds	8	4	2
TPSA	149.01	68.53	136.22
LogP	4.77	4.02	2.38
Solubility (mg/ml)	0.0001	2.5	5.96
Caco-2 (cm/s)	Pe= $211x10^{-6}$	Pe = 129×10^{-6}	Pe = 233×10^{-6}
HIA (%)	100	100	100
F (oral) (%)	8	99	96
PPB (%)	99	99	99
CNS Score	-3.58	-4.32	-5.24
HLM	0.49	0.26	0.32
hERG	0.48	0.23	0.41
AMES	0.7	0.27	0.21

*Determined *in silico* using the ACD/Percepta Program. MW = molecular weight; H-Donors = hydrogen bond-donors; H-Acceptors = hydrogen bond-acceptors; TPSA = topological polar surface area; LogP = the logarithm of the drug partition coefficient between n-octanol and water; Caco-2 = human epithelial cell line Caco-2; HIA= human intestinal absorption; F = Bioavailability; CNS = central nervous system; HLM = human liver microsomes; hERG = the human Ether-à-go-go-Related Gene; AMES = Ames test = *Salmonella typhimurium* reverse mutation assay.

Docking

Docking was validated redocking the originals ligands in the active site of opioid receptors delta and mu, as observed in the crystallography pdb file. Superpositions of poses are representedin Figure 9 and Figure 10, which shows a good match.

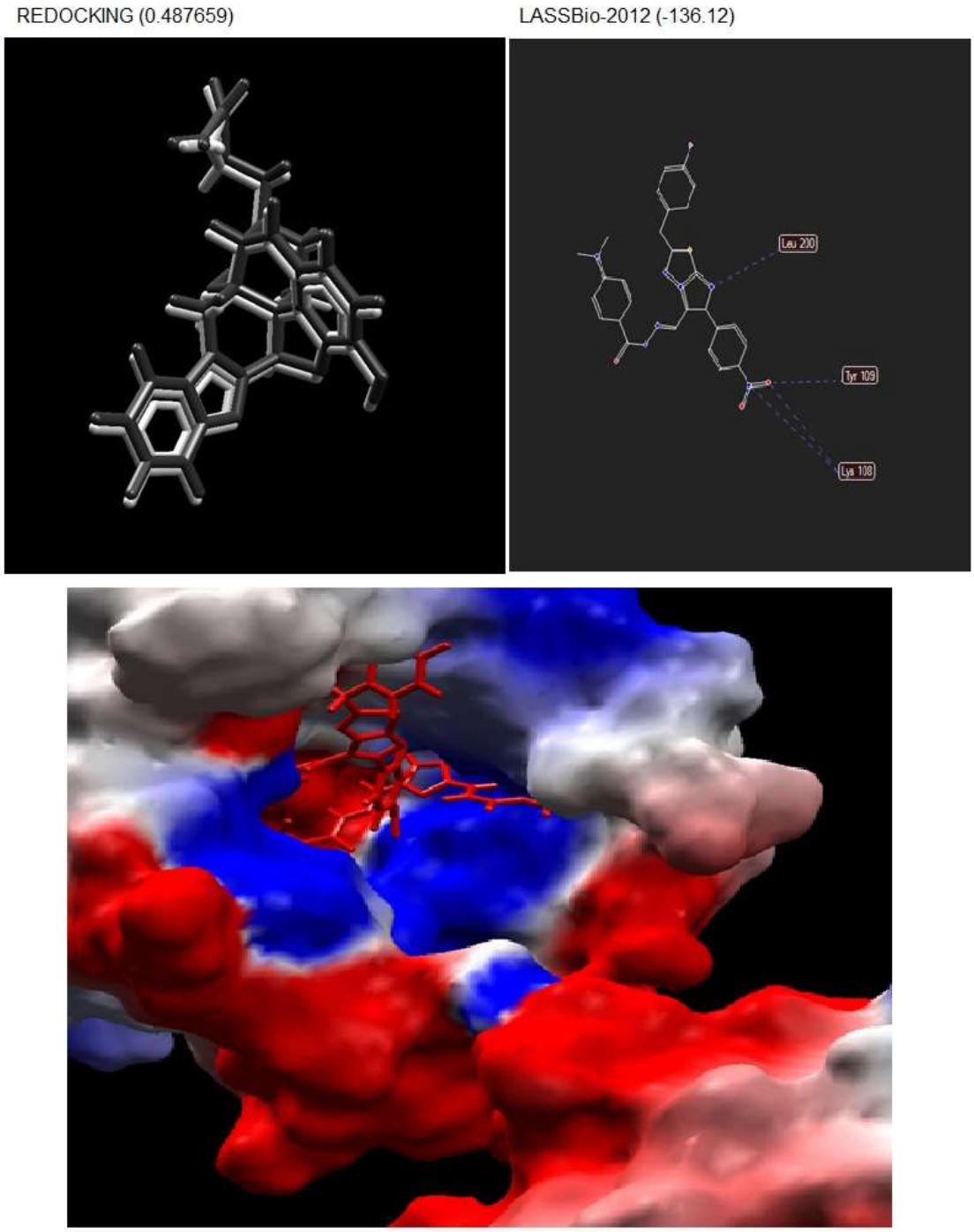

Figure 9 Results of the docking procedures for LASSBio-2012, interaction profile LASSBio-2012 in delta receptor (4N6H) binding site after the redocking process. *RMSD: 0.487659; *Binder energy: -136.12.

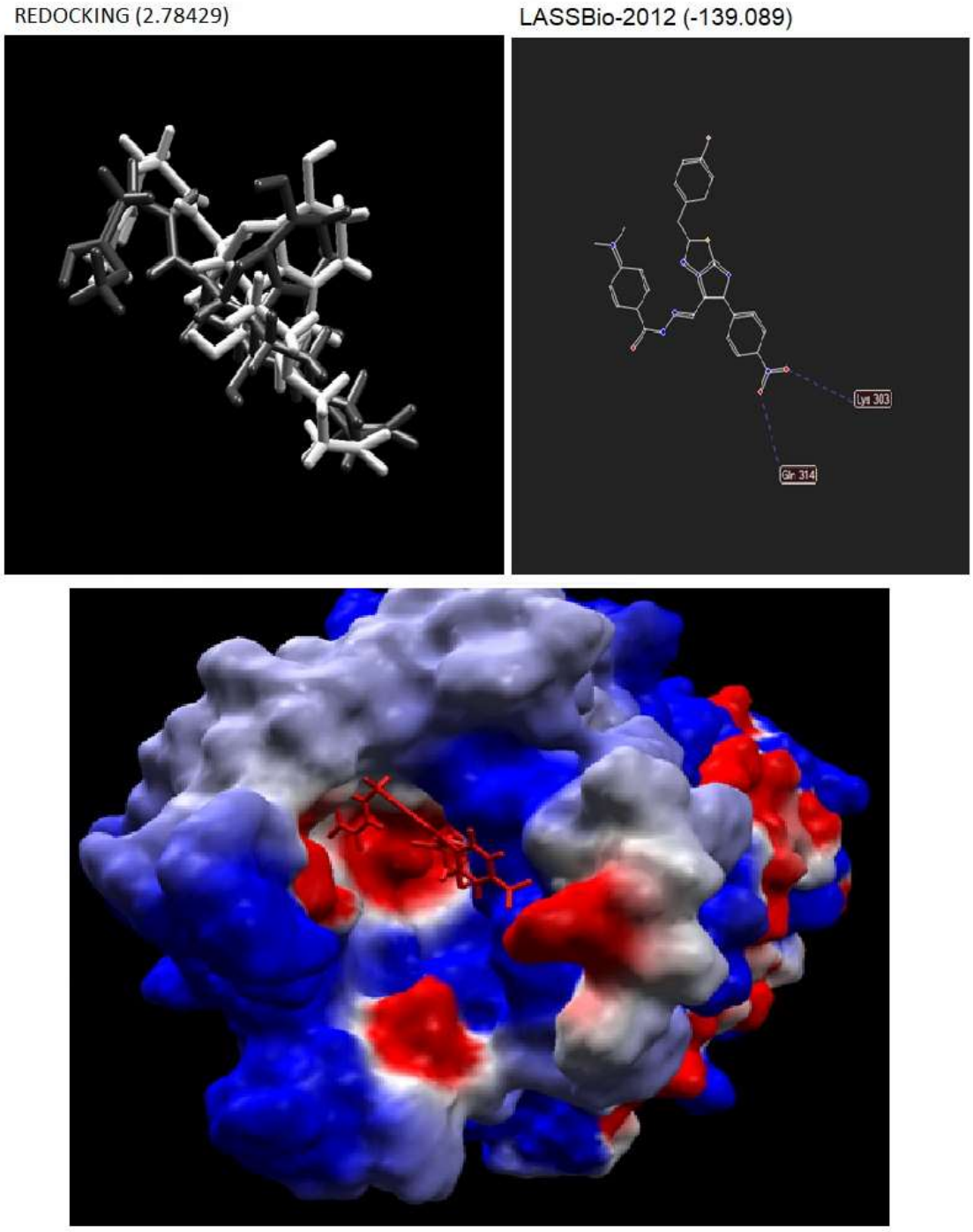

Figure 10 Results of the docking procedures for LASSBio-2012, interaction profile LASSBio-2012 in mu receptor (4DKL) binding site after the redocking process. *RMSD: 2.78429; *Binder energy: -139.089.

Discussion

In the acetic acid-induced writhing test, LASSBio-2012 reduced the number of writhings by up to 79.66% and 85.22% at the doses of 20 mg/kg and 40 mg/kg, similar to *N*-acilhidrazona LASSBio-1586, that presented 88.97% of inhibition at the highest dose of 40 mg/kg.[8]

The acetic acid-induced writhing test is considered an unspecific test. Intraperitoneal administration of acetic acid induces activation of nociceptors that stimulates the release of a variety of painful and inflammatory mediators, being the most important histamine, bradykinin, serotonin, glutamate, noradrenaline, substance P, nitric oxide, and prostaglandins.[20,8] Because of this nonspecificity, it is not possible to accurately indicate the nociceptive pathways in which the molecule acts. For this reason, we performed the formalin-induced nociception test.

The two different phases of a nociceptive event can corroborate with the formalin-induced nociception test. Central-acting mediators elicit the first phase, which include those that activate serotonergic, muscarinic, vanilloid, and glutamatergic receptors. On the other hand, the second phase is characterized by a prevalence of inflammatory mediators, mainly histamine, bradykinin, and prostaglandins.[23,8]

In this test, LASSBio-2012 reduced nociceptive behavior only in the second phase. In this phase, LASSBio-2012 (20 and 40 mg/kg) showed 50.38% and 65.00% of the normal antinociceptive effect, respectively. The statistical analysis revealed that there were no significant differences between the two doses tested. A similar result was observed for indomethacin (97.25%) and LASSBio-1586 (96.74%) at a dose of 20 mg/kg.[8] In the light of these results, LASSBio-2012 does not affect the first phase of the test, suggesting that, like indomethacin, this chemical has a peripheral (not central) effect since it reduces nociception only in the second phase of the formalin test.

Like LASSBio-2012, Meymandi *et* al. (2019) demonstrated that celecoxib (10 –30 mg/kg), a specific COX-2 inhibitor, promoted its anti-inflammatory and antinociceptive activities in mice submitted to the formalin test only in the second phase of the test.[31,32,33]

In the second phase of the formalin test, the pharmacological effect of LASSBio-2012 was completely abolished when animals were pretreated with naloxone, suggesting that its peripheral antinociceptive activity was involved, at least partially, with the opioid system. As an opioid antagonist, naloxone significantly blocks the activity of morphine in both phases of this test. Mehanna *et* al. (2018)[34] demonstrated that tadalafil had its effect totally reversed by

naloxone in the first phase of the same test. Moreover, it partially blocked tadalafil's effect in the second phase, suggesting that this drug has a peripheral antinociceptive effect. Hence, its antinociceptive effect is opioid receptor-dependent, a result thatwas also observed with pyrazole compounds.[35,36,37,34]

It is reported in the scientific literature that the bergamot essential oil (BEO)-induced antinociception is possibly attained through peripheral μ-opioid and κ-opioid receptors.[37] Beyond that, flavonoids'ability to decrease hyperalgesia is probably dependent on the μ and δ-opioid receptors too.[38,39,9] In addition, several studies have shown that the activation of opioid receptors located in the periphery is capable of inhibiting inflammatory pain via *L*-arginine/NO/cGMP pathway.[40,37,34]

For that reason, the involvement of the nitric oxide pathway was investigated. According to our results, LASSBio-2012 (20 mg/kg, p.o.) showed an antinociceptive effect that was not reversed in the second phase of the test, indicating that its peripheral antinociceptive response was not dependent on the nitrergic system.

In order to evaluate the participation of serotonin in the effect of LASSBio-2012, animals were pretreated with ondansetron. The antinociceptive effect of LASSBio-2012 was not affected by this blocker, demonstrating the non-involvement of the serotonergic system. Like LASSBio-1586 in the second phase, LASSBio-2012 also showed no reversibility of its antinociceptive effect.[8] A wide number of serotonin (5-HT) receptors are present in the central and peripheral nervous systems.[41,42] Some studies have shown that 5-HT_1 receptors are involved in antinociception processes, whereas 5-HT_2 receptors are related to pronociceptive out comes.[43,44,42] Our results with LASSBio-2012 also showed no interference of the muscarinic and vanilloid system, nor the K_{ATP} ion channels.

In Table 1 the *in silico* physicochemical properties and ADMET profile LASSBio-2012 and of the anti-inflammatory drugs indomethacin and meloxicam are compared. It can be observed that LASSBio-2012 violated one of Lipinsky's rule of five (Ro5), with a molecular weight about 10% above the required limit. Never the less, with the other properties of this compound, it can be an orally active drug in humans. Also, LASSBio-2012 has been demonstrated to be insoluble when compared to drugs like indomethacin and meloxicam. Concerning their comparative ADMET profile, all compounds were predicted to be highly absorbed (HIA = 100%), highly permeable (Pe > 7 $\times 10^{-6}$ cm/s) and bound very well to plasma protein (PPB > 90%). LASSBio-2012 indicated a poor oral bioavailability (F = 8 %), and the two other compounds have indicated a good oral bioavailability *in silico* (F = 96 and 99%). The main differences amongst LASSBio-2012, indomethacin, and meloxicam rely

on their metabolic stability in human liver microsomes (HLM) and their ability to penetrate in CNS. In Table 1, indomethacin and meloxicam were predicted as stable in HLM (scores of 0.26 and 0.32, respectively), while an undefined result was found for LASSBio-2012 (Score = 0.49). Both anti-inflammatory drugs have been predicted as non-penetrant to CNS (scores of -4.32 and -5.24, respectively) whereas LASSBio-2012 was defined as able to penetrate CNS. To have some notion about the toxicity of LASSBio-2012, the ability of this compound to inhibit hERG (the human Ether-à-go-go-Related Gene), and its mutagenic profile (i.e. probability of a positive Ames test) were predicted. The results were converted into classification scores, and an undefined hERG and mutagenic activities (score > 0.33 and ≤ 0.67) were predicted for LASSBio-2012. Taken together, those *in silico* results suggest a moderate pharmacokinetic profile for LASSBio-2012, mean while, the *in silico* approach was not able to predict its toxicological profile.[8]

In the interaction profile of LASSBio-2012 at the binding site of the pharmacological receptors delta (4N6H) and mu (4DKL) after the redocking process, a strong interaction can be observed, as indicated in the pharmacological test of formalin.

Conclusion

N-acylhydrazones are a versatile class of organic compounds; their derivatives have a wide spectrum of pharmacological properties, which makes the synthesis of these compounds a great interest for the pharmaceutical industry. Based on the results presented here, LASSBio-2012 showed a significant antinociceptive effect in both experimental models of abdominal contortions induced by acetic acid and formalin test. After investigation using several pharmacological blockers (naloxone, *L*-arginine, *L*-NAME, ondansetron, atropine, glibenclamide, ruthenium red), it was observed that its mechanism of action appears to be peripheral, with the involvement of the opioid signaling pathways. Additionally, through redocking procedure, the interaction of LASSBio-2012 with the delta and mu-opioid receptors were confirmed. The physicochemical properties and ADMET profile of molecule were also evaluated, where LASSBio-2012 was orally active in humans, highly absorbable, permeable, with high binding to plasma proteins, capable of penetrating the CNS, which suggests a moderate pharmacokinetic profile. In short, LASSBio-2012 has emerged as a strong candidate for a multi-target antinociceptive drug.

Declarations

Conflict of interest

The authors have no conflicts of interest.

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CONCLUSÃO GERAL

As hidrazonas exibem um amplo espectro de atividades farmacológicas como vasorelaxante, antioxidante, citoprotetora, anti-hipertensiva, analgésica e anti-inflamatória. Por tais razões, o desenvolvimento de novas estruturas moleculares contendo este grupo funcional e a subsequente avaliação de atividades biológicas de interesse, constituem etapas iniciais e essenciais para o desenvolvimento de novos protótipos candidatos a fármacos.

De acordo com os resultados apresentados, as hidrazonas H1, H2, H3, H4, H5, e a *N*-acilhidrazona LASSBio-2012 apresentaram efeito antinociceptivo nos testes das contorções abdominais induzidas por ácido acético, e da formalina. Ambas atuam perifericamente na promoção dos seus efeitos. Sendo que, H5 e LASSBio-2012 foram consideradas com maior potencial antinociceptivo. Portanto, foi investigado o mecanismo de ação de H5 e de LASSBio-2012. Observou-se que o efeito de H5 possui envolvimento de vias de sinalização opióides e nitrérgicas, enquanto LASSBio-2012 possui envolvimento de vias de sinalização opióides.

Além disso, H5 apresentou efeito anti-inflamatório por meio dos testes de migração de leucócitos através da cavidade peritoneal, edema de pata induzido por carragenina e edema de pata induzido por histamina, esta última indicou um envolvimento dos receptores histamínicos na atividade anti-inflamatória de H5. Também foi observado através do teste Rota-Rod, que H5 interfere sobre a coordenação motora. Em relação ao teste de toxicidade com *Artemia salina*, H5 apresentou toxicidade moderada.

Na análise molecular através do docking foi observado que H5 possui uma interação de maneira semelhante ao meloxicam com a enzima COX-2. Ainda na análise molecular, foi observada a interação do LASSBio-2012 com os receptores opioides delta e mu.

Também foram avaliadas as propriedades físico-químicas e perfil ADMET, *in silico*, observou-se que H5 e LASSBio-2012 tem propriedades que os tornariam medicamentos provavelmente ativos por via oral em humanos, altamente absorvidos, altamente permeáveis e extensivamente ligados às proteínas plasmáticas. Além disso, possuem grande biodisponibilidade oral, e com capacidade de penetrarem no SNC. Portanto, H5 e LASSBio-2012 apresentaram perfis farmacocinéticos adequados.

Em resumo, a hidrazona H5 apresentou-se como um forte candidato a fármaco antinociceptivo e anti-inflamatório, e a *N*-acilhidrazona LASSBio-2012 destacou-se como um potente candidato a fármaco antinociceptivo.

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