Research Article

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Acute toxicity and anti-inflammatory activity of bis-thiourea derivatives

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Abstract: In the current work, bis-thiourea derivatives have been synthesized through condensation reaction between isothiocynates and diamines in dry acetone to form **SK1** (1,2-bis(*N*-benzoylthioureido) benzene), **SK2** (1,3-bis(*N*-benzoylthioureido) benzene), and **SK3** (1,4-bis(*N*-benzoylthioureido) benzene). The structures of new synthesized derivatives were confirmed through melting point and spectroscopic technique such as ^{1}HNMR only. The synthesized compounds were assessed for acute toxicity test and are proved free of toxicity. The derivatives were further tested as anti-inflammatory agents by *in vitro* lipoxygenase enzyme inhibition studies, molecular docking, and *in vivo* carrageenan-induced paw edema assay, and histamine-induced edema test. The overall observations presented that compounds **SK1** and **SK3** possess promising anti-inflammatory potential, while compound SK2 is found to be a good anti-inflammatory agent.

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1 Introduction

An inflammation is a defensive response of the immune system to noxious agents like harmful bacteria, chemical toxicants, radiation, and cancerous cells. It is a protective mechanism that identify and neutralize injurious stimuli to initiate the healing process [1]. Inflammation is classified as acute or chronic. The characterized features involved in inflammation are redness, heat, pain, swelling, as well as loss of tissue function that arises from resident immune system responses to damage [2]. Generally, enhancing blood circulation toward areas of tissue injury leads to the restoration of tissue homeostasis, aiding in the resolution of severe inflammation. Subsequently, unchecked acute inflammation has the potential to evolve into a chronic state. In general, the degree and effect of chronic inflammation vary depending on the injury source and capability of body to overcome and heal the injury. Also, inflammation has many hazards, and the patient may suffer from pain and morbidity. Some chronic inflammatory diseases include cancer, anemia, tuberculosis, and arthritis [3,4].

The response mechanism of inflammation involves stimulation of signal pathways to release leukocytes in local tissues and recruitment and accumulation of inflammatory cells from plasma. Responses of inflammation depend on the nature of the stimulus and its position in the body. The main phases in the general mechanism are as follows: (1) cell surface pattern receptors identify injurious stimuli, (2) inflammatory pathways are activated, (3) inflammatory markers are released, and (4) inflammatory cells are recruited [5].

The cyclooxygenase (COX) and lipoxygenase (LOX) are common enzymes that contribute to inflammation. Arachidonic acid metabolism plays an active part in the inflammation mechanism. COX cascade and LOX pathway on appropriate stimulation by neutrophils metabolizes

arachidonic acid to inflammatory mediators such as prostaglandins, thromboxane, and leukotrienes. The activation of local inflammatory mediators causes pain and destruction of tissues. Inhibition of COX and LOX directly decreases leukotrienes and prostaglandins mediator's production [6,7].

Thiourea and derivatives of thiourea are sulfur and nitrogen-containing organic compounds having general formula R4N2CS also known as carbamides. These compounds have a structural resemblance with urea, substituting oxygen atom in urea by sulfur that make thiourea as a markedly different compound from urea. Thioureas are soluble in organic polar solvents like ethanol, chloroform, and dimethyl sulfoxide (DMSO), etc. but difficult to dissolve in hexane-like nonpolar solvents [8]. Thiourea derivatives are very adaptable ligands, which have the capability to coordinate with transition metal ion as neutral, monobasic, and dibasic ligands. The addition of compatible donor sulfur, nitrogen and oxygen atoms, and double bond provide the thiourea multiple bonding potential for coordinated complex formation [9]. The coordination diversity shows the importance of substituted thioureas and their metal complexes in metallurgy, regulation of plant growth, and synthesis of nanomaterials [10,11].

In the nineteenth century, substituted thioureas are often used in plastics, photographic film, dyes, and textiles for commercial purposes [12]. In organic synthesis, thioureas have played a key role as catalysts (thiourea organic catalysis) in Mannich and Biginelli reactions [13]. Apart from synthetic applications, the literature shows that thiourea derivatives play a vital role in pharmaceutical industries and possess significant biological potentials like anti-inflammatory [14], anticancer [15], antidiabetic [16], urease inhibitor [17], antibacterial [18], analgesics [19], and anti-tumor [20].

NSAIDs inhibit the production of prostaglandins [21] and provide anti-inflammatory relief [22]. However, their long-term use has been associated with many side effects such as gastrointestinal ulcer and nephrotoxicity. Therefore, there is an urgent need to design potent and safe novel alternatives to NSAIDs.

The thiourea moiety has emerged as an important pharmacophore and possesses a variety of pharmacological potentials including anti-inflammatory [14,23–27]. However, substituted bis-thiourea derivatives exhibiting anti-inflammatory activities have not been described yet. With the aim to identify small-molecule inhibitors against specific condition, a series of bis-thiourea derivatives (**SK1–SK3**) were synthesized and investigated for their acute toxicity, specific molecular targeting using molecular docking analysis, *in vitro* enzyme inhibition, and *in vivo* anti-inflammatory properties.

2 Materials and methods

Chemicals and solvents including benzoyl chloride, potassium thiocyanate, phenylenediamines, acetone, *n*-hexane, ethyl acetate, DMSO, methanol, and chloroform of analytical grade were purchased from Merck and Sigma Aldrich. The reaction progress and purity of product were examined through TLC (Merck), envisioned under UV light. For resolution of the melting points, electrothermal Gallan Kamp device was used. The ^{1}H-NMR spectrum was recorded at NMR Bruker apparatus (300 MHz) in solvent $CDCl_3$ at Quaid-e-Azam University located in Islamabad.

2.1 Methodology

2.1.1 Synthesis of bis-thiourea derivatives (SK1–SK3)

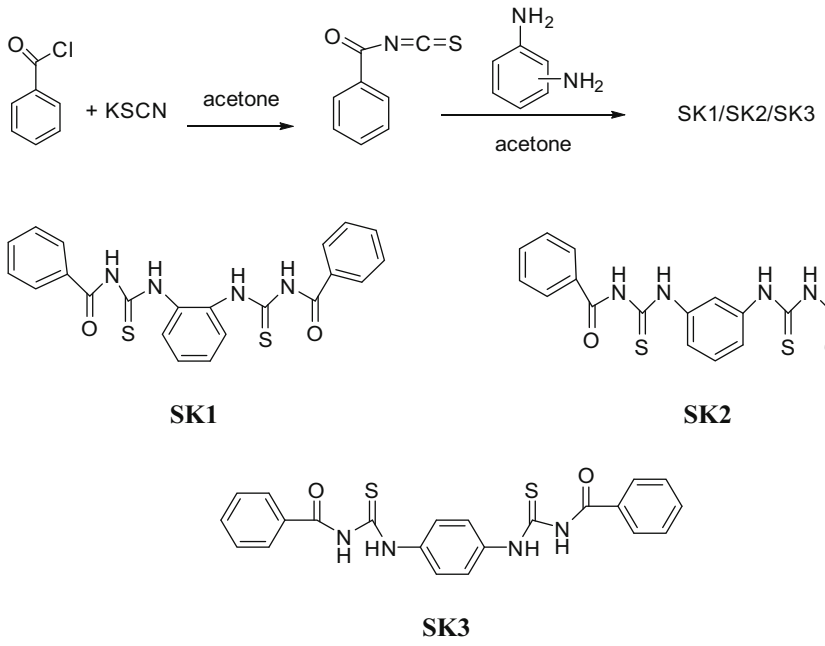
A set of bis-thiourea derivatives was synthesized by using the reflux condensation method, as reported by Shoaib et al. [28]. The reaction was carried out by stirring a 5 mmol (0.58 ml) of benzoyl chloride and 5 mmol (0.48 g) suspension of potassium thiocyanate in dry acetone under reflux for 5–10 min to form intermediate isothiocynate. The KCl precipitate formed was filtered off. Then, 2.5 mmol (0.27 g) of phenylenediamine with 10 ml acetone was poured into isothiocyanate and stirred under reflux for 1–2 h at room temperature. The reaction contents were poured gently into grinded ice, and the precipitate was collected through filtration. The obtained product was dried and recrystallized with ethanol. The product was purified by column chromatography and recrystallization methods (Scheme 1).

2.1.2 Synthesis of SK1 (1,2-bis(*N*-benzoylthioureido) benzene)

Yield: 81.5%, pale yellow powder, melting point: 151–154°C, soluble in chloroform, ethanol, DMSO, and benzene, Rf value: 0.6 (6:4 *n*-hexane and ethylacetate mixture). ^{1}HNMR (300 MHz, $CDCl_3$/ppm): δ 12.38 (2H, s), 9.25 (2H, s), 7.28–7.95 (14H, m). The corresponding MS, ^{13}CNMR, and IR spectroscopic data were reported in the study by Thiam et al. [29].

2.1.3 Synthesis of SK2 (1,3-di(benzoylthioureido) benzene)

Yield: 75.4%, black powder, melting point: 220–223°C, soluble in chloroform, ethanol, DMSO, and benzene, Rf



Scheme 1: Synthesis of bis-thiourea derivatives (**SK1–SK3**).

value: 0.6 (6:4 *n*-hexane and ethylacetate mixture). ^{1}HNMR (300 MHz, $CDCl_3$/ppm): δ 12.69 (2H, s), 9.19(2H, s), 7.23–8.40 (14H, m). The corresponding MS, ^{13}CNMR, and IR spectroscopic data were reported in the study by Wilson et al. [30].

2.1.4 Synthesis of SK3 (1,4-di(benzoylthioureido) benzene)

Yield: 78.7%, black brown powder, melting point: 239–242°C, soluble in chloroform, ethanol, DMSO, and benzene, Rf value: 0.6 (6:4 *n*-hexane and ethylacetate mixture). ^{1}HNMR (δ ppm): 12.72 (2H, s), 9.12 (2H, s), 7.50–7.98 (14H, m). The corresponding MS, ^{13}CNMR, and IR spectroscopic data were reported in the study by Pourshamsian et al. [31].

2.2 Pharmacological activities

2.2.1 *In vitro* LOX inhibition assay

The role of targeted derivatives of bis-thiourea against LOX inhibition assay was examined. Synthesized compounds in various concentrations (μg/mL) were prepared. A sample of 5 μL was mixed with phosphate buffer (970 μL, pH 9) and 17 μL of linoleic acid and maintained at 25° C followed by the addition of 4 μL of the aliquot enzyme. Absorbance was measured using spectrophotometer, and inhibition of LOX was determined by plotting graphically the absorbance against the different concentrations of samples. Nordihydroguaiaretic acid (NDGA) was used as the positive control. The IC_{50} values of respective samples were observed [6].

2.3 Molecular docking

Molecular Operating Environment (MOE) (http://www.chemcomp.com/) software package was used to carry out molecular docking. The Chemical Computing Group has designed MOE to facilitate cheminformatics, bioinformatics, virtual screening, molecular modeling, structure-based drug design, and the capability to construct new applications using SVL (Scientific Vector Language). Ligand plot operation in MOE program was employed for the prediction of orientation and interaction between LOX and ligands [32].

2.3.1 Preparation of ligand molecules

The preparation of ligand molecules for LOX was done by the use of MOE Builder [33], which is a basic tool. The ligand molecules were stabilized through reducing their free energies with the help of an energy minimization algorithm. The key parameters such as energy minimization, force field: MMFF94X, gradient: 0.05, and chiral constraint: current geometry were considered. Reduced ligands were saved in the file of mdb. In the coming phase, the molecules of ligands were ready to use them as a template for MOE docking [34].

2.3.2 Preparation of protein

For examining molecular docking of synthesized compounds, we took the protein molecule (LOX; PDB ID: 3V99) from the Protein Data Bank. The protein molecule was dehydrated by removing water molecules. The 3D structures of the prepared protein molecules were protonated. Energy minimization algorithm was used to reduce free energies of protein molecules. The key parameters such as energy minimization, force field: MMFF94X plus solvation, gradient: 0.05, and chiral constraint: current geometry were considered. When the root mean square gradient value reduces below 0.05, the decreasing of the energy was stopped. The reduced structures of protein molecules were found during the docking analysis [35].

2.3.3 Molecular docking on LOX enzyme

The binding mode between the ligands and protein molecules was investigated using MOE docking software. The docking analysis gives the accurate conformation about the molecules of ligands and thus helps to obtain the stable structure (minimum energy). In the figures of molecular docking, the top poses for H.... (hydrogen Bonding) and π–π interactions are observable. The root-mean-square deviation was calculated using MOE software applications [27].

2.4 Experimental animals and ethical approval

In *in vivo* studies, albino mice with a weight ranging from approximately 20 to 25 g are utilized. These mice were procured from the National Institute of Health, Islamabad, and were maintained and housed 8 per cage under the standard environmental condition with 12 h light–dark cycle, room temperature (25 ± 2°C), 40–50% relative humidity, and free access to food and water. The *in vivo* studies and animal handling were done according to the prescribed instructions outlined in the ethical committee guidelines of the Department of Pharmacy at the University of Malakand. Authorization for the utilization of animals in this study was obtained from the Department of Malakand University, Chakdara, with the reference number pharm/ESS/SM-111/07/21.

2.4.1 Acute toxicity and selection of doses

To explore the safety of synthesized bis-thiourea derivatives, the acute toxicity test was carried out in Albi no mice by using the Lorke model [36] for LD_{50} determination. Mice of both sexes were selected for this test. Experimental mice were classified into six groups ($n = 3$). In phase I, groups 1–6 were orally administered with synthesized compounds at doses of 50, 75, and 100 mg/kg body weight. The animals received drugs at concentrations of 150, 200, and 300 mg/kg in phase II. Animals were examined continuously for the allergic effect of samples for 24 h, and LD_{50} value for each drug was calculated. The dose selection was carried out as per the previous published data [23] and preliminary pharmacological screening.

2.4.2 *In vivo* Carrageenan-induced paw edema test

Anti-inflammatory efficacy of the newly prepared compounds was investigated according to the carrageenan-induced paw edema model. The animals were kept fasted for 12 h before the commencement of experiment. Acute edema was induced in the hind paw by injecting (0.1 or 0.5 m of 1% fresh solution in distilled water) carrageenan. The target bis-thioureas were orally given to mice 30 min or 1-h before the carrageenan injection. There were eight groups of mice. Each group consists of three mice. Group 1 is marked as a vehicle (negative control). The remaining groups (2–7) were treated with tested compounds. The eighth group considered a positive group was cured by the standard drug (10 mg/kg solution of diclofenac sodium in normal saline). After carrageenan injection, the thickness of the paw was recorded at regular intervals of 1 h, till 5th hour by using plethysmometer [37].

The percent increase in volume of paw was measured by using the given formula:

$$\%\text{inhibition} = [V_c - V_t/V_c] \times 100,$$

where V_c = carrageenan group volume and V_t = treated group volume.

2.5 Possible involvement of histaminic pathway

To assess the anti-inflammatory effect of bis-thioureas, Wister Albino mice were exposed to mechanistic approach aimed to know the possible involvement of histaminic pathway in process of inflammation. The new compounds were orally given to experimental mice 30 min/1 h before the injection of histamine. There were divided into eight groups. Group 1 received saline, eighth group was given cetirizine (10 mg/kg in normal saline), and the rest of groups received 12.5 and 25 mg/kg of tested compounds.

The change in the paw volume was noted plethysmometrically [38].

2.6 Data analysis

Results of the study were expressed as mean ± SEM. Graph Pad Prism 5.01 was used to analyze data between the groups and analysis of variance (ANOVA) among groups followed by Dunnet's test for multiple comparisons.

3 Results

Several methods has been reported for the synthesis of thioureas. Bis-thiourea derivatives were synthesized by an efficient and ecofriendly reflux method. The reaction progress and the purity of the product were examined via cleared spots on the TLC plate seen under the UV lamp. The compounds were obtained in very good yield. The appearance of products was powder crystal.

3.1 SK1: 1,2-bis(*N*-benzoylthioureido) benzene

The compound SK1 was obtained as pale-yellow powder crystal from ethanol in very good yield (81.5%), melted at 151–154°C, and is soluble in chloroform, ethanol, DMSO, and benzene having an Rf value of 0.6 (6:4 *n*-hexane and ethylacetate mixture). ^{1}HNMR (300 MHz, $CDCl_3$-*d*): δ ppm 12.38 (2H, s), 9.25(2H, s), 7.28–7.95 (14H, m). The corresponding MS, ^{13}CNMR, and IR spectroscopic data were reported in the study by Nedeljković et al. [24].

^{1}H NMR spectra of compound SK1 in $CDCl_3$ as given in Figure S1 show three main signals. Two single peaks appeared for NH protons. However, 14 protons appeared in the aromatic region gives a multiplet. The signal that was mostly deshielded and appeared at around 12.38 ppm was assigned to NH, which confirmed the formation of thiourea. This high chemical shift value is due to the corresponding protons involved in the formation of hydrogen bonds (H…). The singlet assigned to protons (2NH) was found at around 9.25. The existence of residues 2 NH confirmed bis-thiourea derivatives formation. At shift 7.28–7.95 ppm, the multiplet was found and was assigned to protons of aromatic region in bis-thiourea derivative [24].

3.2 SK2: 1,3-bis(*N*-benzoylthioureido) benzene

The compound SK2 was obtained as block powder crystal from ethanol in very good yield (75.4%), melted at 220–223°C, and soluble in chloroform, ethanol, DMSO, and benzene having an Rf value of 0.6 (6:4 *n*-hexane and ethylacetate mixture). ^{1}HNMR (300 MHz, $CDCl_3$-*d*): δ 12.69 (2H, s), 9.19(2H, s), 7.23–8.40 (14H, m). The corresponding MS, ^{13}CNMR, and IR spectroscopic data were reported in the study by Efeoglu et al. [25].

^{1}H NMR spectra of compound SK2 in $CDCl_3$ in Figure S2 show three main signals. Two single peaks appeared for NH protons. Also, 14 protons appeared in the aromatic region give a multiplet. The signal that was mostly deshielded and appeared at around 12.69 ppm was assigned to NH, which confirmed the formation of thiourea. The high chemical shift value is because of the involvement of the respective protons in the formation of hydrogen bonds (H…). The singlet assigned to protons (2NH) was found at around 9.19. The existence of residues 2 NH confirmed the formation of bis-thiourea derivatives. At shift 7.23–8.40 ppm, the multiplet was found and was assigned to protons of the aromatic region in the bis-thiourea derivative [25].

3.3 SK3: 1,4-bis(*N*-benzoylthioureido) benzene

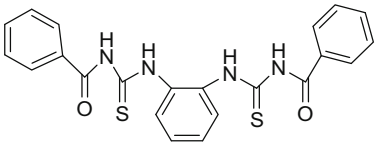
The compound SK3 was obtained as a black brown powder from ethanol in very good yield (78.7%), melted at 238–242°C, and soluble in chloroform, ethanol, DMSO, benzene having an Rf value of 0.6 (6:4 *n*-hexane and ethylacetate mixture). ^{1}HNMR (300 MHz, $CDCl_3$-*d*): δ 12.72 (2H, s), 9.12 (2H, s), 7.98–7.50 (14H, m). The corresponding MS, ^{13}CNMR, and IR spectroscopic data were reported in the study by Nedeljković et al. [26].

^{1}H NMR spectra of compound SK3 in $CDCl_3$ in Figure S3 show three main signals. Two single peaks appeared for NH protons. Also, 14 protons appeared in the aromatic region give a multiplet. The signal that is mostly deshielded appeared at around 12.72 ppm and was assigned to NH, which confirmed the formation of thiourea. This high chemical shift value is because of the corresponding protons involved in the formation of hydrogen bonds (H…). The singlet assigned to protons (2NH) was found at around 9.12. The existence of residues 2 NH confirmed the formation of bis-thiourea derivatives. At shift 7.98–7.50 ppm, the multiplet was found and was assigned to protons of the aromatic region in the bis-thiourea derivative [26].

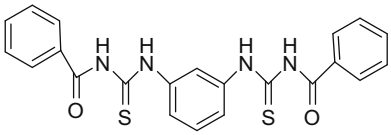
3.4 *In-vitro* enzyme (LOX) inhibition assays

To assess the anti-inflammatory potential of new isomeric bis-thiourea derivatives (**SK1–SK3**), the *in-vitro* LOX enzyme (LOX) inhibition assays was carried out. All three compounds were screened for anti-inflammatory effect at concentration in μg/ml, i.e., 31.25–1,000 μg/ml. The percent enzyme inhibitory response was analyzed. At concentration of 1,000 (μg/ml), maximum response was noted (Table 1).

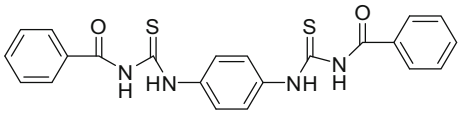
SK1, **SK2**, and **SK3** attained the IC_{50} values 68.06, 105.70, and 63.34 (μg/ml), respectively, at various concentrations compared to the reference NDGA at IC_{50} 19.56 (μg/mL) at the same concentrations.



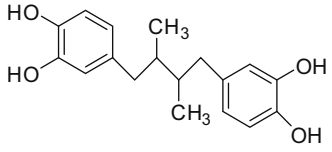
SK1 IC_{50} = 68.06 μg/ml



SK2 IC_{50} = 105.70 μg/ml



SK3 IC_{50} = 63.34 μg/ml



NDGA IC_{50} = 19.56 μg/ml

All the ligand molecules were docked into the binding site of the LOX. The results explained that all the compounds **SK1**, **SK2**, and **SK3** are actively binded in the target site of protein. In the case of compound **SK1**, an active site residue His513 was found to form a hydrogen bond (Figure 1a). However, compound **SK2** was observed to interact with active site residues Gln514, His518, and Arg726 (Figure 1b). Compound **SK3** was predicted to interact with residues His513 and Gln716. Interestingly, active site residue His513 was found to contribute to interaction with all the three compounds docked with LOX. Docking scores and binding energies of ligand molecules are given in Table 2.

The generalized-Born volume integral/weighted surface area (GBVI/WSA) serves as a scoring function designed to estimate the free energy of binding a ligand based on its given pose. In all scoring functions, lower scores signify more favorable poses. In molecular docking investigations, the selection of active and nonactive compounds primarily relies on the binding energies and docking scores.

3.5 Acute toxicity

All the new bis-thioureas were tried to confirm their acute toxicity/safety in mice. In the acute toxicity screening, animals employed were found to be free from harmfulness up to a concentration of 300 mg/kg b.w. No significant difference was noted between the control and treatment groups of SK1–SK3 in terms of mortality and morbidity. The feeding and weight patterns in all groups were found to be normal. Based on the previous finding and the preliminary pharmacological data, 15 and 30 mg/kg b.w were selected for anti-inflammatory activity.

3.6 *In vivo* Carrageenan – induced paw edema model

The di(benzoylthioureido)benzene (SK1, SK2, SK3) was subjected to anti-inflammatory activity by taking diclofenac sodium as a reference for *in vivo* studies via the basic tool carrageenan-induced paw edema test. The data are presented in Table 3. The *in vivo* results proved that the synthesized bis-thiourea is a significant inhibitor of carrageenan. The mean paw volume was monitored at regular intervals of 1 h for 5 h.

Compound SK1 was observed with maximum percentage inhibition on period of the second hour and third hour at a concentration of low dose. At high dose, the efficacy of compound SK1 improved, as well as 56.22% maximum inhibition was noticed after 4 h of carrageenan injection. The synthesized SK2 displayed maximum response, i.e., 36.26% against carrageenan with 15 mg/kg dose at the fifth hour and 45.47% with 30 mg/kg at the third hour.

Table 1: Various concentrations of doses and their effect of on LOX inhibition

Compounds	Conc. (µg/mL)	%Inhibition	IC_{50} (µg/mL)
SK1	1,000	80.11 ± 0.61	68.06
	500	74.73 ± 0.59	
	250	69.18 ± 0.67	
	125	59.34 ± 0.56	
	62.5	45.91 ± 0.61	
	31.25	42.73 ± 0.71	
SK2	1,000	76.92 ± 0.81	105.70
	500	69.81 ± 0.69	
	250	65.17 ± 0.70	
	125	59.08 ± 0.61	
	62.5	41.52 ± 0.46	
	31.25	39.88 ± 0.60	
SK3	1,000	76.81 ± 0.77	63.34
	500	71.45 ± 0.83	
	250	66.30 ± 0.51	
	125	58.94 ± 0.63	
	62.5	49.33 ± 0.51	
	31.25	40.90 ± 0.69	
NDGA	1,000	98.91 ± 0.78	19.56
	500	95.03 ± 0.81	
	250	90.96 ± 0.78	
	125	85.87 ± 0.69	
	62.5	80.67 ± 0.65	
	31.25	79.87 ± 0.41	

Data are presented as mean ± SEM, $n = 3$.

The highest anti-inflammatory action recorded for compound SK3 showed a 55.09 and 57.07% decrease in paw volume on administration of 15 and 30 mg/kg dose, respectively, at the second hour. The SK3 was active at all time intervals, i.e., first to fifth hour appeared as excellent anti-inflammatory agent. Also, a marked inhibiting potential was seen near the diclofenac sodium (Figure 2).

3.7 Possible involvement of histaminic pathway in inflammation

In vivo, the inhibiting property of designed bis-thioureas SK1, SK2, and SK3 was evaluated using cetirizine as standard, and histamine involvement of causing inflammation in mice was studied. The outcomes were presented as mean ± SEM, as depicted in Table 4, which indicated activeness of tested compounds against the effect of histamine.

The compounds were examined to show a marked decrease in the paw volume at the first hour of administration. Also, the high potency of drugs was observed with increasing concentration. Compounds SK1 and SK3 exhibited promising activity against histamine when compared to SK2. The existence of amino substituent at third carbon (m-position) of benzene ring with respect to the first one

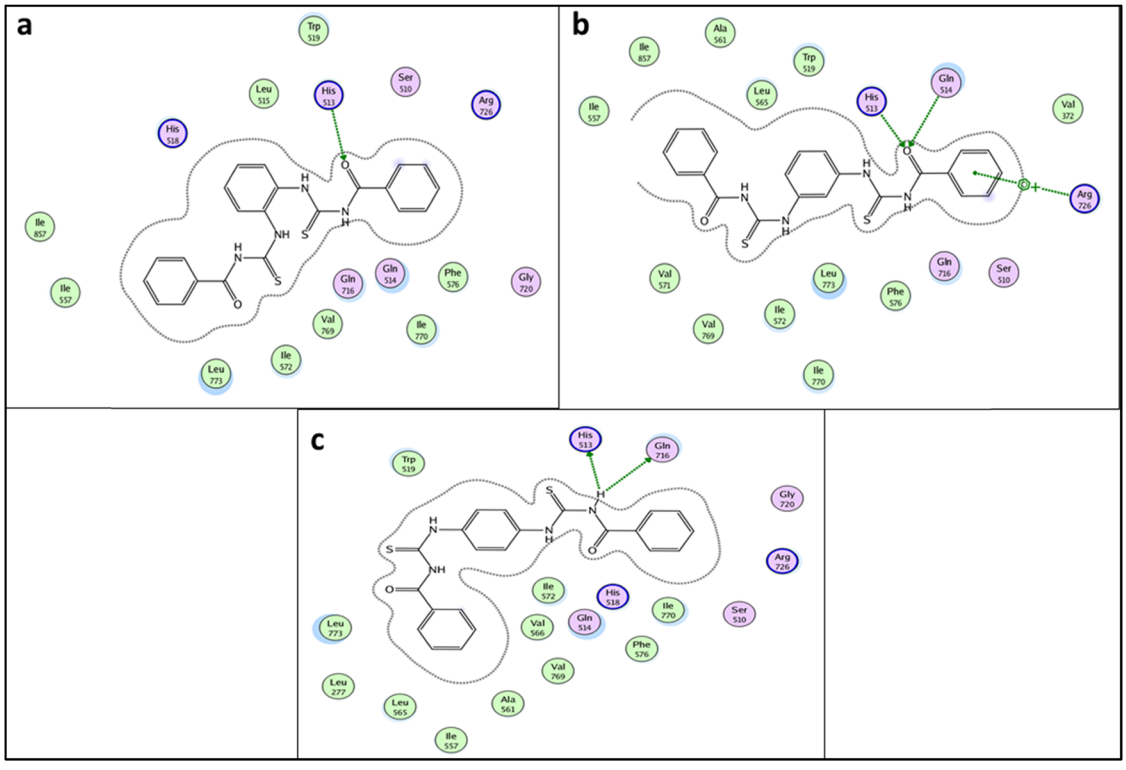


Figure 1: (a) 2D interaction of ligand molecule **SK1** with binding site residues of LOX; (b) 2D interaction of ligand molecule **SK2** with binding site residues of LOX; (c) 2D interaction of ligand molecule **SK3** with binding site residues of LOX.

Table 2: Residue interactions, docking scores, and binding energies of ligand molecules

S.No	Compound	Interacting residues	Docking score	Binding energy (GBVI/WSA)
1	SK1	His513	−13.2354	−25.112
2	SK2	His513, Gln514, Arg726	−15.3783	−28.619
3	SK3	His513, Gln716	−14.7827	−26.555

slightly drops in the action against inflammation of SK2 (Figure 3).

4 Discussion

LOX is a widespread monomeric protein found in cereals and legumes. In human body, LOX stimulates inflammatory reactions. It catalyzes the oxidation of arachidonic acid to produce prostaglandins and leukotrienes [39].

Mahdavi and his coworkers designed a series of novel phenylthiourea derivatives comprising sulfonamide moiety. Most of the derivatives displayed potent inhibition against soybean 15-LOX (IC_{50} < 25 μM). Among the compounds, the 3-methylbenzoyl derivative demonstrated highest inhibition (IC_{50} = 1.8 μM) being 10-folds more than standard quercetin [40]. Doble et al. experienced designed conjugate of thiazole and thiourea targeting LOX. The conjugate inhibitor showed better performance (IC_{50} = 1.4 ± 0.1 μM) than commercial Zileuton (IC_{50} of 1.5 ± 0.3 μM) drug. The potency of conjugate drug against inflammation was further enhanced by incorporating hydrophobic benzoyl pharmacophore and substituting both nitrogens of the thiourea moiety [41].

The *in-vitro* data clearly presented that synthesized bis-thioureas having capability against LOX enzyme. All derivatives SK1, SK2, and SK3 with IC_{50} 68.06, 105.70, and 63.34 μM, respectively, were found to successfully inhibit the LOX enzyme. Statistical data showed that in synthesized bis-thioureas, compounds SK1 and SK3 were found to be more active inhibitors than compound SK2.The existence of amino group at carbon 3 and 4 (ortho and para position) causes significant improvement in inhibition potential of drugs, while its presence at the meta position drops efficacy of SK2.

Naturally, carrageenan is a red seed wood. Chemically, it is a sulfated polysaccharide that induces edema and pain. Researchers employed various edema-inducing agents, including Brewer's yeast, carrageenan, formalin, kaolin, and dextran.

Cheralate et al. synthesized two novel series of triazol urea and triazol thiourea derivatives. The designed compounds were further investigated as anti-inflammatory agent in vivo against carrageenan by the carrageenan-induced paw edema test. It was observed that targeted derivatives significantly reduced the inflammation in paw of rates [32]. Mostafa et al. synthesized some new thiourea derivatives having a well-known sulfonamide moiety. Anti-inflammatory activity was carried out for the targeted compounds using carrageenan-induced rat paw edema assay. The *in vivo* results displayed a regular rise in the inhibitory potentials (decrease in paw thickness) of thioureas observed from 3–4 h. Some compounds were observed as better or similar significant inhibitors comparable to celecoxib and indomethacin, which were used as standards [42].

Here in, the compounds were assessed for the anti-inflammatory activity by using the carrageenan-induced paw edema method. This model is dependent on COX-2 for causing inflammation [41].

In the present study, all the bis-thiourea derivatives showed a significant level of potential against carragenan. The SK1 depicted the highest 53.23%, SK2 35.40%, and SK3 55.09% inhibition response at the second hour, upon 15 mg/kg dose. However, the potency of compounds was folded with concentration. At 30 mg/kg dose, SK1 considerably triggered carrageenan 56.22% at the fourth hour, SK2 56.22% at the third hour, and SK3 57.07% at the second hour.

Per experimental studies, the synthesized derivatives presented moderate (SK2) to good (SK1) and very good (SK3) inhibiting activity. The orientation of the amino group at the o/p position make better the potency of inhibition of SK1 and SK3 compound towards the enzyme LOX. The SK3 remained to be free of steric hindrance towards the enzyme and proved more potent compared to SK1.

To evaluate the underlying anti-inflammatory mechanism of bis-thioureas, the potential involvement of histamine

Table 3: Effects of bis-thiourea on paw edema induced by carrageenan

Test sample/drug	Dosage (mg/kg)	Average thickness of paw (mm)/percentage inhibition				
		1st hour	2nd hour	3rd hour	4th hour	5th hour
Saline	1 ml	0.728 ± 0.037	0.757 ± 0.052	0.750 ± 0.043	0.779 ± 0.050	0.786 ± 0.041
SK1	15	0.377 ± 0.052 (48.21%)	0.354 ± 0.052 (53.23%)	0.354 ± 0.071** (52.80%)	0.376 ± 0.043 (51.73%)	0.418 ± 0.052 (46.81%)
	30	0.358 ± 0.025 (50.82%)	0.344 ± 0.043 (54.55%)	0.336 ± 0.045*** (55.20%)	0.341 ± 0.059 (56.22%)	0.395 ± 0.031 (49.74%)
SK2	15	0.477 ± 0.036 (34.49%)	0.489 ± 0.049 (35.40%)	0.493 ± 0.061* (34.26%)	0.509 ± 0.051 (34.60%)	0.501 ± 0.034 (36.26%)
	30	0.449 ± 0.031 (38.32%)	0.431 ± 0.045 (43.06%)	0.409 ± 0.057** (45.47%)	0.439 ± 0.041 (43.65%)	0.469 ± 0.036 (40.33%)
SK3	15	0.374 ± 0.049 (48.63%)	0.340 ± 0.044 (55.09%)	0.344 ± 0.063*** (51.99%)	0.374 ± 0.039 (51.99%)	0.420 ± 0.061 (46.56%)
	30	0.351 ± 0.033 (51.79%)	0.325 ± 0.039 (57.07%)	0.329 ± 0.051*** (56.13%)	0.349 ± 0.047 (55.20%)	0.389 ± 0.044 (50.51%)
Diclofenac Sodium	10 mg	0.303 ± 0.041 (58.38%)	0.247 ± 0.039 (67.24%)	0.259 ± 0.042*** (65.47%)	0.261 ± 0.045 (66.50%)	0.356 ± 0.038 (54.71%)

Data are presented as mean ± SEM ($n = 6$), * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ significant levels are analyzed by one-way ANOVA followed by the Dunnett test between the sample and control groups.

Figure 2: Effect of bis-thioureas on paw edema induced by carrageenan.

mediator in *in vivo* model was investigated. Upon stimulation, blood releases chemical mediators such as histamine, which triggers the release of prostaglandins and neuropeptides from plasma, resulting in pro-inflammatory effects [2]. Sergey and his coworkers reported the contribution of betulonic triazoles in anti-inflammation. The pharmacological effect of synthesized compounds was screened by using the basic tool histamine-induced paw edema model. It was observed that most of the triazol derivatives of betulonic acid exhibited comparatively low anti-inflammatory activity at a dose range of 20–50 mg/kg compared to that of indomethacin. However, a significant increase in the anti-inflammatory potential of trizoles was noticed by replacing the hexyl substituent on triazol scaffold by aryl, acetyl phenyl, or metoxyphenyl group [43].

In the present study, it was found that tested compounds significantly inhibit histamine at the first hour at 12.5 and 25 mg/kg dose. Also, the effect of drug was found remained significant till 5th hour of the oral administration of inflammation induced by histamine. Thus, the bis-thioureas demonstrated significant inhibitory potentials.

5 Conclusions

This study presents the fruitful synthesis of the bis-thiourea derivatives in very good yields and proved free of toxicity. The designed compounds show that promising anti-inflammatory activity is attributed by pharmacologically active thiourea moiety. The pharmacological screening results also have proved that these compounds are best

Table 4: Inhibition effects of bis-thioureas on edema induced by histamine

Test sample/drug	Dose (mg/kg)	Mean paw edema (mean + SEM) induced by histamine				
		1st hour	2nd hour	3rd hour	4th hour	5th hour
Histamine	1 ml	0.271 ± 0.041	0.251 ± 0.049	0.237 ± 0.038	0.209 ± 0.051	0.221 ± 0.058
SK1	15	0.172 ± 0.039 (36.53%)	0.155 ± 0.037 (38.25%)	0.151 ± 0.035** (36.29%)	0.146 ± 0.044 (30.14%)	0.175 ± 0.039 (20.81%)
	30	0.163 ± 0.048 (39.85%)	0.141 ± 0.033 (43.82%)	0.129 ± 0.039*** (45.57%)	0.141 ± 0.040 (32.54%)	0.172 ± 0.038 (22.17%)
SK2	15	0.194 ± 0.033 (28.41%)	0.169 ± 0.049 (32.67%)	0.145 ± 0.041** (38.82%)	0.149 ± 0.042 (24.875%)	0.183 ± 0.049 (15.044%)
	30	0.175 ± 0.053 (35.42%)	0.159 ± 0.038 (36.65%)	0.140 ± 0.045*** (40.93%)	0.141 ± 0.040 (32.54%)	0.179 ± 0.043 (19.00%)
SK3	15	0.166 ± 0.049 (38.75%)	0.149 ± 0.036 (40.64%)	0.144 ± 0.039* (39.24%)	0.140 ± 0.029 (33.01%)	0.176 ± 0.044 (20.36%)
	30	0.160 ± 0.031 (40.96%)	0.138 ± 0.031 (45.02%)	0.126 ± 0.041*** (46.84%)	0.139 ± 0.040 (33.49%)	0.170 ± 0.049 (23.08%)
Cetirizine	10	0.078 ± 0.024	0.108 ± 0.044	0.081 ± 0.024***	0.119 ± 0.011	0.147 ± 0.069

Data are presented as mean ± SEM ($n = 6$), * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ significant levels are analyzed by one-way ANOVA followed by the Dunnett test between the sample and control groups.

Figure 3: Effect of bis-thioureas on paw edema induced by histamine.

candidates for pain, diabetes, and various types of cancer therapy. Further study is needed to search the whole pharmacologic profile of bis-thioureas, which may provide help to researchers for further exploration in the future.

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