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Anti-inflammatory and analgesic activities of 7-chloro-4-(piperazin-1-yl)quinoline derivative mediated by suppression of inflammatory mediators expression in both RAW 264.7 and mouse models

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Abstract

Background: 4-Aminoquinoline derivatives possess various potential biological properties such as anti-inflammatory and analgesic activity. The introduction of additional piperazine heterocyclic pharmacophoric moiety tends to possess profound impact in increasing the activity. The present work was undertaken to investigate the *in-vitro* and *in-vivo* anti-inflammatory activity as well as the peripheral and central analgesic activities of compound 1-(4-(7-chloroquinoline-4-yl)piperazin-1-yl)-2-(4-phenylpiperazin-1-yl)ethanone (**5**) in experimental models7-chloro-4-(piperazin-1-yl)quinoline derivatives **1-9** demonstrated significant inhibition of nitric oxide (NO) level in RAW 264.7 cells. **Methods:** The percentage inhibition of the LPS-induced NO release of 7-chloro-4-(piperazin-1-yl)quinoline derivatives **1-9** was determined in RAW 264.7 murine macrophage model. Western blot analysis was performed to evaluate the effect of compound **5** on protein expression of iNOS. Gene expression of inflammatory markers (iNOS, COX-2, IL-6, IL-1β and TNF-α) was evaluated using real-time PCR. The peripheral and central analgesic activities of compound **5** were evaluated in mice using writhing and hot-plate tests, respectively. Moreover, the anti-inflammatory activity was assessed using carrageenan-induced paw edema assay in mice. Serum NO and COX-2 levels were detected using ELISA. **Results:** compound **5** demonstrated the highest NO inhibitory activity that was accompanied by inhibition of iNOS protein expression as well as decreased gene expression levels of inflammatory markers iNOS, COX2, IL-6, IL-1β, and TNF-α in RAW 264.7. Compound **5** revealed a potential peripheral analgesic effect through inhibition of abdominal writhing in mice treated with doses of 15mg/kg and 30 mg/kg and its effect was comparable to diclofenac sodium. Compound **5** possessed an analgesic activity starting from 15 min post administration and reached its peak at 45 min that was significantly higher than that of tramadol hydrochloride suggesting its potential as central analgesic agent. Compound **5** showed percentage of inhibition of edema of 34, 50 and 64% at 1, 2, and 3 h respectively, post carrageenan challenge together with a significant decrease in serum NO and COX-2 levels. **Conclusions:** The remarkable anti-inflammatory and analgesic activities of compound 5 could be attributed to the advantageous introduction of the heterocyclic 7-chloro-4-(piperazin1-yl)quinoline scaffold incorporated with N-phenylpiperzine functional groups linked together with the ethanone pharmacophoric chain.

Keywords: Nitric oxide, anti-inflammatory, analgesic, 7-chloro-4-(piperazin-1-yl)quinolines

Introduction

Inflammation is a complex biological defense response that can be triggered by various factors such as infections, tissue injury, and toxic compounds [1]. This defense mechanism acts on the removal of harmful stimuli and initiates the healing process [2]. Chronic inflammation and sepsis could be developed due to the excessive release of cytokines with concurrent migration of inflammatory cells to the affected regions [3].

Upon inflammatory stimuli, macrophages secrete pro-inflammatory mediators such as tumor necrosis factor-alpha (TNF-α), interleukin-1beta (IL-1β), and IL-6 and promote prostaglandin synthesis which leads to up-regulation of inflammatory reactions [4]. In addition, stimulation of the immune cells increases the production of pro-inflammatory proteins/enzymes such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) which induces nitric oxide (NO) and prostaglandin release, respectively. NO and COX subsequently induces inflammatory responses that are involved in the progression of inflammation-related diseases, such as cardiovascular disease and cancer [2,5].

Quinoline moiety derivatives plays vital role in the development of numerous classes of newer drugs having various biological properties such as antimalarial [6], anticancer [7], antibacterial [8], anti-tubercular [9], and anti-inflammatory and analgesic activities [10-13]. Moreover, glafenine, floctafenine and antrafenine (Figure 1) are analgesic and non-steroidal anti-inflammatory drugs (NSAIDs) related to the fenamate class and can be considered as 4-aminoquinolines [14,15]. Introducing another heterocyclic moiety to the quinolone ring initiates the activity[12], as noticed by the piperazine heterocyclic pharmacophoric moiety [16]. Meanwhile, the 7-chloro-4-(piperazin1-yl)quinoline structural skeleton constitutes the principle component of the antimalarial piperaquine, which support this scaffold as an effective candidate with multiple pharmacological potentials [17].

Figure 1.

Figure 2.

Non-steroidal anti-inflammatory and analgesic drugs (NSAIDs) are perscribed for pain relief and improvement of the patient's life style [18], but they are often associated with adverse

effects, therefore studies are still needed for safer candidates. Aboul-Enein *et al.*, [19] disclosed the synthesis and the vascular endothelial growth factor receptor-II (VEGFR-II) inhibitory effect of several 1-(4-(7-chloroquinoline-4-yl)piperazin-1-yl) derivatives (**1-9**, Figure 2). Their structures demonstrated 4-aminoquinoline and aminoacetamide "1,2-diaminoethanone" moieties which are noticed features in many marketed NSAIDs and analgesics (Figure 1). Therefore, in the current work, it was deemed of interest to start with the *in vitro* anti-inflammatory screening of compounds **1-9**. These derivatives demonstrated significant inhibition of NO level in RAW 264.7 cells. It is worth mentioning that, compound **5** namely; 1-(4-(7-chloroquinoline-4-yl)piperazin-1-yl)-2-(4-phenylpiperazin-1-yl)ethanone showed the highest NO inhibitory activity. The structural feature of compound **5** shows a quinoline moiety incorporated with two piperazinyl functional groups linked together with the ethanone pharmacophoric chain (Figure 1). Therefore, as an extension of the previous study [19] and encouraged by the current *in vitro* NO inhibition anti-inflammatory findings, it was of significance to evaluate the analgesic and anti-inflammatory effect of compound **5** both *in vitro* and *in vivo*.

Materials and methods

In vitro assays

Cell culture and treatment

The murine RAW 264.7 machrophage cells (ATCC®), cultured and maintained in Dulbecco's Modified Eagle's Medium (DMEM from Lonza, Verviers, Belgium) in a humidified 5% CO_2 atmosphere (Certomat® CS 20 incubator, Sartorius Stedim Biotech GmbH, Germany). Complete medium contained the following as supplements : 10% FBS, penicillin (100 U/ml), streptomycin sulphate (100 µg/ml) and 4 mM L-glutamine. Sterile scrappers were used to sub-culture the cells off the flasks (Greiner Bio-one, Frickenhausen, Germany).

Inhibition of LPS-induced NO release

RAW 264.7 model was employed to assess the *in vitro* anti-inflammatory potential as previously described [20]. Briefly, cells (5×10^5 cells/ml) were plated overnight in 96-well

microplates. Overnight grwon cultures were treated with either vehicle (0.1% v/v dimethyl sulphoxide vehicle (DMSO, Seva electrophoresis, Catalogue number 39757.02, negative control LPS^-), 100 ng/ml lipopolysaccharide (LPS^+, Sigma-Aldrich, from *E. coli* serotype O111:B4) or with LPS containing 30 µM as a cut off concentration [21] from the studied 7-chloro-4-(piperazin-1-yl)quinoline derivatives **1-9**, dissolved in 0.1% DMSO (v/v). Following 24 h treatment, Griess assay [22] was used to measure NO in triplicate aliquots of culture medium from each treatment group. The assay was performed by mixing 100 µl of culture medium from each well with 100 µl of Griess reagent [equal volumes of solution A (0.1% wt/v N-(1-naphthyl)ethylenediamine hydrochloride) and solution B (1 % wt/v of sulfanilamide in 5% (v/v) phosphoric acid)]. Absorbance was monitored at 540 nm using a Tristar lb 942®microplate reader (Berthold, Germany). NO Inhibition (%) was estimated for each group relative to the LPS only group (LPS^+), normalized to viable cell number as detected with MTT viability assay [23].

Western blot analysis

The relative protein expression of the proinflammatory marker inducible nitric oxide synthase (iNOS) was performed using Western blotting. RAW 264.7 cells were plated and cultured overnight in 6-well plates as 1.5×10^6 cells/well. Cultures were treated with compound **5** (0- 30 µM). After 24 h treatment time, cells were washed using ice-cold phosphate-buffered saline (PBS) and scrapped in RIPA lysis buffer (Catalogue number 89900, Thermoscientific, USA). After incubation for 20 min on ice, cell lysates were centrifuged at 15000 ×g for 20 min at 4°C and protein concentration was measured on a spectrophotometer (Thermo®nano-drop). Samples (80 µg total protein) of cell lysates were resolved on 10% polyacrylamide gel electrophoresis (Bio-Rad Tetra Cell®) and electro-plotted onto nitrocellulose membrane using a Trans-blot mini module (Bio-rad, USA). The membrane was blocked using 5% skim milk (catalogue number 42590.01, Serva Elecrophoresis, Germany) in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 h at room temperature, followed by an overnight incubation at 4°C with 1:1000 dilution of iNOS primary antibody (Merck Millipore, Massachusetts, USA) and β-actin was used as loading control. Following 4×5 min washes with TBST, the membranes were incubated with 1:10,000 dilution of the horseradish peroxidase-conjugated secondary antibody (catalogue number AB97240, Abcam, UK) for 1 h at room temperature, followed by another 4×5 min washes with TBST. Membrane proteins were detected using the ECL western blotting detection substrate (Novex™, Catalogue

number WP20005, Thermoscientific, USA) and imaged using a UVP Biospectrum Imager (analytik Jena, Germany). Densitometric analysis of proteins bands were performed using the imager's built-in software (VisionWorks LS, analytik Jena, Germany) according to manufaturer instructions. Protein Expression of iNOS was normalized to that of β-actin and expressed as fold of normalized expression to the LPS^+ control.

RNA extraction and quantitative real-time polymerase chain reaction (RT-qPCR)

RAW264.7 macrophages were seeded onto 96-well microplates (1.5×10^6 cells/well). Cells were treated with DMSO (0.1% v/v), lipopolysaccharide (100 ng/ml) either alone (LPS^+) or in the presence of compound **5** (30 µM) or indomethacin reference standard (250 µM) [24] for 5 h. Culture supernatants were aspirated off and cells were scraped into 5 volumes RNA*later*® solution (ThermoFisher scientific, CA, USA) and stored at -80C°. Extraction of total RNA from cell lysates was performed as instructed by the manufacturer using Promega SV Total RNA Isolation System (Madison, WI, USA). RNA purity and concentration were assessed using UV spectrophotometric measurements. Complementary DNA (cDNA) was formed through reverse transcription of 1 µg of the isolated RNAs using SuperScript III First-Strand Synthesis kit (Fermentas, Waltham, MA, USA). qPCR was conducted in Applied Biosystem (StepOne™, USA) using SYBR Green PCR Master Mix. The reaction mixture (25 µl) was consisted of 2X SYBR Green PCR Master Mix, cDNA template (2µl), and forward/reverse primers (900 nM).The sequences of primers used for TNF-α, IL-6, IL-1β, β-actin [25], iNOS [26] , COX-2 [27] are represented in Table 1. Reactions were initiated through incubation at 50 °C for 2 min followed by 95 °C for 10 min and then 40 cycles consisted of denaturation (94 °C) for 15 s, different annealing temperatures (56-64 °C) for each pair of primer for 12 s and extension (72 °C) for 25 s. Data from RT-qPCR were analyzed using a specific software (Applied Biosystems) for the detection and analysis of sequences (v1.7). Relative levels of iNOS, COX2, IL-6, IL-1β and TNF-α gene mRNAs were normalized to that of β-actin (internal reference gene) using the comparative Ct method and were reported as fold changes.

Table 1.

Pharmacological studies

Animals

Adult male albino mice weighing 20–25 g were used for the assessment of the analgesic and anti-inflammatory activities. Mice were pruchased from the Animal-Breeding Unit of the National Research Centre (Giza, Egypt). Animals were housed in ventilated cages with *ad libitum* access to tap water and standard pelleted diet under controlled conditions of temperature (23 ± 2°C), light cycle (12 h light/dark), and relative humidity (55 ±5%). All animal procedures were carried out in compliance with the regulations of the ethical committee of the National Research Centre for use of laboratory animals (Number: 16 155).

Analgesic activity assays

The analgesic profile of compound **5** was evaluated in mice (n = 6) by adopting acetic acid-induced writhing test to determine the peripheral analgesic effect at doses of 15 and 30 mg/kg body weight, in addition to the hot-plate technique to determine the central analgesic effect at a dose level of 30 mg/kg body weight.

Writhing test

Peripheral analgesic effect of compound **5** was determined *in-vivo* using the writhing test [28]. Mice were classified as: group I served as the control group and received the vehicle (1% Tween-80 aqueous solution). Group II was injected intraperitoneally (*i.p.*) with diclofenac sodium (10 mg/kg) as reference drug whereas groups III–IV recieved the test compound **5** in two different concentrations (15 and 30 mg/kg, *i.p.*), respectively. Animals were *i.p.* injected with freshly prepared acetic acid [2% (w/v) in saline, 10 ml/kg body weight] as algesic agent 30 min after *i.p.* injection of the vehicle, reference drug, or the different doses of the test compound. The mice were seprated for individual observation for a period of 30 min. The number of writhes produced by each mouse were counted during the observation period. Abdominal writhing is a response described as abdominal muscle contraction and pelvic rotations followed by hind limb extension [29,30]. The percentage inhibition of abdominal writhings was calculated using the following equation [29]:

$$\%\ \text{inhibition of abdominal writhings} = \frac{Nc - Nt}{Nc} \times 100$$

where N_c is the number of writhes of the control group and N_t is the number of writhes of the treated group.

Hot-plate test

The hot-plate method [31] involves comparing the response of mice to pain stimulus in treated and untreated mice at definit time intervals. Mice were classified as: group I served as the control group and received the vehicle (1% Tween-80 aqueous solution); group II received tramadol hydrochloride (25 mg/kg, *i.p.*) as reference standard [29] ; and group III received compound **5** (30 mg/kg *i.p*).. The mice were placed gently into a 1-l dry glass beaker, and the temperature was adjusted to 55–56°C. The mice were pretested, and those having a latency time greater than 15 s were excluded from the testing. The reaction time was measured and it was considered as the time interval (s) starting when the mouse reached the hot beaker until paw licking or jumping occured. The normal reaction time was determined three times at 5-min intervals and the average was calculated for all animals before injection of the vehicle, reference drug, or test compounds. The reaction time was determined at 15-, 30-, 45-, 60-, 90-, and 120-min intervals after vehicle, reference drug, or compound **5** injection.

Anti-inflammatory activity

Carrageenan-induced paw edema assay

Anti-inflammatory activity was assessed in an acute model *via* the carrageenan-induced paw edema assay [32,33]. Adult male albino mice (n = 6) were classified into III groups. Group I (control group) received the vehicle 1% Tween-80 aqueous solution. Group II received indomethacin (5 mg/kg, *i.p.*) as reference drug [34], whereas group III was *i.p.* dosed with 30 mg/kg of compound **5** dissolved in Tween-80 (1% aqueous solution) one hour before carrageenan challenge. The mouse paw edema was induced with subplantar injection of 0.05 ml of 0.5% suspension of carrageenan in saline into the plantar tissue of one hind paw in all groups. Meanwhile, equal volume of saline was injected into the other hind paw for control measurments. Results were recorded by measuring the thickness of both hind paws using a Vernier Caliper (SMEC, Shanghai,China) 1, 2, and 3 h after carrageenan challenge.

The percentage swelling as well as the percent inhibition of edema of the mouse paw was calculated using the following equations [29,35]:

$$\%\ \text{swelling} = \frac{Vc - Vs}{Vs} \times 100$$

where V_c is the thickness of the carrageenan paw and V_s is the thickness of the saline paw at each time interval. The average paw swelling in both compound **5** and indomethacin-treated mice were compared with that of the vehicle treated mice.

$$\%\ \text{inhibition of edema} = \left[1 - \frac{\%\ swelling\ of\ treated\ group}{\%\ swelling\ of\ carrageenan\ group}\right] \times 100$$

Blood collection

Three hours post-carrageenan injection, blood samples were collected from different groups *via* intracardiac puncture, allowed to clot then serum was separated through centrifugation at 3000 rpm at 4°C for 15 min. Collected sera were then aliquoted to be stored at -80°C till next use.

Determination of serum nitric oxide and cyclooxygenase-2 level

Levels of nitric oxide (NO) and cyclooxygenase-2 (COX-2) level were evaluated in the serum of normal mice administered with the vehicle as well as mice receiving the vehicle (control), compound **5** (30 mg/kg, *i.p.*) and indomethacin (5 mg/kg, *i.p.*) then injected with carrageenan, using mouse NO and COX-2 enzyme-linked immunosorbent assay (ELISA), respectively (MyBioSource, Inc., San Diego, CA) according to the manufacturer's instructions.

Acute toxicity test

Compound **5** was administered in different doses (10-500 mg/kg) given *via i.p.* route to respective adult healthy groups of mice followed by continuous observation for any deaths or gross behavioral changes during the first 2 h [36]. The mice were occasionally observed starting from the next 4 to 24 h and for the occurrence of any delayed effects. Examination of mice was further prolonged for 72 hours for any behavioral changes or toxic signs. Mice were kept on observation for a total period of 14 days [37].

Statistical analysis

All results were represented as mean±SEM. Comparison between different groups was performed using one-way analysis of variance (ANOVA). Student–Newman–Keuls method was used as a post-hoc analysis to compare means. Results were considered statistically significant when $P<0.05$.

Results

In vitro anti-inflammatory activity

In the present study, 1-(4-(7-chloroquinoline-4-yl)piperazin-1-yl) derivatives **1-9** were initially tested *in vitro* for their anti-inflammatory potential. We employed RAW 264.7 murine macrophage model as a sensitive and well recognized cells for inflammation induction by LPS, a pattern recognition molecule that is a constituent of the cell wall of Gram negative bacteria [38]. As displayed in Figure 3, the screening revealed differential inhibition of the LPS-induced NO release by the tested compounds at 30 µM. Among the tested compounds, compounds **5** was the most potent to inhibit NO release, recording 74.1% ± 2.2 inhibition of LPS-induced NO, as assessed with Greiss assay.

Figure 3.

To evaluate the impact of compound 5 on the inhibition of LPS-induced iNOS protein expression intracellularly, RAW 264.7 cells were co-treated with LPS and increasing concentrations of compound 5. Western blotting of cell lysate proteins revealed a concentration-dependent inhibition of the iNOS protein expression induced by LPS (LPS+). Densitometric analysis of iNOS bands normalized to β-actin revealed a gradual inhibition by increasing doses of of compound 5, recording only 0.2 fold of LPS^+ group (i.e 80% inhibition of LPS-induced iNOS expression at concentration of 30 µM), as displayed in Figure 4.

Figure 4.

Effect of compound 5 on the gene expression of inflammatory markers in LPS-stimulated RAW 264.7 macrophages

To further explore the anti-inflammatory effect of compound **5**, the influence of compound **5** on the proinflammatory markers iNOS, COX2, IL-6, IL-1β and TNF-α was studied in LPS-induced RAW 264.7 macrophages. As shown in figure 5, LPS (100 ng/ml) markedly increased the transcriptional expression of iNOS (5.6 fold), TNF-α (3.9 fold), COX2 (4 fold), IL-1β (5.3 fold), and IL-6 (3.5 fold), compared with the vehicle-treated macrophages ($p<0.05$). Co-treatment of RAW 264.7 macrophages with compound **5** (30 μM) and LPS for 5h was able to significantly decrease the expression levels of the aforementioned inflammatory markers compared to LPS group ($p<0.05$). It can also be observed that compound 5 has nearly the same effect as indomethacin control on the induction of TNF-α, COX2, and IL-1β expression.

Figure 5.

Pharmacological studies

Analgesic activity

Effect of compound 5 on peripheral analgesic activity (writhing test).

Treatment with compound **5** at doses of 15 mg/kg and 30 mg/kg has resulted in percentage inhibition of abdominal writhing by 33 and 62%, respectively. The decreased the number of writhings in the treated groups at both dose levels was significant compared with acetic acid-induced group that received the vehicle while not significant compared to the diclofenac sodium (10 mg/kg). Furthermore, the maximum effect of compound **5** was observed at 30 mg/kg where it reported a significant decrease in the number of abdominal writhing compared with compound **5** at 15 mg/kg (Figure 6).

Figure 6.

Effect of compound 5 on central analgesic activity (hot-plate test)

The central analgesic activity of compound **5** was evaluated *via* the hot-plate test and results were summarized in Table 2 and Figure 7. In the current study, the analgesic effect was measured at 0, 15, 30, 45, 60, 90, and 120 min post *i.p.* administration of either the vehicle control, tramadol hydrochloride reference drug (25 mg/kg), or compound **5** (30 mg/kg). The analgesic activity started to increase at the 15 min time point where both tramadol and compound **5** possessed

significant analgesic activities compared to control. The highest analgesic activity of tramadol hydrochloride was observed at 30 min post administration which was similar to that achieved by compound **5** at the same time point. Interestingly, compound **5** maintained its analgesic activity and achieved the highest analgesic peak at 45 min post administration which was much higher than tramadol hydrochloride. Although the analgesic activity of compound **5** started to decrease at the 60 min time interval, but it was still significantly higher than that of tramadol. Furthermore, the analgesic activity of compound **5** and tramadol continued till 120 min, where both showed significantly higher effect compared to the control value.

Table 2.

Figure 7.

Effect of compound 5 on *in-vivo* anti-inflammatory activity

Sub-plantar injection of carrageenan in mice has resulted in an increase in the percentage swelling of the mouse paw by 50, 55.6 and 62 % at 1, 2, and 3 h post injection. Compound **5** at dose 30 mg/kg obviously inhibited the carrageenan-induced swelling in a time dependent manner. The percentage of inhibition of edema by compound **5** was 34, 50 and 64% at 1, 2, and 3 h, respectively, post carrageenan challenge while treatment with the reference standard indomethacin exhibited percentage inhibition of edema of 12, 41 and 58% at the same time intervals (Figure 9).

Both compound **5** and indomethacin demonstrated significant decrease in the percentage swelling compared to the carrageenan group at measurements taken 1, 2 and 3 h post carrageenan. In addition, compound **5** showed a significant decrease in percentage swelling compared to the reference standard at the first hour post carrageenan challenge. Interestingly, this significant inhibitory effect persisted in the 2nd and the 3rd hour demonstrating the potential anti-inflammatory response of compound 5.

Figure 8.

Figure 9.

Effect of compound 5 on serum NO and COX-2 levels

In order to evaluate the anti-inflammatory mechanisms of compound 5 *in vivo*, serum NO and COX-2 levels were examined by ELISA. Data represented in Figure 10 demonstrated

significant increase in NO level in serum samples of mice collected 3 hours post carrageenan-injection (control group) compared to the normal (negative control) group. In addition, compound **5** (30 mg/kg) and indomethacin (5 mg/kg) significantly decreased the NO levels in serum compared with the control (carrageenan) group by 57 and 64%, respectively. Furthermore, treatment with compound **5** and indomethacin significantly decreased COX-2 serum level in mice challenged with carrageenan by 55 and 65%, respectively, relative to the control group. No significant differences in serum NO and COX-2 levels were observed between indomethacin and compound **5** treated groups.

Figure 10.

Figure 11.

Acute toxicity

Acute toxicity studies in albino mice with compound **5** at dose up to 500 mg/kg did not cause any signs of toxicity or behavioral changes or mortality during the observation period. In addition, no changes in fur and skin, mucous membranes, and eyes or behavioral pattern. Convulsions, tremors, coma sleep, lethargy, diarrhoea, and salivation were not observed.

Discussion

Nitric oxide (NO) has been previously demonstrated as one of the most important inflammatory mediators. Sensitization with inflammatory inducers such as LPS causes the release of NO from immune cells, a process that is catalyzed by iNOS [39]. Several studies have pointed out the role of NO as a critical regulatory molecule of various physiological processes including host defense and neural coordination [40]. However, overproduction of NO was associated with oxidative damage and inflammatory diseases [41]. The progression of inflammatory diseases involve pro-inflammatory mediators such as COX2, iNOS, TNF-α, IL-6, and IL-1β which are also known to play a substantial role in different autoimmune diseases [42]. Therefore, the levels of NO and the pro-inflammatory markers are regarded as indicators of the degree of inflammation.

In the present study, nine quinoline derivatives **1-9** showed significant inhibition of NO level in RAW 264.7 cells. Remarkably, compound **5** demonstrated the highest NO inhibitory activity which could be attributed to the presence of two privileged piperazinyl heterocyclic

moieties connected by ethanone spacer, and linked to the versatile quinoline scaffold in one molecule. The NO inhibitory activity of compound 5 in LPS-induced RAW 264.7 macrophages was explained to be as result of the inhibition of iNOS protein expression in a concentration-dependent manner as assessed with Western technique.

Furthermore, co-treatment of RAW 264.7 macrophages with compound 5 decreased significantly the gene expression levels of iNOS, COX2, TNF-α, IL-1β and IL-6 in LPS-induced macrophages cells suggesting that compound **5** may exert its anti-inflammatory effect through the suppression of the expression of these proinflammatory mediators. The inhibitory action of compound **5** was much higher for COX2 and IL-6 relative expression levels.

The promising *in vitro* NO inhibitory activity, iNOS protein expression and inflammatory markers gene expression results encouraged to further explore the *in vivo* anti-inflammatory and analgesic potentials of compound **5**.

Acetic acid-induced abdominal writhing test is a well-established, reliable and sensitive *in-vivo* test model that is widely used for the assessment of peripheral analgesic activity [43]. The mechanism of acetic acid-induced pain and abdominal writhing involves the release of endogenous mediators such as arachidonic acid, prostaglandins E2 and F2, COX, bradykinin, serotonin, histamine and cytokines in the peritoneal cavity [44-46]. These mediators prompt local peritoneal nociceptive receptors and cause peripheral nociceptive sensitization as well as inflammatory pain in the peritoneal area [46-48]. In the present study, compound **5** revealed a potential peripheral analgesic effect through possessing a significant decrease in the number of abdominal writhings compared to the control group injected with acetic acid only. The produced analgesic effect of compound **5** was comparable to that produced by diclofenac sodium (10 mg/kg). These results strongly suggest that compound **5** retains peripheral analgesic activity through inhibition of local endogenous mediators involved in pain and inflammation. In agreement with our study, Hassan *et al.*, 2014 reported that the mechanism of peripheral pain inhibition which involves inhibition of prostaglandin biosynthesis is possibly caused by any substance that inhibits acetic acid-induced writhing [45].

In order to investigate the central analgesic activity of compound **5**, the hot-plate test was used. It is a well-established behavioral model of nociception and determination of central

analgesic activity of compounds as it includes higher brain function [49]. The mechanism involved in the elevation of the reaction time of the two behavioral responses integrated in the hot-plate test including; paw licking and jumping reactions, are supraspinal mechanisms [50]. The mechanism of supraspinal/spinal signal integration in neurologic pain includes the release of endogenous opioids, serotonin, noradrenaline, and acetylcholine [51]. In the hot-plate test, centrally acting analgesics exert their action on the spinal cord by acting on the opioid receptors [47].

In general, μ opioid receptors mediate spinal analgesia while in particular, μ opioid receptor subtype 1, mediates supraspinal analgesia. The reference standard "Tramadol" is a μ opioid receptor agonist that also inhibits the neuronal re-uptake of serotonin and norepinephrine resulting in analgesic effect [52]. In the current study, compound **5** possessed an increase in analgesic activity starting from 15 min post administration where it reached its maximum at 45 min time point and was significantly higher than that of tramadol hydrochloride suggesting its potential as central analgesic agent.

The anti-inflammatory activity of compound **5** was evaluated in *in-vivo* using carrageenan-induced paw edema model in mice. This model is commonly used to test the anti-inflammatory activity of compounds acting on mediators of acute inflammation [29]. In carrageenan challenge, inflammation is mediated by the *in situ* libration of proinflammatory mediators such as histamine, bradykinin, tachykinins, complement and reactive oxygen and nitrogen species [29,53]. In addition, carrageenan-induced inflammation results in neutrophils infiltration and migration to sites of inflammation as well as the production of neutrophils-derived free radicals [53,54]. In general, carrageenan injection causes dilation of the postcapillary venules which results in exudation of inflammatory fluid and cells and release of proinflammatory mediators. Thus, inhibition of such events which represent the early exudative inflammatory phase would lead to the inhibition or termination of the inflammatory process [55].

Injection of carrageenan results in edema formation which is considered a biphasic event. The initial phase is a nonphagocytic edema that is associated with trauma and the libration of acute mediators such as serotonin, histamine and bradykinin, and their effect on vascular permeability occurring at 1 or 1.5 h and is primarily. The second phase is mainly ascribed to the overproduction of prostaglandins and release of inducible cyclooxygenase and lysosome enzymes for 2–3 h [29,54,56]. Thus, carrageenan challenge is usually linked with the activation of the cyclooxygenase pathway.

In the current study, the ability of compound **5** to inhibit the carrageenan induced swelling in time dependent manner was demonstrated. The percentage inhibition obtained after 2 and 3 h following carrageenan challenge was 50 and 64%, respectively, indicating that compound **5** possibly exerts its anti-inflammatory effect *via* inhibition of the cyclooxygenase pathway. This postulation was further confirmed through the assessment of the serum levels of NO and COX-2 in mice treated with compound **5** at 3 h post-carrageenan challenge. The results revealed a significant inhibition of serum NO and COX-2 levels demonstrating the potential mechanism of action of compound **5** is *via* inhibition of NO and COX-2 *in vivo*. This finding is in agreement with Mazzoni et al. where maximum activity of the quinolone derivative namely; methyl 1-(4'-methyl-benzoyl)-6-iodo-4-oxo-1,4-dihydroquinoline-2-carboxylate, demonstrated 71% inhibition of edema at the third hour post carrageenan challenge and is also the maximum of the whole experiment. In addition, the most of 4-oxoquinoline-2-carboxylic acid derivatives exhibited good analgesic and anti-inflammatory activities in carrageenan-induced rat paw edema and acetic acid writhing test in mice, respectively [57]. Although compound **5** previously demonstrated no pharmacological impact in terms of *in-vitro* antitumor activity [19], but the current findings suggest that the structure features of compound 5 consists of 7-chloro-4-(piperazin1-yl)quinoline heterocyclic scaffold incorporated with N- phenylpiperzine functional groups linked together with the ethanone pharmacophoric chain potentiated the analgesic and anti-inflammatory activities.

Conclusions

The current study demonstrated the promising anti-inflammatory therapeutic effect of compound 1-(4-(7-chloroquinoline-4-yl)piperazin-1-yl)-2-(4-phenylpiperazin-1-yl)ethanone (**5**) in cellular RAW 264.7 macrophages and animal models. It was capable of inhibiting NO, iNOS protein and gene expression together with the inflammatory mediators: COX-2, IL-6, IL-1β and TNF-α gene expression. In addition, the *in vivo* carrageenan-induced paw edema assay showed a significant decrease in percentage swelling in mice treated with compound **5** compared to indomethacin that was accompanied by a significant inhibition of serum NO and COX-2 levels. Compound **5** revealed a potential peripheral analgesic effect through the significant inhibition of abdominal writhing in mice. Moreover, it possessed a central analgesic activity through raising the

pain threshold. Conclusively, compound **5** could be considered as a promising bioactive anti-inflammatory and analgesic candidate.

Acknowledgment

We are deeply greatful to Prof. Dr. Aboul-Enein, M. N. and Prof. Dr. El-Azzouny A. M. who were responsible for the study entitled: "Design, Synthesis, and Cytotoxic Evaluation of Certain 7-Chloro-4-(piperazin-1-yl)quinoline Derivatives as VEGFR-II Inhibitors" and for providing us with compounds **1**-**9**.

Conflict of interest:

The authors declare no conflict of interest.

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Tables:

Table 1. Nucleotide sequences of primers used in real-time RT-PCR

Gene Name	Sequence (5'-3') Forward/Reverse
β-actin	CCACACCCGCCACCAGTTCG CCCATTCCCACCATCACACC
TNF-α	CCCCTCAGCAAACCACCAAGT CTTGGGCAGATTGACCTCAGC
iNOS	ACAACGTGAAGAAAACCCCTTGTG ACAGTTCCGAGCGTCAAAGACC
COX-2	GCAAATCCTTGCTGTTCCAATC GGAGAAGGCTTCCCAGCTTTTG
IL-1β	AATCTCACAGCAGCACATCAA AGCCCATACTTTAGGAAGACA
IL-6	GGAGGCTTAATTACACATGTT TGATTTCAAGATGAATTGGAT

Table 2. Effect of compound 5 in the hot-plate test in adult male albino mice

	Time (s)						
Treatments	**0**	**15**	**30**	**45**	**60**	**90**	**120**
Control (Vehicle)	10.60±0.60	10.40±0.54$^{\#}$	10.67±0.42$^{\#}$	10.50±0.43$^{\#}$	10.80±0.37$^{\#}$	10.43±0.57$^{\#}$	10.40±0.51$^{\#}$
Tramadol (25 mg/kg)	10.67±0.49	16.13±0.71*	22.21±0.51*	17.65±0.55*	16.47±0.61*	15.29±0.42*	14.71±0.34*
Compound 5 (30 mg/kg)	10.78±0.40	14.14±0.35*$^{\#}$	22.00±0.53*	25.29±0.42*$^{\#}$	18.57±0.51*$^{\#}$	14.45±0.57*	13.82±0.33*

The mice were observed for their response toward pain stimulus at definite time intervals. Each value represents the mean reaction time in second ± SEM. *$P < 0.05$ compared with control; $^{\#}P < 0.05$ compared with tramadol hydrochloride.

Figure legends

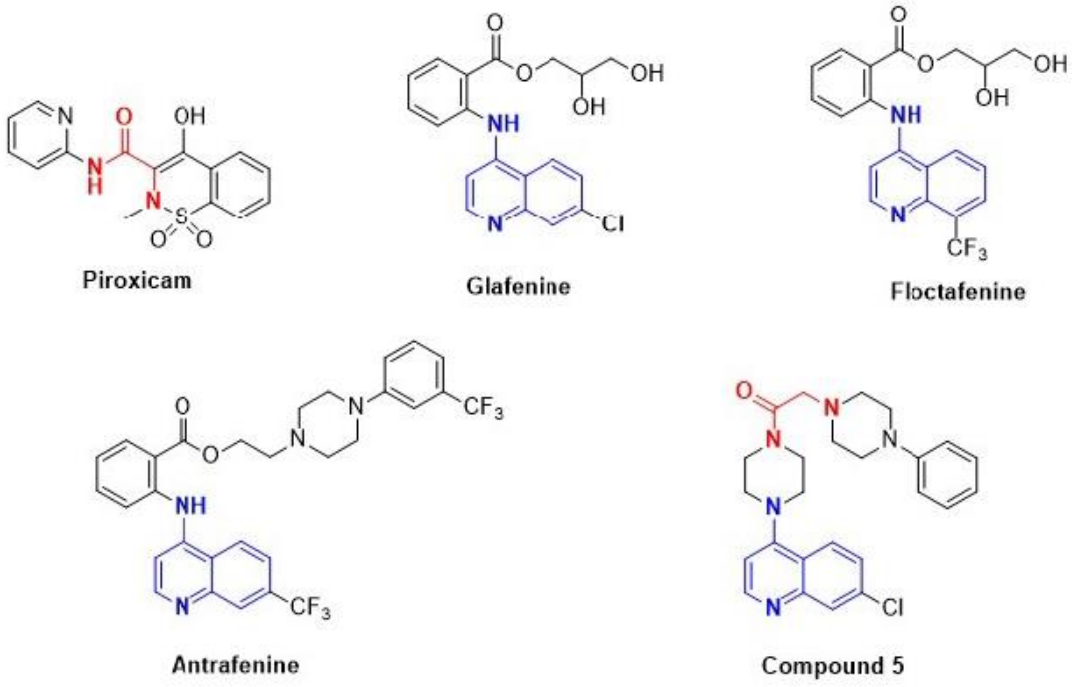
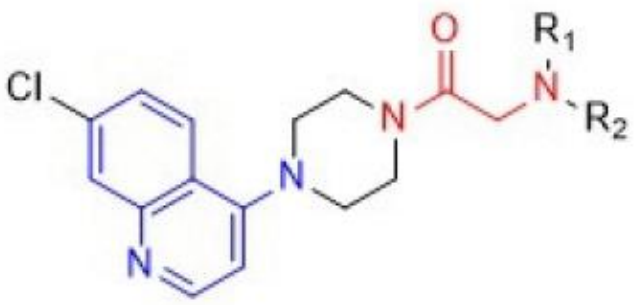


Figure 1. Certain marketed NSAID/analgesic drugs and compound **5**.



Compound 1-9

Compound	NR_1R_2
1	Dimethylamine
2	Piperdine
3	Morpholine
4	*N*-methylpiperazine
5	*N*-phenylpiperazine
6	*N*-(4-methoxyphenyl)piperazine
7	*N*-(4-ethoxyphenyl)piperazine
8	*N*-benzylpiperazine
9	*N*-(4-methylbenzyl)piperazine

Figure 2. The structure features of compound**s 1-9** 1-(4-(7-chloroquinoline-4-yl)piperazin-1-yl) derivatives **(1-9)**.

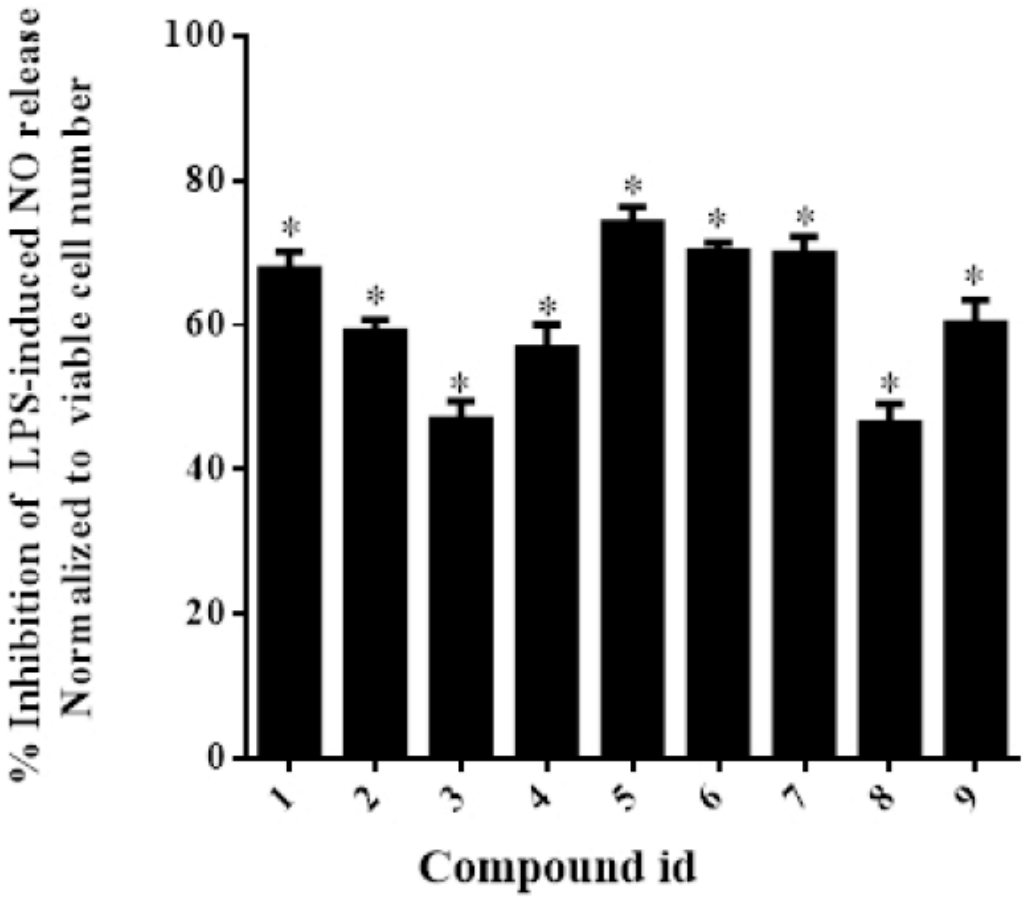


Figure 3. Inhibition of LPS-induced NO release by the indicated compounds in RAW 264.7 cells. Cells were co-treated for 24 h with either LPS (100 ng/ml) alone (LPS+) or with 30 µM of compounds. NO was determined as described in the Methods section. Data are means ± SEM (n=3). **P*<0.05 compared to LPS^{+}control.

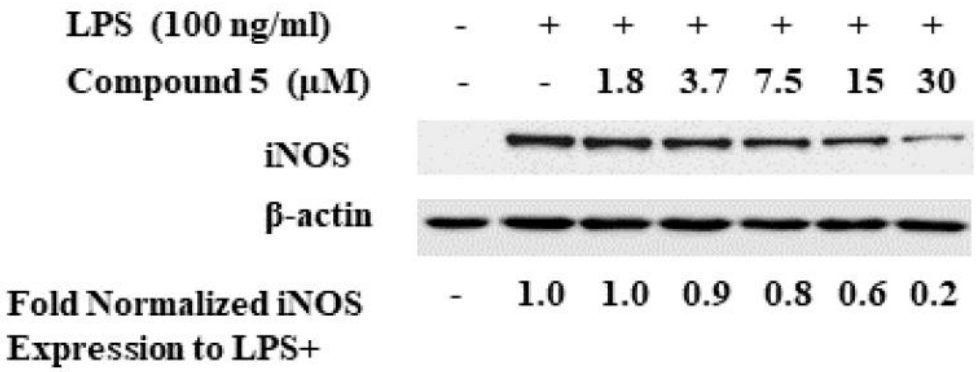


Figure 4. Effect of compound **5** on LPS-induced protein expression of iNOS. RAW 264.7 cells were co-treated for 24 h with either LPS (100 ng/ml) alone (LPS+) or with increasing concentrations of compound 5 (0-30 μM). Protein expression was determined using Western blotting with antibodies against iNOS. β-actin was used as house-keeping protein to normalize iNOS expression. Fold of normalized protein expression of iNOS shown were obtained with densitometric analysis as described in the Materials and Methods section.

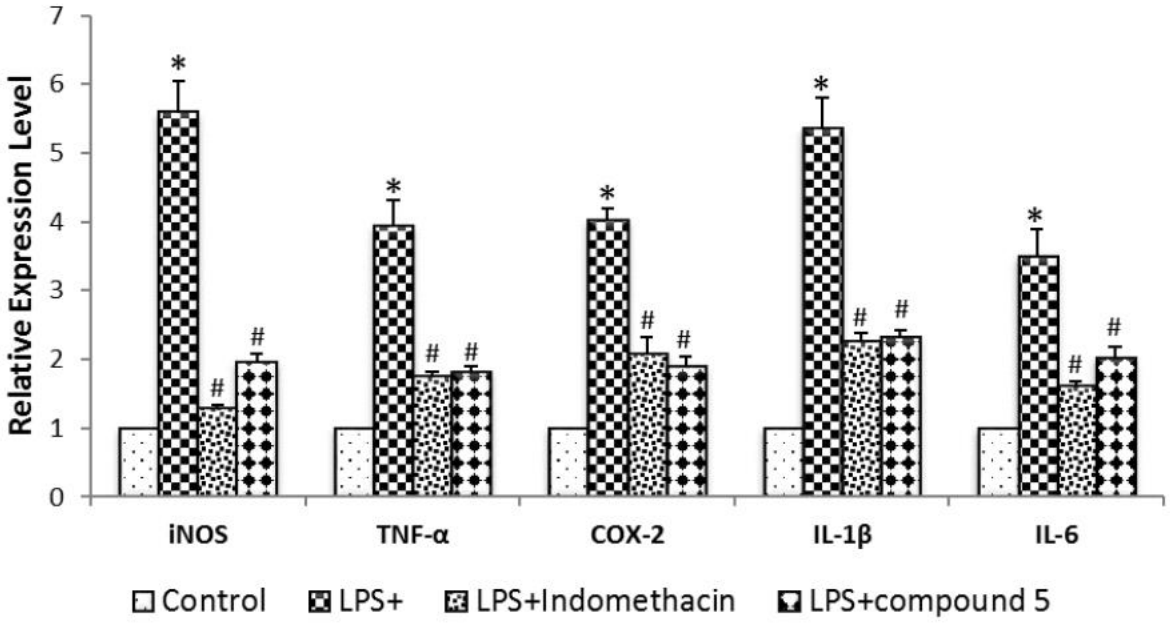


Figure 5. Effect of compound **5** on LPS-induced mRNA relative expression of iNOS, TNF-α, COX-2, IL-1β, and IL-6 in RAW264.7 cells. RAW 264.7 cells were treated for 5 h with either DMSO (control); LPS (100 ng/ml) alone (LPS+); LPS with indomethacin (250 µM); or LPS with compound **5** (30 µM). Relative expression levels were determined using RT-qPCR and are represented as mean fold change related to control ± SEM (n=3). **P*<0.05 compared to control while $^{\#}P$<0.05 compared to LPS.

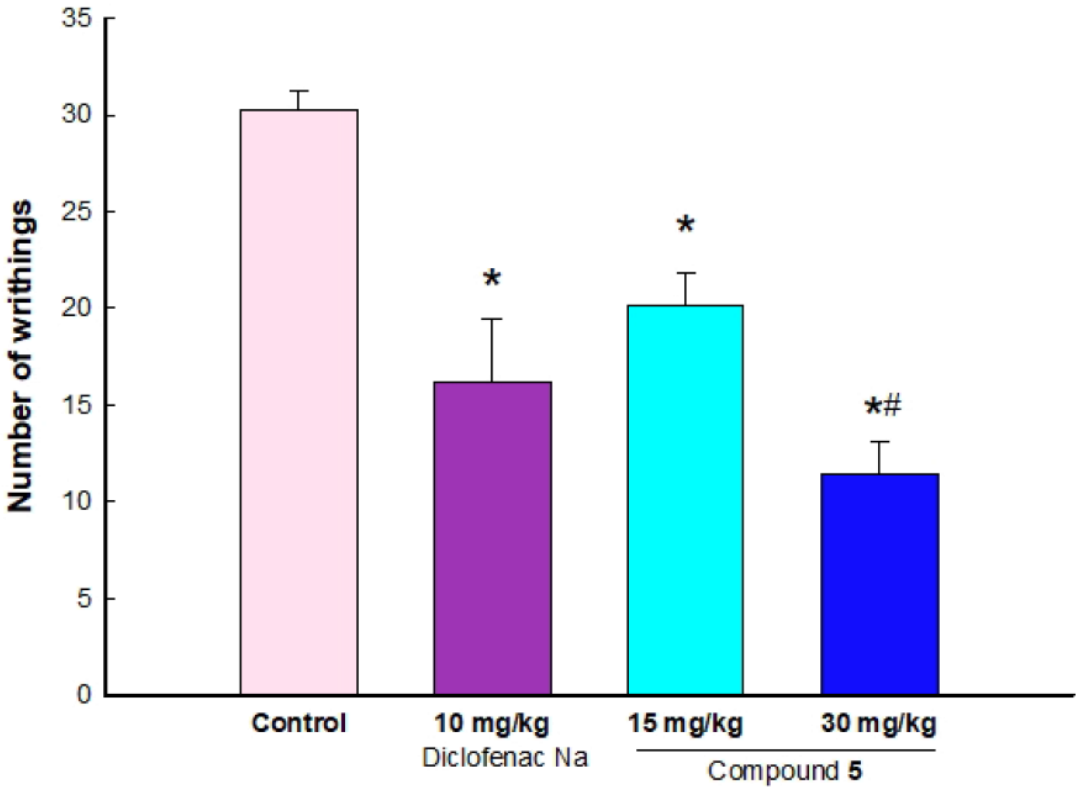


Figure 6. Effect of compound **5** (15 and 30 mg/kg) on acetic acid-induced writhing test. Swiss albino mice were injected with compound **5** (15 and 30 mg/kg, *i.p.*), diclofenac sodium (10 mg/kg), or vehicle (control) 30 min before *i.p.* injection of acetic acid. The mice were individually separated and observed for 30 min. Each value represents the mean of the number of writhes ± SEM. $*P < 0.05$ compared with control; $^{\#}P < 0.05$ compared with compound **5** (15 mg/kg).

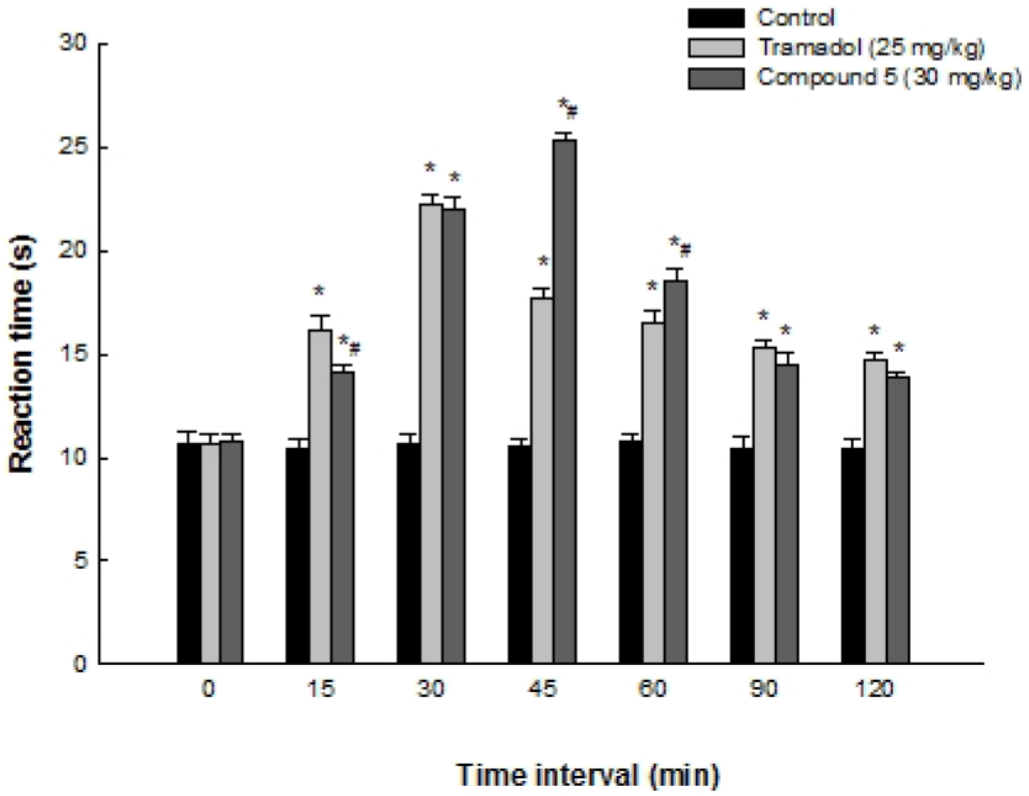


Figure 7. Effect of compound **5** in the hot-plate test in adult male albino mice. Group I served as the control group and received vehicle; Group II received tramadol hydrochloride (25 mg/kg, *i.p.*) body weight; and Group III received compound **5** (30 mg/kg, *i.p.*). Animals were observed for their response toward pain stimulus at definite time intervals. Each value represents the mean reaction time (s) ± SEM. $*P < 0.05$ compared with control; $^{\#}P < 0.05$ compared with tramadol hydrochloride.

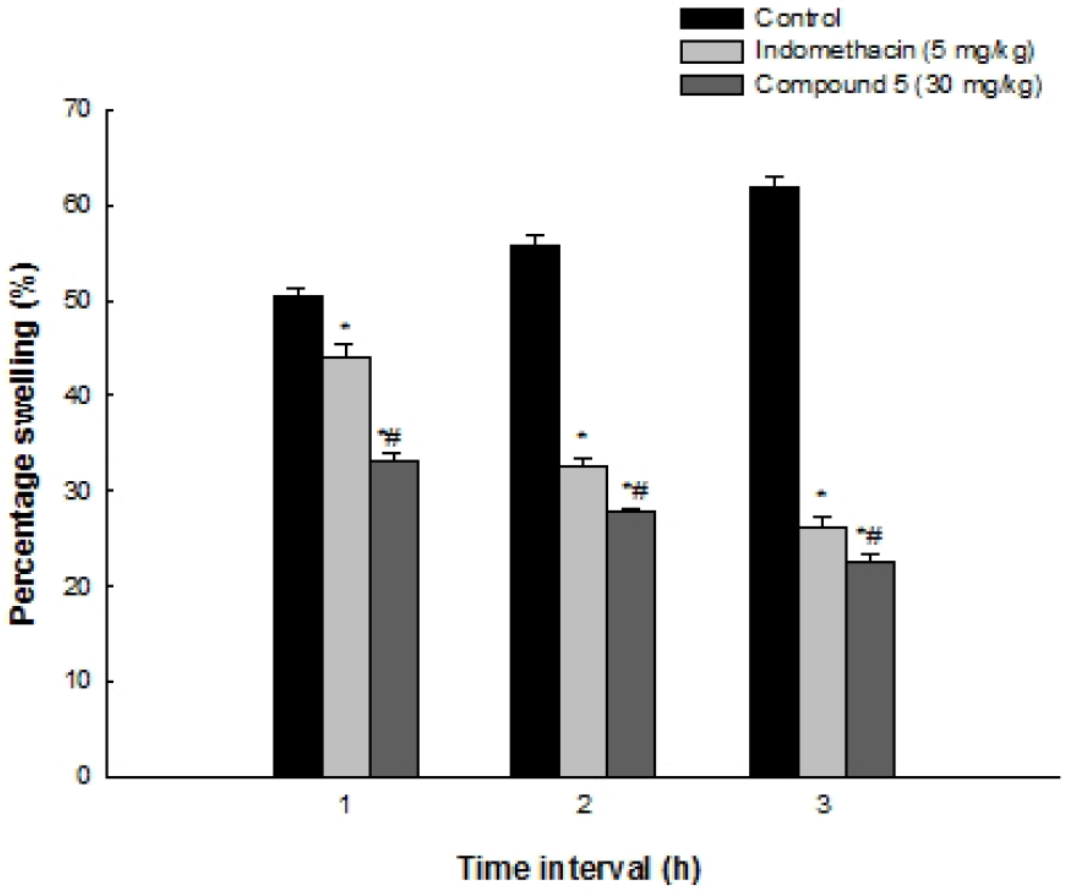


Figure 8. Effect of compound **5** on percentage swelling in carrageenan-induced paw edema. Mice were injected with compound **5** (30 mg/kg, *i.p.*), indomethacin (5 mg/kg, *i.p.*), or vehicle (Control) 1 h before carrageenan challenge. The thickness of the mouse hind paws was measured with a Vernier Caliper 1, 2, and 3 h after carrageenan challenge. The percentage swelling was calculated by measuring the difference between the thicknesses of the two paws. The data represent the mean percentage swelling ± SEM. $^{*}P < 0.05$ compared with control; $^{\#}P < 0.05$ compared with indomethacin.

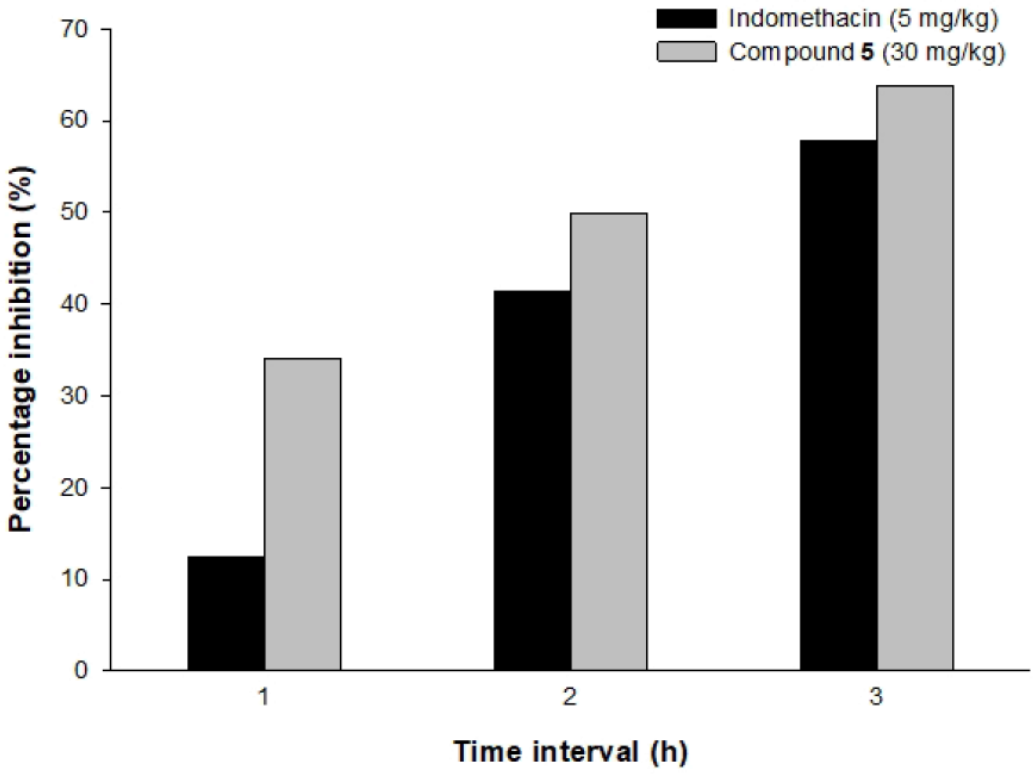


Figure 9. Effect of compound **5** on percentage protection in carrageenan-induced paw edema. Mice were injected with compound **5** (30 mg/k, *i.p.*), indomethacin (5 mg/kg, *i.p.*), or vehicle (Control) 1 h before carrageenan challenge. The thickness of the mouse hind paws was measured with a Vernier Caliper 1, 2, and 3 h after carrageenan challenge. The percentage inhibition of compound 5 as well as indomethacin, used as reference, was calculated relative to carrageenan.

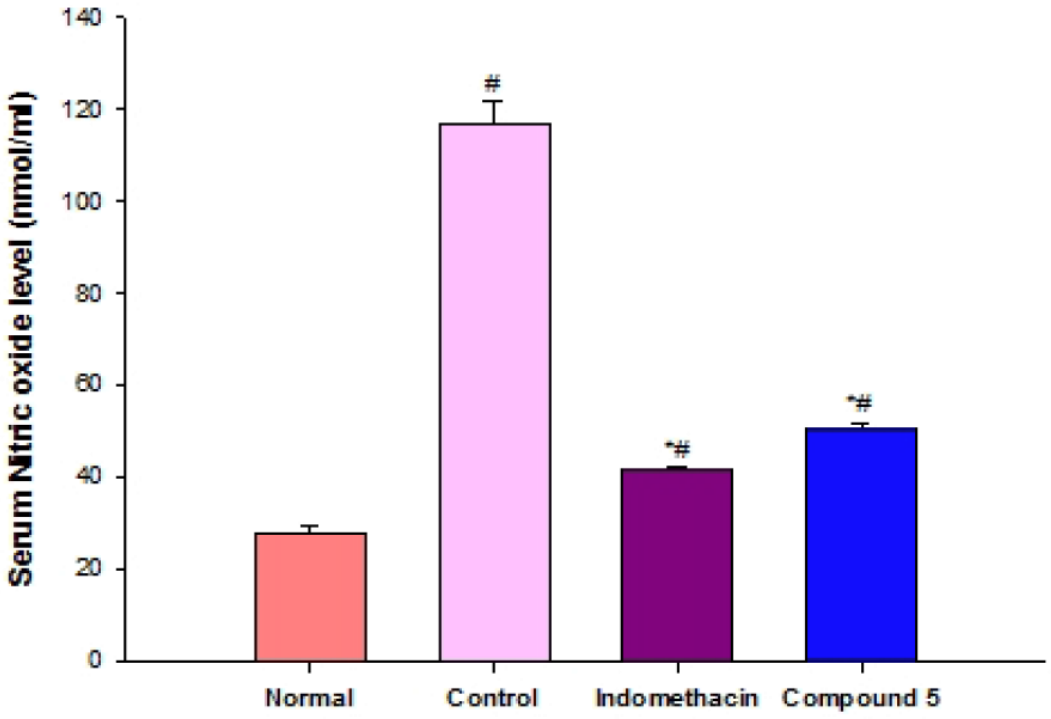


Figure 10. Effect of compound **5** on serum NO. Mice were injected intraperitoneally (*i.p.*) with compound **5** (30 mg/kg, *i.p.*), indomethacin (5 mg/kg, *i.p.*), or vehicle (Control) 1 h before carrageenan challenge. The normal group received *i.p* injection of the vehicle only. Blood samples were collected 3 h post-carrageenan challenge and serum was separated for the determination of NO level using ELISA. The data represent the mean serum NO level ± SEM. $^{\#}P < 0.05$ compared with normal; $^{*}P < 0.05$ compared with control (carrageenan).

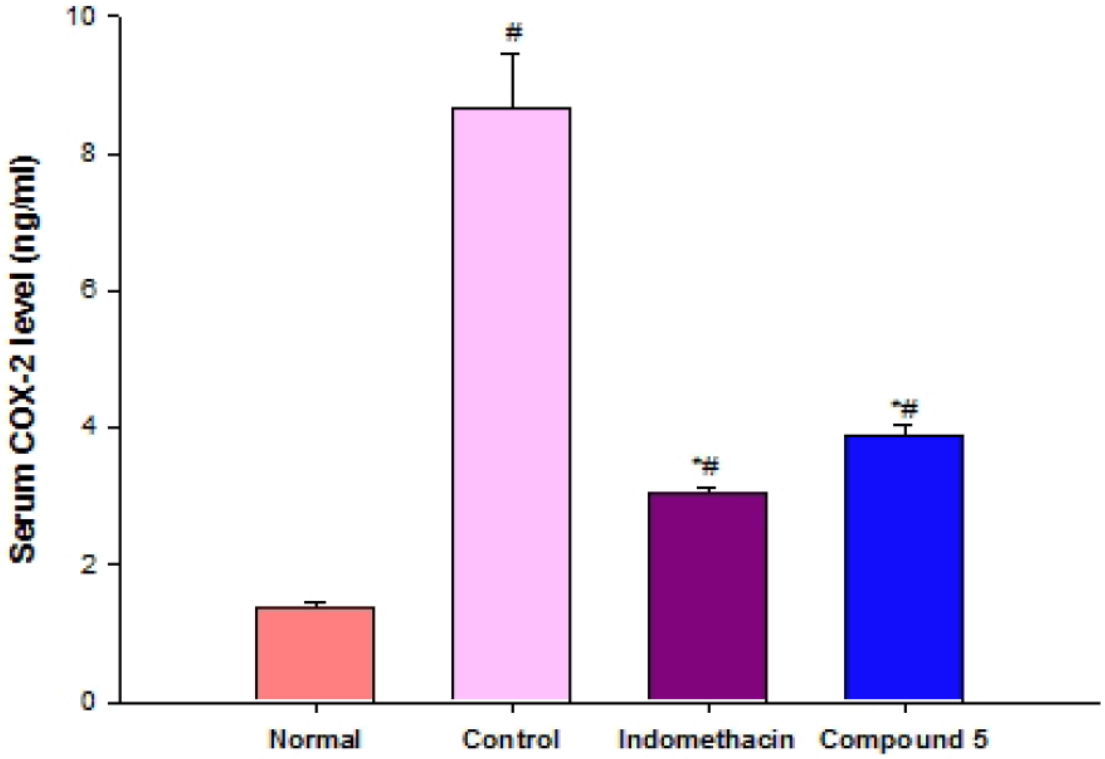


Figure 11. Effect of compound **5** on serum COX-2 level. Mice were injected with compound **5** (30 mg/kg, *i.p.*), indomethacin (5 mg/kg, *i.p.*), or vehicle (Control) 1 h before carrageenan challenge. The normal group received *i.p* injection of the vehicle only. Blood samples were collected 3 h post-carrageenan challenge and serum was separated for the determination of serum COX-2 level using ELISA. The data represent the mean serum COX-2 level ± SEM. $^{\#}P < 0.05$ compared with normal; $^{*}P < 0.05$ compared with control (carrageenan).