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ORIGINAL RESEARCH PAPER

N-Pyrazoloyl and *N*-thiopheneacetyl hydrazone of isatin exhibited potent anti-inflammatory and anti-nociceptive properties through suppression of NF-κB, MAPK and oxidative stress signaling in animal models of inflammation

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Abstract

Background Hydrazide derivatives constitute an important class of compounds for new drug development as they are reported to possess good anti-inflammatory and analgesic activity. The present study was aimed to investigate the role of newly synthesized hydrazide derivatives *N*-pyrazoloyl hydrazone of isatin (PHI) and *N*-thiopheneacetyl hydrazone of isatin (THI) in acute and chronic inflammatory pain models induced by carrageenan and complete Freud's adjuvant (CFA).

Materials PHI and THI (0.1, 1 and 10 mg/kg) pretreatments were provided intraperitoneally to male BALB/c mice prior to inflammatory inducers. Behavioral responses to inflammation and pain were evaluated by assessment of paw edema, mechanical allodynia, mechanical and thermal hyperalgesia. Cytokines production and NF-κB levels were evaluated by ELISA. Western blot analysis was performed for the detection of IκBα, p38, JNK and ERK. Hematoxylin and eosin (H&E) staining and radiographic analysis were performed to evaluate the effect of PHI and THI treatment on bone and soft tissues. Oxidative stress was determined by reduced glutathione, glutathione-*S*-transferase and catalase assays. Evans blue dye was used to monitor vascular protein leakage.

Result PHI and THI dose dependently (0.1, 1 and 10 mg/kg) reduced inflammation and pain in mice, however, the dose of 10 mg/kg exhibited significant activity. The anti-inflammatory and analgesic effects were attributed to suppression of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) production levels. PHI and THI significantly blocked CFA-induced activation of NF-κB and MAPK signaling pathways. Oxidative stress and plasma nitrite levels were reduced remarkably. The PHI and THI (10 mg/kg) treatment did not exhibit any apparent toxicity on the liver, kidney, muscles strength, and motor co-ordination in mice.

Conclusion Both PHI and THI possess significant anti-inflammatory and analgesic activity via inhibition of inflammatory mediators.

Keywords Hydrazide derivatives · Isatin · Allodynia · Hyperalgesia · CFA · Inflammatory pain

Abbreviations

CFA	Complete Freund's adjuvant
COX-2	Cyclooxygenase-2
NF-κB	Nuclear factor kappa B
NO	Nitric oxide
MAPKs	Mitogen activated protein kinase
i.p.	Intraperitoneal
i.pl	Intraplantar
PHI	*N*-Pyrazoloyl hydrazone of isatin
THI	*N*-Thiopheneacetyl hydrazone of isatin
VEGF	Vascular endothelial growth factor
MID#	Mid cells count
CDNB	1-Chloro-2,4-dinitrobenzene

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Introduction

Inflammation is an adaptive response prompted by noxious stimuli like tissue injury and infection [1]. It protects the body against infection and injury, but can itself become deleterious to the host if dysregulated, resulting in chronic disease conditions. Inflammation usually leads to chronic pain which is a public health problem worldwide, affecting about 30% of the world's population [2]. Currently available treatment options for pain management possess higher risks associated with prolonged use and there is an increasing need to develop newer and safer agents [3]. For instance, widely used non-steroidal anti-inflammatory drugs (NSAIDS) are unable to eliminate pain completely and offer serious adverse effects like gastrointestinal bleeding and renal toxicity [3]. Moreover, newer member of NSAIDS like COX-2 selective inhibitors are associated with cardiovascular risks [4] and opioid analgesics have serious adverse effects including dependence and respiratory depression [5]. Thus, there is an urgent need for newer and safer analgesics and anti-inflammatory drugs with better efficacy [6].

Numerous mediators of inflammation such as bioactive lipids, cyclooxygenase-2 (COX-2), reactive nitrogen and oxygen species, and pro-inflammatory cytokines like IL-1β, TNF-α and IL-6 are released during inflammation [6]. Acute inflammation usually subsides, while chronic inflammation persists leading to diseases such as rheumatoid arthritis, Alzheimer's disease, bowel disease, atherosclerosis, and cancer [7].

The pain produced during inflammatory process is triggered due to release of mediators that sensitize the nociceptive nerve endings. In case of peripheral tissue injury, various subchronic pain states occur, characterized by spontaneous pain, allodynia, and hyperalgesia [8]. Stimuli such as pro-inflammatory enzymes, cytokines and algogenic agents can activate NF-κB and have a vital role in regulating the expression of several proteins including pro-inflammatory mediators [9]. Inflammatory mediators like reactive oxygen species (ROS) and cytokines cause sensitization and/or activation of nociceptive neurons [10]. The inflammatory responses are related to transcriptional activation of inflammatory genes by transcription factors (NF-κB, MAPKs and CREB) that cause modulation of expression of various genes involved in immune inflammatory and pain responses [11]. Different pain stimuli activate CREB, NF-κB, and MAPKs intracellular signaling pathways of pain [12]. MAPKs play a vital role in sensitization of inflammatory pain. Studies have proven the anti-nociceptive effects of ERK and p-38 inhibition in several pain models along with confirming that cytokine-induced COX-2 expression is mediated via NF-κB along with ERK and p-38 MAPK in various systems [5, 12, 13].

Hydrazones containing an azometine –NHN=CH– proton are of vital importance in new drug development [14]. Schiff bases of isatin derivatives possess various pharmacological activities such as antibacterial, antiviral, anti-fungal [15], anticancer [16], anti-inflammatory, analgesic [17], anti-convulsant and antidepressant [18]. Hydrazones possess significant anti-inflammatory and analgesic potential as they are reported to be dual inhibitors of both cyclooxygenase (COX) and 5-lipoxygenase (5-LO) [19]. Studies have reported that the hydrazide moiety present in some compounds possess a pharmacophoric character for the inhibition of COX with better safety and efficacy as compared to few available drugs in market [19]. Given the analgesic and anti-inflammatory potential of Schiff bases of isatin, this study involves the pharmacological evaluation of newly synthesized hydrazide derivatives of isatin (PHI and THI) as novel anti-inflammatory and analgesic drug candidates. PHI and THI dose dependently exhibited anti-inflammatory and analgesic activity with no apparent toxicity.

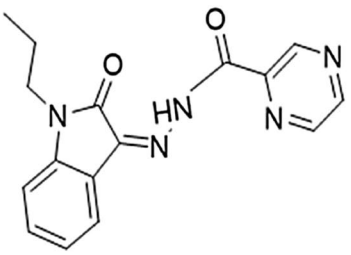
Materials and methods

Chemicals and drugs

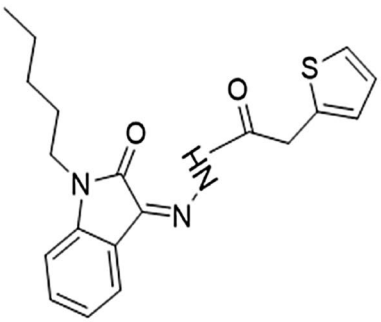
Target compounds (PHI and THI) were synthesized at the Department of Chemistry, University of Azad Jammu and Kashmir Muzaffarabad by condensation reaction between alkylated isatin and respective carboxylic acid hydrazide under ultrasonic radiations. Synthesized compounds (PHI and THI) were purified by thin layer chromatography (TLC), flash column chromatography (FCC) and recrystallization. Finally, structures were confirmed through NMR and IR spectroscopic techniques (Fig. 1). Carrageenan, Complete Freund's Adjuvant, Griess reagent, piroxicam, tramadol, naloxone, gabapentin, flumazenil, diclofenac sodium, and dexamethasone were obtained from Sigma (USA). Chloroform, distilled water and dimethyl sulfoxide (DMSO) used were of research grade. All above-mentioned drugs were dissolved and diluted in 2% DMSO and normal saline. Evans blue dye. Elisa kits of IL-6, TNF- α, IL-1β, NF-κB (eBioscience, Inc., San Diego, CA, United States). Primary and secondary antibodies p-p38, p-ERK, IκBα, p-JNK and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Physicochemical properties of *N*-pyrazoloyl hydrazone of isatin (PHI)

Yellow crystals, Mol. Formula: $C_{16}H_{15}N_5O_2$, Mol. Wt. = 309.32 g/mol, m.p = 183–185°C, yield 75%, R_f = 0.9 (*n*–Hexane: EtOAc), Solubility: chloroform, normal saline and DMSO, IR (FTIR cm^{-1}): 3230 (N–H), 1693 (C=O),



(*Z*)-*N*'-(2-oxo-1-propylindolin-3-ylidene)pyrazine-2-carbohydrazide (PHI)



(*Z*)-*N*'-(2-oxo-1-pentylindolin-3-ylidene)-2-(thiophen-2-yl)acetohydrazide (THI)

Fig. 1 Schematic structure of **a** *N*-pyrazoloyl hydrazone of isatin and **b** *N*-thiopheneacetyl hydrazone of isatin

1614 (C=N), 1458 (CH_2 scissoring), 1359 (C-N stretching), 1049 (C-O Stretching), **^{1}H-NMR** (300 MHz, DMSO-d_6) δ 14.58 (s, 1H, NH), 9.3-7.1 (m, ArH), 3.7 (t, 2H, J = 6.9 Hz), 1.68 (sext., 2H, J = 7.2 Hz), 0.9 (t, 3H, J = 7.2 Hz). **^{13}C-NMR** (100 MHz, DMSO-d_6): δ 165.9, 163.8, 137.8, 133.5, 132.4, 129.0, 127.8, 45.0, 22.0, 11.2.

Physicochemical properties of *N*-thiophene acetyl hydrazone of isatin (THI)

Yellowish brown powder, Mol. Formula: $C_{19}H_{21}N_3O_2S$, Mol. Wt.: 355.45 g/mol, m.p = 88–90 °C, R_f = 058 (*n*-Hexane: EtOAc 7:3), Solubility: chloroform, normal saline and DMSO, IR (FTIR cm^{-1}): 3161 (N–H), 1666 (C=O), 1608 (C=N), 1463 (CH_2 scissoring), 1363 (C–N stretching), 1054 (C–O Stretching). **^{1}H-NMR** (300 MHz, DMSO-d_6) δ (12.4, s, 1H, NH), 7.6–7.1 (m, ArH), 4.3 (s, 2H), 3.69 (t, 2H, J = 7.4 Hz), 1.59 (sext, 2H, J = 7.4 Hz), 1.27 (qui, 4H, J = 7.6 Hz), 0.85 (t, 3H, J = 7.2 Hz. **^{13}C-NMR** (100 MHz, DMSO-d_6): δ 165.9, 163.8, 137.8, 133.5, 132.4, 129.0, 127.8, 45.0, 22.0, 11.2.

Animals

The male BALB/c mice of 3–5 weeks of age were purchased from NIH, Islamabad, Pakistan weighing 22–25 g initially. The mice were kept for more 2 weeks to stabilize, habituate and become more adult. After 7 weeks, all the animals were adults with age of 7 weeks and weights of 28–34 g. All the experimental animals were kept in a controlled environment (23 ± 0.05 °C with 50 ± 10% humidity in a 12-h light–dark cycle). Availability of standard diet and water was ad libitum. All animal experiments were performed as per the requirement of the bio-ethical committee protocols for Care and Use of laboratory Animal (Quaid-i-Azam University, Islamabad) under Ethical Committee code (Approval No. BEC-FBS-QAU2017-65) in compliance with the guidelines for the care and use of laboratory animals provided by the National Institute of Health (NIH publication no. 85–23, revised 1985).

Experimental design and grouping

In the carrageenan-induced acute inflammatory model, animals were divided into the following experimental groups and each group comprised of five animals:

Group I: Vehicle control group (saline with 2% DMSO i.p.)

Group II: Carrageenan-induced group [100 µl/paw, intraplantar (i.pl.)]

Group III: Dexamethasone 10 mg/kg (60 min before carrageenan administration, i.p.)

Group IV: PHI 0.1 mg/kg (60 min before carrageenan administration, i.p.)

Group V: PHI 1 mg/kg (60 min before carrageenan administration, i.p.)

Group VI: PHI 10 mg/kg (60 min before carrageenan administration, i.p.)

Group VII: THI 0.1 mg/kg (60 min before carrageenan administration, i.p.)

Group VIII: THI 1 mg/kg (60 min before carrageenan administration, i.p.)

Group IX: THI 10 mg/kg (60 min before carrageenan administration, i.p.)

In CFA-induced chronic inflammation model, the animals were divided into the following groups and each group comprised of five animals:

Group I: Vehicle control (saline with 2% (DMSO)

Group II: CFA-induced group (20 µl/paw, i.pl.)

Group III: Dexamethasone 10 mg/kg (40 min before CFA administration, i.p.)

Group IV: Piroxicam 10 mg/kg (40 min before CFA administration, i.p.)

Group V: PHI 10 mg/kg (40 min before CFA administration, i.p.)

Group VI: THI 10 mg/kg (40 min before CFA administration, i.p.)

In the carrageenan-induced study for exploring possible mechanisms of pain. In this set of experiment, the animals were divided in the following groups: Vehicle control (saline with 2% DMSO), carrageenan-induced group (100 µl/paw), PHI 10 mg/kg, THI 10 mg/kg, tramadol 50 mg/kg, gabapentin 50 mg/kg, piroxicam 10 mg/kg, flumazenil 0.2 mg/kg, naloxone 4 mg/kg either administered alone or co-administered with PHI and THI. Each group included a minimum of five animals.

Behavioral experiments

Dose selection and optimization

The carrageenan-induced acute inflammatory pain model is a commonly employed method in inflammation and pain studies [20]. Injection of carrageenan into the joint, paw or muscle causes acute inflammation that results in increased sensitivity to thermal and mechanical stimuli at the site of injury [21]. To determine the dose response of *N*-pyrazoloyl hydrazone of isatin (PHI) and *N*-thiopheneacetyl hydrazone of isatin (THI), animals were administered intraplantar (i.pl.) injection of 1% carrageenan solution in the right hind paw and readings were taken at 4 h post-carrageenan administration. Doses of 0.1, 1 and 10 mg/kg were evaluated against carrageenan (100 µl/paw). Treatments were provided 60 min prior to carrageenan injection. As described previously, mice were randomly distributed in equal number in different experimental groups to determine the dose–response. At 4 h post-carrageenan injection, paw edema, mechanical allodynia, thermal hyperalgesia, and mechanical hyperalgesia were measured and the dose with the significant activity was chosen for further experiments [22].

Evaluation of paw edema

The inhibitory effects of PHI and THI pretreatment on inflammatory paw edema were evaluated by measuring paw thickness using a dial thickness gauge (No. 2046F, Mitutoyo, Kawasaki, Japan). Inflammatory paw edema was induced by i.pl. injection of carrageenan (100 µl/paw) or CFA (20 µl/paw, 1 mg/mL of heat killed and dried Mycobacterium tuberculosis, each ml of vehicle contained 0.85 ml paraffin oil plus 0.15 ml mannide monooleate) into the right hind paw of mice as mentioned in previous studies [23]. Paw edema was measured prior to induction of edema and later every 2 h after induction up to 6 h at day 1 for acute effects and on subsequent days at 4 h after the daily treatment until day 6. No treatment was given on day 5 to check any tolerance effects as described previously [23].

Evaluation of mechanical allodynia

To evaluate the effects of PHI and THI on mechanical allodynia, the Von Frey test was performed as previously reported methodology [21]. Threshold to mechanical stimulation was measured after the inflammation of the hind paw was induced with CFA (20 µl/paw). The reflex paw withdrawal was considered as positive pain response. The mechanical allodynia was evaluated before induction and at 2, 4, and 6 h at day 1 after CFA injection. For the remaining days, the evaluation of mechanical withdrawal threshold was done after 4 h of daily treatment. There was an interval in treatment on day 5 to investigate possible tolerance effects [23].

Evaluation of mechanical hyperalgesia

To evaluate the mechanical hyperalgesia, Randall Selitto test (Digital Paw Pressure Randall Selitto Meter, IITC Life Science Inc Woodland Hills, CA, USA) was performed as previously reported methods [24]. The hind paw removal followed by obvious jerk movements was characterized as the nociceptive response and the pressure intensity was automatically recorded. To evaluate the effect of PHI and THI, mice were administered an i.pl. injection of CFA (20 µl/paw) in the right hind paw. The test was performed before and after hind paw inflammation was induced with CFA at different time intervals. An average of three measurements was recorded as the final value for each interval. In the CFA-induced chronic inflammation model, mechanical hyperalgesia was evaluated before CFA injection and at 2, 4, 6 h for day 1 and later at 4 h

of daily treatment for 6 days with the interruption on day 5 to assess any tolerance effects as mentioned earlier [23].

Evaluation of thermal hyperalgesia

To determine thermal hyperalgesia, the hot plate test was used with minor modifications [21]. The animals were placed on the hot plate set at a temperature of 55 °C. The first CFA administered ipsilateral hind paw flexion reflex (nociceptive behavior) along with paw licking and flinching were characterized as the end point [25]. The latency time (s) of paw withdrawal was measured until 60 s. In the CFA-induced inflammatory model, thermal hyperalgesia was evaluated before induction and at 2, 4, 6 h at day 1 post-CFA injection for acute effects and later at 4 h post-daily treatment continuing until day 6 with an interval of day 5 for assessing any tolerance effects as previously described [26]. To ensure the minimal heat damage, animals were exposed to the heat surface for 35 s only [26].

Assessment of possible mechanism of action

A study was conducted to determine the possible mechanism of action of PHI and THI (10 mg/kg) attributed to their antinociceptive effect according to a method described previously [26] with some minor modifications. The carrageenan-induced mice (100 µl/paw) were treated with different classes of antagonists and agonists which were administered both alone and along with PHI (10 mg/kg, i.p.) and THI (10 mg/kg, i.p.) to investigate the possible role of various receptors. Pretreatment was done with cyclooxygenase inhibitor piroxicam (10 mg/kg i.p.) or an analog of g-aminobutyric acid (GABA), gabapentin (50 mg/kg i.p.) or the non-selective opioid receptor agonist, tramadol (50 mg/kg, i.p.) individually, prior to the treatment with PHI (10 mg/kg i.p.) and THI (10 mg/kg, i.p.) alone or in combination. In order to find any involvement of opioid receptors, a group of mice was administered naloxone (2.5 mmol/kg i.p.) which is a non-selective opioid receptor antagonist. Mice were treated with the benzodiazepine receptor antagonist, flumazenil (0.2 mg/kg i.p.) to evaluate the possible role of GABA in pain modulation. Pretreatment was provided with antagonists 15 min prior to treatment with PHI (10 mg/kg i.p.) and THI (10 mg/kg i.p.). Nociception was evaluated after 4 h. The tests were performed (von Frey, Randall Selitto) 4 h after providing treatments [21]. Blood samples were collected by cardiac puncture to measure blood serum nitrite production.

Biochemical assay

Nitric oxide determination

The inhibitory effect of PHI 10 mg/kg and THI 10 mg/kg on nitric oxide (NO) production in CFA-induced blood plasma was determined by performing the Griess reagent test as described previously [27]. The assay was also performed on all groups of animals involved in the mechanistic study.

Western blot analysis

Western blot analysis was performed as described [28]. The paw skin tissue was removed at the end of the experiment and analysis was performed for detection of IκBα, p-p38, p38, p-JNK, JNK, ERK, and p-ERK. The antibodies were detected with the WEST-SAVE Up™ luminol-based ECL reagent (LabFrontier, Seoul, Korea). The target bands were quantified using UN-SCAN-IT™ software Version 6.1 (Silk Scientific Co., Orem, UT).

NF-κB ELISA assay

NF-κB was analyzed using a commercially available ELISA kit (eBioscience, USA). Briefly, the nuclear protein was extracted according to previously described methods [28] and the assay was performed.

TNF- α, IL-1β and IL-6 productions by ELISA kits

The concentrations of IL-1β, IL-6 and TNF- α were determined using commercially available ELISA kits (eBioscience, USA) in tissue samples obtained from all the experimental groups in the chronic study. To prepare the sample, the skin tissue of the paw was removed and 100 mg tissue/ml concentration of phosphate buffer saline (PBS) was used for the extraction of tissue protein, to which 0.05% Tween 20, 0.4 M NaCl and protease inhibitors were added. Samples were centrifuged for 10 min at 3000 rpm as described [23].

Estimation of GSH, GST and catalase

The effect of PHI and THI (10 mg/kg) was determined against oxidative stress by measuring the levels of reduced glutathione (GSH) and activities of glutathione-*S*-transferase (GST) and catalase in paw tissues.

The reduced glutathione level was assayed using Ellman's method [29] and the absorbance was recorded by a UV–visible spectrophotometer at 412 nm [28].

GST activity was determined according to the method described by Jackobson et al. [30], with CDNB as the substrate. CDNB (30 mM) was added to start the reaction and the change in the absorbance was measured by spectrophotometer at 344 nm.

The catalase activity was estimated according to the method established by Aebi [31]. The addition of 100 µl of enzyme started the reaction and the variation in absorbance was noted in triplicate at 240 nm.

Radiological and histological analysis

Radiological analysis of paw tissues was performed to measure the effect of daily pretreatment of PHI (10 mg/kg) and THI (10 mg/kg) on mice inflamed paws. Mice were killed at the end of experiments using chloroform and right hind paws were removed and radiographed [32].

For histopathological evaluation, paws tissues sections of 4 µm thickness were prepared which were stained with hematoxylin and eosin dye and subjected to double blind histological assessment as described [23]. Images were further analyzed by determining the neutrophil infiltration in inflamed paws. The quantification of stained tissue sections was done using image J version 1.46r software, Wayne Rasband, NIH, USA.

Vascular permeability assay

To evaluate the effect of PHI (10 mg/kg) and THI (10 mg/kg) on vascular permeability, the Evans blue dye assay was performed with slight modifications [33]. The evaluation of the vascular permeability effects was done by quantifying the extravasations of dye into the peritoneal cavity [33].

Analysis of liver and kidney function

Toxic effects of PHI and THI chronic treatment on liver and kidney function tests were determined by measuring levels of alanine amino transferase (ALT), aspartate amino transferase (AST), and creatinine. All animals received a single dose of PHI (10 mg/kg), THI (10 mg/kg), dexamethasone (10 mg/kg), piroxicam (10 mg/kg) and vehicle control (normal saline with 2% DMSO) daily. At day 6, blood samples were obtained by cardiac puncture. To obtain the serum, centrifugation of total blood was performed for 10 min at 3000 rpm. These separated serum samples were used for biochemical analyses [34].

Motor performance

Motor coordination and muscle strength test

In order to evaluate the effect of PHI (10 mg/kg) and THI (10 mg/kg) acute and chronic treatment on muscle strength and motor function, Kodzeila's screen test was performed at 0 h, 6 h, and 6th day post-CFA administration as per already described method [26].

For complete assessment of motor deficit weight lifting, a test was performed according to previously reported protocols [26].

Gastric toxicity

To evaluate the effect of PHI and THI treatment on gastric toxicity following oral gavage (p/o) administration of compounds was performed according to previously described method [35]. The animals were divided in 4 groups and each group comprised of minimum 5 mice (vehicle control, diclofenac sodium 10 mg/kg, PHI 10 mg/kg, THI 10 mg/kg). Oral administration was done for 3 days. After 3 days, animals from all groups were killed [35]. The histological analysis and scoring was performed with double blind assessment [32].

Urinary and serum electrolyte measurement

To investigate the effect of PHI and THI on heart, Na^+ and K^+ excretion rate studies were performed. Animals were distributed equally into four groups (vehicle control, diclofenac sodium 10 mg/kg, PHI 10 mg/kg and THI 10 mg/kg). Each group comprised of five animals. Animals were administered drugs for 3 days. On the third day, animals were kept in separate metabolic cages and urine was collected during the last 24-h period. Urine was stored at − 20 °C. At the end of study, blood samples were collected through cardiac puncture. They were then centrifuged to separate the serum which was stored at − 80 °C [36]. Na^+ and K^+ levels were then determined in serum and urine sample.

Hematological analysis

Blood samples were collected for laboratory tests from the retro-orbital plexus to evaluate hematological parameters. The hematological parameters included neutrophils count, lymphocyte count, MID index (MID#), red blood cell count (RBC), and white blood cell count (WBC). [37]

Statistical analysis

All the results were expressed as mean ± standard deviation (S.D) unless otherwise stated. One way analysis of variance (ANOVA) was applied to the data followed by Dunnett's *t* test to determine the statistical significance of the differences between the various study groups (SPSS Version 20.0R). A value of $p < 0.05$ was chosen as the criterion for statistical significance.

Results

Dose selection and optimization

The most effective dose of PHI and THI was determined using the carrageenan-induced acute inflammatory model.

Different doses of 0.1, 1 and 10 mg/kg of both PHI and THI were evaluated by this study. The dose of 0.1 and 1 mg/kg did not produce any prominent reduction in inflammatory and pain behaviors (Figs. 2, 3). However, the dose of 10 mg/kg of PHI and 10 mg/kg of THI resulted in a significant decline in paw edema. Moreover, the results showed remarkable anti-allodynia and anti-hyperalgesic effects in carrageenan-induced inflammation and pain (Figs. 2, 3). Thus, the dose of PHI 10 mg/kg and THI 10 mg/kg was selected for further studies.

Effect of PHI and THI on paw edema

The inhibitory potential of PHI (10 mg/kg, i.p.) and THI (10 mg/kg, i.p.) was evaluated by measuring paw edema. PHI and THI inhibited paw edema induced by CFA in both acute and chronic paw inflammation models (Table 1, 2). Administration of PHI and THI 40 min prior to intraplantar injection of CFA caused significant reduction in the paw thickness at 2, 4, and 6 h after acute edema induced by CFA (Table 1). Similarly PHI and THI (10 mg/kg) treatment for 6 days showed significant inhibition of paw edema as compared to the CFA group. Treatment with dexamethasone (10 mg/kg, i.p.) and piroxicam (10 mg/kg, i.p.) also reduced paw edema significantly. Day 5 showed no considerable effect as treatment was not provided (Table 2).

Effect of PHI and THI on mechanical allodynia

PHI and THI pretreatment significantly increased pain thresholds in mechanical allodynia test at 2, 4 and 6 h post CFA injection, showing notable activity at 4 h of PHI and THI in acute cases (Table 1). Dexamethasone and piroxicam also significantly increased the pain threshold in comparison to negative controls. PHI and THI (10 mg/kg, i.p.) daily treatment effectively increased pain thresholds throughout the treatment period compared with negative controls (Table 2) indicating the effectiveness of PHI and THI in

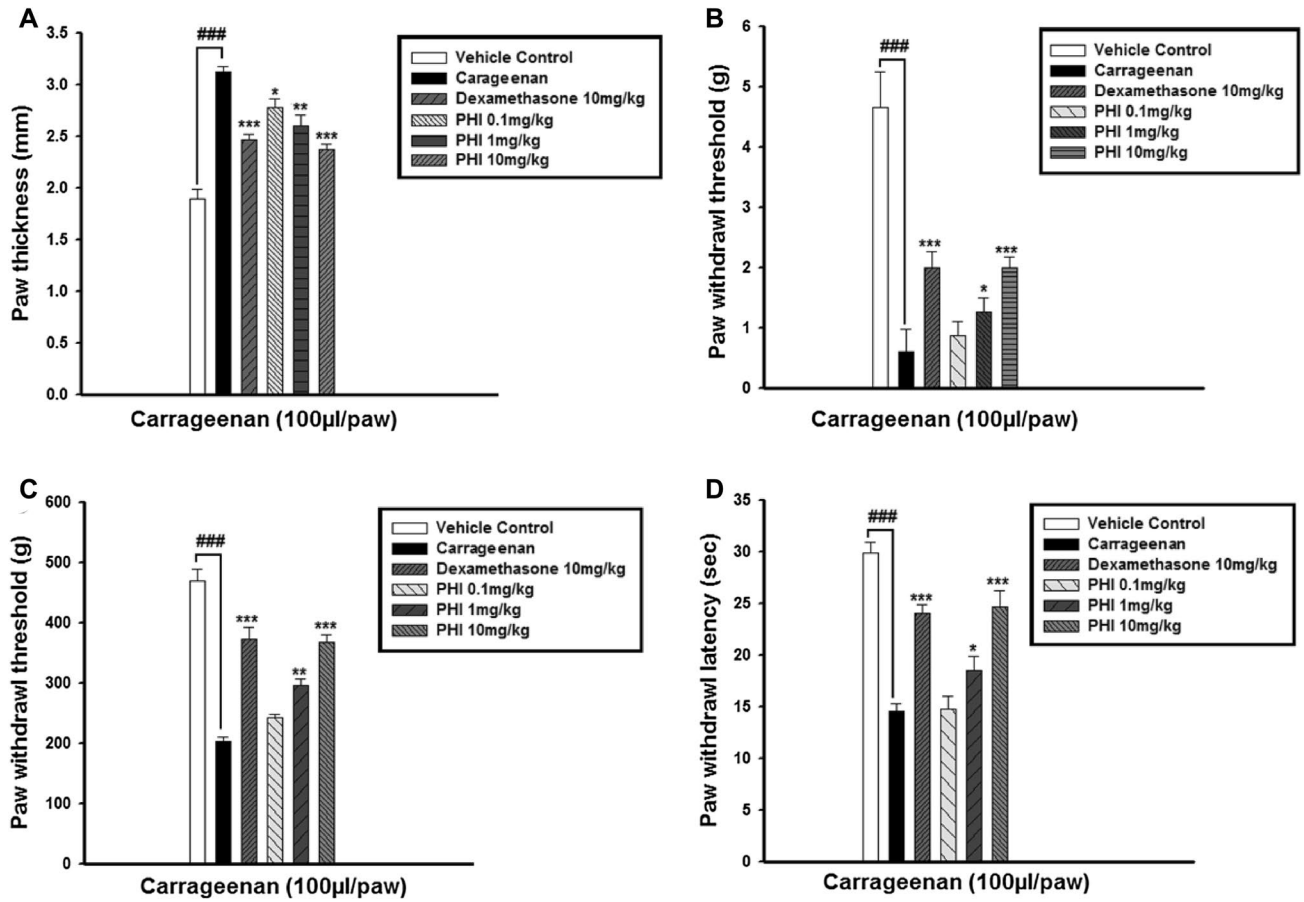


Fig. 2 The dose response of PHI at different doses of 0.1, 1 and 10 mg/kg i.p in carrageenan (100 µl/paw) induced acute inflammatory pain model in mice ($n=5$). To determine the effect of pretreatment on carrageenan induced paw edema, following effects were measured post 4 h carrageenan injection, **a** reduction in paw thickness **b** inhibition of mechanical allodynia **c** inhibition of mechanical hyperalgesia and **d** inhibition of thermal hyperalgesia. The data is given as mean ± SD (*) $p<0.05$, (**) $p<0.01$ and (***) $p<0.001$ and $^{###}$indicates significant differences from the carrageenan-treated group

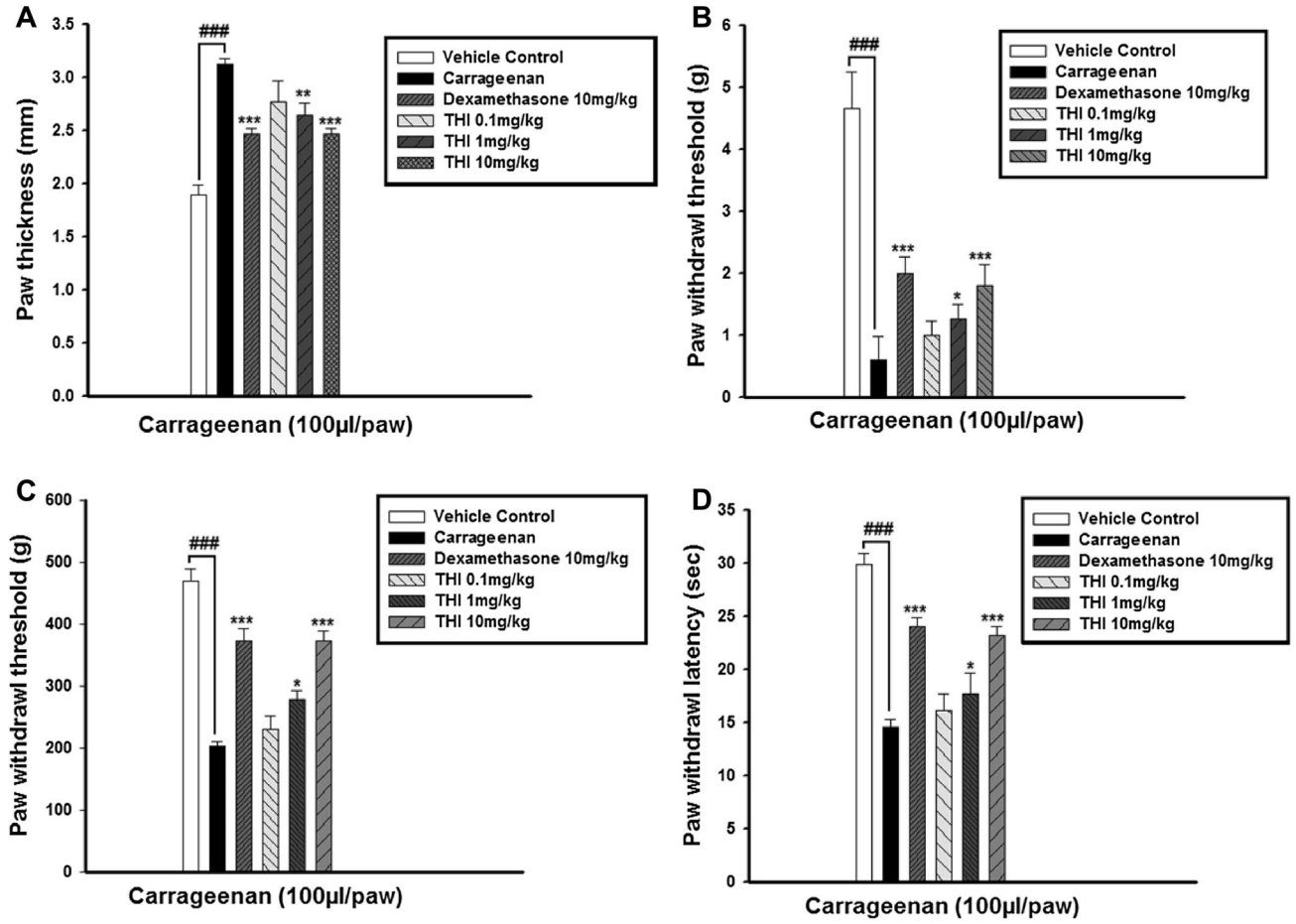


Fig. 3 The dose response of THI at different doses of 0.1, 1 and 10 mg/kg i.p in carrageenan (100 µl/paw) induced acute inflammatory pain model in mice ($n=5$). To determine the effect of pretreatment on carrageenan induced paw edema, following effects were measured post 4 h carrageenan injection, **a** reduction in paw thickness **b** inhibition of mechanical allodynia **c** inhibition of mechanical hyperalgesia and **d** inhibition of thermal hyperalgesia. The data are given as mean ± SD (*) $p<0.05$, (**) $p<0.01$ and (***) $p<0.001$ and $^{###}$indicates significant differences from the carrageenan-treated group

a chronic inflammatory pain model. There was no tolerance effect of PHI and THI on mechanical allodynia as paw withdrawal response was considerably decreased when treatment was interrupted on day 5 which returned when the drug was administered on day 6 (Table 2). Treatment with dexamethasone and piroxicam for 6 days also increased the pain threshold in the positive control group as compared to negative control.

Effect of PHI and THI on mechanical hyperalgesia

The pain threshold was remarkably increased in PHI and THI treated groups in mechanical hyperalgesia test (Table 1) at 2, 4 and 6 h after CFA injection, showing significant activity at 4 h of PHI and THI in acute cases. Dexamethasone and piroxicam treatment showed remarkable anti-hyperalgesic activity. The long-term effects of PHI and THI on mechanical hyperalgesia were evaluated by treating animals for 6 days, skipping day 5 after CFA injection. PHI and THI (10 mg/kg,i.p.) daily treatment increased pain thresholds notably as compared to negative controls (Table 2), indicating the effectiveness of PHI and THI in a chronic inflammatory pain model. PHI and THI showed no tolerance effect on mechanical hyperalgesia as inhibitory hyperalgesic effects were halted when treatment was interrupted on day 5, then restored when the drug was administered on day 6 (Table 2). Six days of treatment with dexamethasone and piroxicam also increased the pain thresholds in the positive control group as compared to the negative controls.

Effect of PHI and THI on thermal hyperalgesia

PHI and THI treatment significantly inhibited acute thermal hyperalgesia (Table 1) at 2, 4 and 6 h post induction with CFA, suggesting promising activity of PHI and THI in the case of acute thermal hyperalgesia. Dexamethasone

Table 1 Effect of acute pretreatment of PHI (10 mg/kg) and THI (10 mg/kg) on CFA-induced inflammation and pain parameters ($n=5$)

Time after CFA injection				
	0 h	2 h	4 h	6 h
Paw Edema				
Vehicle control	2.04 ± 0.06	2.06 ± 0.05	2.09 ± 0.02	2.10 ± 0.01
CFA	2.11 ± 0.08	2.59 ± 0.06###	2.63 ± 0.05###	2.81 ± 0.06###
Dexa 10 mg/kg	2.17 ± 0.09	2.29 ± 0.06***	2.31 ± 0.02***	2.39 ± 0.02***
Pirox 10 mg/kg	2.06 ± 0.04	2.24 ± 0.10***	2.28 ± 0.07***	2.35 ± 0.05***
PHI 10 mg/kg	2.01 ± 0.07	2.21 ± 0.06***	2.26 ± 0.05***	2.31 ± 0.03***
THI 10 mg/kg	2.05 ± 0.06	2.16 ± 0.03***	2.22 ± 0.06***	2.28 ± 0.07***
Mechanical allodynia				
Vehicle control	4.84 ± 0.27	4.50 ± 0.12	4.60 ± 0.15	4.60 ± 0.10
CFA	4.40 ± 0.15	1.13 ± 0.32###	0.77 ± 0.21###	0.80 ± 0.21###
Dexa 10 mg/kg	4.70 ± 1.09	2.00 ± 0.04***	1.70 ± 0.31***	1.90 ± 0.01***
Pirox 10 mg/kg	4.50 ± 1.01	2.00 ± 0.60***	1.60 ± 0.11***	1.73 ± 0.23***
PHI 10 mg/kg	4.80 ± 1.09	1.85 ± 0.30**	1.60 ± 0.31***	1.60 ± 0.34***
THI 10 mg/kg	4.40 ± 0.61	1.80 ± 0.34**	1.44 ± 0.36***	1.40 ± 0.09***
Mechanical hyperalgesia				
Vehicle control	441.75 ± 17.63	424.75 ± 09.04	423.25 ± 08.99	424.25 ± 07.22
CFA	415.20 ± 24.52	217.02 ± 15.65###	169.00 ± 18.38###	167.75 ± 15.71###
Dexa 10 mg/kg	399.60 ± 17.21	345.00 ± 17.32***	335.05 ± 14.74***	355.25 ± 10.62***
Pirox 10 mg/kg	402.00 ± 13.03	337.33 ± 15.89***	327.50 ± 12.58***	319.33 ± 11.37***
PHI 10 mg/kg	407.75 ± 13.17	309.40 ± 08.79***	317.75 ± 17.93***	344.25 ± 11.94***
THI 10 mg/kg	408.40 ± 17.74	345.80 ± 15.80***	311.40 ± 10.45***	316.75 ± 13.32***
Thermal hyperalgesia (hot)				
Vehicle control	30.40 ± 1.14	32.63 ± 1.67	29.89 ± 1.30	31.50 ± 1.00
CFA	28.67 ± 1.03	16.50 ± 0.57###	19.67 ± 0.56###	15.00 ± 1.15###
Dexa 10 mg/kg	29.82 ± 2.36	19.50 ± 0.58***	24.80 ± 0.83***	27.40 ± 1.67***
Pirox 10 mg/kg	30.40 ± 0.80	19.00 ± 0.50**	22.80 ± 0.95***	28.00 ± 1.63***
PHI 10 mg/kg	30.32 ± 0.76	18.76 ± 1.15**	21.80 ± 0.44**	26.00 ± 1.14***
THI 10 mg/kg	30.80 ± 1.29	20.02 ± 1.01***	23.50 ± 1.29***	27.00 ± 1.73***

Readings were measured every 2 h post CFA administration from 0 to 6 h

All values are expressed as mean ± SEM ($n=5$). The data are given as mean ± SD *$p<0.05$, **$p<0.01$ and ***$p<0.001$ and ###indicates significant differences from the CFA-treated group

and piroxicam also increased the pain threshold significantly as compared to the negative control group. For long-term effects of PHI and THI on thermal hyperalgesia, animals were treated daily for 6 days after CFA injection, skipping day 5 for evaluation of tolerance effect of drug on thermal hyperlagesia. PHI and THI (10 mg/kg, i.p.) daily treatment remarkably increased the threshold of pain through the duration of treatment in contrast to negative control (Table 2) indicating the effectiveness of PHI and THI in a chronic inflammatory pain model.

Effect of THI and PHI on NF-κB and MAPK signaling proteins

The molecular anti-inflammatory and anti-hyperalgesic mechanism of THI and PHI was explored by western blot analysis. The results showed (Fig. 4) that THI and PHI remarkably protected the degradation of IkBa in contrast to the CFA-induced animals (Fig. 4a). Supporting these findings, we also found less translocation of NF-κB into the nucleus in animals treated with both the compounds (Fig. 4b), whereas NF-κB translocation was significantly high in CFA-induced animals. Furthermore, the MAPK protein was also suppressed by THI and PHI as compared with CFA treated animals (Fig. 4c). Target bands were quantified as shown in Fig. 4d. Dexamethasone exhibited remarkable reduction in activated NF-κB and MAPK signaling.

Effect of PHI and THI on CFA-induced pro-inflammatory cytokines production

The cytokines productions levels were significantly increased at day 6 after CFA induction. The results showed that compared with vehicle control animals, CFA intra

Table 2 Effect of chronic pretreatment of PHI (10 mg/kg) and THI (10 mg/kg) on CFA-induced inflammation and pain parameters ($n=5$)

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Paw edema						
Vehicle control	2.09 ± 0.06	2.10 ± 0.03	2.11 ± 0.02	2.12 ± 0.03	2.11 ± 0.03	2.11 ± 0.04
CFA	3.18 ± 0.14###	2.95 ± 0.12###	2.98 ± 0.06###	2.99 ± 0.09###	3.03 ± 0.05###	3.13 ± 0.08###
Dexa 10 mg/kg	2.41 ± 0.12***	2.29 ± 0.06***	2.35 ± 0.11***	2.30 ± 0.12***	2.38 ± 0.09***	2.37 ± 0.11***
Pirox 10 mg/kg	2.30 ± 0.11***	2.40 ± 0.10***	2.37 ± 0.13***	2.29 ± 0.15***	2.34 ± 0.13***	2.33 ± 0.08***
PHI 10 mg/kg	2.34 ± 0.07***	2.43 ± 0.06***	2.44 ± 0.05***	2.34 ± 0.03***	2.41 ± 0.08***	2.41 ± 0.10***
THI 10 mg/kg	2.30 ± 0.11***	2.33 ± 0.13***	2.29 ± 0.14***	2.24 ± 0.10***	2.29 ± 0.16***	2.31 ± 0.15***
Mechanical allodynia						
Vehicle control	4.88 ± 0.27	4.82 ± 0.42	4.92 ± 0.70	4.95 ± 0.65	5.06 ± 0.59	5.07 ± 0.63
CFA	0.51 ± 0.10###	0.45 ± 0.09###	0.42 ± 0.07###	0.36 ± 0.09###	0.39 ± 0.12###	0.54 ± 0.20###
Dexa 10 mg/kg	2.23 ± 0.49***	2.68 ± 1.12***	2.80 ± 1.09***	2.90 ± 0.85***	2.00 ± 0.10***	2.20 ± 0.89***
Pirox 10 mg/kg	2.10 ± 0.10***	2.46 ± 1.10***	2.66 ± 1.15***	2.76 ± 0.43***	1.85 ± 0.30***	2.10 ± 1.13***
PHI 10 mg/kg	1.85 ± 0.30***	2.08 ± 0.57***	2.25 ± 0.51***	2.40 ± 0.54***	1.80 ± 0.44***	2.40 ± 1.02***
THI 10 mg/kg	1.68 ± 0.61***	1.80 ± 0.34***	2.40 ± 0.66***	2.48 ± 1.02***	1.52 ± 0.26***	1.80 ± 0.34***
Mechanical hyperalgesia						
Vehicle control	436.25 ± 27.67	438.45 ± 27.62	461.61 ± 37.53	464.25 ± 17.22	471.81 ± 11.98	476.10 ± 21.10
CFA	143.80 ± 22.17###	148.11 ± 21.65###	135.40 ± 27.11###	168.00 ± 14.67###	171.10 ± 21.92###	181.20 ± 11.11###
Dexa 10 mg/kg	380.25 ± 25.21***	352.40 ± 47.32***	367.20 ± 31.87***	393.20 ± 19.61***	356.10 ± 32.47***	375.40 ± 31.35***
Pirox 10 mg/kg	370.50 ± 37.23***	346.53 ± 15.80***	348.75 ± 32.51***	380.73 ± 31.37***	322.67 ± 37.21***	328.25 ± 41.11***
PHI 10 mg/kg	364.65 ± 31.71***	325.00 ± 51.39***	350.75 ± 30.15***	364.20 ± 21.94***	341.01 ± 31.75***	391.25 ± 37.12***
THI 10 mg/kg	361.30 ± 34.46***	337.40 ± 25.51***	341.70 ± 33.42***	346.76 ± 23.61***	324.67 ± 42.39***	344.01 ± 27.79***
Thermal hyperalgesia (hot)						
Vehicle control	31.05 ± 0.81	31.63 ± 1.64	32.40 ± 0.54	32.50 ± 1.29	32.25 ± 0.96	33.40 ± 1.34
CFA	16.75 ± 1.25###	16.67 ± 0.57###	17.06 ± 1.01###	15.25 ± 1.15###	17.50 ± 1.20###	20.10 ± 1.02###
Dexa 10 mg/kg	29.00 ± 1.73***	30.50 ± 0.58***	29.60 ± 1.41***	30.40 ± 1.51***	30.20 ± 0.95***	31.00 ± 0.95***
Pirox 10 mg/kg	26.66 ± 0.57***	28.96 ± 1.15***	27.66 ± 0.57***	29.00 ± 1.01***	26.33 ± 0.57***	29.02 ± 1.25***
PHI 10 mg/kg	27.50 ± 0.76***	29.33 ± 1.15***	28.50 ± 1.41***	29.10 ± 1.08***	29.05 ± 1.22***	30.40 ± 1.00***
THI 10 mg/kg	26.90 ± 0.57***	29.66 ± 0.57***	29.50 ± 1.29***	30.10 ± 1.53***	30.08 ± 1.15***	31.88 ± 0.57***

Readings were measured from 0 to 6 days post-CFA injection with no treatment on day 5

All values are expressed as mean ± SEM ($n=5$). The data are given as mean ± S.D *$p<0.05$, **$p<0.01$ and ***$p<0.001$ and ###indicates significant differences from the CFA-treated group

plantar injection caused significant increases in the levels of cytokines in the negative control group, whereas PHI and THI pretreatment significantly reduced the level of TNF- α (Fig. 5a), IL-1β (Fig. 5b), IL-6 (Fig. 5c) just like in dexamethasone and piroxicam treated groups.

Elucidation of possible mechanism of action

We performed a study to assess the possible mechanism of action. According to the results (Figs. 6, 7) the anti-nociceptive effects in carrageenan-induced inflammatory mice model were significantly reversed with pretreatment with the antagonists naloxone or flumazenil prior to PHI and THI treatment. Pretreatment of animals with tramadol, gabapentin and piroxicam potentiated the effects of PHI and THI (Figs. 6, 7). The behavior results were confirmed by NO data from the same animals which was also in concordance with the behavioral ones.

Effect of PHI and THI on the production of NO in plasma after CFA induction

There was a marked increase in NO levels in CFA-treated mice (Fig. 8a). However, the NO production was significantly reduced after 6 days of treatment with PHI and THI (10 mg/kg). Similarly, dexamethasone and piroxicam treated groups also showed significant reduction in levels of NO in plasma (Fig. 8a).

Effect of PHI and THI on antioxidant enzymes level

CFA reduced the skin levels of endogenous GSH (Fig. 8b) and activities of GST (Fig. 8c) and catalase (Fig. 8d).

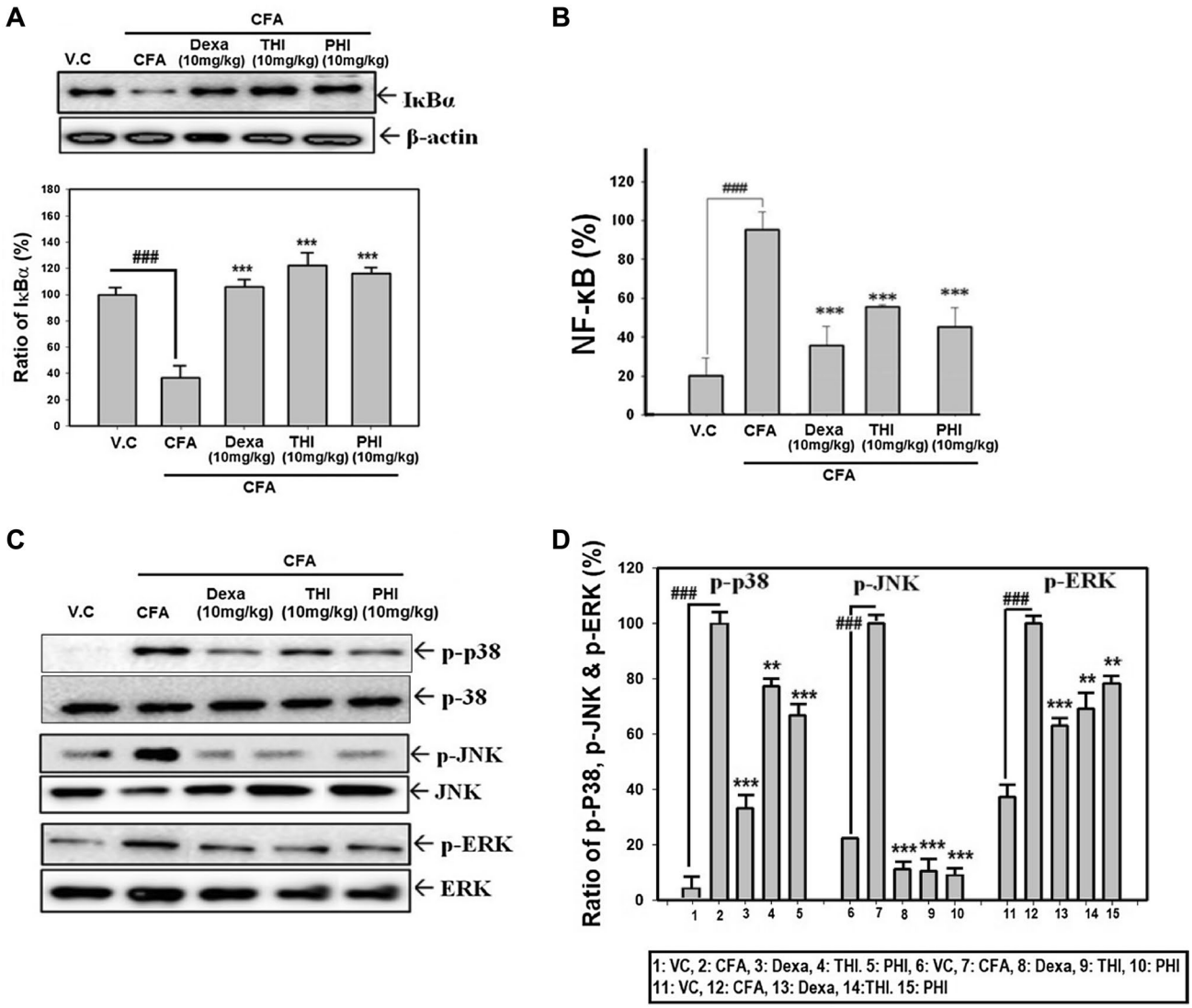


Fig. 4 Effect of PHI and THI on NF-κB and MAPK signaling proteins **a** IκBα **b** NF-κB and **c** p-38, JNK and ERK protein levels in CFA induced paw tissues determined by western blot analysis and ELISA **d** Quantification of target bands. The results are shown as percentage production. The data are given as mean ± SD (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$ and ###indicates significant differences from the CFA-treated group

Treatment with PHI and THI and the standard drugs dexamethasone and piroxicam prevented these reductions of antioxidant defenses in the inflammatory foci (Fig. 8b–d).

Radiological and immune-histological analysis

The radiographic examination of soft tissue showed marked reduction in swelling of right hind paws in PHI and THI treated groups comparable to dexamethasone and piroxicam treated groups (Fig. 9a). There was predominant soft tissue swelling in the CFA treated group. The changes were quantified as shown in Fig. 9c.

Histopathological analysis of right hind paws showed significant infiltration of immune cells and synovial hyperplasia in CFA treated group while PHI and THI treated groups showed reduction of immune cell infiltration and synovial hyperplasia similar to dexamethasone and piroxicam treated groups (Fig. 9b). The histological images were quantified on the basis of neutrophil infiltration at the site of inflammation (Fig. 9d).

PHI and THI suppress acetic acid-induced vascular permeability

As shown in Fig. 9e, acetic acid caused an increase in the permeation of Evans blue from the vasculature to abdominal cavity, while pretreatment with PHI and THI caused significant reductions as compared to the

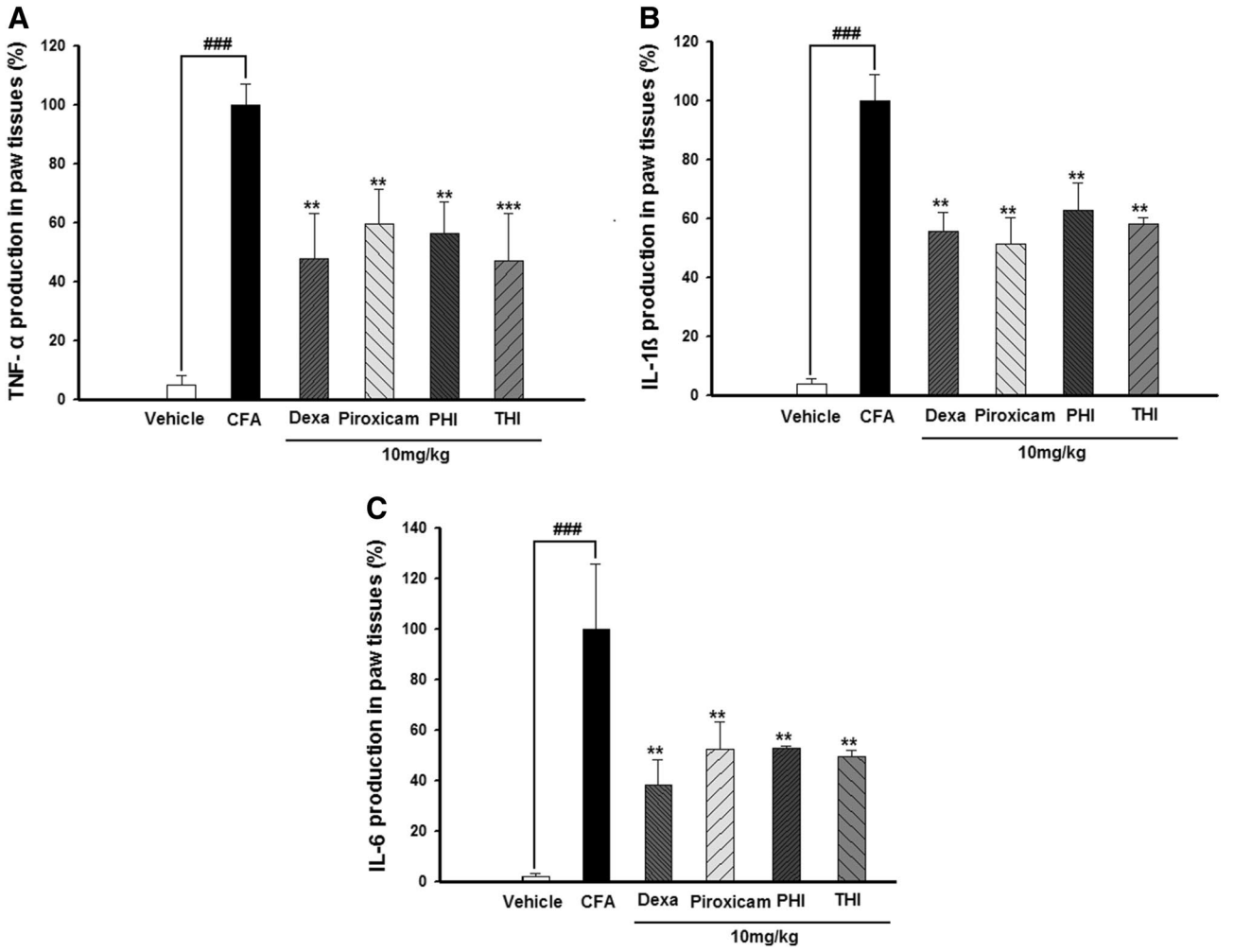


Fig. 5 Effect of PHI and THI on the production levels of inflammatory cytokines **a** TNF-α, **b** IL-1β and **c** IL-6 in CFA treated paw tissues measured by ELISA. The results are shown as percentage production. The data are given as mean ± SD (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$ and ###indicates significant differences from the CFA-treated group

acetic acid-treated group. The inhibition of dye leakage was comparable in treated groups with dexamethasone (10 mg/kg) suggesting that PHI and THI can inhibit vascular permeability and reduce inflammatory effusion (Fig. 9e).

Effect of PHI and THI on hematology profile

The hematological changes related to the chronic inflammatory condition are presented in Table 3. The changes in levels of blood cells were observed and there was increase in platelet count, WBC and MID index in inflamed mice. These values were near to normal levels in PHI and THI (10 mg/kg) treated animals.

Effect of PHI and THI on urinary and serum electrolytes (Na^+ and K^+) excretion rate

Treatment with PHI and THI did not alter serum and urinary Na^+ and K^+ level when compared with vehicle control-treated group (Table 4).

Effect of PHI and THI on hepatic and renal functions

To assess whether PHI and THI induce any liver or kidney damage, plasma levels of AST, ALT and creatinine were measured. The treatment with dexamethasone, piroxicam, PHI and THI (10 mg/kg) did not alter the hepatic and renal function (Table S1), thus establishing its safety profile.

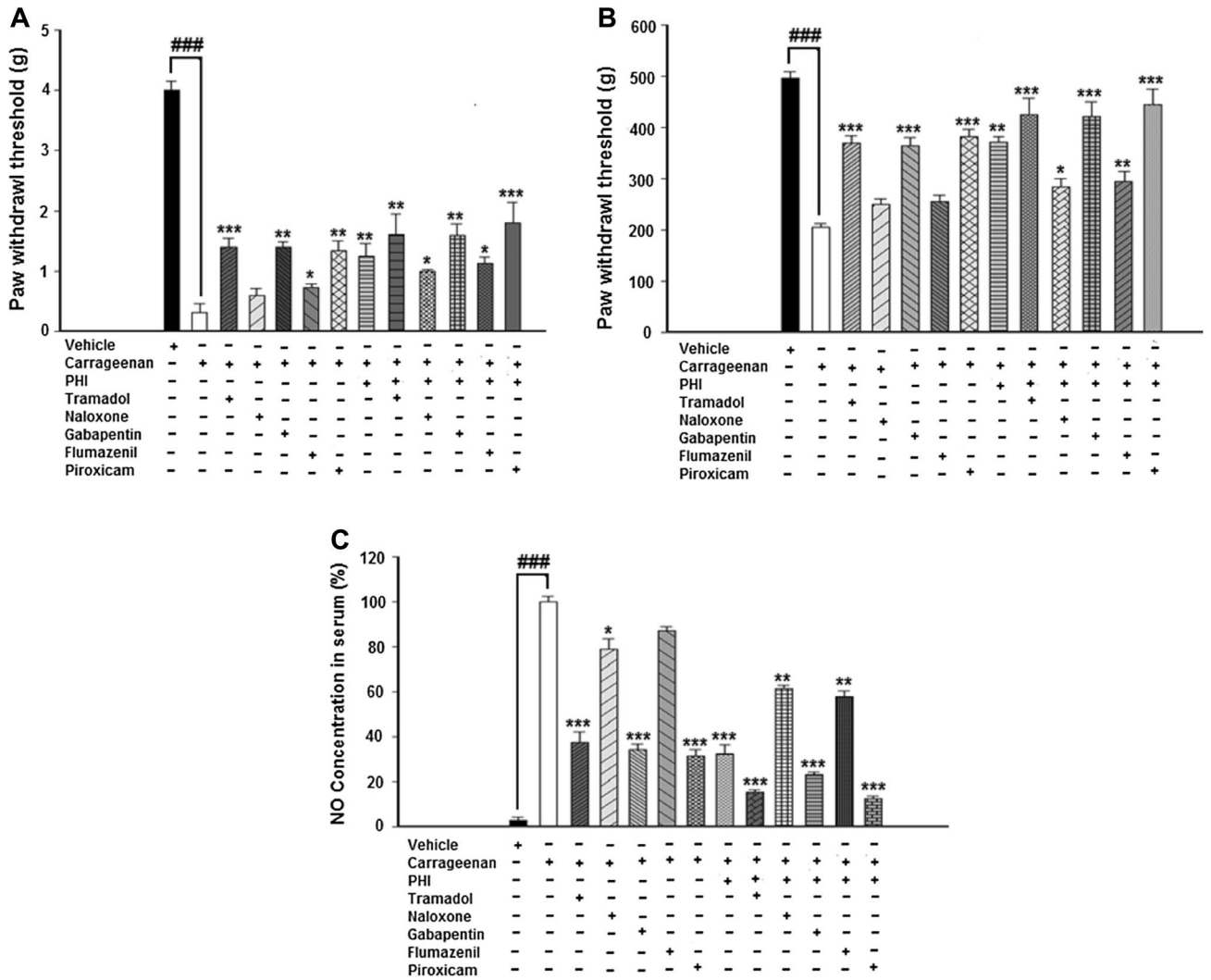


Fig. 6 Effect of pretreatment with agonists tramadol (50 mg/kg i.p.), piroxicam (10 mg/kg, i.p.) and gabapentin (50 mg/kg, i.p.) and antagonists flumazenil (0.2 mg/kg) and naloxone (4 mg/kg) on the anti-nociceptive effects of PHI (10 mg/kg, i.p.) against carrageenan (100 µl/paw) induced acute inflammatory pain model ($n=5$). **a** effect on mechanical allodynia induced by carrageenan **b** effect on mechanical hyperalgesia induced by carrageenan **c** serum concentration of nitrite (NO) production with PHI (10 mg/kg, i.p.) in the carrageenan (100 µl/paw) induced acute inflammatory pain model. The data are given as mean ± SD (*) $p<0.05$, (**) $p<0.01$ and (***) $p<0.001$ and ###indicates significant differences from the carrageenan-treated group

Effect of PHI and THI on the motor activity of mice

Weights test and Kodzeila's screen tests showed that PHI and THI chronic administration did not have any effect on the motor function of the mice (Supplementary file, Fig. S1). The muscle strength and coordination was not effected by acute and long-term administration of PHI and THI (10 mg/kg).

Effect of PHI and THI on gastric mucosa

Microscopic examination (40×) of H&E stained stomach tissues showed intact mucosal lining with no edema, erosion, inflammatory cell infiltrate or necrosis in mice (Supplementary file, Fig. S2) that received a daily oral dose of PHI (10 mg/kg) and THI (10 mg/kg) for 3 days, comparable with normal GIT mucosa. However, the diclofenac sodium (10 mg/kg) treatment exhibited significant toxic effects such as necrosis, immune cell infiltration and hemorrhagic lesions on GIT mucosa as evident from figure S2.

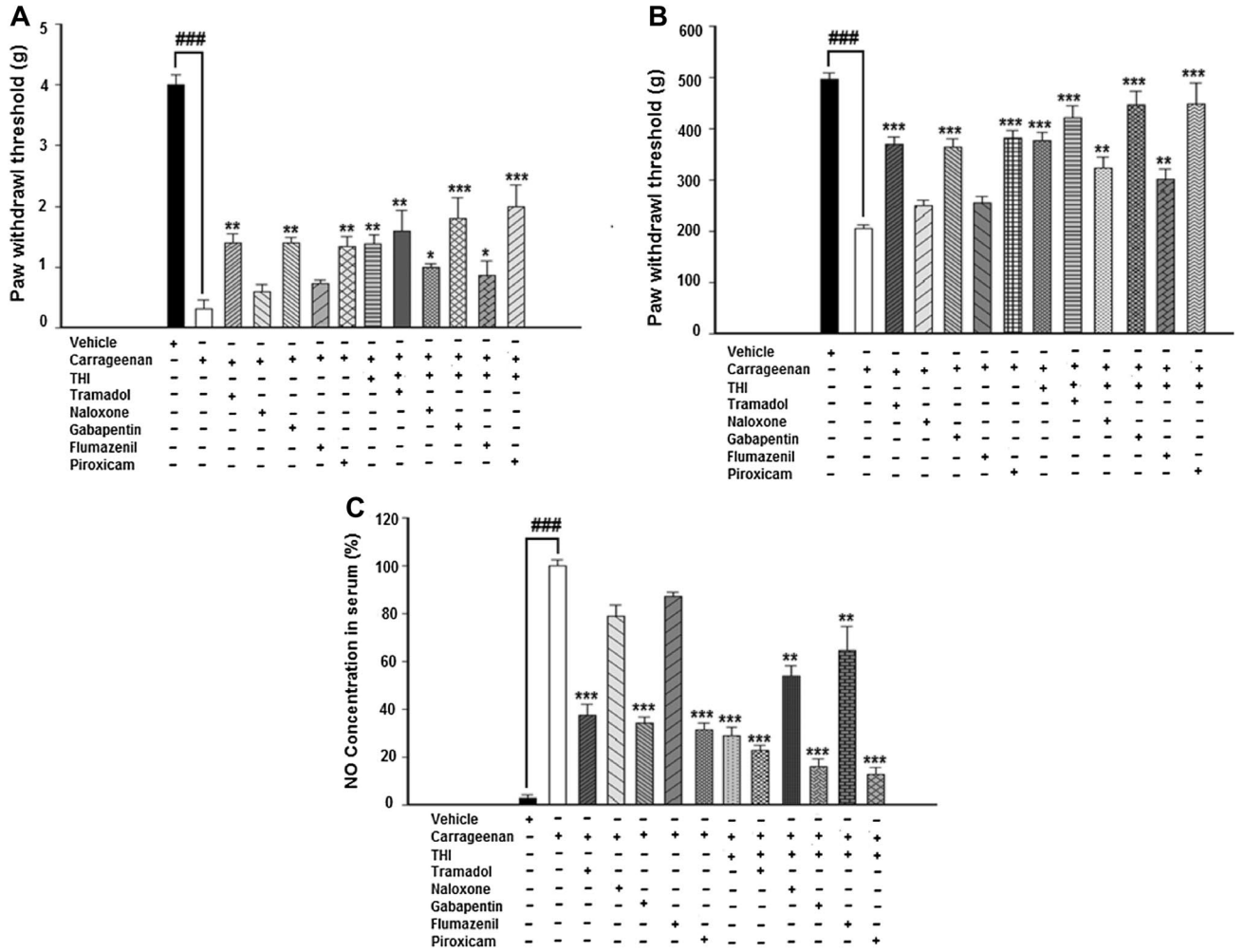


Fig. 7 Effect of pretreatment with agonists tramadol (50 mg/kg i.p.), piroxicam (10 mg/kg, i.p.) and gabapentin (50 mg/kg, i.p.) and antagonists flumazenil (0.2 mg/kg) and naloxone (4 mg/kg) on the anti-nociceptive effects of THI (10 mg/kg, i.p.) against carrageenan (100 µl/paw) induced acute inflammatory pain model ($n=5$). **a** effect on mechanical allodynia induced by carrageenan **b** effect on mechanical hyperalgesia induced by carrageenan **c** serum concentration of nitrite (NO) production with THI (10 mg/kg, i.p.) in the carrageenan (100 µl/paw) induced acute inflammatory pain model. The data are given as mean ± SD (*) $p<0.05$, (**) $p<0.01$ and (***) $p<0.001$ and ###indicates significant differences from the carrageenan-treated group

Discussion

Hydrazones of isatin have been reported to possess various biological activities including significant anti-inflammatory and analgesic potential [17]. The current study evaluated the potential anti-inflammatory and analgesic activity of PHI and THI and their anticipated mechanism of action along with assessment of inflammatory mediators to gain insight into the mechanism responsible for their anti-nociceptive effect. Both compounds were significantly effective in attenuating inflammation and pain in acute and chronic inflammatory models induced by different stimuli such as carrageenan and CFA.

Inflammation induced by subcutaneous injection of carrageenan and CFA immediately results in edema and hyperalgesia [35]. The intraplantar injection of carrageenan and CFA induces inflammation via signaling cascade in mice that triggers the release of various inflammatory and nociceptive mediators such as NO, PGE2, IL-6, TNF-α and IL-1β [22], producing strong immune responses and pain at the site of injection due to local sensitization of nociceptors together with central nervous system modifications [38]. Intraperitoneal administration of PHI (10 mg/kg) and THI (10 mg/kg) prior to carrageenan and CFA injections resulted in significant inhibition of paw edema, mechanical allodynia, mechanical and thermal hyperalgesia along with reduced levels of inflammatory mediator (NO) and cytokines in mice similar to the effects produced by the standard drugs used as positive control, dexamethasone (10 mg/kg) and piroxicam (10 mg/kg).

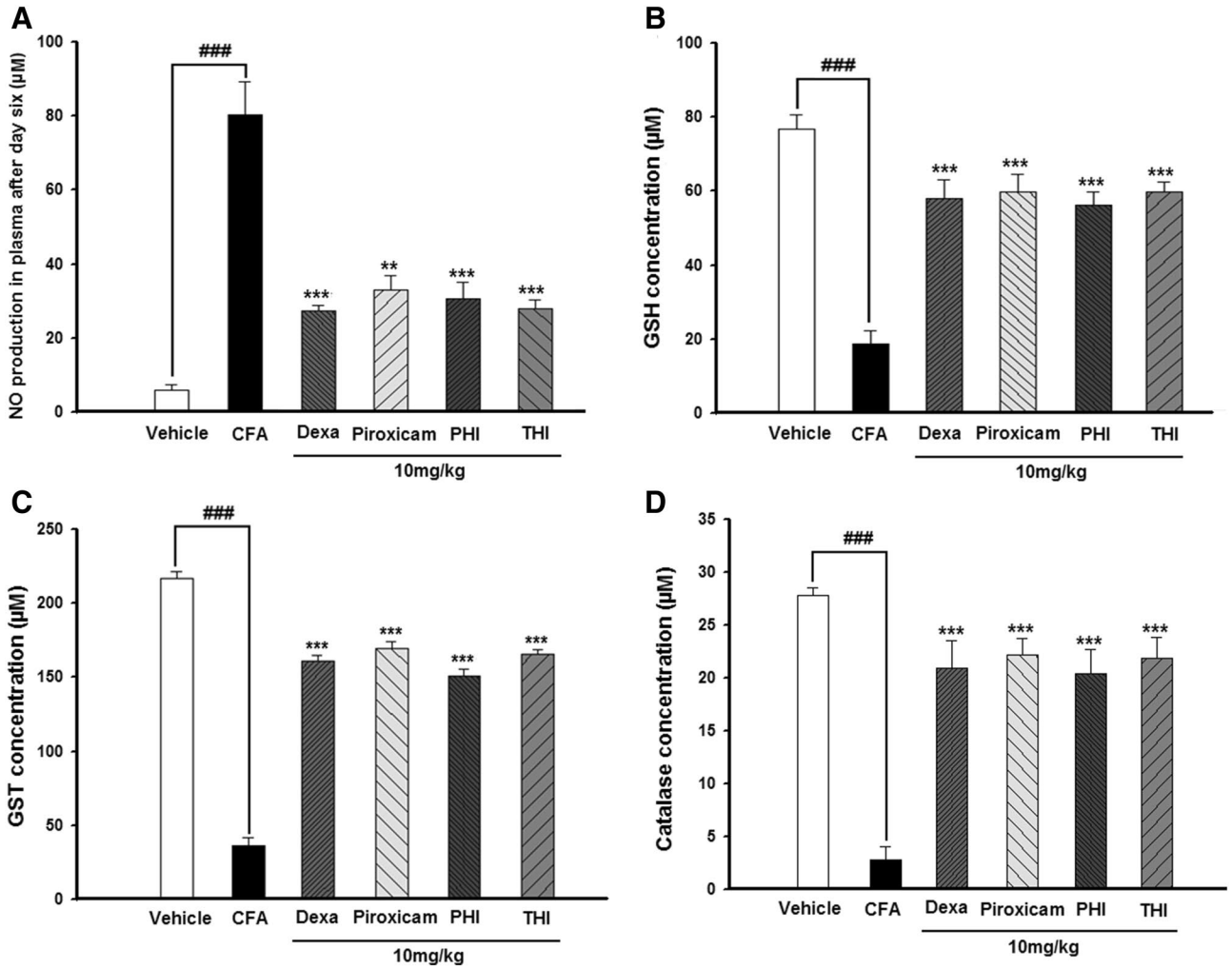


Fig. 8 **a** Effect of chronic pretreatment with PHI and THI on reduction in nitrite (NO) production in blood plasma. The NO concentration was determined using Griess reagent. Animals were administered with CFA, vehicle control PHI (10 mg/kg, i.p.), THI (10 mg/kg), i.p.), dexamethasone (10 mg/kg, i.p.) and piroxicam (10 mg/kg, i.p.). Antioxidant activity of PHI and THI in CFA treated paw tissues. **b** estimation of reduced glutathione (GSH) **c** estimation of enzymatic antioxidant glutathione sulfotransferase (GST) **d** Estimation of catalase activity. The data are given as mean ± SD (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$ and ###indicates significant differences from the CFA-treated group

The cytokines are the diverse group of substances released during inflammation. Among these, TNF-α precedes the release of other inflammatory mediators responsible for cellular recruitment, increased vascular permeability, and hyperalgesia [39]. IL-6 contributes to inflammation and is significantly upregulated during hyperalgesia [40]. IL-1β is associated with exciting nociceptive fibers and triggering apoptosis along with causing nociceptor hypersensitization observed as hyperalgesia due to cyclooxygenase-2 (COX-2) induction [41]. PHI and THI treatment significantly inhibit the release of these cytokines which is an established effect of hydrazones of isatin [42]. NO is a well-established mediator of inflammation and its production is related to the extent of inflammation. It is regulated by TNF-α and produces hyperalgesia due to increased levels of NO during inflammation and pain [43]. In our study, PHI and THI treatment reduced the levels of NO significantly as compared to the CFA-induced group.

To define the molecular mechanism of PHI and THI, the signaling pathways of NF-κB and MAPK were investigated. The effects of both compounds were evaluated on NF-κB and MAPK regulated proteins. The NF-κB pathway has a strong role in pathogenesis of chronic inflammatory diseases [44] while the MAPKs, including the extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK) and p38 mitogen activated protein kinase (p38 MAPK), play a role in transcription of different regulatory proteins and upregulation of cellular responses to cytokines and stress stimuli [45]. The activation of ERK due to nociceptive stimuli regulates central sensitization of pain using different

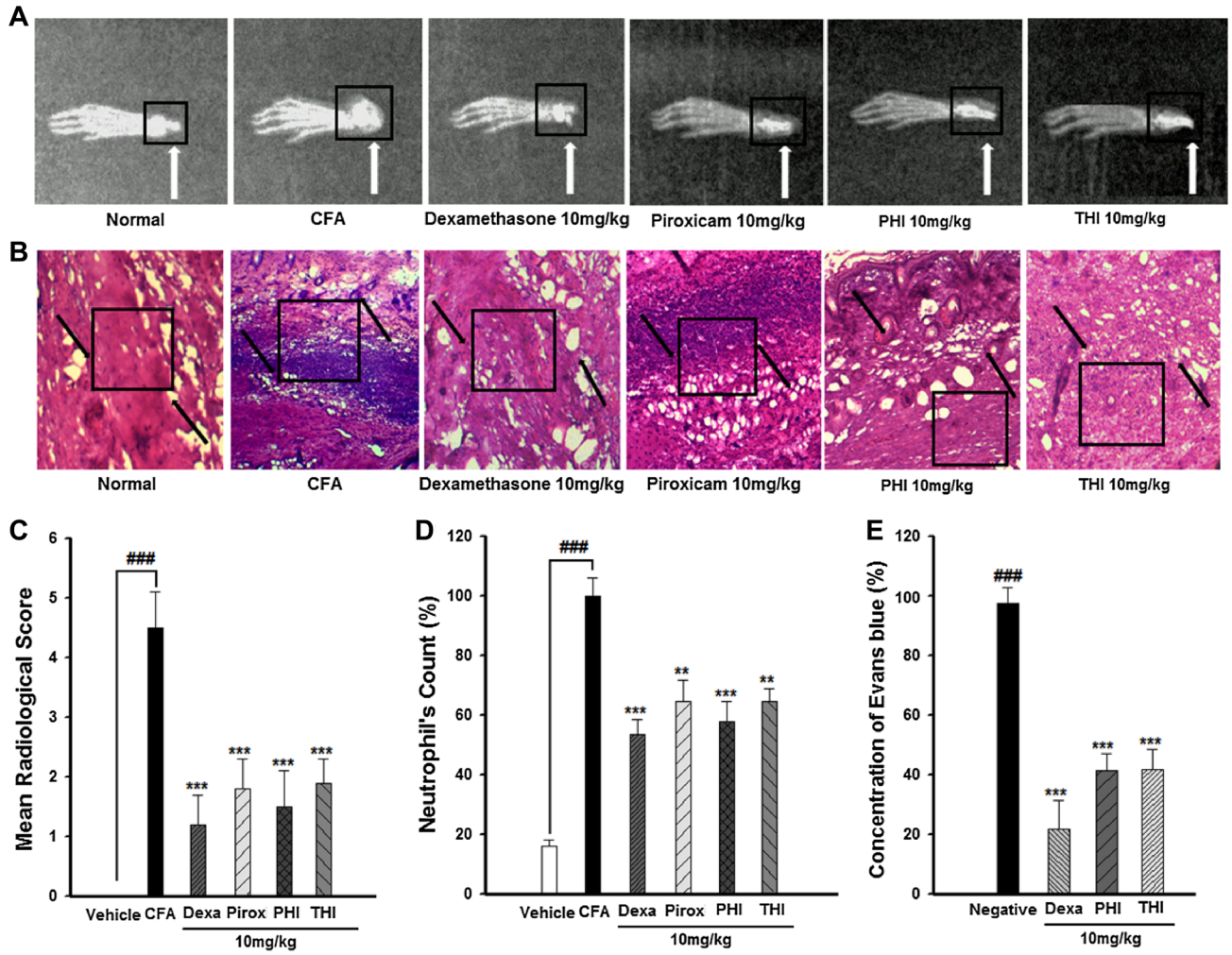


Fig. 9 **a** Effect of 7 days treatment of PHI and THI (10 mg/kg) by radiographic examination of tibio-tarsal joint in CFA-induced inflamed paws of mice from various groups. The pathological changes in the mice paw tissue were quantified **c** using 0–4 scoring as described in the "Materials and methods" **b** histopathological analysis of inflamed paw tissues. Paw tissues were sectioned at 4 µm thickness, stained by hematoxylin–eosin and observed with microscope (×40). The inflamed paw sections were further analyzed **d** determining the number of neutrophils presenting inflammation as described in "Materials and methods" **e** effect of PHI and THI (10 mg/kg) pretreatment on the enhanced vascular permeability induced by acetic acid ($n=5$). The data were derived from three independent experiments and expressed as the mean ± S.D (*) $p<0.05$, (**) $p<0.01$ and (***) $p<0.001 \pm$ S.D and $^{###}$indicates significant differences against the negative control groups

Table 3 Hematology values

Parameters	Neutrophils (10^9/L)	Lymphocytes (10^9/L)	Platelets (10^9/L)	MID# (10^9/L)	WBC (10^9/L)	RBC (10^9/L)
Normal	0.39 ± 0.02	2.03 ± 0.05	263 ± 11.5	0.34 ± 0.02	3.90 ± 0.20	5.66 ± 0.40
Negative	1.30 ± 0.17$^{###}$	5.50 ± 0.18$^{###}$	127 ± 21.9$^{###}$	1.11 ± 0.18$^{###}$	7.43 ± 0.12$^{###}$	5.62 ± 0.28$^{###}$
PHI	0.54 ± 0.05**	2.47 ± 0.21***	266 ± 5.7***	0.45 ± 0.01**	3.73 ± 0.28***	5.92 ± 0.10**
THI	0.43 ± 0.03**	2.51 ± 0.18***	251 ± 2.8***	0.59 ± 0.06*	4.10 ± 0.12***	5.42 ± 0.03*

All values are expressed as mean ± SEM ($n=5$)

$^{###}p<0.001$compared with normal control group

*$p<0.05$, **$p<0.01$, and ***$p<0.001$ compared with negative control group

Table 4 Effect of PHI and THI on sodium and potassium excretion rate from urine and serum

Parameters	Normal	Diclofenac (10 mg/kg)	PHI (10 mg/kg)	THI (10 mg/kg)
Urine Na^+ (mmol/L)	104 ± 2.3	112 ± 1.2	108 ± 3.2	105 ± 1.7
Urine K^+ (mmol/L)	107 ± 2.3	139 ± 1.2	111 ± 3.1	101 ± 3.8
Serum Na^+ (mmol/L)	138 ± 0.7	143 ± 2.8	143 ± 2.1	144 ± 2.1
Serum K^+ (mmol/L)	6.8 ± 0.08	7.15 ± 0.07	7.16 ± 0.09	7.2 ± 0.07

All values are expressed as mean ± SD ($n = 5$) using Student's *t* test

second messenger pathways [46] and activation of p38 is associated with the upregulation of various inflammatory mediators (COX-2, TNF-α) [47]. Our results suggested that both compounds suppressed CFA-induced phosphorylation of IκBα, p38 MAPK, JNK and ERK activations, thus validating their anti-nociceptive effect by blocking the NF-κB and MAPKs signaling pathway and interfering with the production of certain mediators of inflammatory pain. Since activation of NF-κB and MAPK contributes to enhanced hyperalgesia and allodynia due to increased production of certain cytokines [48], downregulation of both pathways produce significant anti-nociceptive effect.

Both carrageenan and CFA induce inflammation and pain by elevating the levels of oxidative stress [25], thus emphasizing the role of anti-inflammatory drugs in preventing free radical mediated damage. The fundamental defense line of the organism against reactive oxygen intermediates includes various antioxidant enzymes such as catalase (CAT), reduced glutathione (GSH) and glutathione-*S*-transferase (GST) mainly [49]. Pretreatment with PHI and THI reduced progression of inflammation by scavenging the free radicals generated during inflammatory processes pertaining to the antioxidant potential of such compounds [50].

An increase in vascular permeability is a prominent feature of many disease states, involving inflammatory mediators (prostaglandins, cytokines) and vascular endothelial growth factor, resulting in plasma protein extravasation, leading to tissue edema. It is usually associated with enhanced perception of pain or hyperalgesic sensitization [33]. Pretreatment with PHI and THI resulted in a protective effect on vascular permeability stimulated in vivo by acetic acid, inhibiting extravasation of plasma from the blood vessel similar to the standard drug dexamethasone.

To assess the possible mechanism of action of PHI and THI, agonists and antagonists of various types were chosen for further study. Both compounds were co-administered with various drugs such as gabapentin, flumazenil, piroxicam, tramadol, and naloxone. NSAIDs are well established anti-nociceptive agents, based on the possible association of inflammation with the process of nociception [51]. Piroxicam is a nonselective inhibitor of cyclooxygenase which belongs to the class of NSAIDs and analgesic drug. The generally described mechanism of action of NSAIDs is attenuation of prostaglandin synthesis by inhibiting cyclooxygenase enzymes, though some central action has also been reported [52]. Notable synergism was observed with the co-administration of PHI and THI with piroxicam, thus, suggesting the possible effect of both compounds on cyclooxygenase and inflammatory cytokines [53]. Anticonvulsants drugs have been an integral part of pain control regimens [54]. The gabapentin is three-substituted g-aminobutyric acid (GABA) derivatives that produces its anti-nociceptive effect via interacting with the α2δ auxiliary subunits of voltage-dependent calcium channels [55]. The interaction of gabapentin with α2δ was confirmed in numerous animal studies and its upregulation within the spinal cord/dorsal root ganglia following gabapentin treatment [55]. GABA antagonist (flumazenil) and agonist (gabapentin) co-administered with PHI and THI interfered considerably with pain, suggesting that PHI and THI may exert their anti-nociceptive effects by interacting with the α2δ auxiliary subunits of voltage-dependent calcium channels. Tramadol is a centrally acting synthetic analgesic, which interacts weakly with μ (mu) opioid receptors. The activation of the opioid receptor in Aδ and C fibers results in inhibition of the calcium channel with subsequent decline in levels of cAMP [56]. This decline in cAMP levels further averts cGRP, substance P and glutamate release [57]. Moreover, tramadol has been reported to produce its anti-nociceptive effect by nonopioids mechanism that causes the enhanced extraneuronal concentrations of the monoamine neurotransmitters serotonin (5-HT) and noradrenaline by interfering with their release and reuptake mechanisms [58]. The monoamine system, predominantly the 5-HT (serotonin), plays major role in modulating the nociception. Several 5-HT receptors have been linked with medullo-spinal modulation of painful sensation. However, 5-HT1A receptor has a significant role as it is expressed in both supra-spinal and spinal regions associated with the modulation of nociception [58]. The coadministration of PHI and THI with tramadol had an augmented anti-nociceptive effect, suggesting that both the compounds may influence opioid and monoamine reuptake system. Blocking of the PHI and THI anti-nociceptive effects via naloxone to some extent further support this hypothesis.

Unlike the detrimental effects of extensively used analgesics and NSAIDs on the gastrointestinal tract resulting in neutrophil activation and inhibition of PGEs, PHI and THI were safe at therapeutic dose levels from the perspective

of ulcer induction and gastric inflammatory events. Sodium and potassium are important in regulating the heart contractions and maintaining blood pressure [59]. The most common complication of NSAIDs is sodium and potassium retention, which leads to complications such as water retention, resulting in edema and hyperkalemia. In turn, this can cause arrhythmia and increased chances of cardiac arrest [60]. Treatment with PHI and THI did not alter renal or liver functions nor showed any change in hematology profile as compared to controls. Moreover, PHI and THI treatment was not associated with any toxic effect on muscle coordination and strength. Thus, both PHI and THI elevated the pain threshold along with considerable improvement in the biochemical parameters and a low toxicity profile, signifying their potential role in the management of pain.

Conclusion

The current study highlighted the anti-inflammatory and analgesic potential of PHI and THI along with their safety profile. The anti-hyperalgesic effects of PHI and THI in inflammatory pain can be attributed to suppression of pro-inflammatory cytokines including IL-6, TNF-α and IL-1β, along with enhanced antioxidant potential. PHI and THI treatment caused inhibition of paw edema with reduced NO levels and suppression of oxidative stress and cytokine production in both acute and chronic inflammatory models. Both compounds acted by blocking IκBα, ERK, JNK and p38 MAPK signaling pathways. Notably, no apparent signs of toxicity were reported by PHI and THI treatment during 6 days, which is a usual time of treatment with anti-inflammatory drugs, supporting both compounds as potential candidates for further studies.

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Compliance with ethical standards

Conflict of interest The authors report no conflict of interest.

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