IJCRT.ORG ISSN : 2320-2882

INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS (IJCRT)

An International Open Access, Peer-reviewed, Refereed Journal

Design, Synthesis, Characterization, and Evaluation of Benzoxazole–Thiazole Hybrids as Anti-inflammatory Agents

Nitika*, Dr. Chinu Kumari, Dr. Bhupendra Singh Tomar, Dr. Abhishek Soni

School of Pharmacy, Department of Pharmaceutical Chemistry, Abhilashi University, Chail Chowk, Distt. Mandi, H.P., India.

ABSTRACT

The long-term use of traditional nonsteroidal anti-inflammatory drugs (NSAIDs) is often limited due to side effects, as inflammation is a major pathological condition associated with numerous acute and chronic diseases. The development of safer and more effective anti-inflammatory agents is an important objective in medicinal chemistry. In this study, we designed, synthesized, characterized, and evaluated benzoxazole–thiazole hybrid derivatives for their anti-inflammatory potential, using a molecular hybridization approach. The synthesized compound was characterized using FT-IR, ^{1}H NMR, and ^{13}C NMR spectroscopy to confirm its chemical structure. The anti-inflammatory activity was evaluated through an in vitro protein denaturation assay, with diclofenac sodium serving as the reference standard. Benzoxazole–thiazole hybrids may be promising lead compounds for developing safer anti-inflammatory agents, as a synthesized hybrid exhibited concentration-dependent anti-inflammatory activity. The results demonstrate that the presence of both thiazole and benzoxazole pharmacophores enhanced biological activity. The synthesized benzoxazole–thiazole hybrid shows potential as a lead molecule for the development of safer, more effective anti-inflammatory agents, warranting further pharmacological and mechanistic investigation.

Keywords: Benzoxazole, Thiazole, Molecular Hybridization, Anti-inflammatory Agents, Protein Denaturation Assay, Diclofenac Sodium.

INTRODUCTION

Overview of Inflammation

The body produces inflammation as a natural defense mechanism against harmful stimuli such as infections, tissue damage, toxins, and chemicals. It is an important part of the immune system and helps in the elimination of harmful substances while promoting tissue repair and healing. Acute inflammation is beneficial and resolves after the injury or infection has been controlled. On the other hand, chronic or uncontrolled inflammation can harm healthy tissues and play an important role in the development of

diseases such as rheumatoid arthritis, osteoarthritis, inflammatory bowel disease, asthma, cardiovascular disease, diabetes, and certain cancers. Non-steroidal anti-inflammatory drugs (NSAIDs) currently available on the market are effective at reducing inflammation, but prolonged use of these may result in adverse effects such as hepatotoxicity, renal toxicity, gastrointestinal ulcers, and cardiovascular complications. Therefore, one of the primary goals of pharmaceutical research is the development of safer and more effective anti-inflammatory agents. Because the combination of these two biologically active heterocyclic scaffolds provides enhanced anti-inflammatory activity along with improved safety and therapeutic potential, benzoxazole–thiazole hybrid compounds have attracted significant attention.[1]

Benzoxazole and Thiazole as Promising Heterocyclic Scaffolds

Heterocyclic compounds are one of the most important classes of organic molecules in medicinal chemistry and pharmaceutical sciences due to their broad biological and pharmacological properties. Their physicochemical and biological properties are enhanced by the presence of heteroatoms such as nitrogen, oxygen, and sulfur, which makes them useful scaffolds for drug discovery.[2] Among these, benzoxazole and thiazole have attracted considerable attention because of their broad therapeutic activities, including anti-inflammatory, antimicrobial, anticancer, antioxidant, antiviral, and analgesic properties.[3] Many marketed drugs and biologically active compounds include these heterocyclic nuclei. An effective method for enhancing biological activity and reducing toxicity is molecular hybridization, which combines two or more pharmacophores into a single molecule. Benzoxazole and thiazole derivatives have attracted considerable attention among heterocyclic systems due to their diverse biological activities and favorable physical and chemical properties.[4] Heterocyclic compounds are typically classified based on several criteria, including ring size, the number of heteroatoms, degree of saturation, and aromaticity. Five-membered heterocycles, such as oxazole, thiazole, imidazole, pyrazole, furan, and thiophene, have garnered significant attention due to their wide range of biological activities. Additionally, six-membered heterocycles, including pyridine, pyrimidine, pyrazine, and triazine, serve as important pharmacophores frequently used in medicinal chemistry. These ring structures, electronic distribution, lipophilicity, hydrogen-bonding ability, and interactions with biological targets are all improved by the presence of heteroatoms. These compounds are known for their enhanced therapeutic efficacy, greater bioavailability, and improved receptor binding. As a result, pharmaceutical research continues to concentrate significantly on the synthesis and biological evaluation of newly developed heterocyclic derivatives. [5,6]

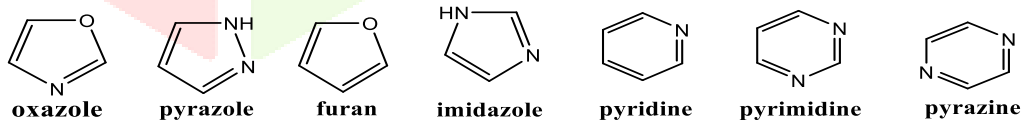


Fig. 1. General Structure of Heterocyclic Compounds

Concept of Molecular Hybridization

Molecular hybridization is recognized as an effective technique for developing therapeutic compounds with diverse applications in modern medicinal chemistry. To enhance pharmacological properties, two or more biologically active pharmacophores combine into a single molecular unit.[7] Molecular hybridization is the process of combining two or more biologically active pharmacophores into a single molecular framework. This approach aims to create hybrid molecules that exhibit enhanced pharmacological activity, improved selectivity, and reduced toxicity. Their hybrid molecule structures are considered promising therapeutic alternatives because both benzoxazole and thiazole nuclei independently exhibit strong antimicrobial and

anti-inflammatory properties. The combination of these pharmacophores has the potential to enhance potency, selectivity, and pharmacokinetic properties.[8]

Benzoxazole

Benzoxazole is a bicyclic aromatic heterocyclic compound formed by the fusion of a benzene ring and an oxazole ring. Fig.2 shows the chemical structure of benzoxazole. The heterocyclic structure features one nitrogen and one oxygen atom, which significantly enhances its biological and chemical reactivity.[9] The structural rigidity, planarity, aromaticity, and strong receptor-binding capabilities of the benzoxazole nucleus have garnered considerable attention. Heteroatoms enhance pharmacological efficacy by increasing hydrogen-bond formation, π-π stacking interactions, and hydrophobic interactions with biological targets. Benzoxazole derivatives possess beneficial physicochemical properties such as metabolic stability, membrane permeability, and moderate lipophilicity.[10] Because of these characteristics, benzoxazole is an important pharmacophore in medicinal chemistry. Various benzoxazole derivatives demonstrate a wide range of biological activities, including anti-inflammatory, antimicrobial, antioxidant, anticancer, antiviral, antitubercular, anticonvulsant, and analgesic effects. The benzoxazole ring's efficacy and selectivity for different therapeutic targets are significantly influenced by structural modifications, as shown in several studies. Benzoxazole serves as a suitable scaffold for developing multifunctional pharmaceutical compounds. Substitution at the 2-position of benzoxazole has been demonstrated in various studies to significantly influence biological activity. Electron-donating and electron-withdrawing groups attached to the benzoxazole nucleus can enhance pharmacological properties such as lipophilicity, potency, and selectivity.[11]

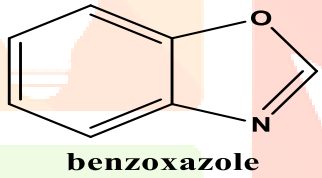


Fig 2. Chemical structure of benzoxazole

Table 1. Physicochemical Properties of Benzoxazole [12]

Property	Benzoxazole
Chemical Formula	C_7H_5NO
Molecular Weight	119.12 g/mol
Structure Type	Fused benzene–oxazole ring
Physical State (at RT)	Colorless to pale yellow Solid
Appearance	White to light yellow solid
Melting Point	27–30°C
Boiling Point	280–300°C
Density	1.20 g/cm³

Thiazole

Thiazole is a five-membered aromatic heterocyclic compound containing one sulfur atom and one nitrogen atom within its ring system. It serves as an important pharmacophore frequently found in both synthetic and natural medicinal compounds. Thiazole derivatives exhibit a broad spectrum of biological activities due to their aromaticity, electrochemical properties, and capacity to interact with various biological receptors.[13] Thiazole-containing compounds have been reported to possess anti-inflammatory, antimicrobial, antifungal, antioxidant, anticancer, antitubercular, anticonvulsant, and antiviral activities. The presence of nitrogen and sulfur atoms in the ring structure enhances pharmacokinetic properties and biological interactions.[14] Several

clinically used drugs, including sulfathiazole, ritonavir, and abafungin, contain the thiazole nucleus. Modification at different ring positions can improve therapeutic potential and modify electrical distribution. Thiazole derivatives remain important targets in medicinal chemistry research due to their wide range of pharmacological effects. Combining thiazole with other biologically active heterocycles could yield stronger, less toxic compounds. The thiazole ring, a five-membered heterocycle containing nitrogen and sulfur, is a key structural motif in numerous bioactive compounds (Fig. 2).[15]

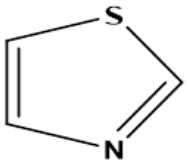


Fig 3. Structure of thiazole

The C-5 position is more reactive to electrophiles; therefore, techniques like sulfonation, halogenation, and nitration are usually used here. At the C-2 position, several groups are added by nucleophilic substitution and coupling processes, such as Suzuki and Heck. Metalation techniques like lithiation further aid in selective substitution. These processes allow the synthesis of several useful thiazole derivatives.[16]

Table 2. Physicochemical Properties of Thiazole [17]

Property	Thiazole
Chemical Formula	C_3H_3NS
Molecular Weight	85.12 g/mol
Structure Type	Five-membered heterocycle with N and S
Physical State (at RT)	Colorless to pale-yellow liquid
Appearance	Pale yellow liquid
Melting Point	−20 to −5°C
Boiling Point	116–118°C
Density	1.10 g/cm³

Anti-inflammatory Activity of Benzoxazole:

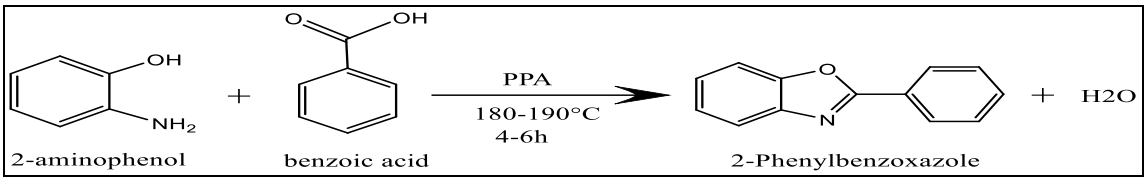
Benzoxazole is an important heterocyclic scaffold with strong anti-inflammatory activity. Inflammatory mediators such as prostaglandins, cytokines, and cyclooxygenase (COX) enzymes are inhibited by many benzoxazole derivatives. It has been shown that structural modifications at different benzoxazole ring positions improve the compounds' anti-inflammatory efficacy and selectivity. The benzoxazole nucleus has received significant attention in the development of new anti-inflammatory agents due to its promising pharmacological profile and low toxicity.[18]

Anti-inflammatory Activity of Thiazole:

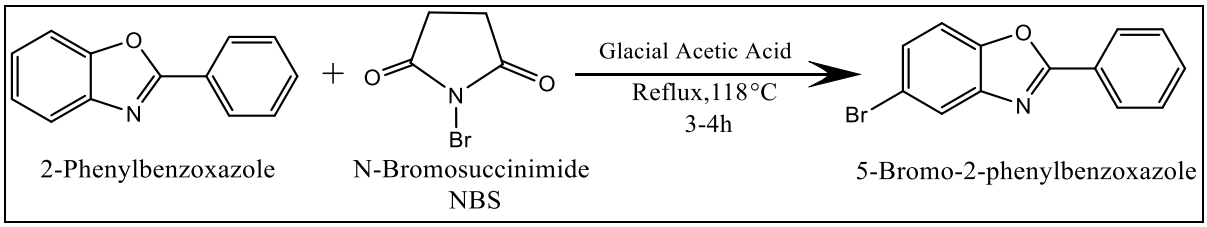
Its thiazole heterocyclic ring is biologically active and exhibits excellent anti-inflammatory properties. By inhibiting cyclooxygenase (COX) enzymes and inhibiting the formation of pro-inflammatory mediators such as prostaglandins and cytokines, thiazole derivatives have been shown to lower inflammation. Improved anti-inflammatory activity results from the thiazole ring's increased interaction with biological targets due to the presence of nitrogen and sulfur atoms. Thus, in developing novel anti-inflammatory drugs, thiazole is an important pharmacophore.[19]

REACTION SCHEME

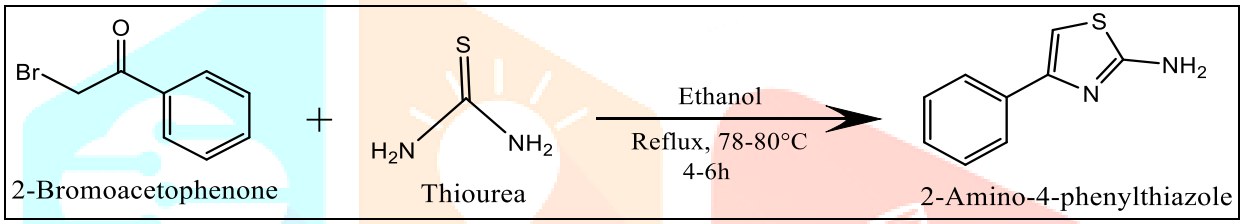
Step 1: Synthesis of 2-Phenylbenzoxazole



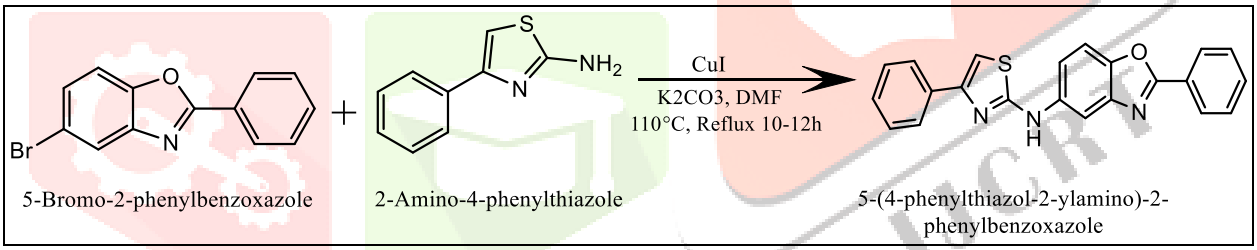
Step 2: Synthesis of Benzoxazole Derivative



Step 3: Synthesis of Thiazole Derivative



Step 4: Synthesis of Benzoxazole-Thiazole Hybrid



RESEARCH METHODOLOGY

Research Design

This work aimed to design, synthesize, characterize, and evaluate new benzoxazole–thiazole hybrid compounds as potential anti-inflammatory agents. Thin Layer Chromatography (TLC), melting point determination, FT-IR, ^{1}H NMR, and ^{13}C NMR spectroscopy were performed to isolate and structurally characterize the synthesized compounds. Diclofenac sodium was used as the reference standard in the in vitro protein denaturation study to evaluate their anti-inflammatory activity. The anti-inflammatory potential of the synthesized benzoxazole–thiazole hybrid compounds was evaluated by comparing the results obtained with the standard drug.[20]

Design of Experimental Research

Standard organic synthesis methods were followed in a multi-step synthetic approach to synthesize the benzoxazole–thiazole hybrid compounds. When appropriate, column chromatography and recrystallization were used to purify the synthesized compounds. Thin Layer Chromatography (TLC) was a method to monitor the compounds' purity, while FT-IR spectroscopy, UV-visible spectroscopy, ^{1}H NMR spectroscopy, and ^{13}C

NMR spectroscopy were used to characterize their structures. The structure–activity relationship (SAR) was determined through analyzing the results of the biological evaluation of the synthesized compounds to evaluate their anti-inflammatory activity.[21]

Design of Biological Evaluation

Standard in vitro anti-inflammatory assays were performed to evaluate the biological activity of the synthesized benzoxazole–thiazole hybrid compounds. Diclofenac sodium was used as the standard drug in the protein denaturation (egg albumin) assay to evaluate its anti-inflammatory activity. In order to determine the structure–activity relationship (SAR) and identify compounds with promising anti-inflammatory potential, the biological activities of the synthesized compounds were compared with the standard drug.[22]

Materials and Methods

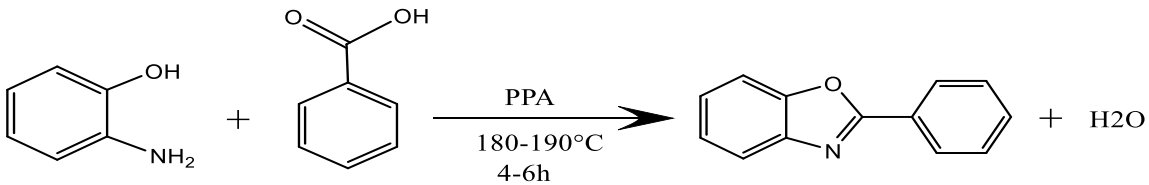
Chemicals and Reagents: The chemicals and reagents used for synthesizing benzoxazole–thiazole hybrid derivatives included 2-aminophenol, benzoic acid, polyphosphoric acid (PPA), 2-phenylbenzoxazole, bromine, glacial acetic acid, N-bromosuccinimide (NBS), sodium bicarbonate, dimethyl sulfoxide (DMSO), 2-amino-4-phenylthiazole, potassium carbonate (K_2CO_3), N, N-dimethylformamide (DMF), ethanol, 2-bromoacetophenone, thiourea, n-hexane, distilled water, silica gel (60–120 mesh), and copper(I) iodide (CuI). All chemicals and reagents were of analytical grade and used without further purification. All chemicals and reagents used in this study were of analytical grade and were employed without additional purification.

Instruments: Benzoxazole–thiazole hybrid compounds were synthesized and characterized using various reagents and standard equipment, including round-bottom flasks (250 mL), reflux condenser, heating mantle, thermometer, thin-layer chromatography (TLC) chamber, column chromatography, Silica Gel 60 F_{21} TLC plates, UV chamber (254 and 366 nm), rotary evaporator, Büchner funnel with vacuum filtration assembly, vacuum pump, hot air oven, melting point apparatus, magnetic stirrer, fume hood, glassware including measuring cylinders, pipettes, volumetric flasks, funnels, and glass rods, beakers, watch glasses, conical flask. During the synthesis, purification, and characterization of the compounds, each piece of glassware was carefully cleaned, dried, and used.

Experimental Procedure

Step 1: Synthesis of 2-Phenylbenzoxazole

Reaction Scheme



2-aminophenol benzoic acid 2-Phenylbenzoxazole

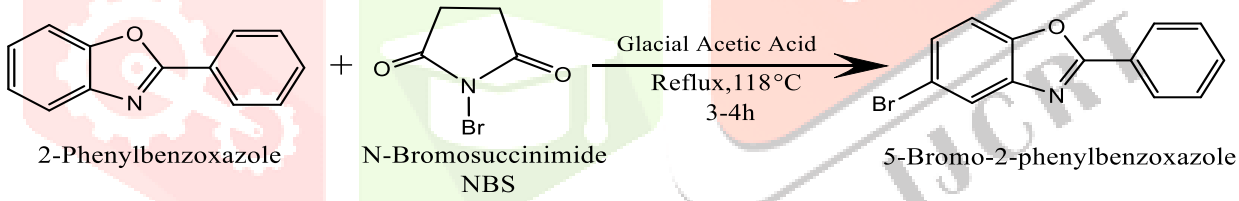
2-Phenylbenzoxazole was synthesized by the cyclocondensation of 2-aminophenol with benzoic acid in the presence of polyphosphoric acid (PPA). The dehydrating agent also served as the reaction medium. First, a dry, clean 250 mL round-bottom flask was filled with 10.91 g of 2-aminophenol and 12.21 g of benzoic acid. To form a homogeneous reaction mixture, 45–50 g of polyphosphoric acid (PPA) was slowly added while being continuously stirred. The high viscosity of PPA facilitated the formation of the benzoxazole ring through intramolecular cyclization and dehydration. A heating mantle was used to slowly raise the temperature of the reaction mixture to 180–190°C. With continuous stirring, the reaction was maintained at

this temperature for 4-6hours. During this period, the condensation reaction yielded an intermediate amide, which was subsequently modified and purified to afford 2-phenylbenzoxazole. TLC (thin-layer chromatography) was used to monitor the reaction's progress. A small amount of the reaction mixture was taken at regular intervals, diluted with ethyl acetate, and spotted on Silica Gel 60 F_{2}[21] TLC plates with samples of the starting material. The plates were prepared using hexane: ethyl acetate (8:2 or 7:3, v/v) as the mobile phase, and they were examined under UV light (254 nm). When the spot for 2-aminophenol disappeared and a spot for the new product appeared, the reaction was considered complete. The reaction mixture was slowly added to 300 mL of crushed ice-cold water while continuously stirred until the reaction was completed. After that, it was allowed to cool to approximately 60-70 °C. The excess polyphosphoric acid was separated, the reaction was quenched, and the crude product was allowed to form. To remove any residual phosphoric acid and other water-soluble impurities, the resulting solid was collected by vacuum filtration using a Büchner funnel and was frequently washed with cold distilled water until the washings were nearly neutral.

Recrystallization from ethanol was used to purify the crude product after it was essentially dried at room temperature. The minimum quantity of hot ethanol was used to dissolve the crude product, and remaining insoluble impurities were filtered out of the heated mixture. After allowing the clear filtrate to cool down to room temperature, it was placed in an ice bath for 30 to 60 minutes to finish crystallizing. Vacuum filtration was used to collect the purified crystals, which were subsequently washed with a small quantity of ice-cold ethanol and dried at 50–60°C in a hot-air oven until they obtained a uniform weight. The final product, 2-phenylbenzoxazole, was obtained as a white to pale yellow crystalline solid. The purified compound was transferred to a clean, dry, airtight container and stored for further characterization by FT-IR, UV–Visible spectroscopy, ^{1}H NMR spectroscopy, ^{13}C NMR spectroscopy, and melting point determination. [23,24]

Step 2: Synthesis of Benzoxazole Derivative

Reaction Scheme



N-bromosuccinimide (NBS) was used for electrophilic bromination of 2-phenylbenzoxazole in glacial acetic acid to produce 5-bromo-2-phenylbenzoxazole. Firstly, a dry 250 mL round-bottom flask with a magnetic stir bar was used to dissolve 19.5 g of 2-phenylbenzoxazole in 100 mL of glacial acetic acid. Until a clear, homogeneous solution was obtained, the reaction mixture was stirred. N-bromosuccinimide (NBS) was used. Initially, a dry 250 mL round-bottom flask with a magnetic stir bar was used to dissolve 19.5 g of 2-phenylbenzoxazole in 100 mL of glacial acetic acid. Until a clear, homogeneous solution was obtained, the reaction mixture was stirred.

The partially exothermic bromination process was then controlled by adding 17.80 g of N-bromosuccinimide (NBS) dropwise over 10 to 15 minutes while stirring continuously and maintaining the reaction temperature below 40°C. After complete addition, a reflux condenser was added, and the reaction mixture was continuously stirred while heated under reflux at 118°C for three to four hours using a heating mantle. 5-bromo-2-phenylbenzoxazole was produced during this period through an electrophilic substitution that mostly occurred at the benzoxazole ring's 5-position.

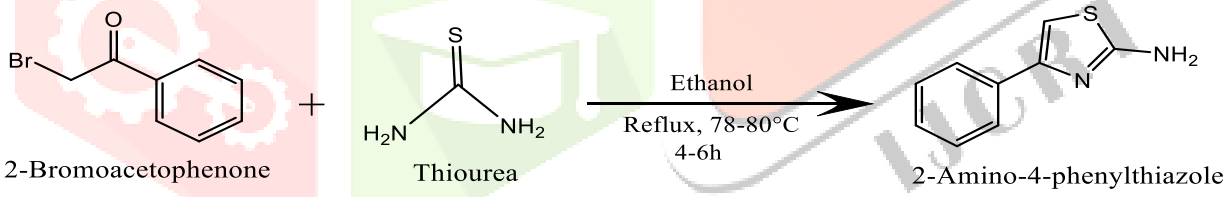
Thin Layer Chromatography (TLC) was employed to monitor the reaction's progress. Samples of the starting material and a small amount of the reaction mixture were taken out at regular intervals, diluted with ethyl acetate, and spotted on Silica Gel 60 F_{254} TLC plates. Hexane: ethyl acetate (8:2, v/v) was used as the mobile phase to develop the plates, and the plates were seen under UV light (254 nm). The reaction was completed when the first material spot disappeared and a new product spot appeared.

After the reaction was completed, the mixture was allowed to stand at room temperature before slowly adding it to 300 mL of crushed ice-cold water while stirring continuously. To get rid of any residual acetic acid, succinimide, and other water-soluble contaminants, the precipitate was collected by vacuum filtering using a Büchner funnel and carefully cleaned with cold distilled water. Recrystallization from ethanol was used to purify the crude product after it had been dried at room temperature. Vacuum filtration was used to collect the purified crystals. The crystals were subsequently rinsed with a small amount of ice-cold ethanol and then dried in a hot-air oven at 50–60°C until they reached a constant weight.

5-bromo-2-phenylbenzoxazole, the final product, was obtained as an off-white to pale yellow crystalline solid. For further characterization by FT-IR, UV-visible spectroscopy, ^{1}H NMR spectroscopy, ^{13}C NMR spectroscopy, mass spectrometry, and melting point determination, the purified compound was transferred to a clean, dry, airtight container.

In glacial acetic acid, the reaction mixture initially appeared as a clear, pale-yellow solution. The solution turned slightly yellow when N-bromosuccinimide was slowly added, accompanied by slight heat evolution. The reaction mixture darkened during reflux, indicating progress of bromination. An off-white to pale yellow precipitate separated from the reaction mixture after being poured into ice-cold water. Pure 5-bromo-2-phenylbenzoxazole was produced as off-white to pale yellow crystals after recrystallization, making it suitable for further synthesis and characterization. [25,26]

Step 3: Synthesis of Thiazole Derivative



2-Bromoacetophenone + Thiourea → 2-Amino-4-phenylthiazole

2-bromoacetophenone with thiourea was synthesized in ethanol to form 2-amino-4-phenylthiazole. Initially, a clean, dry 500 mL round-bottom flask with a magnetic stir bar was used to dissolve 19.90 g of 2-bromoacetophenone in 150–200 mL of ethanol. Until a clear, uniform solution was achieved, the reaction mixture was stirred. With continuous stirring, 7.61 g of thiourea was added gradually. After complete addition, a reflux condenser was added, and the reaction mixture was continuously stirred for four to six hours while being heated under reflux at 78 - 80°C using a heating mantle. During this time, 2-amino-4-phenylthiazole was produced by intramolecular cyclization after nucleophilic substitution.

Thin Layer Chromatography (TLC) was used to monitor the reaction's progress. Samples of the starting material and small aliquots of the reaction mixture were taken out at regular intervals, diluted with ethyl acetate, and spotted on Silica Gel 60 F_{254} TLC plates. Hexane: ethyl acetate (7:3, v/v) served as the mobile phase to develop the plates, which then appeared under UV light (254 nm). The reaction was completed when the spot indicating 2-bromoacetophenone disappeared and a new product spot appeared.

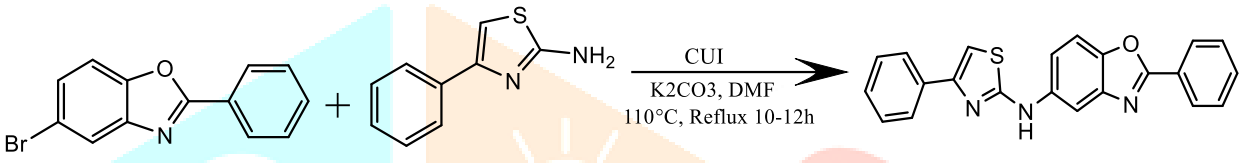
After completion of the reaction, the reaction mixture was allowed to cool at room temperature, then slowly poured into 300 mL of ice-cold distilled water while stirring continuously. A pale-yellow precipitate separated from the reaction mixture. The precipitated solid was collected through vacuum filtration with a Büchner

funnel and washed multiple times with cold distilled water to eliminate any residual thiourea, inorganic impurities, and other soluble materials.

The crude product was dried at room temperature and purified by recrystallization from ethanol. The crude solid was dissolved in the smallest amount of hot ethanol, and the hot solution was filtered, if necessary, to eliminate any insoluble impurities. The clear filtrate was allowed to cool slowly to room temperature and then kept in an ice bath for 30–60 minutes to complete crystallization. The purified crystals were collected by vacuum filtration, washed with a small amount of ice-cold ethanol, and dried in a hot-air oven at 50–60°C until a constant weight was obtained.

2-amino-4-phenylthiazole, the final product, was obtained as a crystalline solid that was white to pale yellow in color. FT-IR, UV–visible spectroscopy, ^{1}H NMR spectroscopy, ^{13}C NMR spectroscopy, and melting point determination will be used to further characterize the purified compound, which was stored in a dry, clean, airtight container.[27]

Step 4: Synthesis of Benzoxazole-Thiazole Hybrid



5-Bromo-2-phenylbenzoxazole 2-Amino-4-phenylthiazole 5-(4-phenylthiazol-2-ylamino)-2-phenylbenzoxazole

Using dry N, N-dimethylformamide (DMF) as the reaction solvent, a copper (I)-catalyzed Ullmann C–N coupling reaction between 5-bromo-2-phenylbenzoxazole and 2-amino-4-phenylthiazole in the presence of potassium carbonate produced 5-(4-Phenylthiazol-2-ylamino)-2-phenylbenzoxazole. Cover a dry, clean 500 mL round-bottom flask with a magnetic stir bar, then add 27.30 g of 5-bromo-2-phenylbenzoxazole and 17.62 g of 2-amino-4-phenylthiazole. The reaction mixture was then stirred until a homogeneous suspension was obtained after adding 150–200 mL of dry DMF. The catalyst, copper (I) iodide (1.90 g), was added after potassium carbonate (20.70 g). To ensure uniform mixing, the reaction mixture was stirred for 10 to 15 minutes at room temperature.

The reaction mixture was heated under reflux at 110°C using a heating mantle for 10–12 hours while continuously stirred, with a reflux condenser attached to the flask. The copper-catalyzed C–N coupling reaction continued at this point, exchanging the amino group of 2-amino-4-phenylthiazole for the bromine atom of 5-bromo-2-phenylbenzoxazole to produce 5-(4-phenylthiazol-2-ylamino)-2-phenylbenzoxazole. Thin Layer Chromatography (TLC) was used to monitor the reaction's progress. Samples of the starting material and small amounts of the reaction mixture were collected at regular intervals, diluted with ethyl acetate, and spotted on Silica Gel 60

F_2[21] TLC plates. Hexane: ethyl acetate (7:3, v/v) was used as the mobile phase to develop the plates, and then appeared under UV light (254 nm). The label corresponding to 5-bromo-2-phenylbenzoxazole disappeared, and the reaction was completed when the spot of 5-bromo-2-phenylbenzoxazole disappeared and a new product spot formed.

Fig 4. Thin-Layer Chromatography (TLC) of the Benzoxazole-Thiazole Hybrid

After the reaction was completed, the mixture was allowed to cool to room temperature before being gently added to 500 mL of ice-cold distilled water while stirring. Immediately, a solid precipitate separated. To remove the residual DMF, potassium carbonate, copper salts, and other water-soluble impurities, the precipitated product was collected by vacuum filtration using a Büchner funnel and carefully washed with cold distilled water until the washings were neutral.

Recrystallization from ethanol to purify the crude product after drying at room temperature. The minimum quantity of hot ethanol was used to dissolve the crude material, and remaining insoluble particles were filtered out of the heated mixture. After cooling the clear filtrate to room temperature, placed in an ice bath for 30 to 60 minutes to complete crystallization. Vacuum filtration was used to collect the purified crystals, which were cleaned with a small quantity of ice-cold ethanol and dried at 50–60°C in a hot-air oven until they reached a constant weight.

Fig 5. Purified Benzoxazole-Thiazole Hybrid Product

5-(4-phenylthiazol-2-ylamino)-2-phenylbenzoxazole, the final product, was obtained as a crystalline solid, yellow to pale brown. FT-IR, UV-visible, ^{1}H NMR, ^{13}C NMR, mass spectrometry, and melting point determination were used to further characterize the purified product after it was transferred to a clean, dry, airtight container. In dry DMF, the reaction mixture appeared as a light brown suspension. The suspension turned brown after copper (I) iodide and potassium carbonate were added. The color eventually turned dark brown during reflux, indicating the coupling reaction's progression. A yellow to pale brown precipitate separated from the solution after the reaction mixture was poured into ice-cold water. Pure 5-(4-phenylthiazol-2-ylamino)-2-phenylbenzoxazole was produced as yellow to pale brown crystals after recrystallization, which is suitable for structural analysis and biological evaluation. [28,29]

Calculation of Retention Factor (Rf)

value was calculated using the following equation:

Rf=Distance travelled by the solvent front/ Distance travelled by the compound (spot)

Distance travelled by the compound = 2.5 cm

Distance travelled by the solvent front = 5.0 cm

Rf=2.5/ 5.0 = 0.50

Rf value= 0.50

Step 5: In Vitro Anti-inflammatory Study of Benzoxazole–Thiazole Hybrids

Chemicals and Reagents: The chemicals and reagents used in the present study included the synthesized benzoxazole–thiazole hybrid as the test compounds, diclofenac sodium as the reference standard, fresh hen's egg albumin (egg white) as the protein source, phosphate buffered saline (PBS, pH 7.4) for maintaining the physiological pH, dimethyl sulfoxide (DMSO, analytical grade) as the solvent, 0.1 N sodium hydroxide (NaOH) and 0.1 N hydrochloric acid (HCl) for pH adjustment, and distilled water for the preparation of solutions and dilution of reagents. All chemicals and reagents used in the study were of analytical reagent (AR) grade and were used without further purification.

Preparation of Solutions

- Fresh 0.5% (v/v) Egg Albumin Solution
- Phosphate Buffered Saline (PBS), pH 7.4
- Stock solution of synthesized benzoxazole–thiazole hybrid derivatives (1 mg/mL in DMSO)
- Diclofenac Sodium Stock Solution (1 mg/mL)
- Working solutions of 50, 100, 200, and 400 µg/mL of both synthesized compounds and Diclofenac sodium

Glassware: The glassware used in the present study included test tubes (15 and 20 mL), a test tube rack, micropipettes (20–200 µL and 100–1000 µL) with sterile micropipette tips, measuring cylinders (10, 50, and 100 mL), volumetric flasks (10, 50, and 100 mL), glass beakers (50, 100, and 250 mL), a glass stirring rod, Whatman filter paper or muslin cloth, quartz or glass cuvettes, and a pH meter. All glassware was thoroughly cleaned, rinsed with distilled water, and dried before use to avoid contamination and ensure the accuracy and reliability of the experimental results.

Instruments and Equipment: The instruments and equipment used during the study included a UV–Visible spectrophotometer (660 nm) for absorbance measurements, a water bath maintained at 70°C, an incubator maintained at 37°C, an analytical balance for precise weighing of chemicals, a magnetic stirrer for uniform mixing of solutions, a vortex mixer for efficient sample homogenization, a refrigerator maintained at 4°C for the storage of reagents and prepared samples, and a stopwatch or timer for accurate monitoring of reaction and incubation times.

Protein Denaturation Assay (Egg Albumin Method)

The in vitro anti-inflammatory activity of the synthesized benzoxazole–thiazole hybrid compound was evaluated using the protein denaturation assay (egg albumin method), based on its ability to inhibit heat-induced protein denaturation, which is considered one of the important mechanisms involved in inflammation. The synthesized hybrid derivatives were prepared using thiazole as the precursor and substituted benzoxazole derivatives through molecular hybridization. Diclofenac sodium was used as the reference standard for comparison.

Fresh 0.5% (v/v) egg albumin solution was prepared immediately before the experiment. A fresh hen's egg was washed thoroughly with distilled water, and the egg white (albumin) was carefully separated from the yolk. The egg white was filtered through muslin cloth or Whatman filter paper to remove fibrous material. 0.5 mL of the filtered egg albumin was diluted to 100 mL with phosphate-buffered saline (PBS, pH 7.4) to prepare a 0.5% (v/v) egg albumin solution. The solution was gently mixed and used immediately.

Stock solutions of the synthesized benzoxazole–thiazole hybrid were prepared by dissolving accurately weighed quantities of each synthesized compound in dimethyl sulfoxide (DMSO) to obtain a concentration of 1 mg/mL. The stock solutions were further diluted with phosphate-buffered saline (PBS, pH 7.4) to prepare working concentrations of 50, 100, 200, and 400 µg/mL. Similarly, a 1 mg/mL Diclofenac sodium stock solution was prepared and diluted to obtain the same concentrations for use as the standard.

Fig 6. pH meter

For each concentration, the reaction mixture consisted of 0.5 mL of freshly prepared 0.5% egg albumin solution, 0.5 mL of phosphate-buffered saline (PBS, pH 7.4), and 1.0 mL of the synthesized benzoxazole–thiazole hybrid derivative solution. The control group contained 0.5 mL egg albumin solution, 0.5 mL PBS, and 1.0 mL DMSO instead of the test compound, whereas the standard group contained 1.0 mL Diclofenac sodium solution in place of the test compound.

The pH of all reaction mixtures was adjusted to 7.4 ± 0.2, when necessary, using 0.1 N NaOH or 0.1 N HCl. The reaction mixtures were incubated at 37°C for 15 minutes to allow interaction between the synthesized compounds and egg albumin. Subsequently, the test tubes were heated in a water bath maintained at 70°C for 5 minutes to induce protein denaturation. After heating, the mixtures were allowed to cool naturally to room temperature. The absorbance of each sample was measured at 660 nm using a UV–Visible spectrophotometer, with PBS serving as the blank. All experiments were carried out in triplicate, and the mean absorbance values were recorded.

The percentage inhibition of protein denaturation was calculated using the following equation:

$$\% \text{ Inhibition } = \frac{A_c - A_t}{A_c} \times 10$$

where **Ac** represents the absorbance of the control and **At** represents the absorbance of the test compound or standard drug. A higher percentage inhibition indicated greater anti-inflammatory activity. The anti-inflammatory activity of the synthesized benzoxazole–thiazole hybrid derivatives was compared with that of

Diclofenac sodium, and the results were expressed as mean ± standard deviation (SD) of three independent experiments. Compounds showing inhibition comparable to or greater than the reference drug were considered promising candidates for further anti-inflammatory investigation. [30,31]

RESULT AND DISCUSSION

Physical Characteristics of the Synthesized Compound

The synthesized benzoxazole–thiazole hybrid (NGH-01) was obtained as a Pale yellow crystalline solid after the final stage of the synthesis and purification. Thin-layer chromatography (TLC) was performed to monitor the reaction's progress and the purity of the synthesized compound, confirming the synthesis of the desired compound. Successful synthesis and effective purification were shown by the isolated compound's uniform crystalline appearance without visible impurities.

Table 3. Physical Characteristics of the Synthesized Benzoxazole-Thiazole Hybrid Compound (NGH-01)

Parameter	Observation
Compound Code	NGH-01
IUPAC Name	N-(2-phenyl-1,3-benzoxazol-5-yl)-4-phenyl-1,3-thiazol-2-amine
Molecular Formula	$C_{22}H_{15}N_3OS$
Molecular Weight	369.44 g/mol
Physical State	Solid
Color	pale yellow
Appearance	Crystalline powder
Rf Value	0.5
TLC Solvent System	Ethyl acetate: n-Hexane
Solubility	Soluble in DMSO and DMF, slightly soluble in methanol and ethanol, and practically insoluble in water.

FTIR Characterization

Compound NGH-01's FTIR spectrum confirmed that the synthesized benzoxazole–thiazole hybrid derivative was successfully synthesized. The N–H stretching vibration was identified as the source of the broad absorption band observed at 3376.59–3263.19 cm^{-1}. Aromatic C–A distinctive absorption band indicated H stretching at 3123.81 cm^{-1} and 1625.19 cm^{-1} (heteroaromatic ring vibration), indicating the presence of the azomethine (C=N) group. Aromatic C=C stretching and C–N stretching were determined as the source of the absorption bands at 1500.21 cm^{-1} and 1368.79 cm^{-1}, respectively. Furthermore, the peaks between 830 and 719 cm^{-1} corresponded to aromatic C–H out-of-plane bending, whereas the bands seen in the 1188–1010 cm^{-1} region were identified as C–O/C–N stretching vibrations. The absorption bands observed at 1365, 1306, 1184, 1041, and 1024 cm^{-1} are attributed to the C–N and C–O–C stretching vibrations within the benzoxazole–thiazole framework. The specific absorption bands observed, which closely match the proposed structure, confirm the successful synthesis of the new benzoxazole–thiazole hybrid derivative (NGH-01).

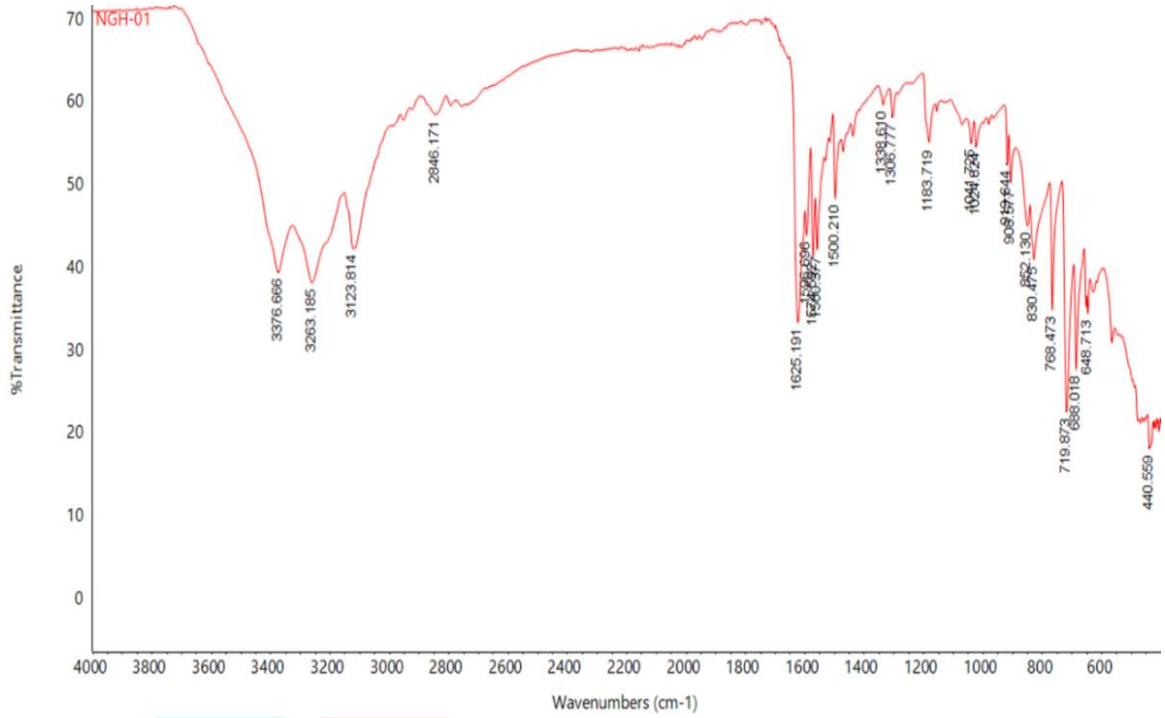


Fig 7. FTIR spectrum of 5-(4-Phenylthiazol-2-ylamino)-2-phenylbenzoxazole

^{1}H NMR Characterization

The proposed benzoxazole–the ^{1}H NMR spectrum analysis of compound NGH-01 supported the proposed structure. The presence of the amine linkage in the synthesized molecule was confirmed as a singlet observed at δ 8.90–9.10 ppm, which corresponded to the secondary amine (–NH–) proton. The aromatic protons of the benzoxazole ring were identified as a doublet at δ 7.72–7.78 ppm (2H). In addition, the aromatic protons of the phenyl rings and the benzoxazole nucleus appeared as a doublet at δ 7.50–7.70 ppm (8H). The remaining aromatic and thiazole ring protons were assigned to a doublet that appeared at δ 7.25–7.50 ppm (3H). The successful synthesis of the proposed benzoxazole–thiazole hybrid derivative (NGH-01) was confirmed by analyzing the chemical shifts, multiplicities, and integration values, which were consistent with the reported molecular structure.

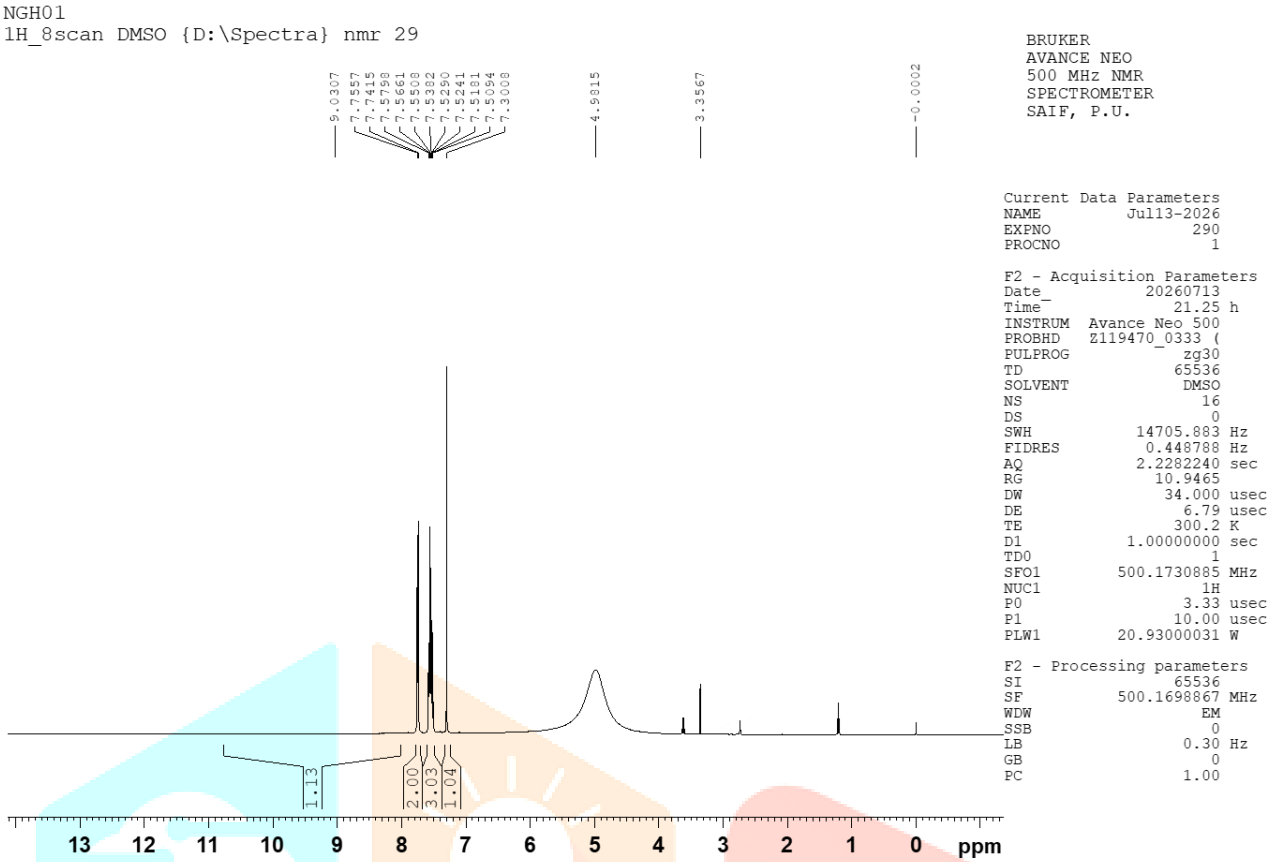


Fig 8.1. ^{1}H NMR spectra of 5-(4-Phenylthiazol-2-ylamino)-2-phenylbenzoxazole

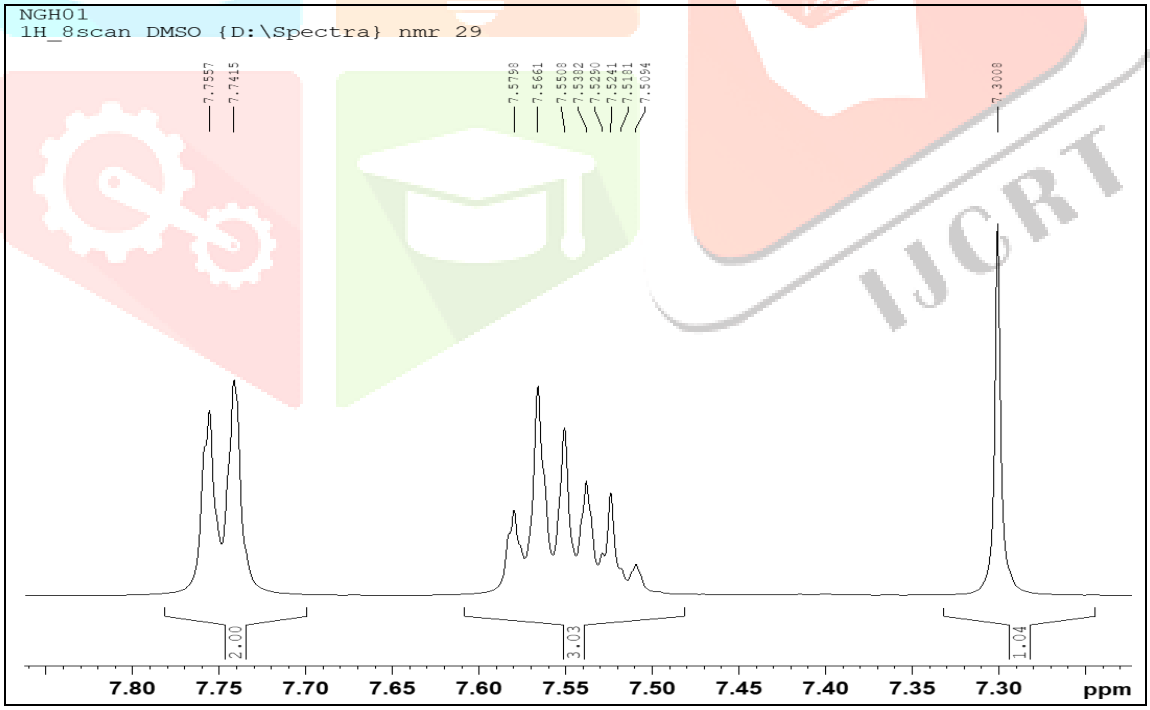


Fig 8.2. ^{1}H NMR Zoom spectra of 5-(4-Phenylthiazol-2-ylamino)-2-phenylbenzoxazole

^{13}C NMR Characterization

The proposed benzoxazole–the ^{13}C NMR spectrum of compound NGH-01 supported the proposed structure. The presence of a heteroaromatic carbon environment was indicated by a distinct resonance at δ 170.5 ppm, which corresponds to the C=N carbon within the benzoxazole–thiazole heterocyclic system. The benzoxazole and phenyl rings' quaternary and aromatic carbons were shown as the basis of the signals at δ 139.2, 130.0, 129.0, 128.6, and 127.4 ppm. The signal at δ 39.5 ppm corresponded to the DMSO-d_3 solvent carbon, but the resonance at δ 103.5 ppm corresponded to the C–O carbon of the benzoxazole ring. The successful synthesis of the proposed benzoxazole–thiazole hybrid derivative (NGH-01) was confirmed by the strong correlation between the observed chemical shifts and the predicted molecular structure.

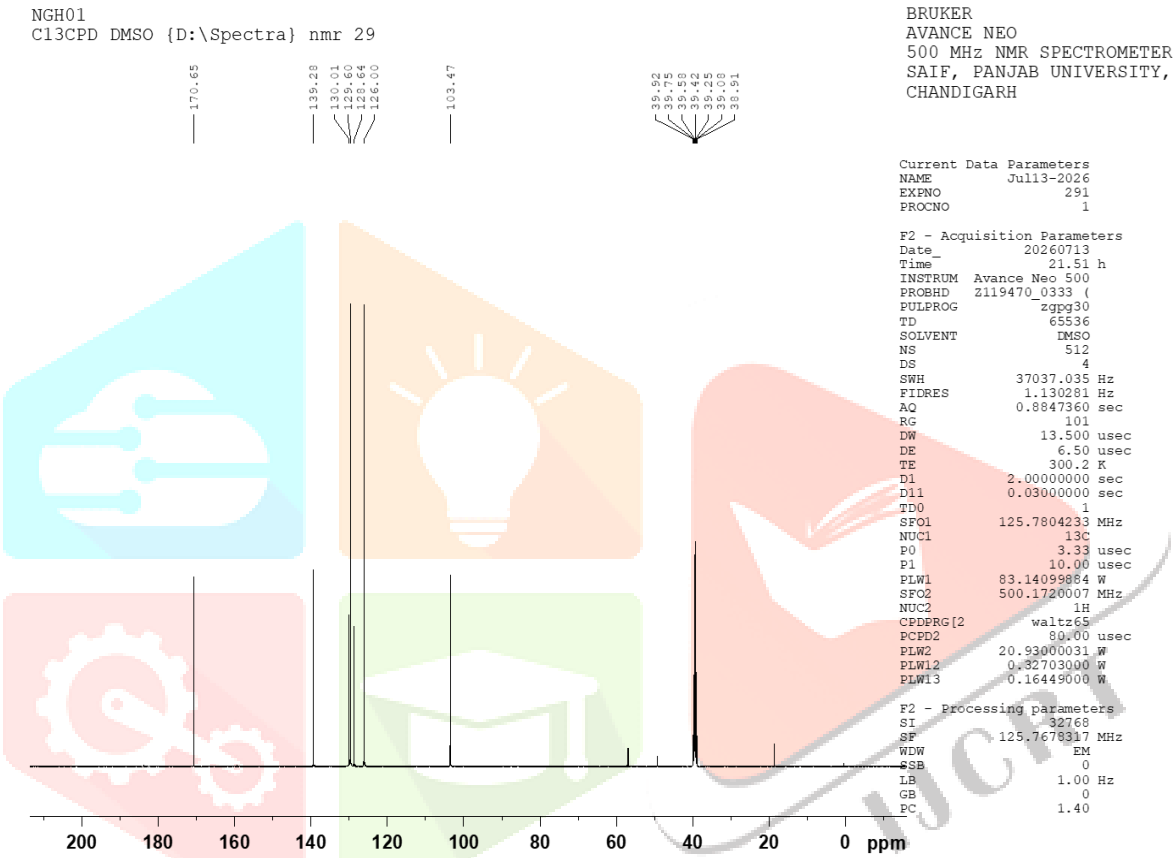


Fig 8.3. ^{13}CNMR spectra of 5-(4-Phenylthiazol-2-ylamino)-2-phenylbenzoxazole

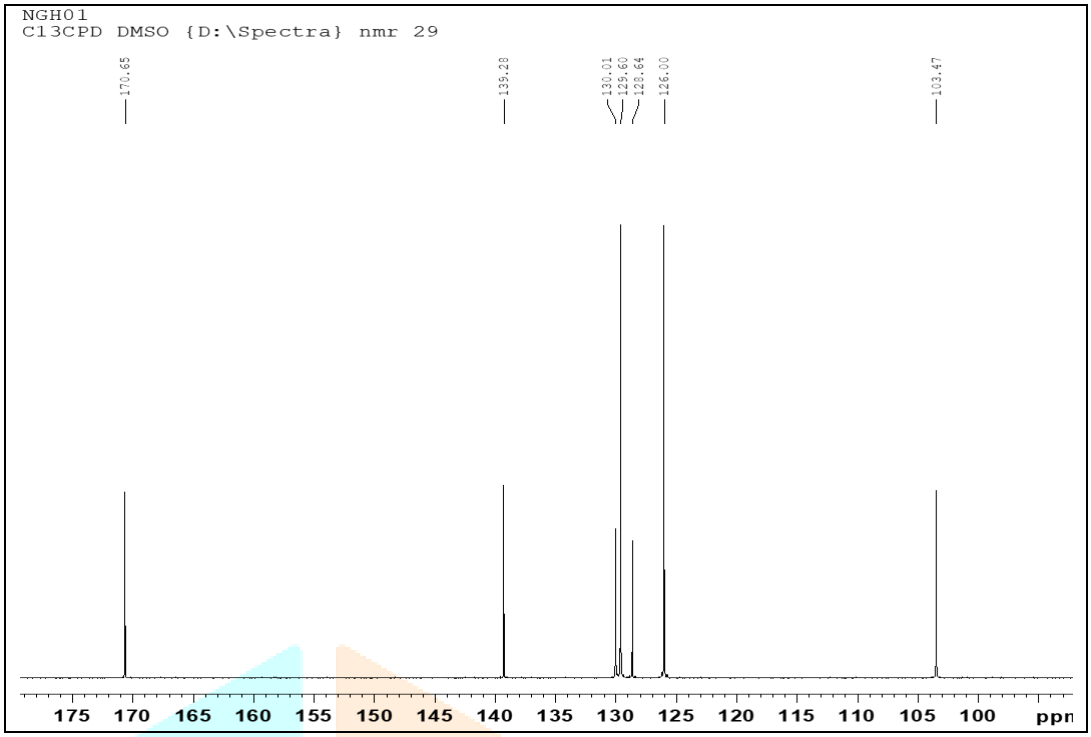


Fig 8.4. ^{13}CNMR Zoom spectra of 5-(4-Phenylthiazol-2-ylamino)-2-phenylbenzoxazole

In Vitro Anti-inflammatory Activity

Inhibition of Protein Denaturation Assay

Diclofenac sodium was used as the standard drug in the egg albumin protein denaturation assay to evaluate the anti-inflammatory activity of the synthesized benzoxazole–thiazole hybrid compound (NGH-01) at concentrations of 50, 100, 200, and 400 µg/mL. Protein denaturation was inhibited by NGH-01 at concentrations of 56.50%, 76.54%, 83.17%, and 86.15%, with the inhibition being concentration-dependent. In comparison, diclofenac sodium demonstrated greater inhibitory activity, achieving 63.30%, 82.83%, 87.03%, and 91.99% inhibition. Overall, NGH-01 showed strong anti-inflammatory activity, particularly at higher concentrations, indicating its potential as a promising anti-inflammatory agent.

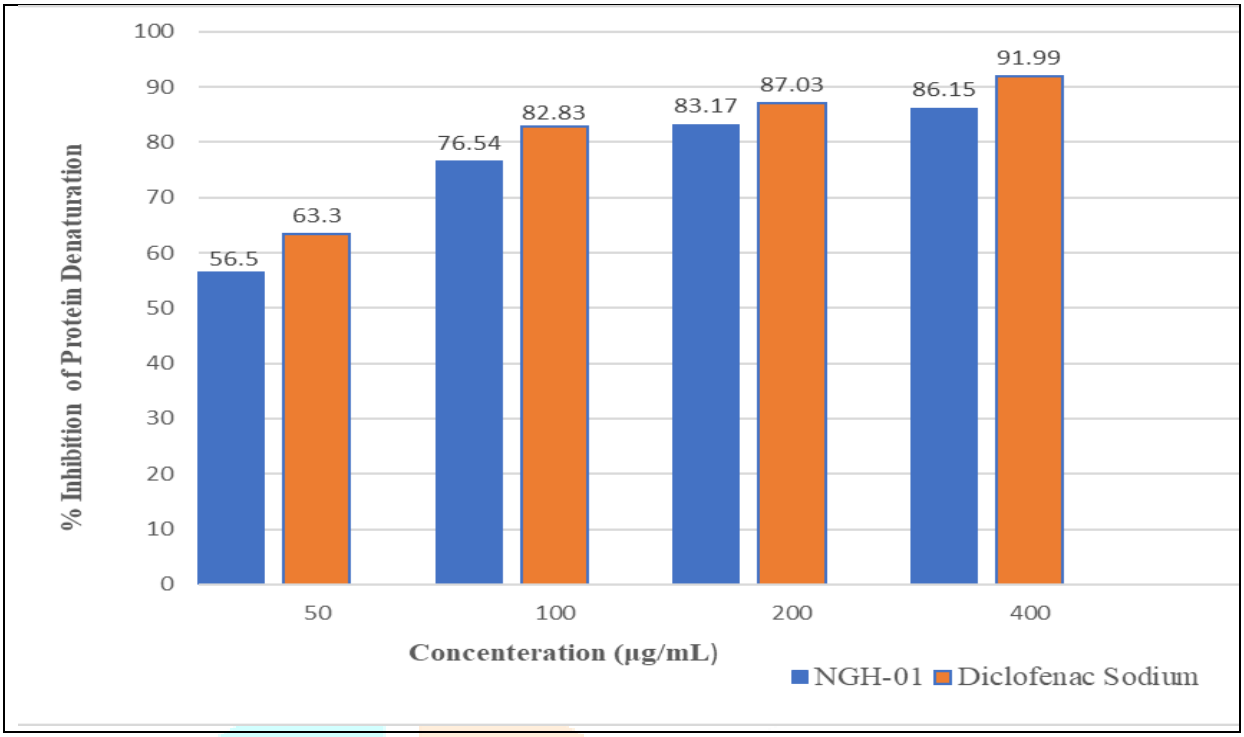


Fig 9. Concentration-dependent inhibition of protein denaturation by NGH-01 and Diclofenac Sodium using the Egg Albumin Method.

S. No.	Concentration (µg/mL)	Absorbance	% Inhibition
1.	50	1.140	56.50%
2.	100	0.615	76.54%
3.	200	0.441	83.17%
4.	400	0.363	86.15%

Table 4. Concentration-dependent inhibition of protein denaturation by NGH-01 in the egg albumin assay

S. No.	Concentration (µg/mL)	Absorbance	Absorbance
1.	50	0.962	63.30%
2.	100	0.450	82.83%
3.	200	0.340	87.03%
4.	400	0.210	91.99%

Table 5. In Vitro Anti-inflammatory Activity of Diclofenac Sodium by Protein Denaturation Assay

The protein denaturation assay was used to evaluate the synthesized benzoxazole–thiazole hybrid derivative (NGH-01) for anti-inflammatory activity. Protein denaturation was inhibited using the compound in a concentration-dependent manner; the percentage inhibition increased from 56.50% at 50 µg/mL to 76.54%, 83.17%, and 86.15% at 100, 200, and 400 µg/mL, respectively. NGH-01 showed strong anti-inflammatory potential at all tested concentrations, while having slightly lower activity than the standard drug diclofenac sodium. The synthesis of benzoxazole and thiazole pharmacophores, which are known for their anti-inflammatory properties along with their ability to stabilize proteins and modulate inflammatory pathways, may origin of the observed activity.

Overall, the results confirm the effective development of the benzoxazole–thiazole hybrid as a promising lead molecule and show that NGH-01 exhibits significant in vitro anti-inflammatory activity. To determine its therapeutic potential, further investigation is suggested, including COX inhibition assays, molecular docking, and in vivo anti-inflammatory activity.

CONCLUSION

To design, synthesis, characterization, and biological evaluation of a novel benzoxazole–thiazole hybrid derivative (NGH-01) using the molecular hybridization approach were successfully demonstrated in this study. TLC, FT-IR, ^{1}H NMR, and ^{13}C NMR spectrum analysis successfully confirmed the chemical structure of the synthesized compound, which was obtained in good purity and confirmed the proposed molecular framework. NGH-01 showed significant concentration-dependent inhibitory activity, achieving 86.15% inhibition at 400 µg/mL, which was comparable to the standard drug diclofenac sodium, according to an in vitro anti-inflammatory evaluation using the egg albumin protein denaturation assay.

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