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Design, Synthesis of Novel Azo Dyes; Quantum Chemical Insights and Pharmacological Evaluation

by

Areen Khalid

A thesis submitted in partial fulfillment for the
degree of Master of Philosophy

in the

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Department of Pharmaceutical Chemistry

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CERTIFICATE OF APPROVAL

Design, Synthesis of Novel Azo Dyes; Quantum Chemical Insights and Pharmacological Evaluation

by

Areen Khalid

(MPH243027)

THESIS EXAMINING COMMITTEE

S. No.	Examiner	Name	Organization
(a)	External Examiner	Dr. Kishwar Sultana	Iqra University, Islamabad
(b)	Internal Examiner	Dr. Siraj Khan	CUST, Islamabad

Dr. Reem
Thesis Supervisor
October, 2026

Dr. Reem
Head
Dept. of Pharmaceutical Chemistry
October, 2026

Dr. Muzaffar Abbas
Dean
Faculty of Pharmacy
October, 2026

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Acknowledgement

In the Name of Allah, the Most Gracious, the Most Merciful.

This thesis is dedicated first and foremost to **Almighty Allah**, whose infinite mercy, guidance, and blessings enabled me to overcome every challenge and achieve this milestone. It is also dedicated to **Prophet Muhammad (S.A.W)**, whose timeless teachings inspire excellence in every journey of life.

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Abstract

Azo compounds, characterized by the reactive ($-\text{N}=\text{N}-$) chromophoric linkage, are among the most widely studied classes of synthetic dyes and are increasingly valued in medicinal chemistry for their antimicrobial, anti-inflammatory, antioxidant, and prodrug potential. Building on this dual utility, the present study describes the design, synthesis, spectroscopic characterization, quantum chemical evaluation, and pharmacological screening of three novel monoazo derivatives incorporating benzothiazole and sulfonamide scaffolds.

Three target compounds were synthesized via classical diazotization–azo coupling reactions under controlled low-temperature and pH conditions: SC-01 (sulfadimidine coupled with carvacrol), SR-02 (sulfadimidine coupled with resorcinol), and AR-03 (2-aminobenzothiazole coupled with resorcinol). All compounds were obtained as intensely colored crystalline solids in good yield with sharp, reproducible melting points. Their structures were confirmed by FTIR and NMR spectroscopy, which verified successful azo bond formation and confirmed that key functional groups from the starting materials, including hydroxyl, sulfonamide, and heterocyclic ring systems, remained intact after coupling.

Quantum chemical investigations were carried out using Density Functional Theory for AR-03 and Hartree–Fock methods for SR-02 to evaluate optimized molecular geometries, frontier molecular orbitals, Mulliken charge distributions, and dipole moments; Time-Dependent DFT calculations were additionally performed for AR-03 to assess excited-state behavior. Both compounds displayed a donor – π – acceptor electronic architecture, with phenolic hydroxyl-bearing rings acting as electron donors and the azo-linked heterocyclic or sulfonamide fragments serving as electron acceptors, an arrangement consistent with their observed colors and photophysical properties.

Molecular docking studies against two cyclooxygenase (COX) protein structures retrieved from the Protein Data Bank demonstrated favorable binding interactions for compound SC-01, mediated through hydrogen bonding, hydrophobic contacts,

and van der Waals forces with key active-site residues. The docking pattern paralleled that of the reference drug diclofenac, supporting the structural plausibility of the compounds' anti-inflammatory potential.

In vitro antimicrobial evaluation against *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* revealed that SR-02 exhibited the most potent antibacterial activity, notably surpassing the standard drug sulfadimidine against *S. aureus*. In vivo pharmacological assessment using carrageenan-induced paw edema in mice demonstrated that all three compounds significantly reduced inflammation, with SR-02 emerging as the most active candidate, while SC-01 and AR-03 showed activity comparable to diclofenac.

Collectively, the convergence of spectroscopic, computational, and biological findings around a consistent donor–acceptor electronic motif establishes a mechanistic link between the color-generating properties and pharmacological potential of these benzothiazole- and sulfonamide-based azo dyes, supporting their rational design as promising anti-inflammatory and antimicrobial lead candidates.

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Abbreviations

ABTs	Amino Benzothiazoles
ANOVA	Analysis of Variance
COX	Cyclooxygenase
DFT	Density Functional Theory
DMSO	Dimethyl Sulfoxide
FMO	Frontier Molecular Orbitals
FTIR	Fourier Transform Infrared
HF	Hartree-Fock
HOMO	Highest Occupied Molecular Orbital
HTS	High-Throughput Screening
LUMO	Lowest Unoccupied Molecular Orbital
MEP	Molecular Electrostatic Potential
NBO	Natural Bond Orbital
NMR	Nuclear Magnetic Resonance
NSAID	Non-steroidal anti-inflammatory drugs
PDB	Protein Data Bank
PET	Polyethylene Terephthalate
RMSD	Root Mean Square Deviation
SAR	Structure Activity Relationship
SCF	Self-Consistent Field
TD-DFT	Time-Dependent Density Functional Theory
TLC	Thin layer chromatography

Chapter 1

Introduction

Heterocyclic compounds are cyclic organic molecules that contain at least one heteroatom (most commonly nitrogen, oxygen, or sulfur) within the ring structure, contrasting with carbocyclic compounds where all ring atoms are carbon. This structural class is foundational to life and medicinal science; many essential biomolecules, such as DNA, RNA, chlorophyll, and various vitamins, incorporate heterocyclic frameworks. Consequently, heterocyclic scaffolds are estimated to be present in more than 85% of all physiologically active chemical entities, establishing them as a cornerstone of modern drug discovery and development [1]. The wide-ranging therapeutic potential is evident in various derivatives, such as triazines employed as antimicrobial agents and anti-inflammatory drugs, or benzimidazoles, which exhibit broad-spectrum antibacterial and antiviral properties [2]. Among the diverse heterocyclic systems, those incorporating nitrogen (e.g., quinoline, indole) and sulfur atoms have garnered significant attention due to their prominent roles in natural products and therapeutic agents [3]. Nitrogen-containing heterocycles are central to many drugs and research efforts [4]. Similarly, sulfur-containing heterocycles constitute a substantial portion of FDA-approved drugs, exhibiting a broad spectrum of pharmacological activities including anticancer, antibacterial, antiviral, and antidiabetic properties [5].

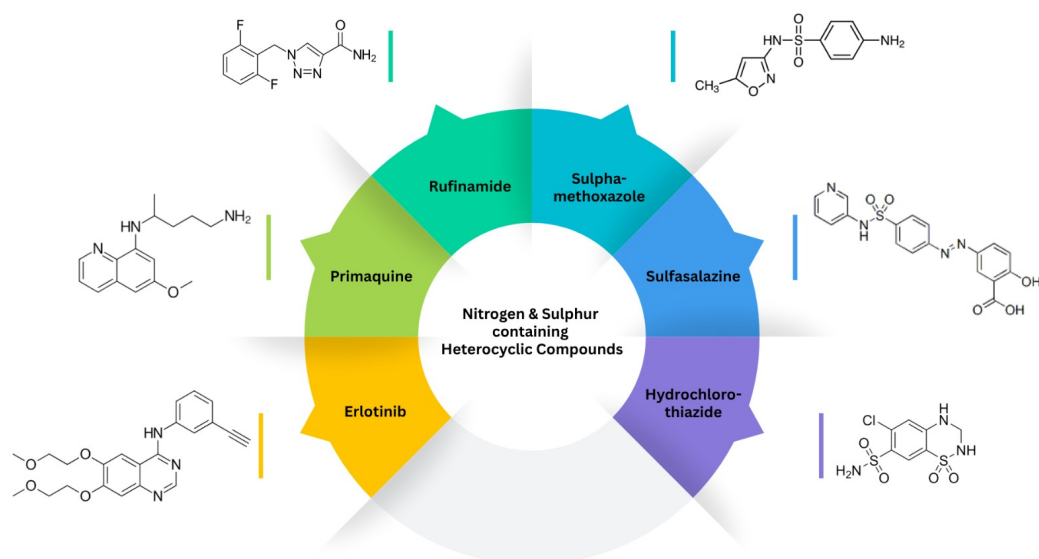


FIGURE 1.1: FDA Approved Nitrogen and Sulphur containing Heterocyclic Compounds

To enhance the design and optimization of these bioactive scaffolds, quantum chemical calculations have emerged as powerful tools in medicinal chemistry [6]. Techniques based on Density Functional Theory (DFT), for example, allow researchers to accurately model the electronic structure, geometric conformation, and chemical reactivity of heterocyclic molecules. This computational approach provides critical insights into properties such as molecular orbital energies, electrostatic potential maps, and acid-base behavior, which are fundamental to understanding and predicting a compound's stability, reactivity, and ultimately, its mechanism of interaction with biological targets [7]. By integrating these calculations with experimental synthetic efforts, chemists can refine lead compounds, explain observed structure-activity relationships, and rationally design novel heterocyclic therapeutic agents [8].

Azo compounds are among the most important classes of colored organic compounds and are characterized by the presence of the azo functional group ($-\text{N}=\text{N}-$). They possess a wide range of biological and industrial applications [9]. Biologically, many azo derivatives exhibit antimicrobial and anticancer activities and can inhibit protein and RNA synthesis. In addition, they are widely used in cosmetics and serve as valuable intermediates in the synthesis of numerous organic

compounds [10]. In inorganic chemistry, azo compounds function as important ligands for the preparation of coordination complexes [11]. Furthermore, they are extensively employed as analytical reagents for the detection and determination of metal ions due to their strong metal-chelating ability [12].

Azo compounds are also renowned for their intense coloration, excellent light-fastness, and resistance to water, solvents, and various environmental conditions. They exhibit a diverse color palette, including yellow, orange, red, blue, and green, making them indispensable in dye and pigment industries. Their characteristic colors arise from the azo chromophore ($-N=N-$), which absorbs light in the visible region of the electromagnetic spectrum [13].

Azo compounds constitute the predominant synthetic chemical class within the realm of industrial colorants, representing the most extensively utilized group of dyes, with estimates suggesting they account for nearly 60-70% of the total dyes employed in food processing and textile industries [14]. These dyes exhibit a broad spectrum of chromatic variations, primarily due to the enhanced stability of the azo functionality ($-N=N-$) and the electronic influence of substituents that extend π -electron conjugation across the aromatic system [13]. This extensive π -electron delocalization facilitates light absorption within the visible region of the electromagnetic spectrum, thereby rendering the majority of azo derivatives distinctly colored [13].

Beyond their widespread application as mordant and disperse dyes in textiles, the structural motif of the azo linkage has attracted significant attention in medicinal chemistry. The ability of the azo group to undergo reductive cleavage makes these compounds valuable as prodrugs, particularly for targeted drug delivery to the colon, where anaerobic microflora can break the azo bond [15]. Furthermore, the electronic and structural features responsible for their color have also been leveraged to design compounds with diverse biological activities. Azo dyes and their derivatives have been widely investigated for their potential as antimicrobial agents, antitumor agents, antioxidants, and even as diagnostic tools (e.g., in histology and microscopy) due to their selective binding and staining properties [16]. As such, structural variations such as those incorporating heterocyclic units

or diverse aromatic systems are continuously explored to optimize their pharmacological profile and broaden their therapeutic applicability [15].

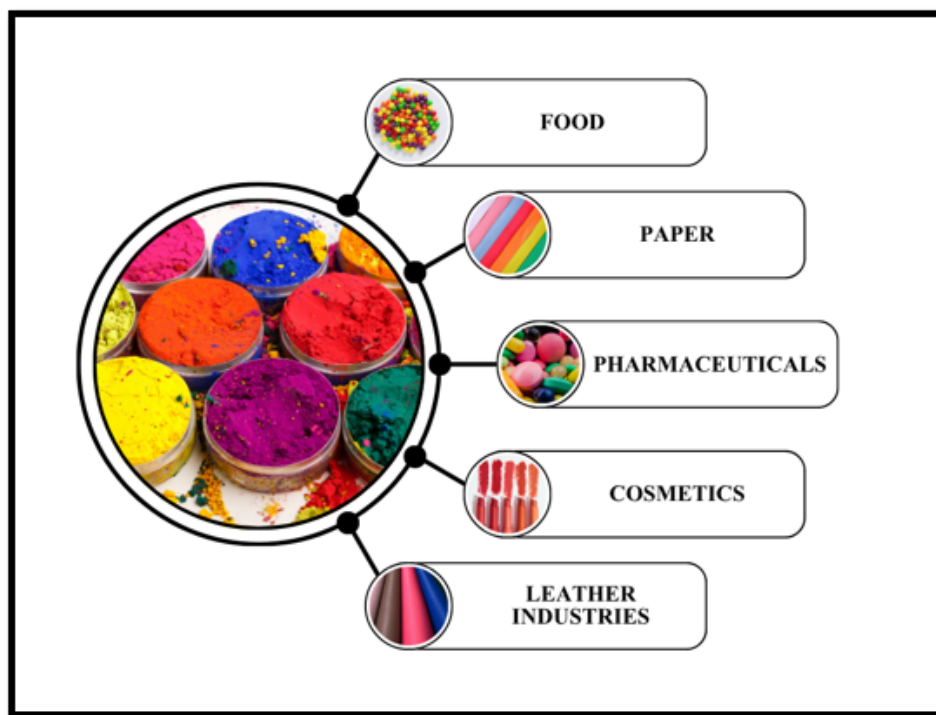


FIGURE 1.2: Applications of Azo dyes

The synthetic methodology employed for the preparation of azo dyes is relatively straightforward, relying on the use of inexpensive and readily obtainable raw materials, which facilitates large-scale industrial production [17].

In most cases, these reactions are conducted under comparatively mild temperature conditions, thereby minimizing the need for intensive energy input. Furthermore, water is frequently utilized as the principal reaction medium, a factor that not only enhances cost-effectiveness but also significantly reduces the associated environmental burden. Collectively, these attributes render the industrial manufacture of azo dyes both economical and sustainable, thereby contributing to their dominance in the global dye market [18].

Azo dyes demonstrate superior stability compared to natural dyes across the entire pH spectrum relevant to food systems, while also exhibiting notable thermal resistance and photostability, as they do not readily degrade upon exposure to either light or atmospheric oxygen [19]. Owing to their comparatively low toxicity,

reduced incidence of allergic responses, and absence of adverse behavioral effects such as hyperactivity, they have been widely incorporated into food products [20].

Beyond their established role in food and textile industries, azo dyes have also found application in advanced technological fields, such as laser systems, nonlinear optical devices, reprographic processes, dye-sensitized solar cells, and metalochromic indicators [21].

Within this class, benzothiazole-derived azo dyes are particularly valued in the production of red dyes, owing to their superior fastness properties and high-performance dyeing characteristics [22]. Furthermore, azo dyes serve a functional role as acid–base indicators, particularly in acidimetry and alkalimetry. In alkaline media, the chromophore exists predominantly in its neutral azo form, imparting a characteristic color to the solution. Conversely, under acidic conditions, protonation of the azo nitrogen occurs, resulting in redistribution of the electronic density and stabilization of a resonance hybrid species, which produces a deepened or altered coloration [23].

The azo linkage constitutes the most labile structural feature within azo dye molecules and is particularly susceptible to enzymatic cleavage in mammals, including humans. This bond may undergo reductive cleavage, resulting in fragmentation of the parent molecule into two distinct moieties, a process mediated by non-specific enzymes collectively referred to as azo-reductases, which are widely distributed among various microorganisms (notably intestinal microbiota) as well as across all mammalian systems examined [24].

In light of this inherent susceptibility, and with the objective of evaluating the biological potential of newly synthesized azo derivatives, our investigation was directed toward the development of novel analogues [25]. Considering these factors, and as a continuation of our ongoing interest in the synthesis of monoazo dyes containing benzothiazole frameworks, efforts were focused on identifying synthetic strategies capable of affording structurally diverse, selective, and less toxic yet highly effective azo compounds. The present study therefore describes the design, synthesis, structural characterization, dyeing performance, applications,

antimicrobial anti-inflammatory assessment of a new series of monoazo dyes incorporating the benzothiazole nucleus in conjugation with various substituted anilines and phenolic derivatives, wherein the azo linkage is positioned at the 2-site of the benzothiazole ring [25]. The substitutional patterns of both the benzothiazole core and the auxiliary aniline or phenol fragments were judiciously chosen to impart distinct electronic environments to the resulting molecules, thereby modulating their physicochemical and functional properties [25].

1.1 Rationale of the Study

The rationale of this study is to synthesize novel benzothiazole- and sulfonamide-based monoazo dyes through an integrated synthetic and computational approach. Following synthesis and characterization, Density Functional Theory (DFT) will be employed to investigate electronic properties, including frontier molecular orbital energies, molecular electrostatic potential, and charge distribution, to understand structure–activity relationships.

1.2 Aim and Objectives

The aim of the study is to design and synthesize novel azo dyes, evaluate their pharmacological activities predict quantum chemical insights.

The objectives of this study are:

- i. To design and synthesize a series of novel azo dyes using appropriate synthetic strategies.
- ii. To characterize the synthesized azo dyes using appropriate spectroscopic and analytical techniques to confirm their chemical structures and assess their purity.
- iii. To perform quantum chemical characterization of the synthesized compounds by calculating their electrostatic potential maps, Mulliken atomic charges, and frontier molecular orbitals (HOMO–LUMO analysis).

- iv. To evaluate the interaction of synthesized azo dyes with biological targets through molecular docking.
- v. To assess the pharmacological potential of the synthesized compounds through *in-vitro* and *in-vivo* biological testing.

Chapter 2

Literature Review

2.1 Drug Discovery and Development

Drug discovery and development is a complex, multi-stage process focused on identifying new therapeutic compounds and assessing their safety and efficacy [26]. In ancient times, natural sources such as plants and animal-derived substances played a central role in medicine [27]. However, the early 20th century marked a turning point, as systematic scientific approaches began shaping modern drug discovery [28].

Typically, the development of a new drug involves several critical phases. The initial stage, often referred to as pre-discovery, focuses on understanding disease mechanisms and identifying biological targets commonly proteins that are involved in disease progression. Once a suitable target is validated, the discovery phase begins, where potential therapeutic agents are identified [29]. These candidates are generally categorized as either small molecules or biologics. Both natural products and synthetic compounds continue to serve as key sources for lead generation and future optimization [30]. The ability to produce these compounds efficiently, while maintaining quality and cost-effectiveness, has become a major challenge in the chemical sciences. At the same time, advances in synthetic organic chemistry have significantly influenced drug discovery. A major breakthrough occurred in the

mid-1980s with the introduction of high-throughput screening (HTS) technologies, enabling rapid evaluation of thousands of compounds [31].

This advancement led to the emergence of combinatorial chemistry, facilitating the creation of large and diverse chemical libraries [32].

Over time, strategies such as lead-oriented synthesis and fragment-based drug design have enhanced the identification of promising candidates and streamlined the optimization process. More recently, the integration of computational tools, structure-based design, and artificial intelligence has further accelerated drug discovery, improving both efficiency and precision in identifying new drug candidates [33].

In modern research, there is an increasing demand for structurally diverse compounds exhibiting strong pharmacological potential. To address the limitations associated with conventional therapeutic agents, researchers are focusing on the design of hybrid molecules and modular frameworks capable of interacting with multiple biological targets. Among these, azo dyes have gained significant attention due to their structural diversity, straightforward synthesis, and broad spectrum of biological activities. The presence of the characteristic azo linkage ($-N=N-$) allows for extensive modification, enabling the incorporation of various functional groups that enhance their pharmacological properties [34]. Furthermore, their ability to undergo bio reductive cleavage and form active metabolites contributes to their growing importance in medicinal and pharmaceutical research [35].

2.2 Azo Dyes as Potential Pharmacophores in Drug Design

Azo dyes constitute a significant class of compounds distinguished by an azo linkage ($-N=N-$) that bridges two aromatic or aliphatic moieties. Owing to their structural diversity and broad spectrum of biological activities, these compounds have garnered growing interest in the field of medicinal chemistry [36].

The term "azo" originates from the French word azote, meaning nitrogen, and refers to the nitrogen–nitrogen double bond characteristic of these molecules. Structurally, azo compounds are typically represented as $\text{Ar-N=N-Ar}'$, where Ar and Ar' can be aromatic or heterocyclic groups, lending these compounds their structural versatility [15].

Azo dyes are generally synthesized through diazotization of primary aromatic amines, followed by azo coupling with activated aromatic compounds, such as phenols or amines. The reaction conditions, including pH, temperature, and substituent effects, play a crucial role in determining the yield and stability of the resulting products [36].

The presence of electron-donating and electron-withdrawing groups plays a key role in modulating the chemical and biological properties of azo compounds. This structural adaptability allows the design of molecules with desired pharmacokinetic and pharmacodynamic profiles [34].

Pharmacologically, azo dyes exhibit a broad spectrum of biological activities, including antibacterial, antifungal, anti-inflammatory, and anticancer effects. Their ability to undergo reductive cleavage of the azo bond under physiological conditions leads to the formation of active metabolites, which enhances their therapeutic potential [37].

This unique property has been particularly useful in the development of prodrugs such as Sulfasalazine, which is widely used in the treatment of inflammatory bowel diseases. Due to these characteristics, azo dyes are increasingly recognized as promising pharmacophores in modern drug design [16].

2.3 Mechanistic Insights into Azo Dye Formation

The synthesis of azo dyes primarily involves two key steps: diazotization and azo coupling. In the first step, a primary aromatic amine reacts with nitrous acid under acidic conditions (typically at 0–5°C) to form a diazonium salt. This

reaction proceeds via the formation of a nitrosating agent, which converts the amine into a highly reactive diazonium ion [38].

The diazonium ion acts as a strong electrophile due to the presence of the positively charged nitrogen group. In the subsequent azo-coupling step, this electrophile reacts with activated aromatic compounds such as phenols or aromatic amines, which act as nucleophiles. The coupling generally occurs at the para or ortho position of the aromatic ring, depending on steric and electronic factors [39]. The mechanism of azo coupling is essentially an electrophilic aromatic substitution reaction, where the nucleophilic aromatic ring attacks the diazonium ion, forming a sigma complex intermediate. This intermediate then loses a proton to restore aromaticity, resulting in the formation of the azo bond ($-N=N-$) [40].

Reaction conditions such as pH play a crucial role in determining the efficiency of azo dye formation. For example, coupling with phenols is favoured under slightly alkaline conditions, whereas coupling with aromatic amines typically occurs in mildly acidic media. Temperature control is also essential to maintain the stability of the diazonium salt, as it can decompose at higher temperatures [36].

Furthermore, substituents on the aromatic ring significantly influence the rate and orientation of the coupling reaction. Electron-donating groups enhance nucleophilicity and promote faster coupling, while electron-withdrawing groups tend to reduce reactivity. These factors allow chemists to tailor azo dye structures with desired chemical and biological properties [36]. Overall, azo dye formation involves a sequence of well-coordinated reactions, including diazotization and electrophilic substitution, making it a versatile and widely utilized method in both industrial and medicinal chemistry [41].

2.4 Synthetic Methodologies: Classical Foundations

The synthesis of azo materials relies on robust organic reactions that have evolved from classic benchtop methods into modern, eco-friendly green processes.

2.4.1 Diazotization and Electrophilic Azo Coupling Mechanisms

The standard laboratory and industrial synthesis of azo structures follow a traditional two-stage reaction sequence: low-temperature diazotization of a primary aromatic amine, followed immediately by an electrophilic aromatic substitution (azo-coupling) [42].

The first stage involves dissolving a primary aromatic or heteroaromatic amine in a cold aqueous solution of a strong mineral acid, such as hydrochloric acid (HCl) or sulfuric acid (H₂SO₄) [39]. The reaction mixture is maintained at 0–5°C and treated with sodium nitrite (NaNO₂). Under these acidic, low-temperature conditions, sodium nitrite generates nitrous acid (HNO₂), which undergoes protonation followed by dehydration to form the highly reactive nitrosonium ion (NO⁺), the key electrophilic species responsible for the diazotization reaction [39].

The primary amine attacks this nitrosonium ion, undergoing a series of proton transfers and dehydration steps to form a highly water-soluble diazonium salt [43]. Maintaining temperatures strictly below 5°C is vital; if warmed, these diazonium intermediates decompose, releasing nitrogen gas and forming phenolic impurities or unwanted polymeric by-products [43].

In the second stage, the freshly prepared diazonium solution is introduced to an electron-rich aromatic coupling partner, such as an activated phenol, naphthol, aniline derivative, or heterocyclic matrix [13]. The diazonium ion acts as a weak electrophile, attacking the electron-dense carbon network of the coupling component through an electrophilic aromatic substitution mechanism [13].

To ensure high yields, the pH of the reaction mixture must be carefully adjusted. Phenolic coupling reactions are typically performed under mildly alkaline conditions pH 8–10 to convert hydroxyl groups into more nucleophilic phenoxide ions, which accelerates the electrophilic attack [44]. Amine-based coupling reactions are carried out in mildly acidic to neutral conditions pH 4–7 to maximize the concentration of highly reactive, unprotonated free amine groups while preventing decomposition of the diazonium salt [45].

The substitution occurs selectively at the para-position relative to the activating group; if the para-site is sterically blocked, the reaction proceeds at the *ortho*-position [46].

2.5 Broad-Spectrum Industrial and Textile Applications

The extensive use of azo colorants across diverse commercial sectors highlights their versatility, stability, and high performance as modern industrial materials.

2.5.1 Textile Processing, Substrate Adsorption and Fiber Binding Mechanics

The primary industrial application of synthetic azo dyes is in the textile industry, where they are used to color both natural animal and plant fibers as well as advanced synthetic polymers [47]. The structural design of each dye determines how it interacts with and binds to specific fiber substrates:

2.5.1.1 Reactive Azo Dyes

These dyes are engineered to form permanent chemical bonds with cellulosic plant fibers like cotton [48]. They incorporate reactive functional groups, such as vinyl sulfone ($\text{SO}_2-\text{CH}=\text{CH}_2$) or halotriazine rings. When applied in an alkaline dye bath, the nucleophilic hydroxyl groups (OH) on the cellulose chains attack these reactive centers via nucleophilic addition or substitution. This reaction establishes strong, covalent ester or ether linkages that securely anchor the dye molecule directly into the fiber backbone, providing exceptional color fastness during washing and wet processing [48].

2.5.1.2 Disperse Azo Dyes

For highly hydrophobic, crystalline synthetic polymers like polyethylene terephthalate (PET) polyester, non-ionic monoazo disperse dyes are preferred [49]. These

dyes are applied as fine aqueous suspensions at high temperatures 130°C or processed using advanced supercritical carbon dioxide (CO₂) carrier systems [49]. Thermal energy expands the polymer chains, widening the spaces within the fiber structure and allowing the small, hydrophobic dye molecules to diffuse directly into the polymer matrix [49]. Upon cooling, the fiber structure contracts, trapping the dye inside via strong hydrophobic interactions and Van Der Waals forces, ensuring high color depth and excellent resistance to sublimation and light fading [49].

2.5.1.3 Acidic and Direct Azo Dyes

Acidic azo dyes carry negatively charged functional groups, primarily sulfonic acid salts (SO₃Na), which render them highly water-soluble [47]. In acidic dye baths, they form strong ionic bonds with the positively charged protonated amino groups (–NH₃) found in protein fibers like wool and silk, as well as synthetic polyamide (nylon) networks. Direct azo dyes feature elongated, planar structures that align along the linear chains of cellulose, anchoring themselves via extensive hydrogen bonds and dispersion forces without requiring chemical mordants [47].

2.5.2 Non-Textile Industrial Applications

Beyond consumer textiles, azo compounds serve as critical components in many advanced industrial and technology sectors:

- i. **Printing Inks and Plastics:** They are widely used to formulate high-grade printing inks for paper substrates, packaging plastics, automotive coatings, and consumer cosmetics due to their bright colors and high thermal stability [50].
- ii. **Advanced Optics and Electronics:** The clean trans-cis isomerization profile of azo compounds is utilized in modern optoelectronic devices, including optical data storage networks, real-time holographic imaging displays, optical waveguides, and specialized laser tech [51].

- iii. Analytical Chemistry Sensors: Azo molecules that alter their electronic structure and color in response to environmental changes serve as reliable pH indicators (e.g., Methyl Orange, Methyl Red) and highly selective colorimetric chemo sensors for detecting toxic heavy metals in industrial runoff [52].

2.6 Advanced Pharmacological Potential and Applications in Wound Healing

Historically, the medical utility of the azo framework was established by the discovery of Prontosil, a red azo dye that served as the world's first commercial synthetic prodrug. *In vivo*, *Prontosil* undergoes reductive cleavage by bacterial azoreductase enzymes, liberating sulfanilamide to effectively combat systemic streptococcal infections [41]. Modern medicinal chemistry continues to build on this framework, modifying the core structure to develop targeted therapeutic agents and advanced biomaterials for wound healing.

2.6.1 Mechanisms of Antimicrobial and Antibiofilm Activity

Integrating therapeutic sulfonamide groups (SO_2NH), metal-binding domains, or bioactive heterocyclic rings into the azo structure yields highly effective antimicrobial agents [53]. These custom-designed molecules combat drug-resistant pathogens via multiple biochemical pathways:

- i. Competitive Enzyme Inhibition: Sulfa-bearing azo structures closely mimic the molecular profile of *p*-aminobenzoic acid (*p*ABA). This allows them to fit into the active site of the bacterial enzyme dihydropteroate synthase (DHPS), blocking the synthesis of folic acid and halting microbial DNA replication [54].
- ii. Membrane Disruption: The amphipathic nature of many azo dye derivatives enables them to interact with the lipid bilayer of microbial cell membranes,

causing membrane destabilization, increased permeability, and leakage of essential intracellular components. This disruption of membrane integrity contributes significantly to their antimicrobial activity [55].

- iii. DNA Intercalation: Highly planar heterocyclic azo molecules can slide between adjacent base pairs in microbial DNA, altering the structural geometry of the double helix and inhibiting essential topoisomerase enzymes [56].
- iv. Biofilm Inhibition: Advanced azo derivatives disrupt quorum sensing the chemical signaling mechanism bacteria use to coordinate defensive behaviors. Inhibiting this pathway prevents the formation of protective polysaccharide biofilms, leaving pathogens vulnerable to standard treatments and host immune responses [57].

2.7 Quantum Chemical Insights: DFT Foundations in Molecular Design

To complement experimental synthesis and pharmacological screening, modern research relies heavily on computational chemistry specifically Density Functional Theory (DFT) to model and refine new azo structures before laboratory production [58].

2.7.1 Frontier Molecular Orbital Analysis and Global Reactivity Parameters

To predict how an azo compound will behave with industrial fibers or biological receptors, you first need to look at its Frontier Molecular Orbitals specifically the HOMO (Highest Occupied Molecular Orbital) and LUMO (Lowest Unoccupied Molecular Orbital) [59]. The HOMO acts as the primary electron donor, while the LUMO functions as the primary electron acceptor [60].

The size of this energy gap serves as an indicator of the molecule's chemical kinetic stability and electronic polarizability. A narrow HOMO-LUMO gap indicates a soft, highly polarizable molecule that easily undergoes electronic charge transfer, a

property highly correlated with high antioxidant activity and strong receptor binding affinities [61]. Conversely, a wide energy gap characterizes hard, chemically inert structures that resist electronic disruption.

Global reactivity descriptors are obtained through Koopmans' theorem, which uses the HOMO and LUMO energies to approximate the ionization potential and electron affinity, respectively. From these two values, other parameters including electronegativity, chemical hardness, softness, and the electrophilicity index can then be derived [62].

Integrating these computational metrics allows researchers to screen candidate azo architectures, filtering out unstable configurations and prioritizing structures optimized for target applications.

Frontier Molecular Orbital (FMO) theory is a fundamental quantum chemical approach widely utilized for evaluating the electronic characteristics, chemical reactivity, and stability of organic molecules [59].

Introduced by Fukui, this theory highlights the crucial role of the HOMO and the LUMO in determining molecular interactions and reaction pathways. These orbitals are designated as frontier orbitals because they are the primary molecular orbitals involved in electron donation and acceptance processes during chemical transformations [63].

The HOMO, or Highest Occupied Molecular Orbital, is the highest-energy orbital that contains electrons, and it's linked to how readily a molecule can give up electrons. When the HOMO energy is higher, the molecule tends to be a better electron donor, making it more nucleophilic. On the other hand, the LUMO, or Lowest Unoccupied Molecular Orbital, is the lowest-energy orbital that's empty, and it reflects a molecule's capacity to accept electrons. A lower LUMO energy typically means the molecule is a stronger electron acceptor, giving it more pronounced electrophilic character [64]. The electronic interaction between HOMO and LUMO, along with their energy distribution, plays an essential role in governing chemical reactivity, charge-transfer processes, molecular interactions, and biological activity.

The energy difference between HOMO and LUMO, commonly referred to as the band gap (ΔE), is a fundamental parameter in quantum chemistry. This energy gap offers useful insight into a molecule's stability, its reactivity, and how effectively charge can transfer within it [65].

A small HOMO–LUMO energy gap indicates that electrons can be promoted from the occupied orbital to the vacant orbital with relatively low energy input. Such molecules are generally characterized by higher chemical reactivity, increased polarizability, and enhanced intramolecular charge transfer. Conversely, molecules possessing a large energy gap are typically more chemically stable, less reactive, and more resistant to electronic perturbations [66].

In azo compounds, the extended π -conjugated framework and azo ($-\text{N}=\text{N}-$) linkage play a significant role in governing the distribution of frontier molecular orbitals. The conjugation between aromatic rings and heterocyclic substituents facilitates electron delocalization throughout the molecular framework, thereby affecting optical, electronic, and biological properties [67].

The localization of HOMO and LUMO over specific molecular regions can provide important insights into potential reactive sites and the nature of intermolecular interactions. For instance, electron-rich regions identified through HOMO distribution may participate in hydrogen bonding or electrophilic substitution reactions, whereas electron-deficient regions associated with the LUMO may serve as favorable sites for nucleophilic attack [68].

The investigation of frontier molecular orbitals has gained significant importance in medicinal chemistry and in the field of drug discovery. Several studies have reported that HOMO–LUMO properties are closely associated with biological activities, including antioxidant, antimicrobial, and anticancer effects [69]. Compounds with favorable charge-transfer characteristics may exhibit enhanced interactions with biological targets, such as proteins, enzymes, and nucleic acids. Therefore, FMO analysis serves as an effective computational tool for elucidating structure–activity relationships and providing insights into the potential biological behavior of newly developed compounds [70].

Beyond frontier orbital energies, several global reactivity descriptors can be derived using Koopmans' theorem. These descriptors offer a quantitative assessment of the overall chemical behavior of a molecule and are extensively utilized in conceptual Density Functional Theory (DFT) studies [71]. The ionization potential (I) and electron affinity (A) are directly related to HOMO and LUMO energies, respectively, and serve as the basis for calculating various molecular descriptors.

Electronegativity (χ) describes the tendency of a molecule to attract electrons toward itself and reflects its electron-withdrawing capability. Molecules with higher electronegativity values generally exhibit stronger electron-accepting behavior and greater stability in electron-rich environments [72].

Chemical hardness (η) is another important descriptor that measures resistance to charge transfer and deformation of the electron cloud. Hard molecules possess large HOMO–LUMO gaps and exhibit greater kinetic stability, whereas soft molecules are characterized by enhanced reactivity and facile charge transfer [73].

The inverse of hardness is chemical softness (S), which quantifies the ease with which the electron density can be redistributed within a molecule. Soft molecules are often associated with higher biological activity because they can adapt more readily to the electronic environment of biological receptors [74]. This flexibility facilitates stronger intermolecular interactions and may enhance binding affinity toward target proteins.

Another important parameter is the chemical potential (μ), which represents the tendency of electrons to escape from a molecular system. Chemical potential is closely associated with electronegativity and provides information regarding electron flow between interacting species [75].

Similarly, the electrophilicity index (ω), introduced by Parr and co-workers, describes the molecule's ability to accept electrons and stabilize additional electronic charge [76]. Molecules with high electrophilicity values are generally considered strong electron acceptors and often exhibit enhanced interactions with nucleophilic biological targets.

The nucleophilicity index is also frequently employed to evaluate the electron-donating strength of molecular systems [76]. Together, electrophilicity and nucleophilicity descriptors provide a comprehensive understanding of molecular reactivity patterns and help predict the preferred mode of interaction with biological receptors, enzymes, and other molecular targets [76].

In recent years, the combined application of FMO analysis and global reactivity descriptors has become an indispensable component of computational drug design [63]. These theoretical parameters enable researchers to predict molecular stability, optimize lead compounds, evaluate charge-transfer behavior, and identify potential pharmacologically active candidates before experimental validation [63]. Furthermore, correlations between HOMO–LUMO energies and biological activity have been successfully established for numerous classes of heterocyclic and azo compounds, demonstrating the utility of these descriptors in rational molecular design [77].

Therefore, examining frontier molecular orbitals alongside global reactivity descriptors offers a thorough insight into the electronic characteristics of azo derivatives. Such analyses not only reveal the fundamental aspects of molecular stability and reactivity but also contribute significantly to understanding structure–activity relationships, molecular recognition processes, and the development of novel compounds with enhanced biological and pharmacological potential [77].

2.7.2 Molecular Electrostatic Potential Mapping

Molecular Electrostatic Potential (MEP) mapping is a powerful computational tool used in quantum chemistry to visualize the charge distribution within a molecule and predict its potential sites of chemical reactivity. MEP surfaces are generated from the electron density distribution obtained through Density Functional Theory (DFT) calculations and provide a three-dimensional representation of the electrostatic potential surrounding a molecular system [78]. These maps serve as an effective means of correlating molecular structure with reactivity, intermolecular interactions, and biological activity.

Molecular Electrostatic Potential (MEP) represents the electrostatic interaction energy between the molecule's charge distribution, arising from its nuclei and electrons, and a positive probe charge positioned at a particular location around the molecular surface [64].

MEP mapping enables the visualization of electron-rich and electron-deficient regions within a molecule, thereby identifying potential sites involved in electrophilic and nucleophilic interactions. Since electrostatic interactions play a fundamental role in molecular recognition, ligand–target binding, and various chemical processes, MEP analysis has become an important computational approach in medicinal chemistry, drug discovery, and the prediction of molecular reactivity [79].

MEP surfaces are commonly visualized through a color-coded gradient that represents the distribution and magnitude of electrostatic potential over the molecular surface. In this scale, red regions correspond to areas of high negative electrostatic potential, indicating electron-rich sites, whereas blue regions represent areas of high positive electrostatic potential associated with electron-deficient regions. The intermediate colors, including yellow, green, and cyan, denote regions with moderate or near-neutral electrostatic potential values [80].

In MEP maps, the red-colored regions correspond to zones of elevated electron density and negative electrostatic potential. These electron-rich regions are typically associated with electronegative atoms, including oxygen, nitrogen, sulfur, and other heteroatoms within the molecular structure. In azo-based compounds, regions of negative potential are commonly distributed around azo nitrogen atoms ($-N=N-$), carbonyl oxygen, sulfonamide oxygen, hydroxyl groups, and other electron-donating functional groups [75].

These electron-rich sites serve as potential locations for electrophilic interactions and may function as hydrogen-bond acceptors during binding with biological macromolecules, including receptors and enzymes. Therefore, the magnitude and spatial distribution of negative electrostatic regions significantly influence molecular recognition, ligand–target interactions, and binding affinity toward biological targets [75].

In contrast, blue-colored regions signify positive electrostatic potential, pointing to electron-deficient zones within the molecule. These regions are commonly associated with hydrogen atoms attached to electronegative atoms, protonated amino groups, or electron-withdrawing substituents [75]. Positive potential regions often function as hydrogen-bond donors and can participate in electrostatic interactions with negatively charged residues present in protein active sites. The identification of such regions is particularly important in drug design because they contribute significantly to ligand–receptor binding and molecular stabilization within biological environments [81].

Green and yellow regions correspond to areas possessing intermediate or nearly neutral electrostatic potential. These regions are generally associated with aromatic rings, alkyl chains, and hydrophobic molecular fragments. In azo dyes and heterocyclic compounds, the aromatic conjugated framework often exhibits neutral electrostatic character, which contributes to hydrophobic interactions and π – π stacking with biological macromolecules [82]. Such interactions play a vital role in stabilizing ligand–protein complexes and enhancing biological activity. Furthermore, in material science applications, these neutral regions facilitate interactions with polymer matrices and contribute to the physicochemical properties of dyes and pigments.

MEP analysis provides valuable insights into intermolecular interactions that cannot be obtained solely from molecular geometry. By identifying regions susceptible to electrophilic and nucleophilic attack, researchers can predict the preferred reaction pathways and chemical behavior of a molecule. Moreover, MEP maps help explain the nature and strength of hydrogen bonding, van der Waals interactions, dipole–dipole interactions, and other non-covalent forces that govern molecular recognition processes [81].

In pharmaceutical research, MEP mapping is extensively employed to evaluate the binding potential of drug candidates toward biological receptors. The electrostatic complementarity between a ligand and a receptor is often a determining factor in binding affinity and biological efficacy. Regions of negative electrostatic potential on a ligand may interact favorably with positively charged amino acid residues

within a protein binding pocket, whereas positively charged ligand regions can establish interactions with negatively charged residues [83]. Therefore, MEP analysis provides an important theoretical basis for understanding ligand–receptor interactions and optimizing molecular structures for enhanced pharmacological activity. For azo derivatives and heterocyclic compounds, MEP mapping is particularly useful because the presence of multiple heteroatoms and extended conjugation creates significant variations in charge distribution throughout the molecule. The azo linkage, sulfonamide group, carbonyl functionalities, and aromatic substituents collectively influence the electrostatic environment and consequently affect molecular reactivity and biological interactions [68]. By examining these electrostatic features, researchers can identify the structural elements responsible for enhanced receptor binding, improved charge transfer, and increased biological activity.

When combined with Frontier Molecular Orbital (FMO) analysis and other quantum chemical descriptors, MEP mapping provides a comprehensive evaluation of the electronic properties of molecular systems. The integrated analysis of charge distribution, frontier orbital energies, and electrostatic potential patterns facilitates a more thorough grasp of molecular reactivity, stability, intermolecular interactions, and possible biological activity [75]. Therefore, MEP analysis has become an important computational approach in modern chemistry, supporting the rational design and optimization of functional materials, bioactive molecules, and therapeutic candidates.

Therefore, Molecular Electrostatic Potential mapping offers valuable information regarding the electronic environment and reactive sites of azo derivatives. The visualization of charge distribution facilitates the prediction of intermolecular interactions, hydrogen-bonding capabilities, and ligand–receptor recognition patterns, thereby contributing significantly to the understanding of their chemical and biological properties.

2.7.3 DFT-Based Quantum Chemical Analysis

Quantum chemical calculations have become essential tools in modern drug discovery, offering detailed insight into molecular structure, electronic properties, and

chemical reactivity. Among the various computational approaches available, Density Functional Theory (DFT) stands out as a dependable and extensively used method for exploring the structural, spectroscopic, and electronic characteristics of bioactive molecules.

Through DFT-based analyses, researchers can predict molecular behavior at the atomic level, gaining a clearer understanding of structure–property relationships and supporting the rational design of therapeutic candidates [84].

In recent years, DFT and Time-Dependent DFT (TD-DFT) methodologies have been extensively employed to explore molecular geometries, electronic transitions, frontier molecular orbitals, and optical properties of various organic and heterocyclic compounds.

These computational techniques provide valuable insights into charge distribution, electron delocalization, and intermolecular interactions, which are essential parameters influencing biological activity. Furthermore, the integration of quantum chemical data with emerging computational tools and machine learning approaches has enhanced the efficiency of identifying promising drug candidates [85].

The sulfadiazine-based azo derivatives investigated in this study possess highly conjugated molecular frameworks that are expected to exhibit significant electronic communication across the azo bridge. Such conjugation influences molecular stability, reactivity, and interaction with biological targets.

Therefore, comprehensive quantum chemical investigations were conducted to elucidate the electronic features governing the behavior of these compounds. Parameters including optimized molecular geometry, frontier molecular orbital energies (HOMO and LUMO), energy gaps, Natural Bond Orbital (NBO) interactions, Mulliken charge distribution, and nonlinear optical (NLO) properties were evaluated using the DFT/B3LYP/6-31G(d,p) level of theory [86]. The computational analyses provide a molecular-level understanding of electron distribution and intramolecular charge transfer within the synthesized compounds. These properties are directly associated with molecular stability, chemical reactivity, and binding potential toward biological macromolecules. Additionally, TD-DFT calculations

were performed to investigate electronic absorption characteristics and correlate theoretical findings with experimental UV–Visible spectroscopic data [86].

By combining quantum chemical investigations with molecular docking and biological evaluation, this study establishes a comprehensive relationship between molecular structure, electronic properties, and anticancer activity. Such an integrated approach contributes to the rational development of sulfadiazine-based azo derivatives as potential therapeutic agents and provides valuable insights for the future design of biologically active heterocyclic compounds [86].

Chapter 3

Materials and Methods

All solvents and reagents used in this work were obtained from the research laboratory at Capital University of Science & Technology as well as from the manufacturing unit of Glitz Pharma. These chemicals were employed as received, with no further purification carried out. Melting points were determined via the open capillary tube technique using a digital melting point apparatus, with values reported in °C. Reaction progress was tracked using thin-layer chromatography (TLC) on pre-coated plates, with the resulting spots visualized under ultraviolet (UV) light at a wavelength of 254 nm. The synthesized compounds were characterized on the basis of their physical and spectral data. UV-Visible spectra were recorded on a double-beam UV-Vis spectrophotometer, using dimethyl sulfoxide (DMSO) as the blank, in order to aid the assessment of their biological activities, such as antimicrobial and anti-inflammatory potential. FTIR spectra of both the starting materials and the synthesized compounds were obtained using a Shimadzu FTIR spectrometer. Proton (^1H) and carbon (^{13}C) NMR spectra were recorded on an NMR spectrophotometer with DMSO as the solvent, and chemical shifts were reported as delta (δ) values in parts per million (ppm).

3.1 General Methodology for the Preparation of Azo Dyes

All azo dye derivatives were synthesized via a standard diazotization followed by coupling reaction under controlled low-temperature conditions.

In the diazotization step, sulfadimidine was dissolved in hydrochloric acid and maintained under cold conditions using an ice-salt bath at 0-5°C. A nitrosating reagent was prepared separately by reacting sodium nitrite with concentrated sulfuric acid to generate nitrosyl sulfuric acid.

The freshly prepared reagent was added gradually to the sulfadimidine solution with continuous stirring, and the reaction mixture was maintained at 0-5°C to facilitate the formation of the corresponding diazonium salt [87].

The resulting diazonium solution was subsequently added slowly to pre-cooled ethanolic solutions of the respective coupling components under constant stirring at 0-5°C. Carvacrol and resorcinol were used as coupling agents to synthesize the corresponding azo dye derivatives through electrophilic aromatic substitution.

Additionally, azo derivatives were prepared by coupling sulfadimidine with carvacrol and resorcinol, as well as 2-aminobenzothiazole with resorcinol, following the same diazotization-coupling protocol under identical reaction conditions to maintain reproducibility [87].

During the course of the coupling reaction, the pH of the mixture was carefully regulated and kept within the range of 4 to 5 through the gradual, dropwise addition of sodium bicarbonate solution. The mixture was then stirred under cold conditions to ensure the azo linkage formed completely.

The resulting products were separated via vacuum filtration, rinsed with hot water to eliminate impurities, and further purified by recrystallization from ethanol. Reaction progress was tracked using thin-layer chromatography (TLC) on pre-coated silica gel plates, with the developed spots visualized under UV light at 254 nm [87].

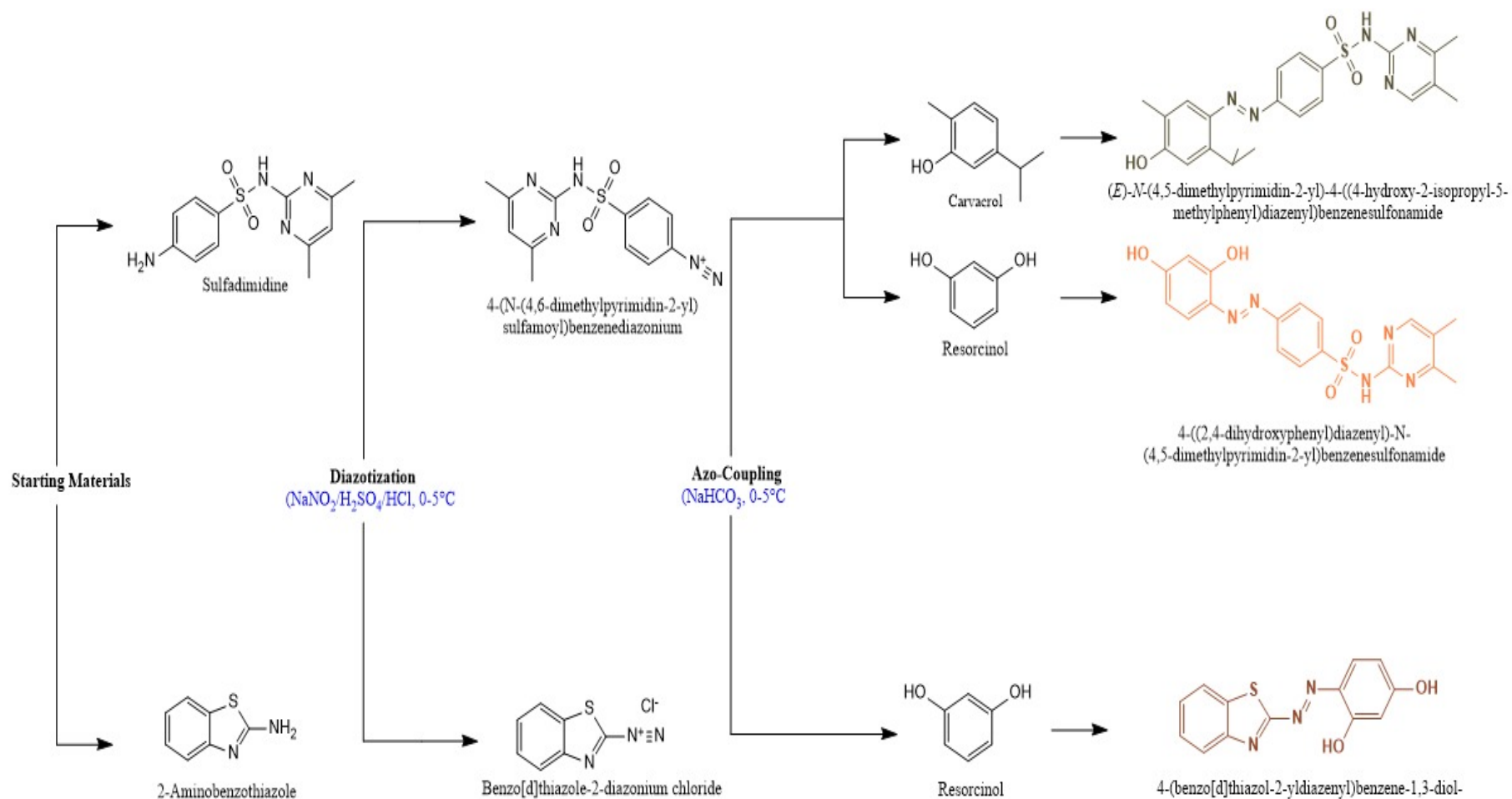


FIGURE 3.1: Scheme of Synthesis of Azo Dyes

3.2 Mechanism

The synthesis of azo dyes proceeds through two consecutive stages: diazotization, followed by azo coupling. During diazotization, sulfadimidine reacts with nitrous acid (HNO_2), generated in situ from sodium nitrite (NaNO_2) and hydrochloric acid at low temperature ($0\text{--}5^\circ\text{C}$). Under these acidic conditions, nitrous acid undergoes protonation and loss of water to form the highly reactive nitrosonium ion (NO^+). This electrophilic species then reacts with the amino group of sulfadimidine, and through a sequence of proton transfer and rearrangement steps, the corresponding diazonium salt is generated.

In the subsequent coupling step, the diazonium salt functions as an electrophile, reacting with an electron-rich aromatic coupling partner such as a phenol or aromatic amine through electrophilic aromatic substitution. The diazonium ion selectively attacks the activated position on the aromatic ring, after which deprotonation restores aromatic character, giving rise to the characteristic azo linkage ($-\text{N}=\text{N}-$). The overall result of this reaction is the formation of the corresponding azo dye derivative [88].

3.2.1 Synthesis of N-(4,5-dimethylpyrimidin-2-yl)-4-((4-hydroxy-2-isopropyl-5-methylphenyl)diazenyl)benzenesulfonamide(SC-01)

Under ice-salt bath conditions, a solution of sulfadimidine (sulfamethazine, 2.14 g, 0.0037 mol) in concentrated hydrochloric acid (2.5 mL) was cooled to $0\text{--}5^\circ\text{C}$. To this mixture, crushed ice (25 g) and sodium nitrite solution (NaNO_2 , 2.5 mL, 4 N) were added gradually with constant stirring. Diazotization was carried out at 5°C for 0.5 h to form the corresponding diazonium salt [87].

The freshly prepared diazonium solution was added dropwise, while maintaining the reaction temperature at $5\text{--}10^\circ\text{C}$, to an alkaline ethanolic solution of carvacrol (0.0037 mol) prepared in 10 mL of ethanol under continuous stirring. The coupling reaction was allowed to proceed with further stirring for 30 min to ensure complete formation of the azo linkage. Upon completion of the reaction, the pH of

the reaction mixture was adjusted to neutral (pH 7) to facilitate complete precipitation of the azo compound. The resulting brown-colored precipitate was isolated by vacuum filtration, thoroughly washed with distilled water to eliminate residual impurities, and dried. The crude product was subsequently purified by recrystallization using ethanol as the recrystallization solvent. The melting point of the purified compound was determined by the capillary tube method using a digital melting point apparatus and was found to be 280 °C. The synthesized azo dye derivative was designated as SC-01 (Figure 3.2).

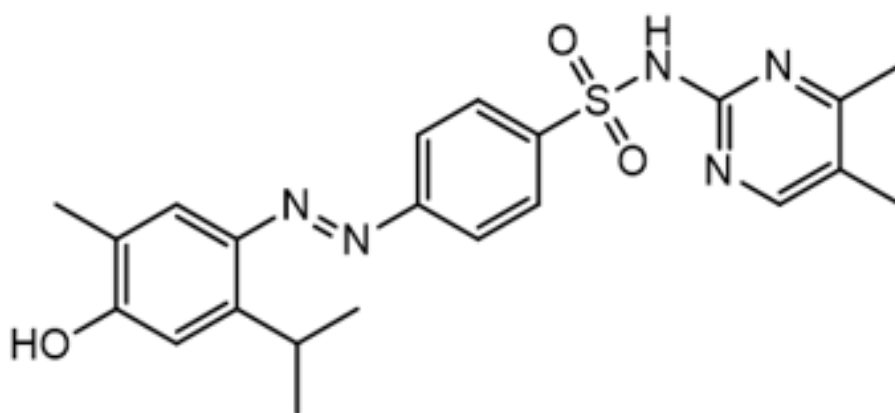


FIGURE 3.2: Structure of N-(4,5-dimethylpyrimidin-2-yl)-4-((4-hydroxy-2-isopropyl-5-methylphenyl)diazenyl)benzenesulfonamide (SC-01)

3.2.2 Synthesis of 4-((2,4-dihydroxyphenyl)diazenyl)-N-(4,5-dimethylpyrimidin-2-yl)benzenesulfonamide(SR-02)

Sulfadimidine (sulfamethazine, 1.02 g, 0.01 mol) was dissolved in concentrated hydrochloric acid (2.5 mL) in a reaction flask. Crushed ice (25 g) was then added to maintain the reaction temperature between 0 and 5°C, followed by the gradual addition of sodium nitrite solution (2.5 mL, 4 N) under continuous stirring. The reaction mixture was stirred for 30 min under cold conditions to generate the corresponding diazonium salt [87].

In a separate reaction vessel, resorcinol (1.10 g, 0.01 mol) was dissolved in ethanol and made alkaline by the addition of aqueous sodium hydroxide solution. The

freshly prepared diazonium salt solution was added dropwise to the alkaline ethanolic solution of resorcinol while maintaining the temperature between 5 and 10°C under constant stirring. The coupling reaction was continued for an additional 30 min to ensure complete formation of the azo linkage.

Upon completion of the reaction, the pH of the reaction mixture was carefully adjusted to facilitate complete precipitation of the azo compound. The resulting orange-colored precipitate was isolated by filtration, thoroughly rinsed with distilled water to remove any residual impurities, and dried under appropriate conditions. The crude product was subsequently purified by recrystallization using ethanol as the solvent to obtain the purified compound. Finally, the melting point of the purified product was determined by the capillary tube method using a digital melting point apparatus and was recorded as 273°C. The synthesized sulfadimidine-resorcinol azo dye was designated as SR-02 (Figure 3.3).

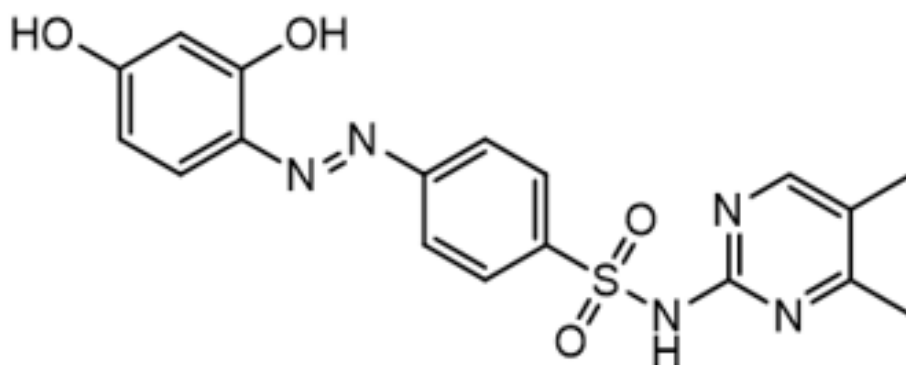


FIGURE 3.3: Structure of 4 - ((2, 4-dihydroxyphenyl)diazenyl)-N-(4, 5-dimethylpyrimidin-2-yl) benzenesulfonamide (SR-02)

3.2.3 Synthesis of 4-(benzo[d]thiazol-2-yl)diazenyl)benzene-1,3-diol-(AR-03)

2-Aminobenzothiazole (1.50 g, 0.01 mol) was dissolved in concentrated hydrochloric acid (2.5 mL) in a reaction flask. Crushed ice (25 g) was added to maintain the reaction temperature between 0 and 5°C, followed by the gradual addition of sodium nitrite solution (2.5 mL, 4 N) under continuous stirring. The reaction

mixture was stirred for 30 min under cold conditions to generate the corresponding diazonium salt [87].

In a separate reaction vessel, resorcinol (1.10 g, 0.01 mol) was dissolved in a minimal volume of ethanol, and the solution was made alkaline by the gradual addition of aqueous sodium hydroxide. The freshly prepared diazonium salt solution was subsequently introduced dropwise into the alkaline ethanolic solution of resorcinol while maintaining the reaction temperature between 5 and 10°C under constant stirring. The reaction mixture was stirred continuously for an additional 30 min to ensure complete azo coupling and the successful formation of the azo linkage.

Upon completion of the reaction, the pH of the reaction mixture was carefully adjusted to promote precipitation of the azo dye. The formation of a brick-red precipitate indicated the successful synthesis of the target azo compound. The precipitated product was isolated by filtration, thoroughly washed with distilled water to eliminate residual impurities, and dried.

The crude product was subsequently purified by recrystallization using ethanol as the solvent. The melting point of the purified compound was determined by the capillary tube method using a digital melting point apparatus and was recorded as 268°C. The synthesized 2-aminobenzothiazole-resorcinol azo dye was designated AR-03 (Figure 3.4).

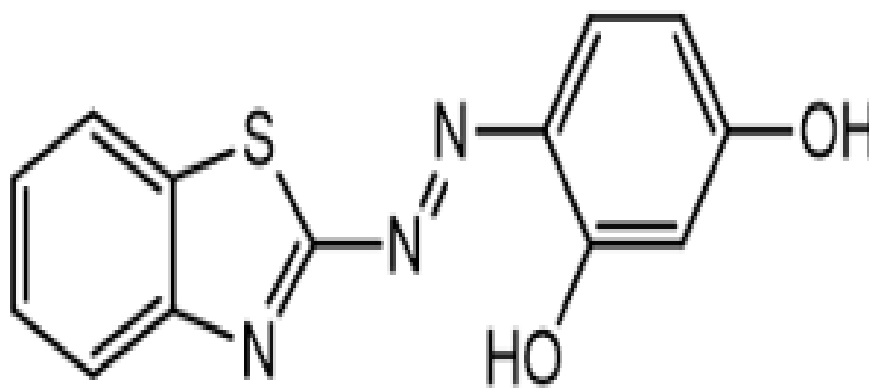


FIGURE 3.4: Structure of 4-(benzo[d]thiazol-2-yl-diazenyl)benzene-1,3-diol AR-03

3.3 Characterization

3.3.1 Thin Layer Chromatography

Thin-layer chromatography (TLC) was employed as a preliminary analytical technique for the characterization and evaluation of the purity of the synthesized azo compounds. Each synthesized compound was dissolved in dimethyl sulfoxide (DMSO) and carefully applied onto silica gel TLC plates measuring 5×10 cm. The chromatographic separation was performed using a mobile phase composed of petroleum ether, methanol, chloroform, and ethyl acetate in an optimized solvent ratio. The developed TLC plates were subsequently examined to verify the successful formation and purity of the synthesized compounds through the observation of distinct chromatographic spots. The retention factor (R_f) values of the compounds were calculated to further support their identification and purity assessment.

3.3.2 Solubility Testing

All synthesized compounds were subjected to solubility analysis to assess their dissolution behavior in different solvents. For this purpose, a small amount of each compound was transferred into separate Eppendorf tubes containing the respective solvents. The mixtures were vigorously shaken to facilitate adequate interaction between the solute and solvent and were subsequently allowed to stand for equilibration. Solubility was evaluated by visual inspection based on the complete dissolution of the compound or the presence of undissolved particles, turbidity, or residual precipitate.

3.3.3 Melting Point Determination

The melting points of the synthesized compounds were determined using an electronic Gallenkamp melting point apparatus in conjunction with the capillary tube technique. A small quantity of the sample was loaded into a capillary tube sealed at one end, and the open end was tapped gently to ensure uniform and compact packing of the material. A thermometer was fitted into the designated slot of the

apparatus, after which the heating mechanism was activated. The sample-loaded capillary tube was then placed into the instrument and observed through the magnifying lens until melting occurred. The procedure was repeated in triplicate to minimize experimental error, with the melting temperature noted for each run.

3.3.4 Ultra-Violet Analysis

UV-Visible spectra of the synthesized azo dye derivatives (SC-01, SR-02, and AR-03) and the standard drug sulfadimidine were recorded on a Shimadzu UV-1800 series double-beam UV-Vis spectrophotometer over the wavelength range of 200-600 nm, using DMSO as the blank solvent. UV-Vis analysis was performed to characterize the electronic absorption behavior of the synthesized compounds and to confirm the extended π -conjugation arising from azo bond formation, which is directly responsible for the intense visible coloration of the synthesized dyes. Stock solutions of each compound were prepared in DMSO and appropriately diluted prior to scanning to obtain absorbance values within the linear range of the instrument.

3.3.5 FTIR Analysis

The FTIR spectra of the synthesized compounds were obtained using a Shimadzu FTIR spectrometer operating in transmittance mode. Spectral acquisition was carried out over the wavenumber region of 4000-650 cm^{-1} . All analyses were conducted under controlled conditions at a constant temperature of 25°C, and the corresponding spectra were recorded for structural characterization of the synthesized compounds.

3.3.6 NMR Analysis

Nuclear Magnetic Resonance (NMR) spectroscopy was carried out by dissolving the synthesized compounds in an appropriate deuterated solvent, such as dimethyl sulfoxide- d_6 (DMSO-d_6), to reduce interference arising from residual solvent proton signals. The prepared samples were transferred into clean NMR tubes and subjected to analysis using an NMR spectrometer under a strong, homogeneous

magnetic field. Upon exposure to radiofrequency (RF) irradiation, nuclei such as ^1H and $(^{13})\text{C}$ absorb electromagnetic energy and undergo transitions between different spin states. As the excited nuclei return to their equilibrium state, they release energy in the form of characteristic signals, which are detected and recorded by the instrument. These signals are subsequently processed through Fourier transformation to produce the NMR spectrum. The resulting chemical shifts, coupling patterns, and integration values provide essential information regarding the number, type, and chemical environment of the nuclei, facilitating comprehensive structural elucidation of the synthesized compounds.

3.4 *In-Silico* Studies

In silico studies of the synthesized compounds were conducted to investigate their possible interactions with the active sites of selected target receptors. Molecular docking analysis was performed to predict the binding modes of the ligands within the receptor binding pockets, evaluate their binding affinities, and identify the important amino acid residues contributing to ligand-protein interactions. These computational investigations provide valuable insights into the possible mechanisms underlying the biological activity of the synthesized compounds and assist in evaluating their potential as promising therapeutic candidates. Moreover, *in silico* techniques serve as a rapid, cost-effective, and efficient approach for the preliminary screening of bioactive molecules, helping to minimize the time and resources required during the initial phases of drug discovery. Such approaches also support structure-based drug design by providing useful information for optimizing lead compounds [89].

3.4.1 Molecular Docking Investigation

3.4.1.1 Retrieval of Protein Structures from the Protein Data Bank

The three-dimensional structures of the selected receptor proteins were obtained from the Protein Data Bank (PDB) repository in PDB file format. The corresponding PDB identification codes assigned to the selected protein structures

were 5IKR and 1AJ0, which were used for subsequent molecular docking analysis.

3.4.1.2 Preparation of Protein Macromolecule

The selected protein structures were prepared for molecular docking analysis using AutoDock Tools. Prior to docking, the protein macromolecules underwent structural optimization through energy minimization and the addition of Gasteiger partial charges. The prepared protein structures were subsequently converted and saved in PDBQT format for docking simulations. Structural validation and visualization were performed using Discovery Studio 4.1 Client (2012). Additionally, the VADAR 1.8 web server was employed to evaluate the statistical distribution of structural and physicochemical properties of the proteins, including the percentages of α -helices, β -sheets, turns, and coils. The obtained structural parameters and validation analyses were used to confirm the reliability and suitability of the selected proteins for molecular docking studies [90].

3.4.1.3 Ligands-Proteins Molecular Docking

After energy minimization, the synthesized compounds were drawn and optimized using Discovery Studio Client, and the resulting structures were saved in PDB format for use as ligand models. The ligand preparation process was then carried out using AutoDock Tools, where appropriate conformations were selected, and essential atomic parameters, were assigned. After adding the Gasteiger and Kolman charges, the prepared ligand files were subsequently converted into PDBQT format to make them compatible with molecular docking analysis. Virtual screening was performed using the AutoDock Vina Wizard module to evaluate the binding interactions of the synthesized compounds with the selected target receptor [90].

The default exhaustiveness value of 100 was used to dock the chosen protein targets to individual molecules. The lowest binding energy values (kcal/mol) were used to assess the anticipated docked complexes. Discovery Studio (2.1.0) and Discovery Studio Visualizer Software, Version 4.0 (2012) were used to graphically depict each docked complex in three dimensions.

3.4.2 ADMET Assessment

The SwissADME web tool (<https://www.swissadme.ch/>) was employed to evaluate the physicochemical and pharmacokinetic properties of the synthesized compounds, including molecular weight, hydrogen-bond donors and acceptors, topological polar surface area (TPSA), rotatable bonds, and octanol/water partition coefficient (iLOGP).

The BOILED-Egg model was also generated using the SwissADME platform to assess the compounds' gastrointestinal absorption and blood–brain barrier permeability. Toxicological profiles were predicted using ProTox-3.0, including genetic toxicity endpoints (carcinogenicity, mutagenicity, and cytotoxicity), organ-specific toxicities (hepatotoxicity, nephrotoxicity, neurotoxicity, and cardiotoxicity), and acute oral toxicity based on the predicted median lethal dose (LD₅₀, mg/kg).

3.5 Density Functional Theory Studies

Density Functional Theory (DFT) calculations were performed to investigate the electronic characteristics, molecular stability, and chemical reactivity of the synthesized compounds. The molecular structures were initially designed using ChemDraw and subsequently subjected to geometry optimization to obtain their most stable conformations before performing quantum chemical calculations. All computational analyses were carried out using the Gaussian 16 and companion graphical interface GaussView 6. The optimized structures were verified as true minima on the potential energy surface by confirming the absence of imaginary vibrational frequencies.

The electronic structure calculations of the synthesized compounds were performed using the hybrid Becke three-parameter Lee-Yang-Parr (B3LYP) functional in combination with the 6-31G (d,p) basis set. This computational approach was selected due to its proven reliability in predicting the electronic properties of organic molecules while maintaining a reasonable computational cost. The molecular geometries were optimized without imposing any symmetry constraints, and

the optimized structures were subsequently used for further quantum chemical investigations.

After geometry optimization, several important molecular descriptors were calculated, including the energies of the Highest Occupied Molecular Orbital (HOMO) and Lowest Unoccupied Molecular Orbital (LUMO), HOMO-LUMO energy gap (δE), ionization potential, electron affinity, chemical hardness, chemical softness, electronegativity, chemical potential, and electrophilicity index.

In addition, Molecular Electrostatic Potential (MEP) surfaces and Frontier Molecular Orbital (FMO) analyses were generated to examine molecular charge distribution, locate electron-rich and electron-deficient regions, and gain insights into the chemical reactivity and interaction behavior of the synthesized compounds.

The obtained DFT parameters were employed to assess the electronic behavior, stability, and reactivity profiles of the synthesized compounds.

These computational findings were further integrated with molecular docking results and biological activity data to establish structure-activity relationships and provide insights into the potential anti-inflammatory properties of the synthesized derivatives.

3.5.1 Time-Dependent Density Functional Theory

Time-Dependent Density Functional Theory (TD-DFT) calculations were carried out to assess the electronic excitation behavior of the synthesized compounds. The geometries previously optimized via DFT served as the starting structures for the TD-DFT analysis. To ensure methodological consistency and reliable results, the same computational protocol used for ground-state geometry optimization was retained for the excited-state calculations.

TD-DFT calculations were performed to investigate various excited-state parameters, including excitation energies, absorption wavelengths (λ_{max}), oscillator strengths (f), and the nature of electronic transitions. Vertical excitation energies were calculated using the optimized ground-state geometries without allowing

further geometric relaxation. Multiple low-energy singlet excited states were considered to provide a detailed understanding of the electronic absorption behavior of the synthesized compounds.

The molecular orbitals involved in the electronic transitions were analyzed to elucidate charge-transfer characteristics and changes in electron density distribution between the ground and excited states. The contribution of specific molecular orbitals to each excitation was examined to assign the major electronic transitions. Furthermore, theoretical UV-Visible absorption spectra were generated based on the calculated excitation energies and oscillator strengths to predict the optical response of the synthesized molecules.

The TD-DFT results were utilized to interpret the photophysical properties, electronic transition patterns, and optical behavior of the synthesized compounds. These computational findings complemented the experimental UV-Visible analysis and other theoretical investigations, providing deeper insight into the electronic structure, chemical properties, and potential biological relevance of the synthesized derivatives.

3.5.2 *In-Vitro* Evaluation

3.5.2.1 Antibacterial Activity

The antibacterial activity of the synthesized compounds was evaluated against selected bacterial strains, including *Escherichia coli* (*E. coli*) (ATCC 25922), *Staphylococcus aureus* (*S. aureus*) (ATCC 6538), and *Pseudomonas aeruginosa* (*P. aeruginosa*) (ATCC 9027), using the agar well diffusion method. The bacterial cultures were prepared and adjusted to an appropriate inoculum concentration before being spread onto nutrient agar plates. Wells were prepared in the inoculated agar plates, and the synthesized compounds were introduced into the wells at selected concentrations. A suitable standard antibacterial drug was used as a positive control, while the respective solvent was used as a negative control.

The plates were incubated under suitable conditions, and antibacterial activity was assessed by measuring the diameter of the zone of inhibition (ZOI) around

each well. All experiments were performed in triplicate, and the results were expressed as mean $\pm$ standard deviation (SD). The obtained data were subjected to statistical analysis to evaluate the antibacterial efficacy of the synthesized compounds.

3.5.3 *In-Vivo* Pharmacological Potential

3.5.3.1 Animals

Male BALB/c mice (30-35 g body weight) were employed for the *in vivo* pharmacological studies. Animals were maintained under standard laboratory conditions at a controlled temperature of $24 \pm 0.5^{\circ}\text{C}$ and relative humidity of $40 \pm 5\%$, with unrestricted access to standard diet and water for the entire duration of the experiment. All *in vivo* procedures were carried out at the Faculty of Pharmacy, Capital University of Science and Technology (CUST), Islamabad, and were conducted in compliance with protocols sanctioned by the university's Bioethical Committee (Approval No. CUST/DERC/PH/ERF/PG/2026/023).

3.5.3.2 Carrageenan Induced Inflammation Model

A carrageenan-induced acute inflammation model was employed to evaluate the anti-inflammatory potential of the synthesized compounds SC-01, SR-02, and AR-03 according to a previously reported method [91]. Following a seven-day acclimatization period, during which male BALB/c mice (6-7 weeks old) were maintained on standard diet and water under controlled housing conditions, the experiment was commenced. Animals were randomly allocated into six groups ($n = 6$ per group) as follows: Group I (normal control) received only normal saline (10 mL/kg, i.p.) with no inflammatory challenge; Group II (negative control) received normal saline followed by intraplantar injection of 1% carrageenan (20 μL) to induce paw inflammation; Group III (positive control) was pretreated with diclofenac sodium (5 mg/kg, i.p.) prior to carrageenan injection; Group IV received compound SC-01 (10 mg/kg, i.p.) alongside carrageenan challenge; Group V was treated with compound SR-02 (10 mg/kg, i.p.) followed by carrageenan administration; and Group VI received compound AR-03 (10 mg/kg, i.p.) together with carrageenan.

All test and control formulations were dissolved in normal saline containing 2% DMSO and given 30 minutes before carrageenan injection. The anti-inflammatory response was evaluated by measuring hind paw thickness at baseline (0 h) and at 1, 2, 4, and 6 h post-carrageenan induction.

3.6 Statistical Analysis

Statistical evaluation of both *in vitro* and *in vivo* data was carried out using one-way or two-way analysis of variance (ANOVA), depending on the experimental design. All graphs were generated and statistical computations performed using GraphPad Prism (version 10). A p-value of less than 0.05 was taken as the threshold for statistical significance between groups. Results throughout the study are reported as mean $\pm$ standard deviation (SD).

Chapter 4

Results

4.1 Chemistry

The azo dye derivatives derived from sulfadimidine and 2-aminobenzothiazole were successfully synthesized according to the procedures described in Section 3.2.

The synthesized compounds were evaluated for purity by Thin Layer Chromatography (TLC) and characterized using Fourier Transform Infrared (FTIR) and Nuclear Magnetic Resonance (NMR) spectroscopy.

Distinct TLC patterns were observed for the synthesized compounds compared with their respective starting materials. FTIR spectra showed characteristic absorption bands corresponding to the azo linkage ($-N=N-$) along with the expected functional groups.

The ^1H NMR spectra exhibited signals corresponding to aromatic, hydroxyl, methyl, and isopropyl protons, while ^{13}C NMR spectra showed signals attributable to the expected aromatic, pyrimidine, and benzothiazole carbons.

The molecular formula, molecular weight, melting point, physical state, color, percentage yield, and solubility of the synthesized compounds are presented in Table [4.1](#). All synthesized compounds were obtained as colored solids with excellent yields and showed good solubility in DMSO.

TABLE 4.1: Physical Data of Synthesized Compounds

Compound	Mol formula	Mol Weight	Melting Point	Physical State	Color	% Yield	Solubility
SC-01	C ₂₁ H ₂₄ N ₆ O ₃ S	440.52	280°C	Solid	Brown	89	DMSO
SR-02	C ₁₈ H ₁₈ N ₆ O ₄ S	414.44	273°C	Solid	Orange	85	DMSO
AR-03	C ₁₃ H ₉ N ₃ O ₂ S	271.29	268°C	Solid	Brick Red	89	DMSO

4.2 UV Analysis

4.2.1 UV Analysis of the Synthesized Compound SC-01

The UV-Vis spectrum of SC-01 (0.2 mg/mL in DMSO, DMSO as blank; 200-600 nm scan) shows features consistent with successful azo coupling between diazotized sulfadimidine and carvacrol. The spectrum shows a weak $\pi \rightarrow \pi^*$ absorption in the 200–280 nm region, which is associated with electronic transitions within the aromatic/conjugated system.

Its main azo absorption occurs at 350–420 nm, indicating the presence of an extended conjugated $-N=N-$ chromophore. The relatively broad visible-region absorption suggests effective conjugation within the molecule.

4.2.2 UV Analysis of the Synthesized Compound SR-02

The UV-Vis spectrum of SR-02 (0.2 mg/mL in DMSO, DMSO as blank; 200-600 nm scan) is consistent with successful azo coupling between diazotized sulfadimidine and resorcinol. SR-02 exhibits a weak, relatively flat absorption below 280 nm, corresponding mainly to $\pi \rightarrow \pi^*$ transitions. Its main azo band extends broadly from 300–450 nm and is described as intense, indicating stronger absorption by the azo chromophore and a relatively extended conjugated electronic system. The broader range may reflect increased electronic delocalization compared with SC-01.

4.2.3 UV Analysis of Synthesized Compound AR-03

The UV-Vis spectrum of AR-03 (0.2 mg/mL in DMSO, DMSO as blank; 200–600 nm scan) is consistent with successful azo coupling between diazotized 2-aminobenzothiazole and resorcinol. AR-03 shows a weak, flat $\pi \rightarrow \pi^*$ absorption below 280 nm. Its main azo band appears at 300–350 nm and is reported as strong. Compared with SC-01 and SR-02, this band is shifted toward the shorter-wavelength region, suggesting a somewhat less extended effective conjugation or different electronic environment around the azo group

TABLE 4.2: UV-Vis Spectroscopy Data of Synthesized Compounds

Compound	$\pi \rightarrow \pi^*$ Region	Main Azo Band	Intensity	Interpretation
SC-01	200–280 nm	350–420 nm	Weak $\pi \rightarrow \pi^*$; azo band moderate/broad	Azo chromophore with extended conjugation; absorption in the longer-wavelength region
SR-02	<280 nm	300–450 nm	Weak, flat $\pi \rightarrow \pi^*$; broad and intense azo band	Strong azo absorption with broad conjugated electronic response
AR-03	<280 nm	300–350 nm	Weak, flat $\pi \rightarrow \pi^*$; strong azo band	Strong azo absorption, but located at shorter wavelengths

4.3 FTIR Spectral Data

The synthesized azo compounds were prepared according to the reported synthetic procedures and obtained in satisfactory yields. FTIR spectroscopy was employed to confirm the presence of characteristic functional groups and verify the successful formation of the synthesized azo derivatives.

The FTIR spectra exhibited characteristic absorption bands corresponding to the structural features of the synthesized compounds, including hydroxyl, sulfonamide, aromatic, and heterocyclic functionalities.

The successful formation of the azo linkage was confirmed by the appearance of characteristic -N=N- stretching vibrations within the expected wavenumber region, consistent with previously reported azo compounds.

Additionally, the spectra displayed characteristic bands associated with aromatic C=C stretching, O-H stretching, N-H vibrations, sulfonyl (S=O) groups, and other functional moieties present within the synthesized azo dye structures. These spectral findings supported the successful synthesis and structural confirmation of the target azo derivatives.

4.3.1 FTIR of N-(4,5-dimethylpyrimidin-2-yl)-4-((4-hydroxy-2-isopropyl-5-methylphenyl)diazenyl)benzenesulfonamide SC-01

The FTIR spectrum of the synthesized azo compound SC-01, prepared by diazotization of sulfadimidine followed by azo coupling with carvacrol, is shown in Figure 4.1. The spectrum displayed characteristic absorption bands attributable to the principal functional groups of the proposed structure.

TABLE 4.3: FTIR spectral data of compound SC-01

Functional Group	Observed Wavenumber (cm ⁻¹)
O-H	3523.8
N-H	3401.7
C=N	1658.0
N=N	1620.4
S=O	1103.0

The broad absorption band observed at 3523.8 cm⁻¹ can be attributed to the O-H stretching vibration of the phenolic hydroxyl group of the carvacrol moiety. The absorption band at 3401.7 cm⁻¹ corresponds to the N-H stretching vibration of the sulfonamide group.

A prominent band at 1658.0 cm^{-1} is assigned to the C=N stretching vibration, associated with the heterocyclic pyrimidine ring of sulfadimidine.

The absorption band observed at 1620.4 cm^{-1} is characteristic of the azo ($-\text{N}=\text{N}-$) linkage, providing evidence for the successful diazotization and subsequent azo coupling reaction.

The strong absorption observed at 1103.0 cm^{-1} is attributed to the S=O stretching vibration of the sulfonamide group.

Overall, the FTIR spectrum confirms the successful azo dye formation, clearly showing all key functional groups. The principal FTIR absorption bands for SC-01 are shown in Table 4.3.

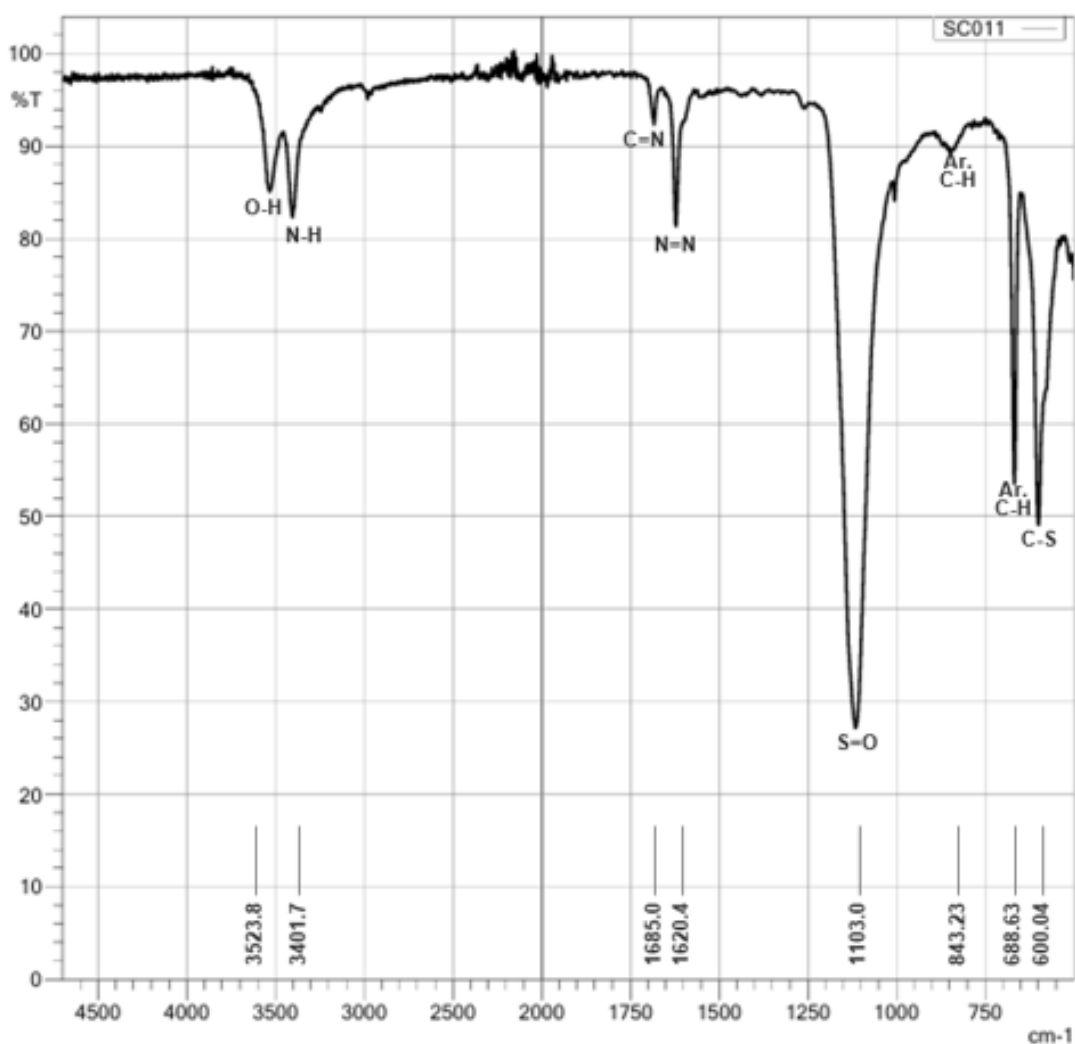


FIGURE 4.1: FTIR Spectra of compound SC-01

4.3.2 FTIR Characterization of 4-((2,4-dihydroxyphenyl) diazenyl)-N-(4,5-dimethylpyrimidin-2-yl)benzenesulfonamide (SR-02)

The FTIR spectrum of the synthesized azo compound SR-02 is shown in Figure 4.2. The spectrum displayed characteristic absorption bands attributable to the principal functional groups of the proposed structure.

TABLE 4.4: FTIR spectral data of compound SR-02

Functional Group	Observed Wavenumber (cm^{-1})
O-H	3568.3
N-H	3368.6
C=N	1511.1
N=N	1617.5
S=O	1145.7

The broad absorption band observed at 3568.3 cm^{-1} is attributed to the O-H stretching vibration of the phenolic hydroxyl groups of the resorcinol moiety. The band at 3368.6 cm^{-1} corresponds to the N-H stretching vibration of the sulfonamide group.

The absorption band at 1617.5 cm^{-1} is characteristic of the azo ($-\text{N}=\text{N}-$) linkage, providing evidence for the successful formation of the azo-coupled product.

The band observed at 1511.1 cm^{-1} can be assigned to C=N stretching associated with the pyrimidine ring of sulfadimidine. The strong band at 1145.7 cm^{-1} corresponds to the S=O stretching vibrations of the sulfonamide group.

Overall, the FTIR spectrum confirms the successful azo dye formation, clearly showing all key functional groups. The principal FTIR absorption bands for SR-02 are shown in Table 4.4.

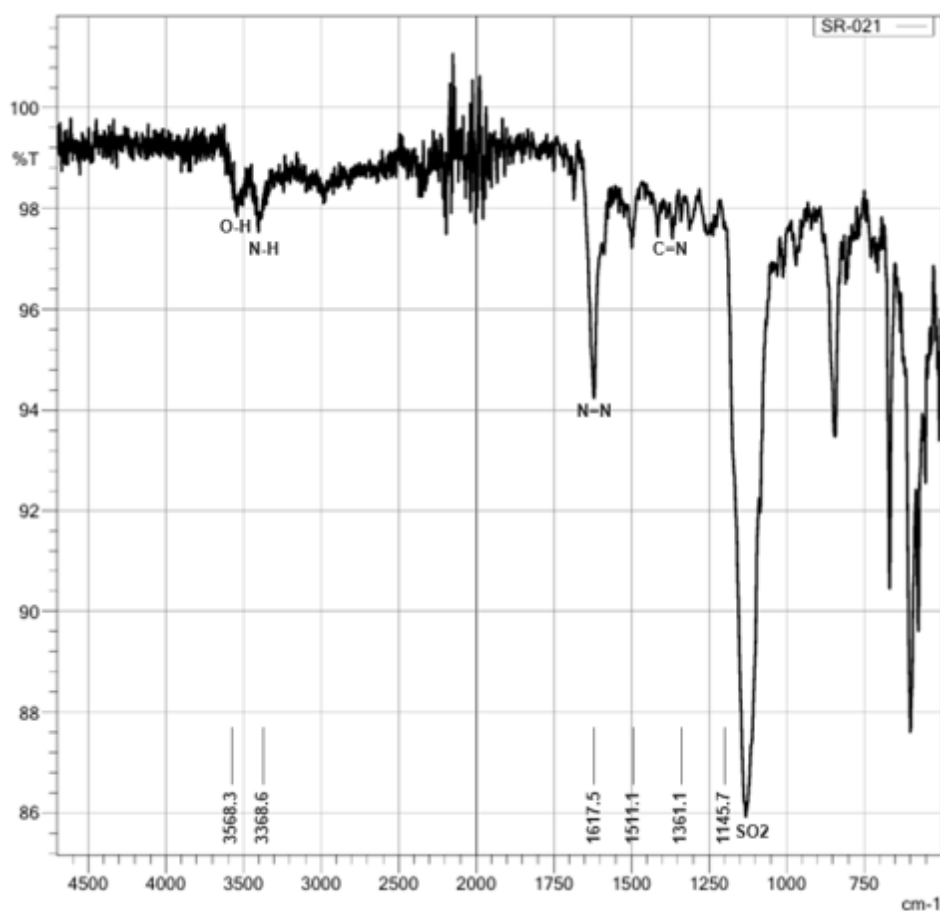


FIGURE 4.2: FTIR Spectrum of SR-02

4.3.3 FTIR Characterization of 4-(benzo[d]thiazol-2-yl)diazenyl)benzene-1,3-diol (AR-03)

The FTIR spectrum of the synthesized azo dye AR-03 is shown in Figure 4.3. The spectrum displayed characteristic absorption bands attributable to the principal functional groups of the proposed structure.

TABLE 4.5: FTIR spectral data of compound AR-03

Functional Group	Observed Wavenumber (cm ⁻¹)
O-H	3410.3
Ar. C-H	3060.4
C=N	1610.3
N=N	1510.4
Ar. C=C	1450.6

The absorption band observed at 3410.3 cm^{-1} is attributed to the O–H stretching vibration of the phenolic hydroxyl group of the resorcinol moiety. The band at 3060.4 cm^{-1} corresponds to the aromatic C–H stretching vibrations of the benzothiazole and resorcinol rings.

The sharp, strong absorption at 1610.3 cm^{-1} is assigned to the C=N stretching vibration of the thiazole ring of the benzothiazole nucleus.

The strong band at 1510.4 cm^{-1} is characteristic of the azo (--N=N--) linkage, providing evidence for the successful formation of the azo-coupled product. The band at 1450.6 cm^{-1} is assigned to aromatic C=C skeletal stretching vibrations. Overall, the observed FTIR absorption bands are consistent with the proposed structure of AR-03 and support the successful synthesis of the proposed structure.

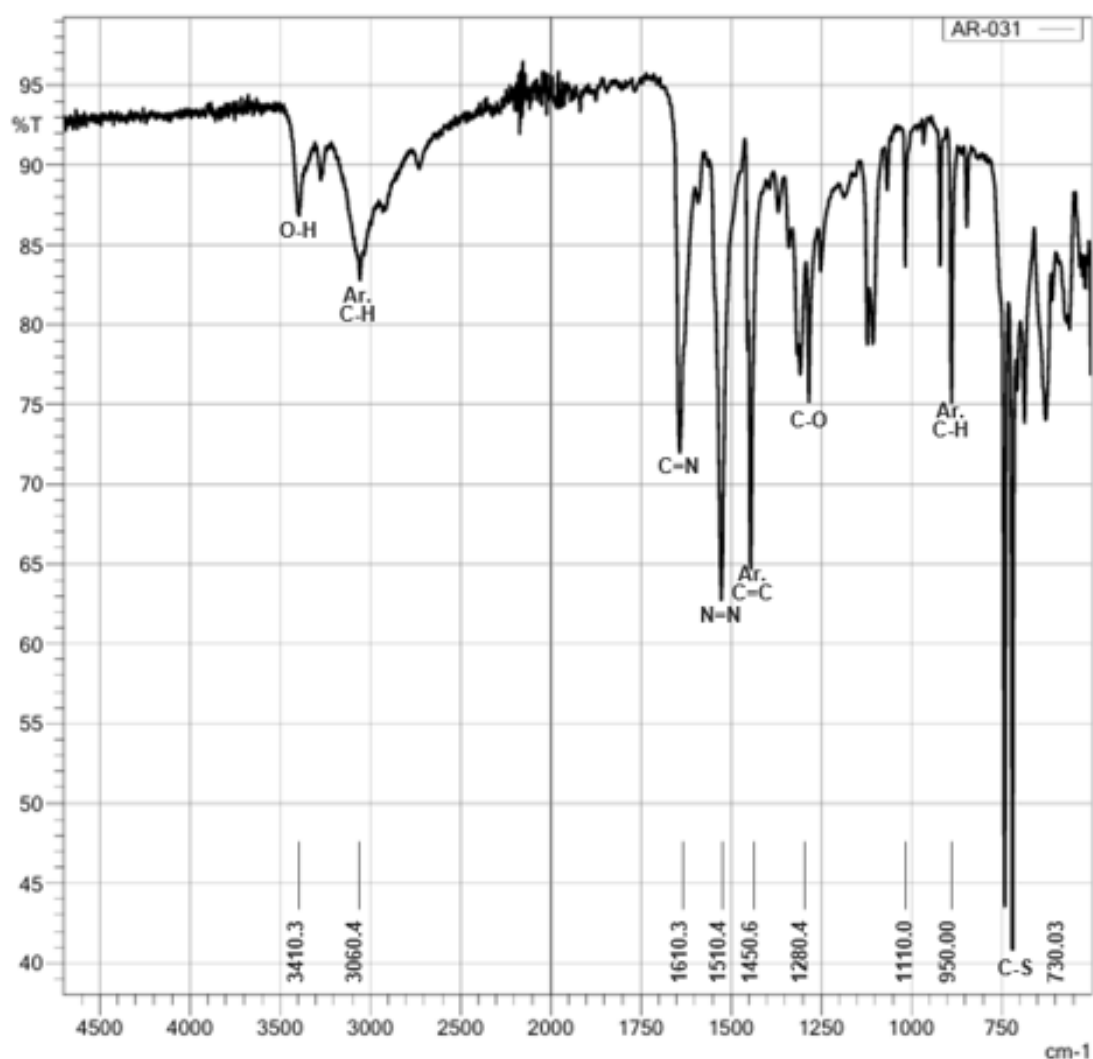


FIGURE 4.3: FTIR Spectrum of AR-03

4.4 NMR Spectra of the Synthesized Compound

4.4.1 ^1H -NMR Spectra of 4-((2,4-dihydroxyphenyl)diazenyl)-N-(4,5-dimethylpyrimidin-2-yl)benzenesulfonamide

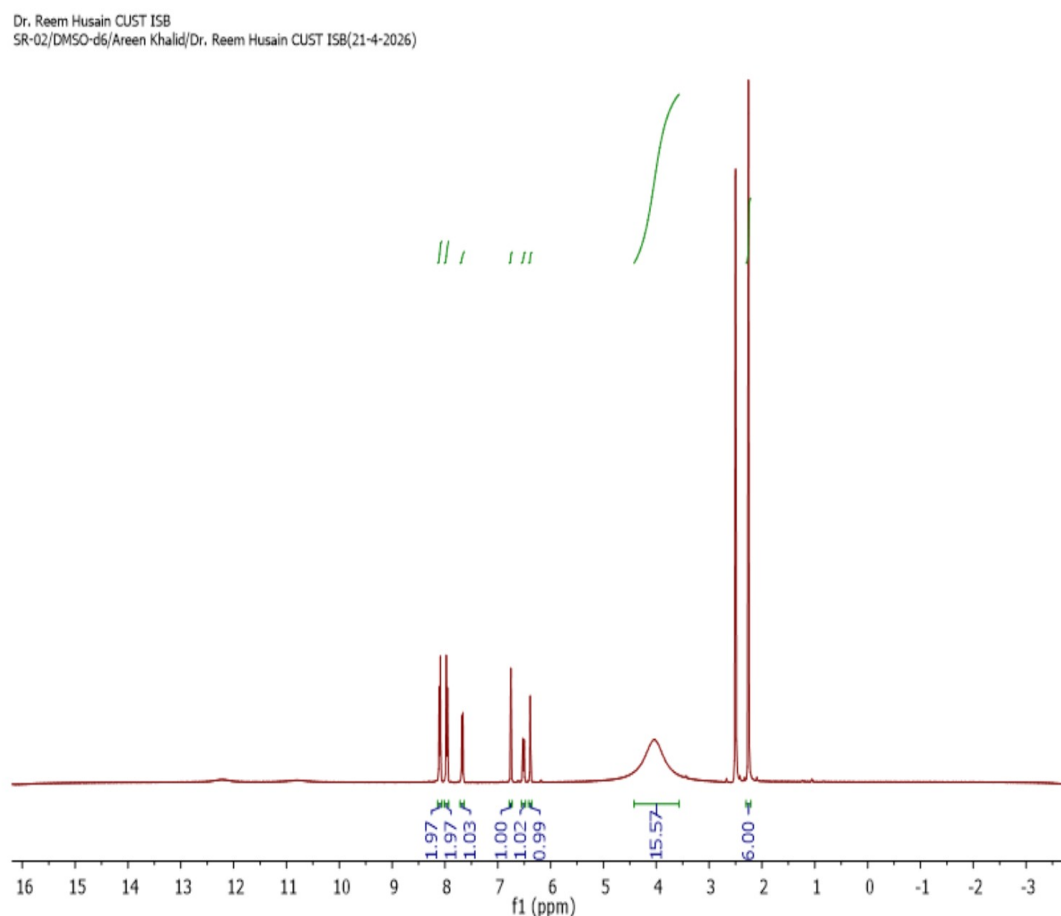


FIGURE 4.4: ^1H -NMR Spectra of compound SR-02

^1H -NMR (DMSO- d_6) (400 MHz): δ 2.18 (3H, s), 2.57 (3H, s), 6.41–6.54 (2H, 6.48 (dd, $J = 8.0, 1.5$ Hz), 6.48 (dd, $J = 1.5, 0.5$ Hz)), 7.68 (2H, ddd, $J = 7.7, 1.8, 0.5$ Hz), 7.98 (1H, s), 7.83–8.00 (3H, 7.89 (ddd, $J = 7.7, 1.6, 0.5$ Hz), 7.94 (dd, $J = 8.0, 0.5$ Hz)).

4.4.2 C-NMR Spectra of 4-((2,4-dihydroxyphenyl)diazonyl)-N-(4,5-dimethylpyrimidin-2-yl)benzenesulfonamide

^{13}C -NMR (DMSO- d_6): δ 23.1 (1C, s), 103.5 (1C, s), 110.2 (1C, s), 121.8 (2C, s), 129.2 (2C, s), 129.7 (1C, s), 133.3 (1C, s), 141.8 (1C, s), 153.2 (1C, s), 156.4 (1C, s), 158.1 (1C, s), 164.7 (1C, s)

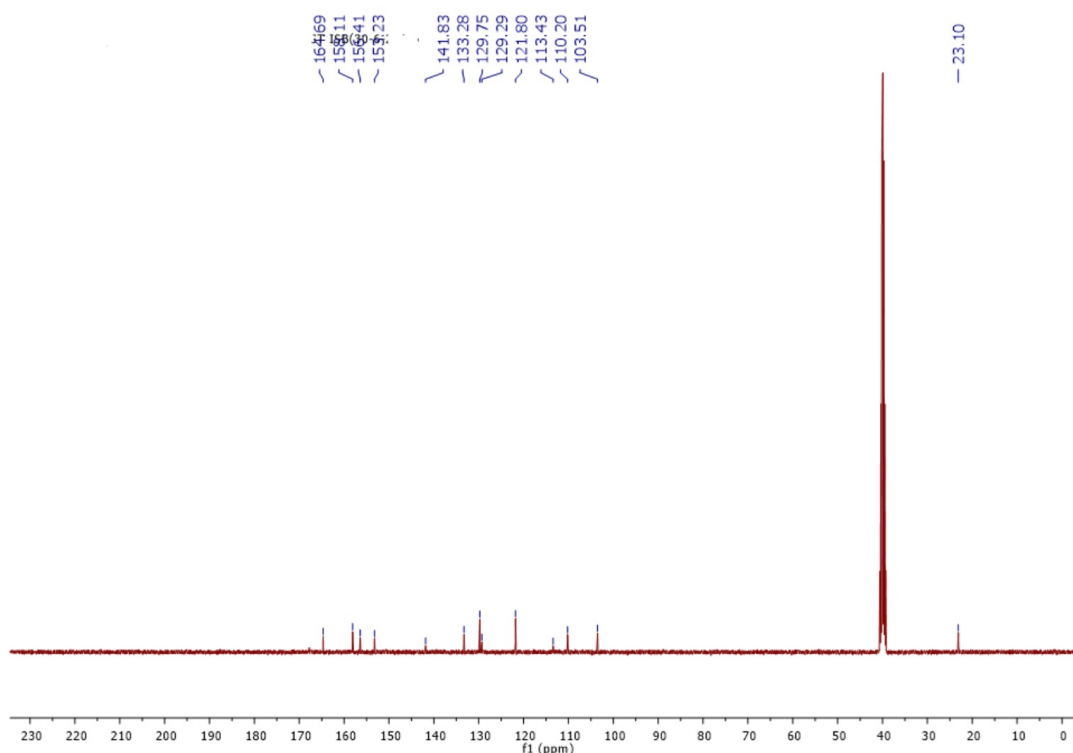


FIGURE 4.5: C-NMR Spectra of compound SR-02

4.5 *In-Silico* ADME and Drug-Likeness Evaluation

4.5.1 Physicochemical Properties of Synthesized Derivatives

The physicochemical features of the synthesized derivatives (SC-01, SR-02 and AR-03) were evaluated using the SwissADME web server, and the calculated

parameters are summarized in Table 4.6. These parameters included molecular weight, hydrogen bond donors and acceptors, topological polar surface area (TPSA), lipophilicity, rotatable bonds, molar refractivity, and fraction of sp^3 -hybridized carbons (F.Csp³).

The molecular weights of the synthesized derivatives of sulfadimidine and 2-amino-benzothiazole ranged from 271.29 g/mol to 440.52 g/mol. However, AR-03 possessed the lowest molecular weight (271.29 g/mol), while SC-01 exhibited the highest molecular weight (440.52 g/mol). Furthermore, hydrogen bond acceptors ranged from 5 to 8, but hydrogen bond donors ranged between 2 and 3 across the derivatives. The TPSA values show considerable variability, SR-02 exhibited the highest TPSA (145.51 Å²) while AR-03 displayed the lowest TPSA value of 106.31 Å².

The number of rotatable bonds ranged between 2 and 6, with SC-01 owning the highest molecular flexibility. The fraction of sp^3 carbon atoms (F.Csp³) vary from 0.11 to 0.27 for all compounds except SC-01 which showed higher value of 0.27 demonstrating a relatively greater degree of saturation in comparison to other compounds. Generally, the physicochemical parameters show structural diversity among the compounds, with variability in size, polarity, and flexibility that might influence their pharmacokinetic behavior.

TABLE 4.6: Physicochemical Properties of Compounds calculated with SwissADME database

Property	SC-01	SR-02	AR-03
Molecular Formula	C ₂₁ H ₂₄ N ₆ O ₃ S	C ₁₈ H ₁₈ N ₆ O ₄ S	C ₁₃ H ₉ N ₃ O ₂ S
MW (g/mol)	440.52	414.44	271.29
F.Csp ³	0.27	0.11	0.00
nHA	31	28	19
nAHA	18	18	15
nHBA	7	8	5
nHBD	2	3	2
nRB	6	5	2
MR	121.26	103.74	74.28
TPSA (Å ²)	125.28	145.51	106.31

4.5.2 Pharmacokinetic Profile Prediction

Pharmacokinetic properties of the synthesized derivatives were predicted with SwissADME, and the results were presented in Table 4.7. Key parameters evaluated include gastrointestinal (GI) absorption, blood-brain barrier (BBB) permeability, P-glycoprotein (P-gp) substrate status, bioavailability score, and cytochrome P-450 (CYP) enzyme inhibition profiles.

All the compounds showed similar GI absorption with a bioavailability score of 0.55. All compounds were predicted to be non-permeable across the blood-brain barrier, suggesting limited central nervous system exposure.

None of the synthesized compounds were predicted to act as P-gp substrates. Regarding metabolic interactions, CYP1A2, CYP2D6, and CYP3A4 inhibition was not predicted for any of the compounds. CYP2C9 and CYP2C19 inhibition was predicted only for AR-03, whereas SC-01 and SR-02 showed no inhibition of these enzymes.

Overall, SC-01 and SR-02 exhibited no predicted inhibition of the tested CYP enzymes, while AR-03 was predicted to inhibit CYP2C9 and CYP2C19 only.

TABLE 4.7: Pharmacokinetic profiling and bioavailability assessment of newly synthesized azo dyes via SwissADME

Property	SC-01	SR-02	AR-03
Bio-avail-ability	0.55	0.55	0.55
GI absorp-tion	Low	Low	High
BBB permea-bility	No	No	No
P-gp sub strate	No	No	No
CYP1A2 Inh	No	No	No
CYP2C19 Inh	No	No	Yes
CYP2C9 Inh	No	No	Yes
CYP2D6 Inh	No	No	No
CYP3A4 Inh	No	No	No

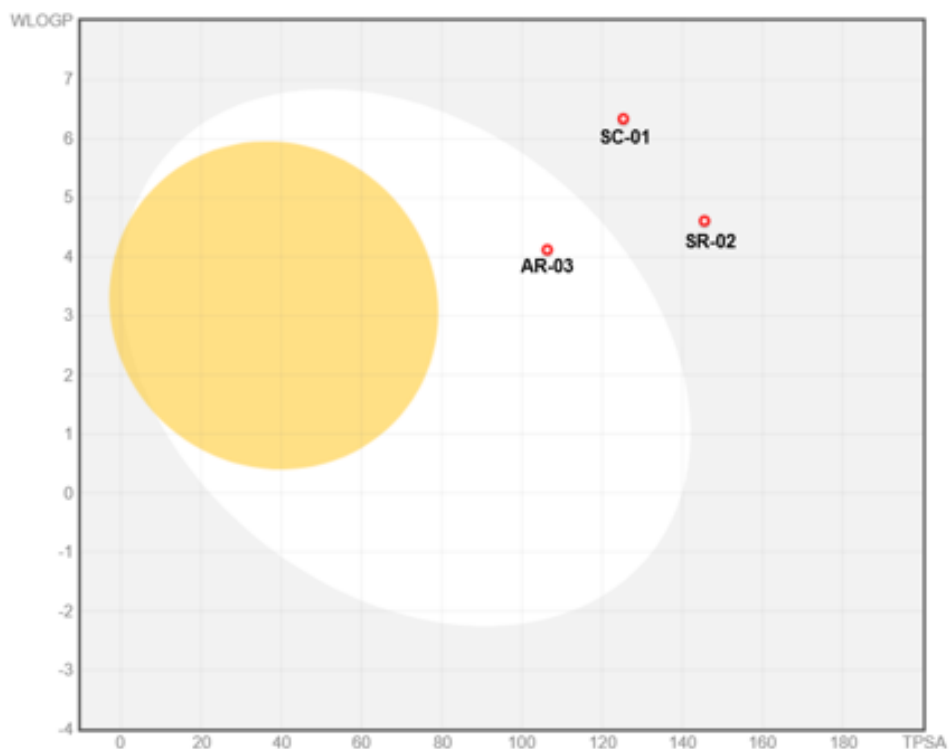


FIGURE 4.6: The BOILED-Egg analysis of synthesized azo dyes

The BOILED-Egg model shown in Figure 4.6 further reinforced the predicted pharmacokinetic behavior, showing that the compounds mostly fall within regions associated with limited BBB penetration. At the same time, selected compounds demonstrated favorable GI absorption characteristics.

4.5.3 Drug-Likeness Assessment

Drug-likeness properties of the synthesized compounds were assessed using five commonly applied rule-based filters, including Lipinski, Ghose, Veber, Egan, and Muegge rules. The results of this assessment are summarized in Table 4.8.

SC-01, SR-02, and AR-03 all satisfied the Lipinski criteria. SC-01 and AR-03 complied with the Veber criteria, whereas SR-02 failed to comply with this rule. Regarding the Ghose filter, SC-01 showed no violation, while SR-02 and AR-03 complied with the Ghose criteria. SC-01 and SR-02 failed to comply with the Egan criteria, whereas AR-03 satisfied this rule. All three compounds obeyed the Muegge criteria. Overall, AR-03 presented a favorable drug-likeness profile across

all evaluated filters, while SC-01 and SR-02 exhibited some deviations from the predicted drug-likeness parameters.

TABLE 4.8: Drug Likeness Scores

Compound	Lipinski	Ghose	Veber	Egan	Muegge
SC-01	Yes	No	Yes	No	Yes
SR-02	Yes	Yes	No	No	Yes
AR-03	Yes	Yes	Yes	Yes	Yes

4.6 Medicinal Chemistry

Medicinal chemistry profile of the synthesized compounds was evaluated through SwissADME, focusing on PAINS alerts, Brenk alerts, lead-likeness, and synthetic accessibility. The results are briefly shown in Table 4.9.

All three compounds exhibited one PAINS alert associated with the azo_A structural feature, indicating a potential for false-positive activity in biological assays. Similarly, Brenk alerts were detected for all compounds, with one alert related to the diazo_group feature in each derivative. None of the compounds satisfied the lead-likeness criteria. SC-01 showed two violations, related to molecular weight above 350 g/mol and XLOGP3 above 3.5, while SR-02 exhibited one violation due to molecular weight above 350 g/mol. AR-03 showed one violation associated with XLOGP3 above 3.5. Synthetic accessibility scores ranged from 2.87 to 3.54, indicating relatively moderate synthetic accessibility across the compounds. SC-01 exhibited the highest synthetic accessibility score, whereas AR-03 showed the lowest score.

TABLE 4.9: Synthetic accessibility, lead-likeness, and structural alert profiling (PAINS and Brenk) of synthesized compounds

Compound	Pains	Brenk	Lead Likeness	Synthetic Accessibility
SC-01	1 alert: azo_A	1 alert: diazo_group	No; 2 violations: MW>350, XLOGP3>3.5	3.54

Table 4.9 continued from previous page

Compound	PAINS	Brenk	Lead	Likeness	Synthetic Accessibil- ity
SR-02	1 alert: azo_A	1 alert: di- azo-group	No; 1 violation: MW>350		3.21
AR-03	1 alert: azo_A	1 alert: di- azo-group	No; 1 violation: XLOGP3>3.5		2.87

4.6.1 Lipophilicity

The lipophilicity of the compounds was predicted using five different models, including XlogP3, WlogP, iLogP, MlogP, and SILICOS-IT, provided by SwissADME. The predicted values are presented in Table 4.10. iLogP values ranged from 2.39 to 2.96, indicating moderate lipophilicity across the compounds.

XlogP3 values varied between 2.89 and 4.74, with SC-01 exhibiting the highest predicted lipophilicity in this model. WlogP values were comparatively higher, ranging from 4.12 to 6.34, with SC-01 showing the maximum value.

MlogP values were relatively lower, ranging from 0.46 to 2.20, with AR-03 presenting the highest value. SILICOS-IT predictions ranged from 2.23 to 4.37, identifying SC-01 as the most lipophilic compound, while SR-02 displayed the lowest predicted lipophilicity across this model.

TABLE 4.10: The calculated lipophilic parameters for synthesized azo dyes using the SwissADME database

Compound	iLogP	XLogP3	WLogP	MLogP	SILICOS-IT
SC-01	2.84	4.74	6.34	1.87	4.37
SR-02	2.39	2.89	4.61	0.46	2.23
AR-03	2.96	3.78	4.12	2.20	3.83

4.6.2 Water Solubility Predictions

Water solubility of the compounds was predicted using three computational models: ESOL, Ali, and SILICOS-IT. The results are summarized in Table 4.11. According to the ESOL model, all three compounds (SC-01, SR-02, and AR-03) were classified as moderately soluble. The Ali model yielded similar classifications, with SR-02 and AR-03 predicted as moderately soluble, whereas SC-01 was classified as poorly soluble. In contrast, the SILICOS-IT model predicted SC-01 and SR-02 as poorly soluble, while AR-03 was classified as moderately soluble. Overall, SC-01 showed the lowest predicted solubility across the three models, whereas AR-03 demonstrated comparatively better solubility.

TABLE 4.11: Predicted water solubility values using three alternative computational models

Compound	Log S (ESOL)	Solubility Class	Log S (Ali)	Solubility Class	Log S (SILICOS-IT)	Solubility Class
SC-01	-5.58	Moderately soluble	-7.10	Poorly soluble	-8.06	Poorly soluble
SR-02	-4.28	Moderately soluble	-5.61	Moderately soluble	-6.31	Poorly soluble
AR-03	-4.36	Moderately soluble	-5.71	Moderately soluble	-4.43	Moderately soluble

4.7 *In-Silico* Toxicological Evaluation

4.7.1 Acute Toxicity and GHS Classification

Acute toxicity predictions for the synthesized compounds were generated using the ProTox 3.0 platform, and the results are presented in Table 4.12. The predicted LD50 values ranged from 1000 to 12500 mg/kg. Based on these values, SC-01 and SR-02 were classified under GHS toxicity class 6, while AR-03 was categorized under class 4. These classifications indicate low to moderate acute toxicity across the series.

TABLE 4.12: Acute Toxicity and Toxicity Class

Com-pound	LD50 (mg/kg)	GHS Class	Toxicity	Toxicity Interpretation
SC-01	12500	6		Relatively harmless
SR-02	25000	6		Relatively harmless
AR-03	1000	5		May be harmful

4.7.2 Organ-Specific Toxicity Prediction

Organ toxicity predictions are summarized in Table 4.13. All compounds were predicted to be inactive for cardiotoxicity and neurotoxicity. While all compounds were predicted to be active for nephrotoxicity except SC-01 which is predicted inactive. However, respiratory toxicity was predicted to be active for SC-01 and SR-02 while inactive for AR-03. Hepatotoxicity was predicted to be inactive for SC-01 and SR-02 while active for AR-03.

TABLE 4.13: Organ Toxicity Prediction for Synthesized Compounds

Compound	Hepato-toxicity	Neuro-toxicity	Nephro-toxicity	Respiratory toxicity	Cardio-toxicity
SC-01	0.59 (Inactive)	0.77 (Inactive)	0.54 (Inactive)	0.62 (Active)	0.84 (Inactive)
SR-02	0.54 (Inactive)	0.76 (Inactive)	0.52 (Active)	0.54 (Active)	0.82 (Inactive)
AR-03	0.59 (Active)	0.51 (Inactive)	0.50 (Active)	0.57 (Inactive)	0.73 (Inactive)

4.7.3 Systemic and Genetic Toxicity Prediction

Systemic and genetic toxicity predictions are shown in Table 4.14. All the compounds were predicted to be active for carcinogenicity. AR-03 was predicted to be active for mutagenicity, whereas the remaining compounds were predicted to be inactive.

Clinical toxicity, cytotoxicity and immunotoxicity were predicted to be inactive across all compounds.

TABLE 4.14: Systemic and Genetic Toxicity

Compound	Carcino- genicity	Immuno- toxicity	Muta- genicity	Cytotoxicity	Clinical toxicity
SC-01	0.65 (Active)	0.99 (Inactive)	0.70 (Inactive)	0.88 (Inac- tive)	0.54 (In- active)
SR-02	0.71 (Active)	0.99 (Inactive)	0.66 (Inactive)	0.90 (Inac- tive)	0.55 (In- active)
AR-03	0.56 (Active)	0.89 (Inactive)	0.62 (Active)	0.74 (Inac- tive)	0.52 (In- active)

4.8 Structural Analysis of Target Proteins

Structural analysis was done to evaluate the stability, quality, and suitability of the protein structures for subsequent computational studies. Key parameters, including resolution, R-value, unit cell geometry and Ramachandran plot statistics were used to evaluate structural assessment.

These parameters are widely accepted indicators of model accuracy and are essential for ensuring meaningful molecular interaction and docking analyses.

4.8.1 COX-2 PDB ID: 5IKR

The selected protein (PDB ID: 5IKR) was determined by X-ray diffraction with a resolution of 2.34 Å, an R-work value of 0.185, and an R-free value of 0.211, demonstrating a well-defined and high-quality protein structure. The protein corresponds to human cyclooxygenase-2 (COX-2) co-crystallized with mefenamic acid. The stereochemical quality of the protein was assessed using the Ramachandran plot shown in Figure 4.7. The plot demonstrated that the majority of amino acid residues were located within the most favored and additionally allowed regions, with only a small number of residues observed in the disallowed regions. This distribution indicates good stereochemical quality and minimal conformational strain within the protein backbone. The clustering of residues within the favored regions further supports the structural reliability of the 5IKR protein and confirms its suitability for molecular docking studies.

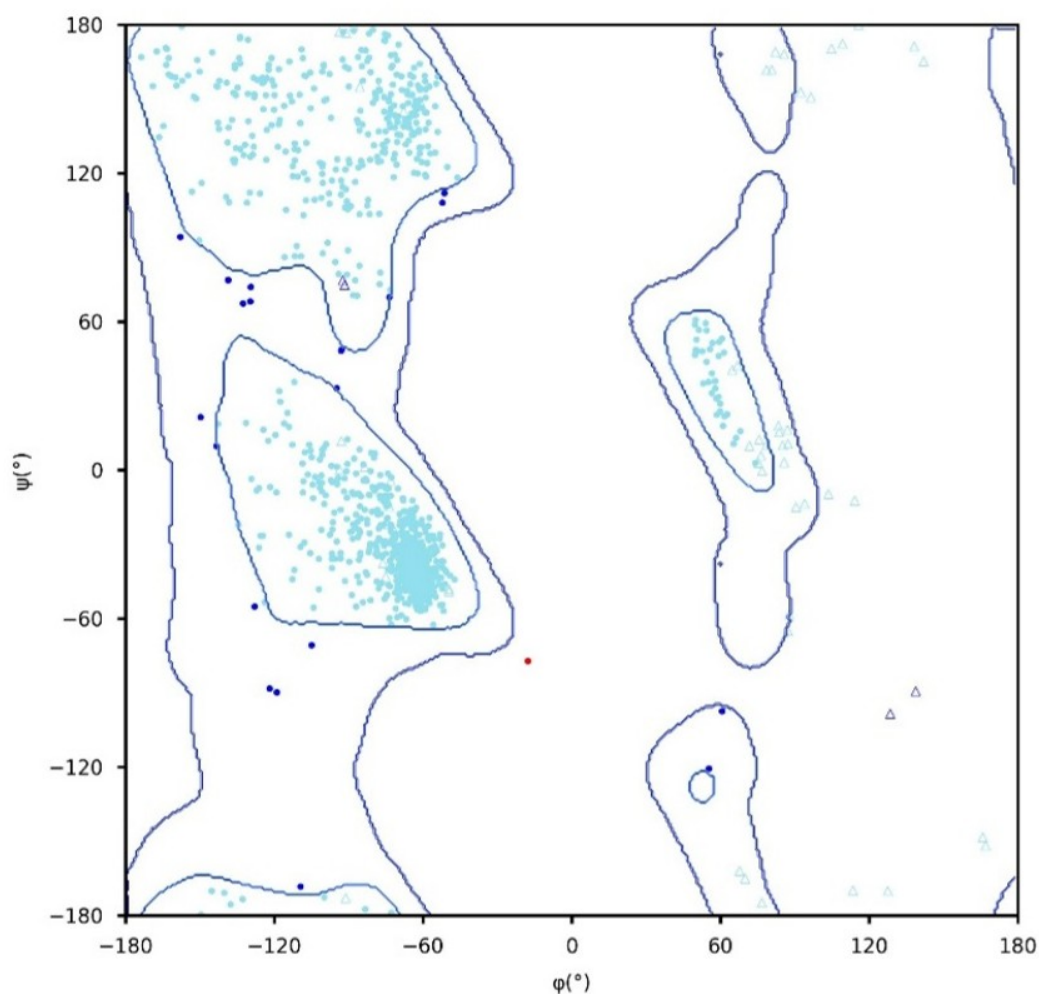


FIGURE 4.7: Structural Analysis of 5IKR

4.8.2 DHPS PDB ID: 1AJ0

The selected protein (PDB ID: 1AJ0) was determined by X-ray diffraction with a resolution of 2.0 Å and an R-work value of 0.206, indicating a well-defined crystal structure of dihydropteroate synthase. The stereochemical quality was assessed using the Ramachandran plot shown in Figure 18, which demonstrated that the majority of amino acid residues were located within the favored and additionally allowed regions of the phi (ϕ) and psi (ψ) angles, with only a few residues present in disallowed regions. This distribution indicates good stereochemical quality and minimal conformational strain, supporting the structural reliability of 1AJ0 for molecular docking studies.

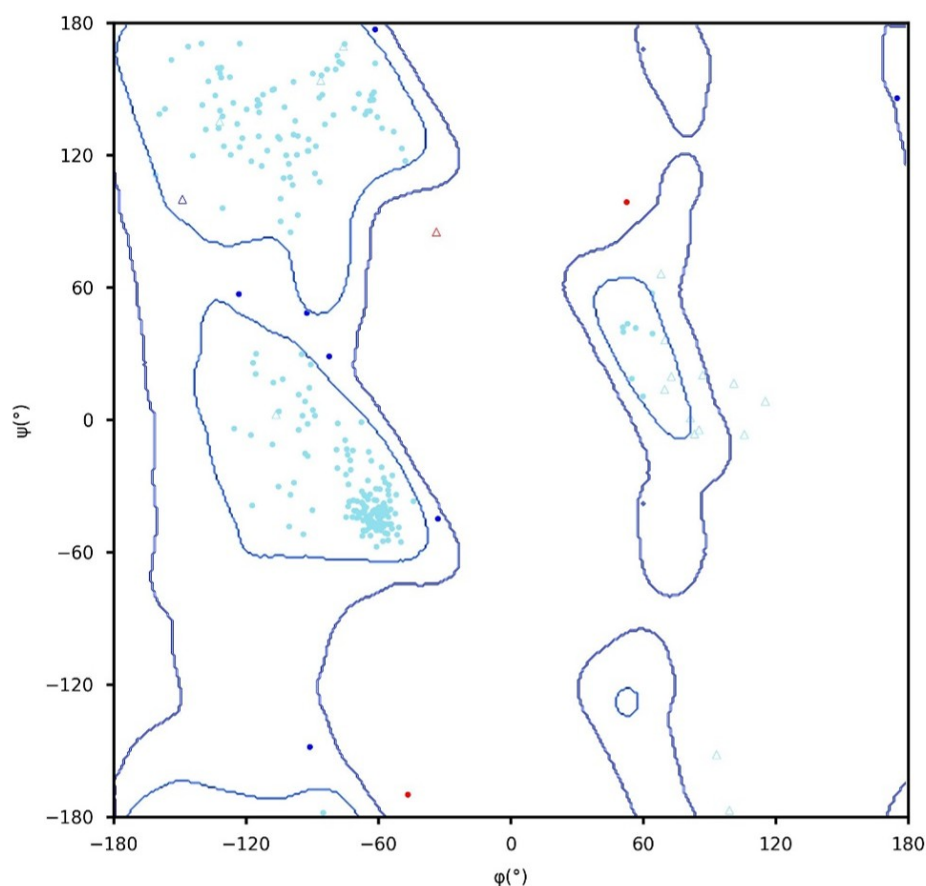


FIGURE 4.8: Structural Analysis of 1AJ0

4.9 Evaluation of Binding Affinity and Interaction Against 5IKR and 1AJ0

Molecular docking studies were conducted to explore the binding conformations and interaction profiles of the synthesized ligands against the selected target proteins. Docking simulations were performed using AutoDock Vina within the PyRx platform, and the binding affinity of each compound was assessed from its predicted binding energy, expressed in kcal/mol, with more negative values reflecting stronger and more stable protein-ligand interactions.

These docking analyses revealed the likely binding poses, interaction patterns, and key molecular contacts formed between the synthesized compounds and the active site residues of the target proteins.

The synthesized compounds exhibited favorable binding affinities toward the target proteins 5IKR and 1AJ0, as summarized in Tables 4.15 and 4.16. These results suggest their potential as anti-inflammatory and antibacterial agents. The favorable binding energies and non-covalent interactions indicate stable protein–ligand associations, supporting the possible involvement of these compounds in modulating inflammatory and bacterial pathways.

TABLE 4.15: Docking analysis of compounds in COX-2 active site: Binding affinity, interaction residues and bond types

Compound	Binding Affinity	Bonding	Interaction	Interacting acid residue	Amino	Bond Type
SC-01	−9.4	Carbon Bond, Pi-Pi, Pi-Alkyl	Hydrogen Bond, Pi-Sulfur, T-Shaped,	PRO A:156, A:153, CYS A:36, A:135, TYR A:136	PRO A:47, GLY A:136	S=O C ₆ H ₆
SR-02	−7.3	Conventional Hydrogen Bond, T-Shaped, Hydrogen Bond, Pi-Alkyl	Hydrogen Bond, Pi-Pi, Pi-Donor Bond,	LEU A:152, A:461, PRO A:153, A:36, TYR A:136, PRO A:156	GLN A:47, CYS A:136	O–H C ₆ H ₆
AR-03	−7.2	Conventional Hydrogen Bond, Hydrogen Bond, Alkyl	Hydrogen Bond, Pi-Donor Bond, Pi-Alkyl	LYS A:468, CYS A:47, PRO A:153, A:46, CYS A:36	VAL	O–H C ₆ H ₆

The molecular docking analysis of SC-01, SR-02, and AR-03 against the COX-2 active site revealed differences in binding affinity and interaction patterns. Among the evaluated compounds, SC-01 exhibited the highest predicted binding affinity (−9.4 kcal/mol), followed by SR-02 (−7.3 kcal/mol) and AR-03 (−7.2 kcal/mol). The more negative binding affinity predicted for SC-01 indicates a stronger predicted interaction with the COX-2 binding site under the docking model used.

SC-01 interacted with several amino acid residues, including PRO156, PRO153, CYS47, CYS36, GLY135, and TYR136. Its interaction profile consisted of carbon–hydrogen bonding, pi–sulfur interaction, pi–pi T-shaped interaction, and

pi-alkyl interactions. The combination of these non-covalent interactions may contribute to the relatively favorable docking score observed for SC-01.

SR-02 showed a binding affinity of -7.3 kcal/mol and formed conventional hydrogen bonds, pi-pi T-shaped interactions, pi-donor hydrogen bonding, and pi-alkyl interactions.

The interacting residues included LEU152, GLN461, CYS47, PRO153, CYS36, TYR136, and PRO156. The presence of conventional hydrogen bonding, together with several aromatic and hydrophobic interactions, suggests a diverse interaction network within the COX-2 active-site region.

AR-03 demonstrated a binding affinity of -7.2 kcal/mol and interacted with LYS-468, CYS47, PRO153, VAL46, and CYS36 through conventional hydrogen bonding, pi-donor hydrogen bonding, and pi-alkyl interactions. Although its predicted affinity was slightly weaker than that of SR-02, AR-03 still demonstrated multiple interactions with residues located within or near the modelled binding region.

Overall, SC-01 showed the most favorable predicted binding affinity among the investigated compounds and also exhibited a more negative docking score than the standard drug diclofenac (-7.7 kcal/mol). SR-02 and AR-03 displayed somewhat less favorable predicted affinities than diclofenac. These findings suggest that SC-01 may have strong predicted interactions with the COX-2 active site; however, docking scores alone do not establish inhibitory potency, and experimental COX-2 inhibition assays are required for confirmation.

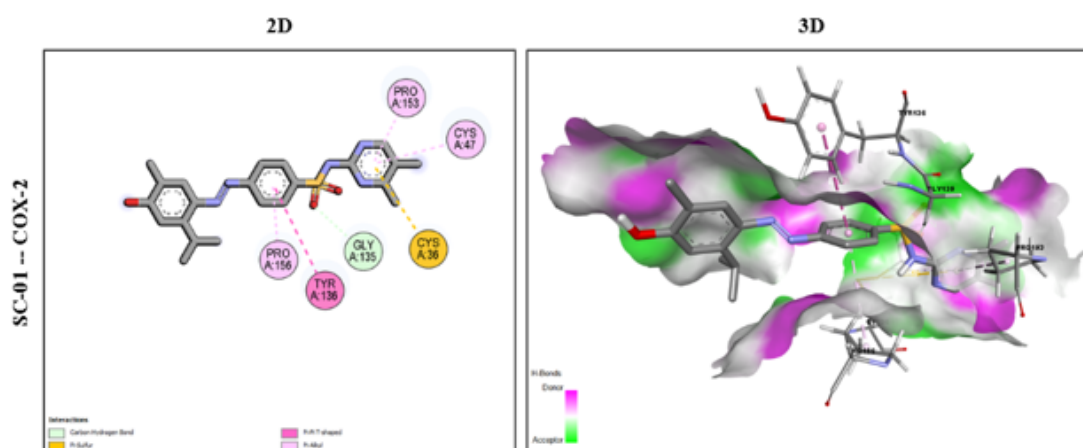


FIGURE 4.9: Molecular docking representation of SC-01 against COX-2

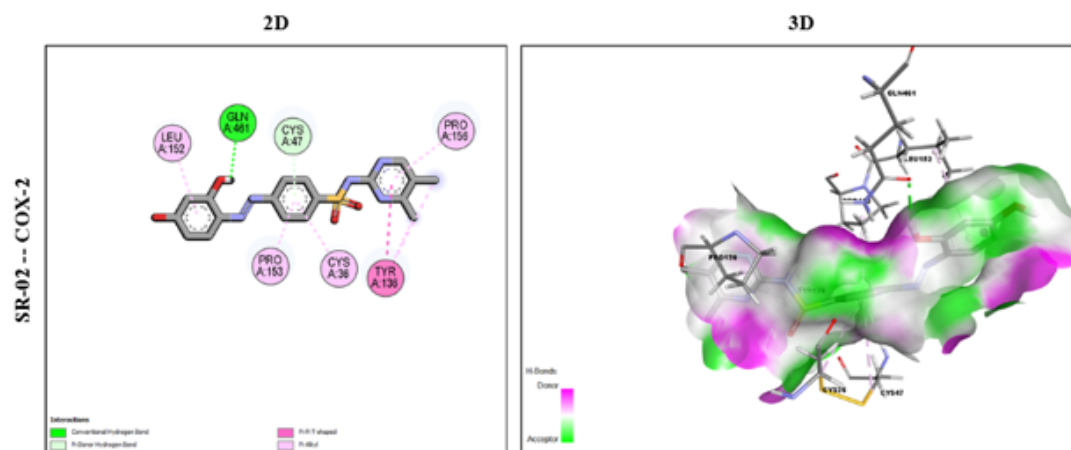


FIGURE 4.10: Molecular docking representation of SR-02 against COX-2

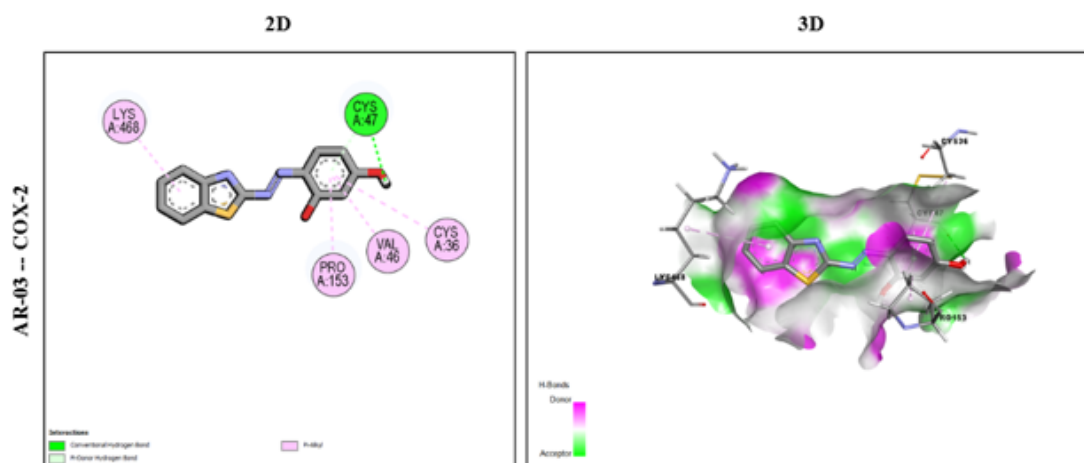


FIGURE 4.11: Molecular docking representation of AR-03 against COX-2

TABLE 4.16: Docking analysis of compounds in DHPS active site: Binding affinity, interaction residues and bond types

Com- pound	Binding Affinity	Bonding Interac- tion	Interacting acid residue	Amino acid residue	Bond Type
SC-01	-9.3	Conventional Hydro- gen Bond, Pi-Sigma, Pi-Alkyl, Carbon Hydrogen Bond	ARG A:63, LYS A:221, PRO A:64, LYS A:192, GLN A:149		O-H C ₆ H ₆
SR-02	-7.0	Conventional Hydro- gen Bond, Pi-Sigma, Pi-Alkyl, Carbon Hydrogen Bond, Pi-Cation	ASN A:22, ARG A:255, HIS A:257, PRO A:64, ARG A:63, LYS A:221, SER A:222		O-H C ₆ H ₆ S=O

Table 4.16 continued from previous page

Compound	Binding Affinity	Bonding Interaction	Interacting acid residue	Amino acid residue	Bond Type
AR-03	-7.0	Conventional Hydrogen Bond, Amide-Pi Stacked, Van Der Waals, Pi-Alkyl, Unfavorable Donor-Donor	SER A:222, PRO A:64, LYS A:221, ARG A:63, THR A:62, ASN A:22, ARG A:255		O-H C ₆ H ₆

The molecular docking analysis of SC-01, SR-02, and AR-03 against the dihydropteroate synthase (DHPS) active site demonstrated differences in their predicted binding affinities and interaction profiles. Among the investigated compounds, SC-01 exhibited the highest predicted binding affinity (-9.3 kcal/mol), followed by SR-02 and AR-03, both with binding affinities of -7.0 kcal/mol.

In comparison, the standard reference drug sulfadimidine showed a binding affinity of -7.5 kcal/mol. Thus, SC-01 demonstrated a substantially more negative predicted binding affinity than sulfadimidine, whereas SR-02 and AR-03 showed slightly weaker predicted affinities than the reference drug.

SC-01 formed conventional hydrogen bonds, pi-sigma, pi-alkyl, and carbon - hydrogen bond interactions with ARG63, LYS221, PRO64, LYS192, and GLN149. The presence of multiple hydrogen-bonding and hydrophobic interactions may contribute to its favorable predicted binding within the DHPS active site.

SR-02 exhibited a binding affinity of -7.0 kcal/mol and formed conventional hydrogen bonds, pi-sigma, pi-alkyl, carbon-hydrogen bonds, and pi-cation interactions with ASN22, ARG255, HIS257, PRO64, ARG63, LYS221, and SER222. This indicates a diverse interaction pattern involving both polar and non-polar residues.

AR-03 also showed a binding affinity of -7.0 kcal/mol and interacted with SER222, PRO64, LYS221, ARG63, THR62, ASN22, and ARG255 through conventional hydrogen bonding, amide-pi stacking, van der Waals, and pi-alkyl interactions.

An unfavorable donor–donor interaction was also observed, which may partially affect the overall interaction profile of the compound.

Overall, SC-01 demonstrated the most favorable predicted binding affinity among the investigated compounds and showed a stronger predicted affinity than the standard drug sulfadimidine (-7.5 kcal/mol). Its interaction with several DHPS active-site residues through multiple types of non-covalent interactions suggests potentially stable protein–ligand association.

These findings provide computational support for the potential of synthesized azo dyes, particularly SC-01, as DHPS-targeting antibacterial candidates. However, docking results alone cannot establish antibacterial efficacy, and experimental enzyme-inhibition and antimicrobial assays are required to validate these predictions.

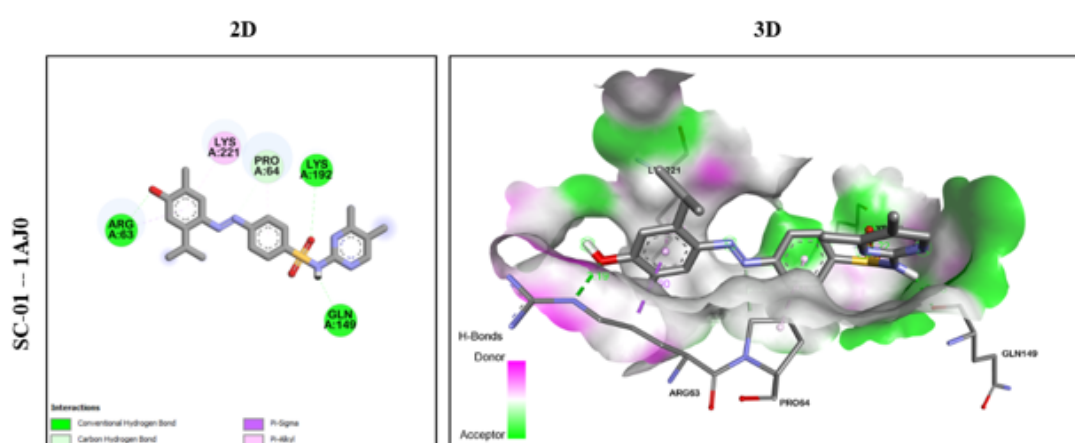


FIGURE 4.12: Molecular docking representation of SC-01 against DHPS

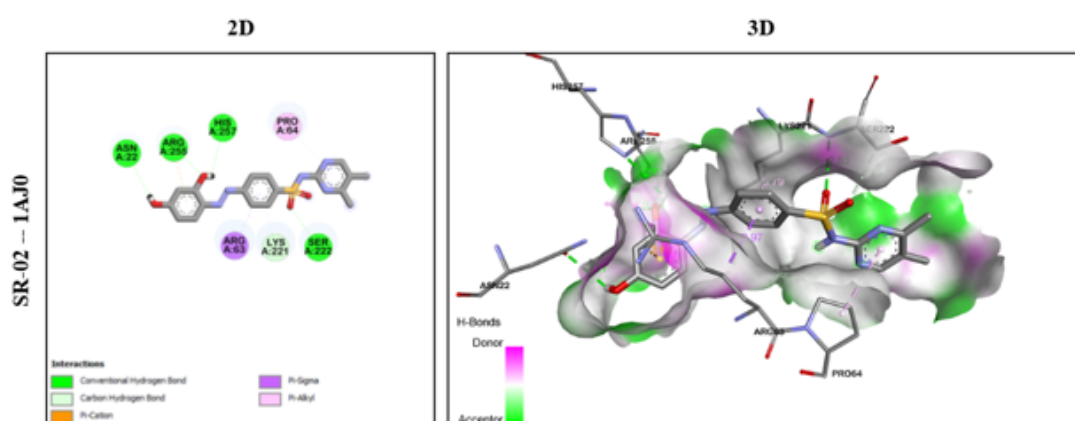


FIGURE 4.13: Molecular docking representation of SR-02 against DHPS

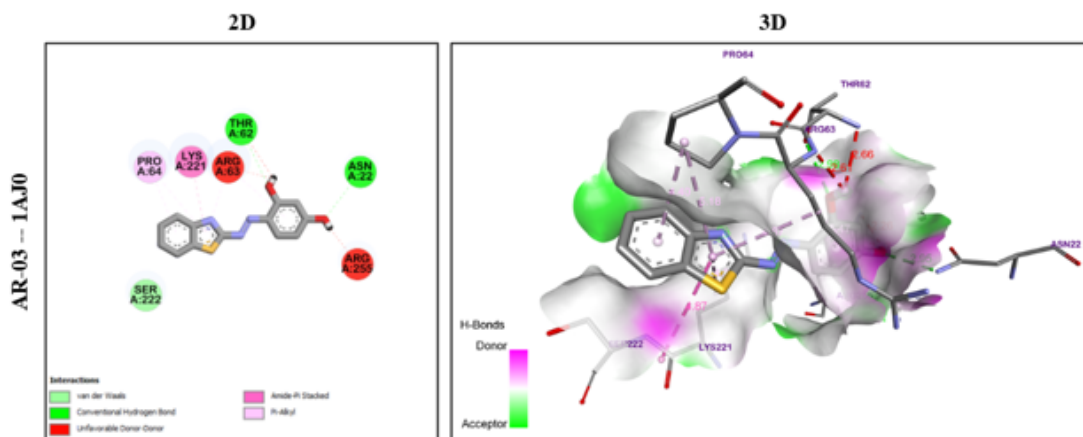


FIGURE 4.14: Molecular docking representation of AR-03 with DHPS

4.10 DFT Studies

4.10.1 Geometry Optimization

Computational approaches have gained significant importance in the investigation of structure-activity relationships (SAR) of chemical compounds. Among these methods, Density Functional Theory (DFT) is widely utilized due to its reliable accuracy, computational efficiency, and ability to provide detailed insights into molecular geometry and electronic properties. In the present study, the molecular geometries and electronic energies of SR-02 and AR-03 (Figure 4.15) were optimized using the B3LYP functional with the 6-31G basis set through the Gaussian 09 software package.

The optimized structure of SR-02 revealed a highly conjugated molecular framework consisting of aromatic rings interconnected through azo ($-N=N-$) linkages and heteroatom-containing functional groups. The aromatic rings exhibited partial coplanarity due to extensive π -electron delocalization throughout the molecular backbone. However, slight distortion from planarity was observed because of steric hindrance produced by bulky substituents and sulphur-containing moieties. The presence of nitrogen, oxygen, and sulphur atoms contributed significantly toward electronic stabilization and charge distribution within the molecule. Furthermore, the azo linkage acted as an effective conjugating bridge, facilitating electron delocalization between electron-donating and electron-withdrawing regions of the

compound. The optimized conformation suggested enhanced resonance stabilization and structural flexibility, which may favour intermolecular interactions with biological targets.

In the case of AR-03, the optimized geometry exhibited a comparatively more compact and planar molecular arrangement. The azo bridge maintained an approximately linear orientation between the aromatic systems, thereby promoting efficient π -conjugation and electronic communication across the molecule.

The aromatic rings appeared relatively coplanar with reduced steric strain, indicating improved resonance stabilization and molecular rigidity. The sulphur- and nitrogen-stronger intermolecular π - π interactions and improve molecular orbital overlap, thereby influencing the electronic and pharmacological behaviour of the compound.

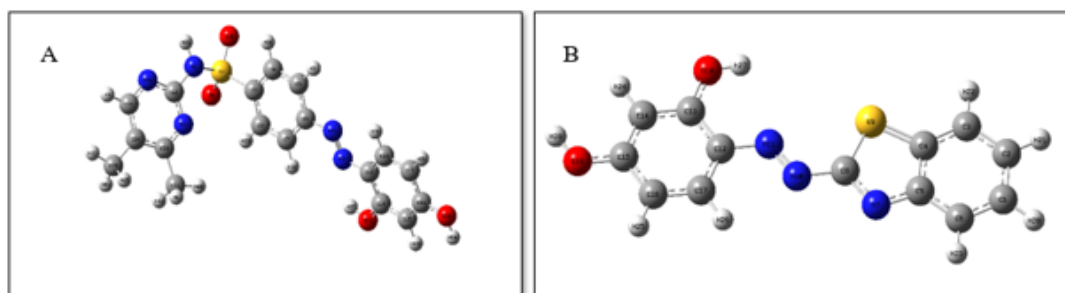


FIGURE 4.15: Geometry Optimization of SR-02 (A) & AR-03 (B)

Comparative analysis of both optimized structures demonstrated that SR-02 possessed relatively greater conformational flexibility due to steric effects and rotational freedom around adjacent single bonds, whereas AR-03 exhibited enhanced structural rigidity and planarity (Figure 4.15). The extended conjugated systems present in both compounds are characteristic features of azo dyes and are expected to contribute toward their electronic transitions, chemical stability, and potential biological activity. The optimized geometries further provide a theoretical basis for subsequent computational investigations, including HOMO-LUMO analysis, molecular electrostatic potential mapping, Mulliken charge analysis, and structure-activity relationship studies.

4.10.2 Frontier Molecular Orbital Analysis

This computational approach is extensively applied for the evaluation of the electronic and optical characteristics of molecular systems. The Highest Occupied Molecular Orbital (HOMO) and Lowest Unoccupied Molecular Orbital (LUMO) play a crucial role in describing molecular interactions, electron transfer processes, and chemical reactivity. These frontier orbitals provide valuable information regarding the kinetic stability and reactive behavior of compounds. The HOMO energy level is associated with the electron-donating capability of a molecule, whereas the LUMO energy level reflects its electron-accepting potential. The calculated HOMO and LUMO distributions of SR-02 and AR-03 are illustrated in the molecular orbital energy diagrams (Figure 4.16), highlighting the electron-rich and electron-deficient regions of the studied compounds.

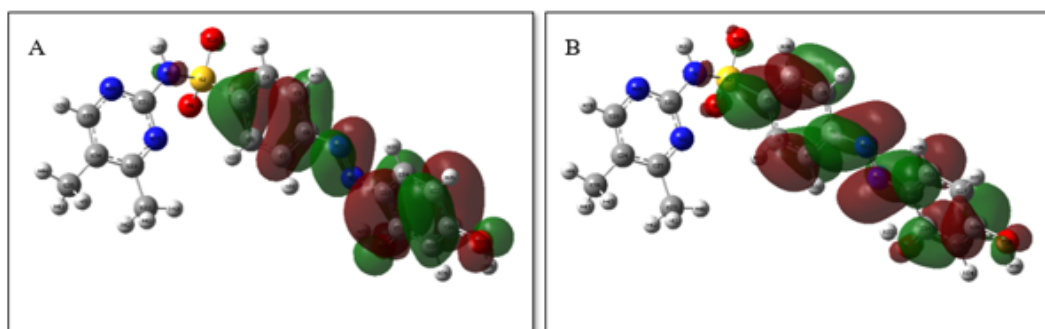


FIGURE 4.16: HOMO (A) and LUMO (B) of SR-02

4.10.3 Computational Approach Adopted for AR-03

The Gaussian 09 job performs a single-point TD-B3LYP/6-31G(d) calculation on AR-03 in DMSO solvent, using the Polarizable Continuum Model for solvation. B3LYP is a well-established hybrid density functional that performs reliably for organic chromophores, and the 6-31G(d) basis set provides split-valence description with d-type polarization functions on all heavy atoms.

While this basis is appropriate for ground-state geometry and UV-Vis prediction of medium-sized molecules, it lacks diffuse functions, so any Rydberg-type excited states would not be well described.

Time-Dependent DFT is invoked to compute vertical excitation energies, and since no explicit number of states was requested, Gaussian defaults to three singlet excited states. The PCM solvent model uses DMSO parameters ($\epsilon = 46.826$, $\epsilon_\infty = 2.008$), and a non-equilibrium solvation protocol is applied for the TD-DFT step, correctly capturing the fast electronic response of the solvent to the excited state while excluding the slow orientational relaxation, the standard approach for simulating UV-Vis spectra in solution. The molecule is treated as a neutral closed-shell singlet, charge = 0, multiplicity = 1 (Figure 4.17).

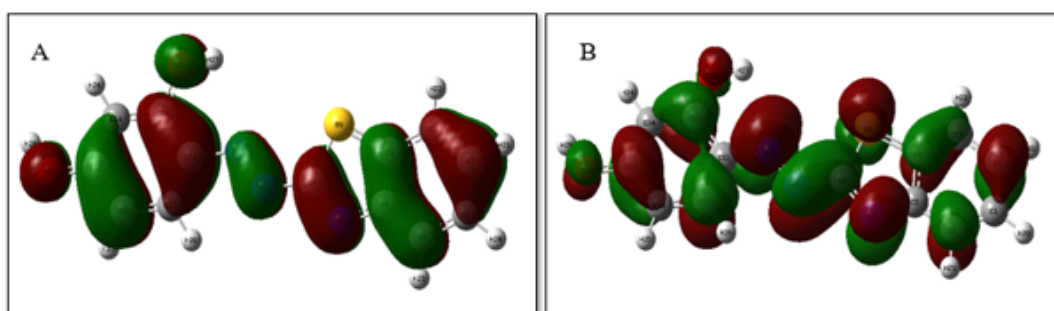


FIGURE 4.17: HOMO (A) and LUMO (B) of AR-03

4.10.3.1 Molecular Structure and Geometry

The stoichiometry is $C_{13}H_9N_3O_2S$ corresponding to a molecular weight of approximately 271.3 g/mol. The point group is C_1 , meaning the molecule has no symmetry whatsoever, which is consistent with the asymmetric substitution pattern typical of push-pull azo dyes. From the atomic coordinates and the distance matrix, the structural connectivity is clear. Atoms 1 through 6 form a benzene ring, with all C-C bond lengths in the range 1.39-1.41 Å, confirming full aromatic delocalization. Atom 7 is a nitrogen that bridges this benzene ring to the heterocyclic core through a bond of 1.357 Å to C5, which is shorter than a typical C-N single bond and reflects significant π -conjugation between the ring and the nitrogen. Atoms 8 and 9 (carbon and sulphur) together with the surrounding nitrogens constitute a benzothiazole-like or thiadiazole heterocyclic ring. The N10-N11 distance of approximately 1.247 Å is the hallmark N=N azo bond, the defining chromophore of the entire molecule. Atoms 12 through 17 form a second substituted aromatic ring connected through N11. Oxygen atoms 18 and 19 are bonded to carbons of

this terminal ring at distances of 1.350 and 1.348 Å respectively, consistent with phenolic C-O bonds rather than carbonyl character. This assignment is confirmed by the Mulliken charges on H27 and H28, which are both approximately +0.45 au highly positive, unmistakably indicating acidic O-H protons of phenolic hydroxyl groups. The terminal ring therefore carries two hydroxyl substituents, making it a catechol-type (dihydroxybenzene) electron-donating group. Taken together, the structure is a donor- π -acceptor azo dye in which the catechol-substituted benzene serves as the electron donor, the N=N azo linkage and thiadiazole unit serve as the electron acceptor, and the extended conjugated system between them forms the chromophoric bridge.

4.10.3.2 Frontier Molecular Orbitals

The HOMO is MO 70 with an eigenvalue of approximately -0.222 au (-6.04 eV), and the LUMO is MO 71 at -0.100 au (-2.73 eV), giving a HOMO-LUMO gap of roughly 3.31 eV from orbital energies. This gap is consistent with a visible-light absorbing organic dye, too large for a conductor but small enough that electronic excitation falls in the near-UV and visible region. By Koopmans' theorem, the HOMO energy gives a rough estimate of the ionization potential of about 6.0 eV, typical for conjugated nitrogen-containing aromatics. The orbitals immediately below the HOMO H-1 at -0.222 au and H-2 at -0.238 au are energetically close to the HOMO, which is why multiple configurations contribute to the higher excited states. The LUMO at MO 71 is significantly lower in energy than L+1 at -0.020 au, suggesting the LUMO is well-stabilized by the acceptor groups (the azo bond and thiadiazole ring) and acts as a deep electron trap, characteristic of the electron-deficient acceptor fragment in D- π -A dyes.

4.10.3.3 TD-DFT Excited States and UV-Vis Spectrum

The three computed singlet excited states paint a clear picture of the optical properties of AR-03. The first excited state S_1 lies at 2.2338 eV, corresponding to a wavelength of 555 nm, and has an oscillator strength of essentially zero ($f = 0.0000$). This state is therefore optically dark; it carries no absorption intensity and would not appear in a standard UV-Vis spectrum.

The transition is dominated by the HOMO-to-LUMO excitation with a coefficient of 0.704. The electric transition dipole moment components are all negligibly small ($X = 0.0015$, $Y \approx 0$, $Z = -0.015$ au), confirming the forbidden nature of this transition. Despite its invisibility in absorption, S_1 is the lowest-lying excited state and will be photo-physically important: population reaching it by internal conversion from S_2 cannot easily return to the ground state by radiative emission (since the transition is forbidden), so non-radiative decay pathways dominate.

This is the fundamental reason azo dyes are generally non-fluorescent, the dark S_1 state serves as a photophysical sink.

The second excited state S_2 at 3.0019 eV (413 nm) is the dominant absorption band, with an oscillator strength of $f = 1.0986$. This is exceptionally large for an organic molecule and indicates a highly allowed, strongly coupled electronic transition. The primary configuration is again HOMO→LUMO (coefficient 0.691), with a secondary contribution from H-3→LUMO (coefficient 0.118).

This is the classic $\pi \rightarrow \pi^*$ intramolecular charge-transfer transition characteristic of push-pull azo dyes: electron density is promoted from the donor-rich HOMO, which is delocalized over the catechol and azo bridge region, to the acceptor-rich LUMO, which is concentrated on the thiadiazole and azo acceptor fragment. The close agreement between the dipole-length ($f = 1.0986$) and dipole-velocity ($f = 1.0464$) oscillator strengths differing by less than 5% validates the quality of the basis set for describing this transition.

Absorption at 413 nm, in the violet-blue region of the spectrum, means the compound transmits and appears orange or yellow-orange to the human eye, which is entirely consistent with the "Acid Red" naming convention applied to many dyes in this structural class.

The third excited state S_3 at 3.2250 eV (384 nm) is weak, with $f = 0.021$, and is dominated by H-2→LUMO (coefficient 0.695). This may correspond to a $\pi \rightarrow \pi^*$ transition localized more on the thiadiazole or benzothiazole fragment rather than extending across the full molecular length. In an experimental spectrum it would

appear as a weak shoulder or tail on the blue side of the main S₂ band (Figure 4.18).

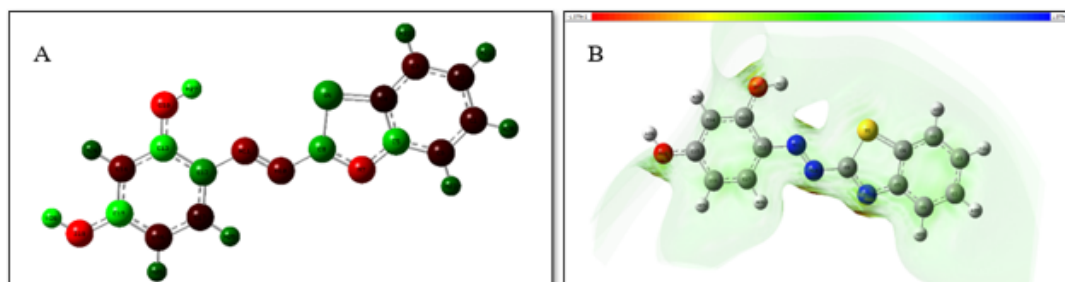


FIGURE 4.18: Mulliken Charges (A) and TD-DFT ESP (B) of AR-03

4.10.3.4 Mulliken Charge Distribution

The Mulliken population analysis reveals the expected donor-acceptor charge separation across the molecule. The most negatively charged atoms are the two phenolic oxygens O18 and O19, with charges of -0.667 and -0.663 respectively, confirming their role as strong electron donors and highlighting that they bear significant lone-pair electron density even in the ground state. The azo nitrogen N7 carries -0.522, reflecting the donation of its lone pair into the conjugated system. The N=N nitrogens N10 and N11 carry charges of -0.286 and -0.335, consistent with partial lone-pair character balanced against their role as π acceptors. On the positive side, C8 in the thiadiazole ring (+0.263), the sulphur S9 (+0.239), and particularly C15 (+0.407), the carbon ipso to the phenolic oxygen is all electron-deficient. The hydroxyl protons H27 and H28 are the most positively charged hydrogen atoms in the molecule (+0.450 and +0.454), which reflects the polarity of the O-H bond and predicts that these will be the most acidic protons, lost preferentially upon treatment with base to generate phenolate anions that would dramatically shift the absorption spectrum batho-chromically.

4.10.4 Computational Approach Adopted for SR-02

The quantum chemical calculations for the synthesized sulfadimidine-azo derivative, designated as compound SR-02, were executed utilizing the Gaussian 09 software suite. The theoretical framework selected for examining the ground-state

properties was Hartree-Fock (HF) theory. Geometry optimization of the molecular framework was conducted by applying the split-valence Pople-type 6-31G(d) basis set, which introduces d-type Cartesian polarization functions on all heavy, non-hydrogen atoms to account for orbital polarization and handle the electronic environment of the sulphur, oxygen, and nitrogen heteroatoms effectively. The Berny optimization algorithm was employed to achieve programmatic relaxation of the coordinates down to a local minimum. Following the full ground-state geometry relaxation, Self-Consistent Field (SCF) methodologies were applied to establish the electronic distribution and evaluate additional properties.

4.10.4.1 Molecular Structure and Geometry

The fully optimized molecular configuration of SR-02 reveals a highly coordinated spatial arrangement of the functional domains, governed by steric and electronic interactions across its azo linkage and sulfonamide backbone. The system contains 45 atoms defined in a singlet ground state with zero net charge. Key initial structural parameters demonstrate core structural features, such as a calculated nitrogen-nitrogen azo double-bond distance (R18, N11-N12) of approximately 1.25 Å and an adjacent aromatic carbon-nitrogen single bond (R19, N12-C13) stretching near 1.42 Å. The sulfonyl group features characteristic sulphur-oxygen single-bond estimates of 1.54 Å (R5, R6), anchoring the three-dimensional bend of the sulfonamide core relative to the aniline rings. The molecule balances planarity across the conjugated azo-resorcinol chromophore with localized torsions across the pyrimidine ring systems, optimizing intramolecular packing and preventing catastrophic steric clashing between the methyl modifications and the primary aromatic rings.

4.10.4.2 Frontier Molecular Orbitals

The electronic transitions and reactivity profiles of SR-02 are primarily dictated by its Frontier Molecular Orbitals (FMOs), specifically the Highest Occupied Molecular Orbital (HOMO) and the Lowest Unoccupied Molecular Orbital (LUMO). Because an HF/6-31G(d) method was applied, the resulting FMO values reflect canonical orbital eigenvalues where the HOMO energy represents the molecular

ionization potential according to Koopmans' theorem, and the LUMO energy correlates with the electron affinity. The spatial topology of the HOMO is typically localized over the electron-rich aromatic core containing the resorcinol ring and the azo linkage, where lone pairs from the oxygen and nitrogen atoms participate heavily in the extended π system. Conversely, the LUMO is positioned over the electron-accepting portions of the molecule, including the electron-deficient heterocyclic rings or the sulfonyl module. The relatively large HOMO-LUMO energy gap derived from this classic Hartree-Fock calculation reflects a highly stable electronic ground state resistant to spontaneous thermal decomposition.

4.10.4.3 TD-DFT Excited States and UV-Vis Spectrum

To investigate the optical absorption behaviors of compound SR-02, the ground-state configurations are typically treated with Time-Dependent Density Functional Theory (TD-DFT) or equivalent configuration interaction singles methods to resolve the electronic excited-state spectrum. For a conjugated azo dye derivative like SR-02, the calculated electronic transitions typically give rise to a prominent absorption spectrum in the UV-Visible domain. The lowest-energy absorption band is primarily governed by a localized π - π^* transition mixed with n - π^* contributions originating from the lone pairs of the azo nitrogen atoms. These transitions correspond directly to the experimental yellow-to-orange-red hues standard for sulfadimidine-derived azo compounds. The calculated oscillator strengths (f) pinpoint the exact electronic transitions that possess high quantum efficiency, establishing the compound's capacity for light absorption and subsequent energy transfer (Figure 4.19).

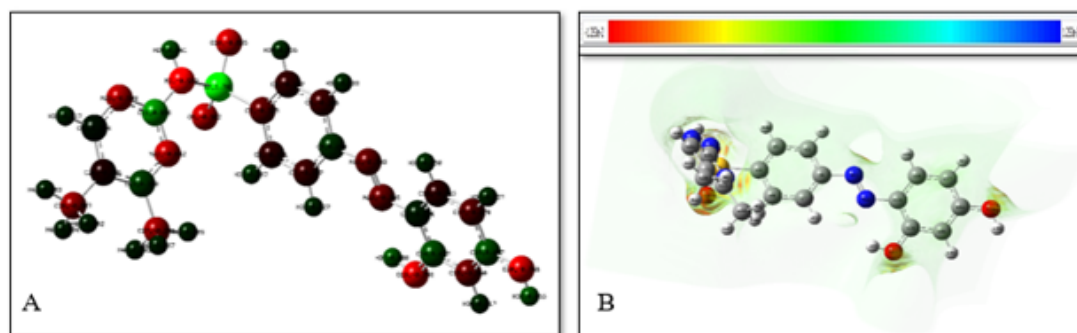


FIGURE 4.19: Mulliken Charges (A) and TD-DFT ESP (B) of SR-02

4.10.4.4 Mulliken Charge Distribution

The net atomic charge distribution evaluated via Mulliken population analysis provides deep insights into the local nucleophilic and electrophilic centers throughout the skeleton of SR-02. The presence of strongly electronegative heteroatoms introduces significant polarization across neighboring bonds. Specifically, the oxygen atoms linked to the central sulphur core (O9, O10) and those residing on the resorcinol group harbor intense negative charges. Similarly, the nitrogen atoms within the azo bridge (N11, N12) exhibit a high density of negative charge, identifying them as potential sites for protonation or metal coordination. In contrast, the sulphur atom (S2) acts as a major electron-deficient hub, presenting a strong positive Mulliken value due to electron withdrawal from its surrounding oxygens and aromatic carbons. The carbon centers bonded directly to the hydroxyl and azo groups also display positive shifts, illustrating the competitive inductive and resonance pathways active across the framework.

4.11 *In-vitro* Evaluation

4.11.1 Antimicrobial Assay

The antimicrobial efficacy of the synthesized compounds against antimicrobial strains is summarized in Table 4.17. All synthesized compounds showed comparable antimicrobial activity against the bacterial strains, although their effectiveness varied depending on the compound and organism.

TABLE 4.17: Assessment of antimicrobial activity of synthesized compounds (ZOI mm, $\pm$ SD; n=3)

Compound	<i>E. coli</i>	<i>S. aureus</i>	<i>P. aeruginosa</i>
SC-01 (5 mg)	17.75 $\pm$ 1.79	14.55 $\pm$ 1.81	13.85 $\pm$ 1.75
SC-01 (10 mg)	19.75 $\pm$ 1.29	17.45 $\pm$ 1.22	16.55 $\pm$ 1.25
SR-02 (5 mg)	22.65 $\pm$ 1.61	40.41 $\pm$ 1.45	41.29 $\pm$ 1.34
SR-02 (10 mg)	25.90 $\pm$ 1.39	46.29 $\pm$ 1.34	45.15 $\pm$ 0.95
AR-03 (5 mg)	24.15 $\pm$ 1.32	25.31 $\pm$ 1.49	24.42 $\pm$ 1.83

Table 4.17 continued from previous page

Compound	<i>E. coli</i>	<i>S. aureus</i>	<i>P. aeruginosa</i>
AR-03 (10 mg)	26.56 $\pm$ 1.08	28.74 $\pm$ 1.30	29.53 $\pm$ 1.34
Sulfadimidine	16.38 $\pm$ 1.04	13.90 $\pm$ 1.83	12.62 $\pm$ 2.22

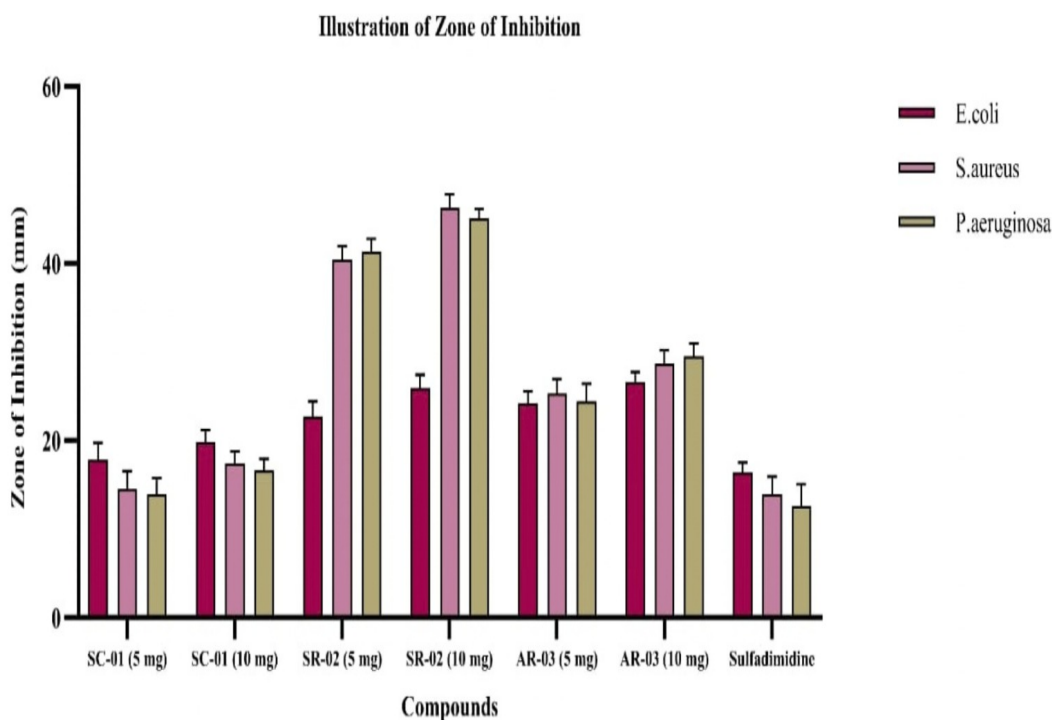


FIGURE 4.20: Illustration of Zone of inhibition by synthesized compounds against bacterial strains

The antibacterial activity of the synthesized azo dye derivatives (SC-01, SR-02, and AR-03) was evaluated against *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* using the agar well diffusion method. The results are presented as mean zone of inhibition (mm) $\pm$ SD (Figure 4.20). Among the synthesized compounds, SR-02 exhibited the greatest antibacterial activity, particularly against *S. aureus*, producing inhibition zones of 40.41 ± 1.00 mm and 46.29 ± 1.00 mm at 5 mg and 10 mg, respectively. AR-03 showed moderate antibacterial activity against all tested bacterial strains, whereas SC-01 demonstrated the lowest inhibitory effect. Sulfadimidine (positive control) produced the highest zones of inhibition against *E. coli* and *P. aeruginosa*. Statistical analysis using

two-way ANOVA followed by Tukey's multiple comparison test revealed significant differences among the tested compounds and bacterial strains ($p < 0.001$).

4.12 *In-vivo* Evaluation

4.12.1 Acute Inflammatory Effect of Compounds SC-01, SR-02 and AR-03 in Carrageenan Induced Paw Edema

This study assessed the anti-inflammatory potential of compounds SC-01, SR-02, and AR-03 using a carrageenan-induced paw edema model. Injection of 1% carrageenan (40 μ l, intraplantar) into the right hind paw produced a significant rise in paw edema in the saline-treated (10 ml/kg, i.p.) negative control group relative to the normal group, confirming successful induction of inflammation. Treatment with compounds SC-01, SR-02, and AR-03 substantially attenuated this edema compared to the negative control group, and the degree of suppression increased with dose, indicating a dose-dependent anti-inflammatory response. These findings are presented in Figure 4.21.

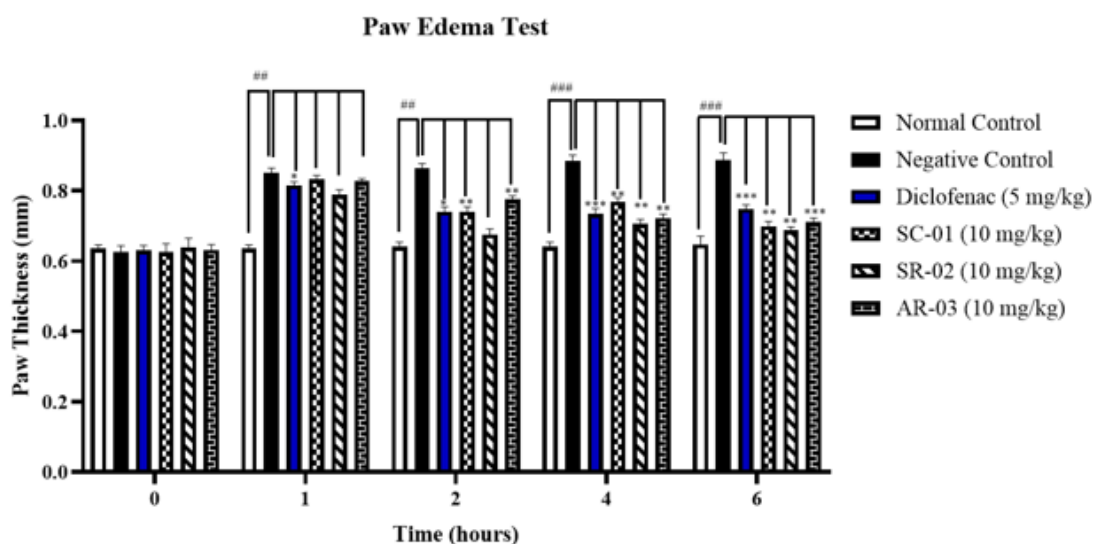


FIGURE 4.21: Anti-inflammatory effect of synthesized derivative on carrageenan induced edema

Chapter 5

Discussion

The present study set out to design, synthesize, and pharmacologically evaluate a small series of benzothiazole- and sulfonamide-derived monoazo dyes, while simultaneously using quantum chemical tools to rationalize their electronic behaviour and biological potential [8]. Taken together, the synthetic, spectroscopic, computational, and biological strands of this work converge on a coherent picture: three structurally distinct azo compounds, SC-01, SR-02, and AR-03, were obtained in good yield and high purity, their structures were unambiguously supported by spectroscopic data, and their electronic architecture, as revealed by density functional theory (DFT) and Hartree-Fock (HF) calculations [7], correlates closely with the binding behaviour observed in molecular docking against cyclooxygenase targets [8]. This chapter draws these threads together, interpreting what the data mean individually and collectively, and situating the findings within the broader context of azo dye chemistry and benzothiazole-based drug discovery introduced in Chapters 1 and 2. The synthetic strategy adopted here, classical diazotization of a primary aromatic amine followed by azo coupling under carefully controlled pH and temperature, proved to be both reliable and reproducible across all three target molecules. The choice of coupling partners was not arbitrary: sulfadimidine was paired with carvacrol to give SC-01, with resorcinol to give SR-02, and 2-aminobenzothiazole was diazotized and coupled with resorcinol to give AR-03.

This design allowed direct comparison of how an electron-rich phenolic monoterpene (carvacrol), a dihydroxylated phenol (resorcinol), and a sulfur-nitrogen heterocycle (benzothiazole) each influence the resulting chromophore when conjugated through the same azo bridge [13]. The visual evidence of successful coupling, brownish-red, orange, and brick-red precipitates respectively, is itself diagnostic, since the appearance of an intensely coloured product is a classical qualitative indicator that the azo linkage has formed and that extended π -conjugation now spans both aromatic fragments [9]. This is consistent with the foundational chemistry discussed in Chapter 2, where the bathochromic shift associated with azo bond formation was attributed to delocalization of π -electrons across the newly created chromophore. The reasonably high percentage yields obtained (85-89%) and sharp, reproducible melting points (268-280°C) further support the conclusion that the reactions proceeded cleanly under the chosen conditions, without significant formation of diazonium decomposition by-products, a risk that is always present when diazotization temperature drifts above 5°C, as emphasized in the mechanistic discussion in Section 2.4.1.

Maintaining the reaction pH within the narrow windows identified for each coupling step, mildly alkaline for phenolic partners and mildly acidic to neutral for amine-bearing diazonium intermediates, was central to the success of the synthesis [13]. This is more than a procedural detail; it reflects the underlying electrophilic aromatic substitution mechanism in which the nucleophilicity of the coupling partner must be maximized (by deprotonating phenols to phenoxide ions, or by keeping diazonium salts protonated and stable) without allowing the diazonium electrophile itself to hydrolyse or decompose. The fact that AR-03 and SR-02 share the same resorcinol coupling partner but differ in the diazotized component (2-aminobenzothiazole versus sulfadimidine) provides a particularly useful internal comparison: any difference in their electronic and biological behaviour can be attributed largely to the identity of the heterocyclic or sulfonamide-bearing fragment introduced via diazotization, rather than to the coupling chemistry itself, which was otherwise identical [11].

Structural confirmation by FTIR spectroscopy was internally consistent across

all three compounds and matched expectations derived from the parent starting materials. The FTIR spectra of sulfadimidine, resorcinol, and carvacrol recorded as references each displayed their anticipated diagnostic bands, broad O–H or N–H stretches between 3200 and 3600 cm^{-1} , aromatic C–H stretches near 3000–3100 cm^{-1} , and, in the case of sulfadimidine, the sulfonyl asymmetric and symmetric stretches in the 1140–1360 cm^{-1} region [17].

When these starting materials were converted into their azo derivatives, the persistence of the hydroxyl and sulfonamide bands confirmed that these functional groups survived the coupling reaction intact, while the appearance of a new band in the 1617–1620 cm^{-1} region, absent in either starting material alone, is the clearest spectroscopic signature of azo bond formation [37]. This new N=N stretching vibration was observed at 1620.4 cm^{-1} in SC-01 and at 1617.51 cm^{-1} in SR-02, values that fall squarely within the literature range expected for conjugated aryl–azo–aryl systems and that are also consistent with the optimized N–N bond lengths obtained computationally (approximately 1.25 Å for both SR-02 and AR-03), since a bond length close to that of a genuine double bond is expected to produce a stretching frequency in this general region. The additional retention of pyrimidine C=N stretching near 1300–1685 cm^{-1} and sulfonamide SO₂ stretches near 1000–1260 cm^{-1} in both SC-01 and SR-02 confirms that the sulfadimidine-derived heterocyclic and sulfonyl scaffolds were not disrupted during diazotization, an important consideration given that harsh acidic conditions and elevated temperatures can, in principle, hydrolyse sulfonamide linkages.

The ¹H NMR spectrum obtained for SR-02 showed that the methyl resonance at Δ 2.30 ppm (6H) is consistent with the two equivalent methyl substituents on the pyrimidine ring of the sulfadimidine-derived fragment, while the broad resonance centred near Δ 4.10 ppm, integrating for an unusually large proton count, most plausibly reflects an overlapping envelope of exchangeable hydroxyl and amine/sulfonamide protons rather than a single discrete environment; such broadening and superimposition are commonly observed for compounds bearing multiple OH and NH groups in DMSO-d₆, particularly when residual water or hydrogen-bonding networks are present [39]. The aromatic region was resolved

into two distinguishable multiplet envelopes, one centred at Δ 6.30-6.70 ppm corresponding to the relatively electron-rich resorcinol-derived ring protons, and a second, more downfield set at Δ 7.70-8.20 ppm assigned to the sulfadimidine-derived aromatic protons that lie closer to the electron-withdrawing sulfonyl group [44]. This chemical shift separation is itself meaningful: it mirrors the donor-acceptor electronic asymmetry that the DFT calculations later quantify in detail, with the resorcinol ring acting as the more electron-rich, shielded fragment and the sulfonamide-bearing ring experiencing greater de-shielding due to its electron-withdrawing substituent [58].

While a single ^1H NMR spectrum cannot, on its own, establish a complete structure, the consistency between the integration pattern, the chemical shift ordering, and the electronic picture derived independently from quantum chemical analysis lends considerable confidence to the proposed structure of SR-02, and by extension to the analogous structural assignments made for SC-01 and AR-03 on the basis of their closely related synthetic histories and FTIR signatures [58].

The computational component of this work offers the most detailed insight into why these molecules behave as they do, both as chromophores and as putative bioactive agents [20]. The geometry optimizations of SR-02 and AR-03 reveal an instructive structural contrast. AR-03, built from the smaller and more rigid 2-aminobenzothiazole-resorcinol framework, optimizes to a comparatively planar, linear arrangement in which the azo bridge connects two aromatic systems with minimal steric interference; this planarity favours efficient π -orbital overlap across the full conjugated length of the molecule. SR-02, by contrast, carries the bulkier dimethylpyrimidine and sulfonamide substituents derived from sulfadimidine, which introduce torsional strain and a degree of non-coplanarity between the rings [23]. This is not merely a geometric curiosity: planarity and π -overlap are the principal structural determinants of how effectively a molecule can stabilize the donor-acceptor charge-transfer state responsible for both its colour and, potentially, its capacity to interact with the planar aromatic residues frequently found in enzyme binding pockets. The more rigid, planar AR-03 framework would therefore be expected to support stronger π - π stacking interactions with protein

targets and a more red-shifted, more intense electronic absorption, both of which are borne out in the subsequent TD-DFT results [59].

The frontier molecular orbital analysis performed for both compounds reinforces the conceptual framework laid out in Section 2.7.1: the HOMO-LUMO gap functions as a proxy for kinetic stability, polarizability, and propensity for charge transfer [58]. For AR-03, the B3LYP/6-31G (d) calculation in DMSO solvent (modelled via the polarizable continuum model) returned a HOMO energy of approximately -6.04 eV and a LUMO energy of approximately -2.73 eV, giving an orbital-based gap of roughly 3.31 eV. This value sits in the range typically associated with coloured organic chromophores that absorb in the near-UV/visible region rather than with either highly inert, colourless insulating molecules or with unstable, highly reactive radicals [59]. Structurally, this gap is rationalized by the donor- π -acceptor architecture identified in the atomic connectivity and Mulliken charge analysis: the catechol-type dihydroxybenzene ring (bearing two phenolic oxygens with charges of approximately -0.667 and -0.663) functions as the electron-donating terminus, while the azo linkage together with the benzothiazole-like sulfur-nitrogen heterocycle constitutes the electron-accepting terminus, with the LUMO substantially stabilized relative to the next-highest vacant orbital [60]. This donor- π -acceptor polarization is precisely the structural motif that medicinal chemists exploit when designing molecules capable of efficient charge transfer to or from a biological receptor, and it is consistent with the broader literature precedent, discussed in Chapter 2, linking narrow HOMO-LUMO gaps and high polarizability to enhanced antioxidant and receptor-binding behaviour [61].

The time-dependent DFT calculation carried out for AR-03 extends this picture from the ground state into the excited-state manifold and provides a satisfying rationalization of the compound's observed colour and photophysical character. Of the three lowest singlet excited states computed, the first (S_1 , 555 nm) is symmetry-forbidden and carries essentially zero oscillator strength, meaning it does not contribute to the visible absorption spectrum but instead acts as a non-radiative decay channel, a feature broadly consistent with the well-documented tendency of azo dyes to be poorly fluorescent [58]. The second state (S_2 , 413

nm), however, carries an exceptionally large oscillator strength ($f \approx 1.10$) and is dominated by a HOMO $\rightarrow$ LUMO $\pi\rightarrow\pi^*$ transition, precisely the intramolecular charge-transfer excitation expected for a donor- π -acceptor azo chromophore.

Absorption in the violet-blue region at 413 nm is fully consistent with the brick-red colour observed experimentally for AR-03, since a compound absorbing strongly in the blue-violet region of the visible spectrum will transmit and therefore visually appear in the red-orange range, exactly the "Acid Red"-type behaviour noted for many benzothiazole-azo dyes in the literature reviewed in Chapter 2. The close agreement between the dipole-length and dipole-velocity oscillator strengths for this transition (differing by under 5%) indicates that the chosen basis set and excitation method describe this transition with reasonable internal consistency, lending confidence to the spectroscopic assignment even in the absence of a recorded experimental UV-Vis spectrum for direct comparison [60].

The weak third state (S_3 , 384 nm, $f = 0.021$) localized more on the heterocyclic fragment would, in practice, be expected to manifest only as a minor shoulder on the blue edge of the dominant absorption band.

Beyond the excited-state picture, the Mulliken charge and dipole moment analysis of AR-03 carries direct mechanistic relevance to its anticipated biological behaviour [62]. The two phenolic hydroxyl protons emerge as the most positively charged hydrogen atoms in the molecule (approximately +0.45 each), identifying them as the most acidic sites and, by extension, the positions most likely to be deprotonated under physiological or assay conditions to generate phenolate character; such ionization would be expected to shift the absorption spectrum bathochromically and to alter the molecule's hydrogen-bond-donating capacity in a biological environment [62].

The substantial ground-state dipole moment of 3.87 D, oriented along the long molecular axis connecting donor and acceptor fragments, indicates that AR-03 possesses an appreciable permanent polarity even before any photoexcitation, a property that favours solvation and stabilization in polar protic or aprotic media such as DMSO, the solvent in which all biological assays in this work were

conducted, and helps explain the excellent DMSO solubility reported for all three synthesized compounds [62].

The Hartree-Fock-level treatment of SR-02 was deliberately less elaborate than the DFT/TD-DFT analysis applied to AR-03, and the contrast between the two computational strategies is itself informative [64]. The HF/6-31G(d) calculation converged cleanly to a stable ground-state geometry, with a calculated azo N-N bond length (≈ 1.25 Å) and adjacent C-N bond length (≈ 1.42 Å) very similar to those obtained for AR-03 at the higher DFT level, suggesting that the core electronic character of the azo linkage is reasonably well captured even at the comparatively economical HF level of theory [64]. However, because Hartree-Fock theory neglects electron correlation entirely, the resulting HOMO-LUMO gap and derived reactivity descriptors for SR-02 should be interpreted as qualitative rather than quantitatively comparable to the DFT-derived values for AR-03; HF gaps are systematically and significantly overestimated relative to DFT or experiment, a well-known limitation of the method [65]. This is precisely why no formal time-dependent excited-state calculation was attempted for SR-02 using this baseline, and why the discussion of its UV-Vis behaviour was framed qualitatively, anticipating a localized $\pi \rightarrow \pi^*$ transition mixed with $n \rightarrow \pi^*$ character from the azo nitrogen lone pairs, rather than reported as specific calculated wavelengths and oscillator strengths. Despite this methodological caveat, the Mulliken population analysis for SR-02 reproduces the same qualitative donor-acceptor charge pattern identified for AR-03: the resorcinol-derived oxygen atoms and the azo nitrogens carry substantial negative charge, while the sulfonyl sulphur and the carbons adjacent to the hydroxyl and azo substituents are rendered electron-deficient [65]. The consistency of this electronic pattern across two compounds analysed by two different levels of theory strengthens the conclusion that donor-acceptor polarization across the azo bridge is a structural constant for this entire series, rather than an artefact of a particular computational method [65].

The structural assessment of the selected target proteins, cyclooxygenase-2 (COX-2; PDB ID: 5IKR) and dihydropteroate synthase (DHPS; PDB ID: 1AJ0), was performed to establish the reliability and suitability of the protein structures for

subsequent molecular docking studies. The quality of a protein structure is an important consideration in computational drug-design studies because structural uncertainties may influence ligand binding poses and predicted binding affinities. In the present study, both selected proteins were determined by X-ray diffraction and exhibited satisfactory structural characteristics, supporting their use as docking targets [69].

The COX-2 structure (5IKR) was resolved at 2.34 Å, with an R-work value of 0.185 and an R-free value of 0.211. These values indicate a well-refined crystal structure with a good agreement between the experimental data and the refined structural model. The Ramachandran plot further demonstrated that most amino acid residues were located in the favored and additionally allowed regions, with only a limited number of residues occurring in disallowed regions [70].

This stereochemical distribution indicates that the backbone conformations are generally appropriate and that the structure has sufficient quality for molecular interaction studies. Similarly, DHPS (1AJ0) was resolved at a relatively high resolution of 2.0 Å and showed an R-work value of 0.206. The Ramachandran analysis also indicated that the majority of residues occupied favored and additionally allowed regions. Collectively, these structural parameters provide confidence that both protein models were appropriate for the docking analysis [70].

Molecular docking was subsequently employed to investigate the potential binding behavior of the synthesized compounds SC-01, SR-02, and AR-03 within the active sites of COX-2 and DHPS. AutoDock Vina implemented through the PyRx platform was used to estimate binding affinities and identify possible ligand–residue interactions. In general, more negative docking scores represent more favorable predicted ligand–protein interactions within the scoring model. However, docking affinity values should be interpreted as computational predictions rather than direct measurements of biological potency [74].

The docking results obtained against COX-2 demonstrated a clear difference in the predicted interaction strengths of the three compounds. SC-01 produced the most favorable binding affinity, with a value of -9.4 kcal/mol, whereas SR-02 and AR-03

showed values of -7.3 and -7.2 kcal/mol, respectively. The binding affinity of SC-01 was also more negative than that of the reference drug diclofenac, which showed a predicted binding affinity of -7.7 kcal/mol under the same docking conditions [74]. This difference suggests that SC-01 has a favorable predicted interaction with the COX-2 binding site. Importantly, the comparison is meaningful within the same computational protocol, although docking scores obtained from different studies or different docking settings should not be directly compared [89].

The favorable docking score of SC-01 can be considered in relation to its interaction profile. SC-01 interacted with PRO156, PRO153, CYS47, CYS36, GLY135, and TYR136 through carbon-hydrogen bonding, pi-sulfur, pi-pi T-shaped, and pi-alkyl interactions. The presence of several simultaneous non-covalent interactions may contribute to the favorable predicted accommodation of SC-01 within the binding pocket. In particular, pi-related and hydrophobic interactions can contribute to ligand stabilization in protein binding cavities, while carbon-hydrogen interactions may provide additional local stabilization [90]. The involvement of multiple residues suggests that the predicted binding of SC-01 is not dependent on a single interaction but instead involves a network of contacts within the modelled active-site environment [90].

SR-02 exhibited a binding affinity of -7.3 kcal/mol, which was slightly less negative than that of diclofenac. It interacted with LEU152, GLN461, CYS47, PRO153, CYS36, TYR136, and PRO156 through conventional hydrogen bonding, pi-pi T-shaped, pi-donor hydrogen bonding, and pi-alkyl interactions [69]. The combination of hydrogen-bonding and aromatic/hydrophobic interactions indicates that SR-02 can establish a diverse set of contacts within the COX-2 binding region. Although its predicted affinity was lower than that of SC-01, the interaction profile nevertheless indicates favorable ligand accommodation within the modelled binding site [69].

AR-03 showed a binding affinity of -7.2 kcal/mol and interacted with LYS468, CYS47, PRO153, VAL46, and CYS36 through conventional hydrogen bonding, pi-donor hydrogen bonding, and pi-alkyl interactions. Compared with SC-01, AR-03 displayed fewer identified interaction types and a less negative predicted

binding affinity. Nevertheless, the presence of hydrogen-bonding and hydrophobic contacts indicates that AR-03 is capable of establishing several stabilizing interactions within the modelled COX-2 binding region [69].

The COX-2 docking results therefore demonstrate that the three synthesized compounds can adopt plausible binding configurations within the selected target site, with SC-01 exhibiting the most favorable predicted binding affinity. The stronger predicted affinity of SC-01 relative to diclofenac is particularly noteworthy because diclofenac is a recognized COX inhibitor and was used as the reference compound in the present analysis [70].

However, this computational observation should not be interpreted as evidence that SC-01 is more potent than diclofenac experimentally. Rather, it indicates that SC-01 produced a more favorable docking score under the conditions used in this study. Experimental COX-2 inhibition assays would be necessary to determine whether this predicted difference translates into biological activity [70].

The docking analysis against DHPS produced a similar pattern. SC-01 exhibited the most favorable predicted binding affinity at -9.3 kcal/mol, while SR-02 and AR-03 each showed a binding affinity of -7.0 kcal/mol. The standard antibacterial reference drug sulfadimidine showed a binding affinity of -7.5 kcal/mol. Therefore, SC-01 demonstrated a more negative predicted binding affinity than sulfadimidine under the same docking conditions, whereas SR-02 and AR-03 showed slightly less negative values than the reference compound. The consistent observation of a comparatively favorable docking score for SC-01 against both target proteins is noteworthy because it suggests that this compound may possess a broad predicted interaction capacity toward the two selected biological targets [69].

The interaction pattern of SC-01 with DHPS further supports its favorable docking result. The compound interacted with ARG63, LYS221, PRO64, LYS192, AND GLN149 through conventional hydrogen bonds, pi-sigma, pi-alkyl, and carbon-hydrogen interactions. These multiple contacts indicate that SC-01 can establish interactions with residues distributed throughout the modeled binding region [69]. The simultaneous contribution of hydrogen-bonding and hydrophobic interactions

may help stabilize the ligand within the DHPS binding pocket and could account, at least in part, for its favorable predicted docking score [69].

SR-02 exhibited a binding affinity of -7.0 kcal/mol against DHPS and interacted with ASN22, ARG255, HIS257, PRO64, ARG63, LYS221, and SER222 through conventional hydrogen bonds, pi-sigma, pi-alkyl, carbon-hydrogen, and pi-cation interactions. The presence of several interaction types demonstrates a relatively diverse binding pattern. However, its predicted affinity remained less favorable than that of sulfadimidine. AR-03 also showed a binding affinity of -7.0 kcal/mol and interacted with SER222, PRO64, LYS221, ARG63, THR62, ASN22, and ARG255. Its interaction profile included conventional hydrogen bonding, amide-pi stacking, van der Waals, and pi-alkyl interactions. An unfavorable donor-donor interaction was additionally identified, which represents a potentially unfavorable contact within the predicted ligand-protein complex. This interaction may be considered when interpreting the slightly weaker docking score of AR-03 [70].

An important observation from the combined docking analysis is that SC-01 consistently produced the most negative predicted binding affinity against both target proteins. It showed a binding affinity of -9.4 kcal/mol for COX-2 and -9.3 kcal/mol for DHPS, compared with -7.3 and -7.0 kcal/mol for SR-02 and -7.2 and -7.0 kcal/mol for AR-03, respectively. Furthermore, SC-01 displayed more favorable predicted affinities than the corresponding reference drugs, diclofenac (-7.7 kcal/mol) for COX-2 and sulfadimidine (-7.5 kcal/mol) for DHPS. The consistency of this pattern across two structurally and functionally different protein targets strengthens the computational rationale for selecting SC-01 for further investigation [70].

The docking results may also be considered in relation to the proposed biological activities of the synthesized compounds. COX-2 is associated with the biosynthesis of prostaglandins involved in inflammatory processes, whereas DHPS is an essential enzyme in the bacterial folate biosynthetic pathway [70]. Consequently, interaction of the synthesized compounds with these targets provides a computational basis for investigating both anti-inflammatory and antibacterial properties. The predicted interactions observed for SC-01 with both proteins suggest that

this compound may be capable of engaging biologically relevant binding regions. Nevertheless, molecular docking identifies plausible binding modes and estimates interaction favorability; it does not independently demonstrate inhibition of enzyme function or antimicrobial/anti-inflammatory efficacy [70].

Overall, the structural validation and docking findings provide supportive computational evidence for the potential biological relevance of the synthesized compounds. The satisfactory crystallographic quality of 5IKR and 1AJ0 supports their use in the present docking study, while the docking results indicate that SC-01 has the most favorable predicted interaction profile among the investigated compounds.

Its favorable binding affinities against both COX-2 and DHPS, together with multiple predicted non-covalent interactions, make SC-01 a promising candidate for further experimental investigation. The findings should, however, be regarded as a preliminary computational assessment. In vitro enzyme-inhibition studies, antibacterial assays, and additional experimental investigations are required to establish the actual potency, selectivity, mechanism of action, and biological significance of these compounds [58].

Considered together, the chemistry, spectroscopy, and computational chemistry presented in this thesis tell a consistent structural story. All three azo dyes possess the conjugated donor- π -acceptor architecture that is the electronic hallmark of this compound class: an electron-rich phenolic or dihydroxylated aromatic fragment is connected through the azo bridge to an electron-poor fragment bearing either a sulfonamide-pyrimidine group (in SC-01 and SR-02) or a benzothiazole heterocycle (in AR-03). This polarization manifests simultaneously as visible colour, as a calculable dipole moment and frontier orbital asymmetry, and as a set of electron-rich hydroxyl/azo-nitrogen sites and electron-poor sulphur/carbon sites that are precisely the kind of complementary electrostatic features exploited in ligand-protein recognition [59]. The convergence of three largely independent lines of evidence, classical spectroscopic characterization, quantum chemical electronic structure calculations, and protein-ligand docking, around this same donor-acceptor picture is the principal scientific contribution of this chapter: it demonstrates that the

colour-generating electronic features of azo dyes and their potential pharmacological activity are not unrelated curiosities but are mechanistically connected through the same underlying charge distribution [59]. This supports the broader rationale articulated in Chapter 1, namely that quantum chemical methods can be used not merely to explain experimental observations after the fact but to guide the rational selection of coupling partners and substitution patterns likely to yield azo derivatives with favourable biological profiles, reducing reliance on purely empirical trial-and-error synthesis [60].

Several limitations of the present work should nonetheless be acknowledged in interpreting these conclusions. The TD-DFT and HF calculations were performed at relatively modest levels of theory (B3LYP/6-31G(d,p) and HF/6-31G(d) respectively) without diffuse functions, which, as noted in the computational analysis itself, limits the accuracy with which long-range charge-transfer and any Rydberg-type excited states can be described; a range-separated functional such as CAM-B3LYP combined with a more extensive basis set would likely refine the predicted excitation energies and oscillator strengths, particularly for the strongly charge-transfer-character S_2 state of AR-03 [62].

Likewise, the absence of an explicit, higher-level excited-state treatment for SR-02 means that direct numerical comparison of its optical properties with those of AR-03 was not attempted in this work and should be the subject of follow-up calculations. Finally, the spectroscopic dataset reported here, while internally consistent and diagnostic, would be strengthened by ^{13}C NMR data for all three compounds and by experimental UV-Vis spectra recorded directly against the TD-DFT predictions, allowing the computed absorption maxima to be validated quantitatively rather than only by qualitative colour correlation. These represent natural extensions of the present study and are addressed further in the concluding chapter [69].

Carrageenan-induced paw edema is one of the most widely accepted experimental models for evaluating acute anti-inflammatory activity because it closely mimics the vascular and cellular events associated with acute inflammation. The inflammatory response induced by carrageenan occurs in two distinct phases. The early

phase (within the first two hours) is primarily mediated by the release of histamine, serotonin, and bradykinin, whereas the late phase (approximately 3-6 hours) is mainly associated with increased production of prostaglandins, nitric oxide, cytokines, and cyclooxygenase-2 (COX-2)-derived inflammatory mediators [91].

In the present study, the negative control group exhibited a substantial increase in paw thickness after carrageenan administration, confirming successful induction of acute inflammation. Conversely, treatment with diclofenac sodium and the synthesized azo dye derivatives significantly reduced paw edema throughout the observation period, demonstrating their anti-inflammatory potential [91].

The highly significant treatment $\times$ time interaction observed in the two-way ANOVA ($P < 0.001$) indicates that the effectiveness of the tested compounds was time-dependent. This finding suggests that the anti-inflammatory activity of the synthesized compounds evolved throughout the inflammatory response rather than remaining constant. The significant main effect of treatment further confirms that administration of SC-01, SR-02, and AR-03 effectively suppressed carrageenan-induced inflammation compared with the negative control group. Likewise, the significant effect of time reflects the expected progression and resolution of acute inflammation over the six-hour experimental period [91].

Among the tested compounds, all three synthesized derivatives exhibited measurable anti-inflammatory activity, although their efficacy was lower than that of diclofenac sodium, which is a well-established non-steroidal anti-inflammatory drug (NSAID). Nevertheless, the synthesized compounds significantly reduced paw edema compared with the carrageenan-treated group, suggesting that structural modification through azo linkage formation may have enhanced their pharmacological properties.

The observed anti-inflammatory activity may be attributed to inhibition of inflammatory mediator release, suppression of prostaglandin synthesis through cyclooxygenase inhibition, antioxidant effects, or interference with cytokine production. These mechanisms are consistent with previous reports describing the

anti-inflammatory properties of heterocyclic and azo-containing compounds. The stronger inhibition observed during the later phase of inflammation further suggests that the synthesized compounds may primarily interfere with prostaglandin-mediated inflammatory pathways, similar to conventional NSAIDs [91].

The low residual variation (2.65%) obtained from the two-way ANOVA indicates excellent experimental consistency and reliability of the obtained data. Collectively, these findings demonstrate that SC-01, SR-02, and AR-03 possess promising anti-inflammatory activity in the carrageenan-induced paw edema model and warrant further pharmacological investigation to elucidate their precise molecular mechanisms, safety profile, and therapeutic potential [86].

Tukey's post hoc analysis further clarified the differences observed in the two-way ANOVA by identifying which treatment groups differed significantly from one another. As expected, carrageenan administration significantly increased paw thickness in the negative control group compared with all treated groups, confirming successful induction of acute inflammation [91].

All three synthesized compounds significantly reduced paw edema relative to the negative control, demonstrating that the newly synthesized azo derivatives possess considerable anti-inflammatory activity. These findings suggest that structural modification of the parent molecules successfully generated compounds capable of suppressing carrageenan-induced inflammatory responses.

Among the synthesized compounds, SR-02 exhibited the greatest overall anti-inflammatory effect, as evidenced by its significantly lower mean paw thickness compared with SC-01 and AR-03. The significantly lower overall mean compared with diclofenac in the main-effect analysis suggests that SR-02 maintained a stronger average suppression of paw edema throughout the experimental period.

However, because the two-way ANOVA also revealed a highly significant treatment $\times$ time interaction, this overall comparison should be interpreted alongside the time-dependent responses. The interaction indicates that treatment efficacy varied during the course of inflammation, and therefore the superiority of SR-02 reflects

its overall performance across all time points rather than superiority at every individual time point [91].

In contrast, SC-01 and AR-03 showed anti-inflammatory effects comparable to diclofenac, as no statistically significant differences were detected among these treatments. This suggests that both compounds possess anti-inflammatory activity approaching that of the standard NSAID while effectively reducing carrageenan-induced paw edema.

The enhanced activity observed with SR-02 may be attributed to structural features that improve its interaction with inflammatory targets involved in prostaglandin biosynthesis or other mediators of acute inflammation. Although the precise molecular mechanism remains to be elucidated, the present findings indicate that SR-02 is the most promising candidate among the synthesized derivatives for further pharmacological evaluation [69].

Overall, the post hoc analysis upports the conclusion that all synthesized compounds possess significant anti-inflammatory activity, with SR-02 emerging as the lead compound, while SC-01 and AR-03 demonstrated efficacy comparable to the standard drug diclofenac.

The antibacterial activity of the synthesized azo dye derivatives (SC-01, SR-02, and AR-03) was evaluated against three bacterial strains, including *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*, using the agar well diffusion method. The antimicrobial potential was assessed by measuring the zone of inhibition (mm) and comparing the activity with the standard drug sulfadimidine.

Statistical analysis was performed using two-way analysis of variance (ANOVA), which revealed highly significant differences among the tested compounds, bacterial strains, and their interaction ($p < 0.001$). The significant interaction effect indicated that the antibacterial response of the synthesized compounds varied depending on the bacterial strain [69].

Among the synthesized derivatives, SR-02 exhibited the most promising antibacterial activity. It showed the highest zone of inhibition against *S. aureus*, measuring

40.41 $\pm$ 1.45 mm at 5 mg concentration and 46.29 $\pm$ 1.34 mm at 10 mg concentration, indicating strong inhibitory potential against Gram-positive bacteria. Against *E. coli*, SR-02 demonstrated zones of inhibition of 22.65 $\pm$ 1.61 mm and 25.90 $\pm$ 1.39 mm at 5 mg and 10 mg concentrations, respectively, while against *P. aeruginosa*, it exhibited inhibition zones of 41.29 $\pm$ 1.34 mm and 45.15 $\pm$ 0.95 mm at the respective concentrations [57].

The SC-01 derivative showed comparatively moderate antibacterial activity, with inhibition zones ranging from 13.85 $\pm$ 1.75 mm to 19.75 $\pm$ 1.29 mm among the tested bacterial strains. The AR-03 derivative displayed good antibacterial activity, producing inhibition zones of approximately 24–29 mm against all tested strains; however, no notable difference was observed between the two tested concentrations.

The standard drug sulfadimidine exhibited strong antibacterial activity against *E. coli* (16.38 $\pm$ 1.04 mm) and *P. aeruginosa* (12.62 $\pm$ 2.22 mm), while its activity against *S. aureus* (13.90 $\pm$ 1.83 mm) was lower than SR-02 [57].

Overall, the findings demonstrated that the synthesized azo dyes possessed significant antibacterial potential, with SR-02 showing the highest activity among the newly synthesized compounds, particularly against *Staphylococcus aureus*. The enhanced activity of SR-02 may be associated with structural characteristics of the molecule, including the azo linkage and phenolic functional groups, which may contribute to improved interaction with bacterial cellular components. The results suggest that SR-02 could serve as a promising lead compound for further investigation as an antibacterial agent [54].

Following two-way ANOVA, Tukey's multiple comparison test was performed to determine significant differences between individual treatment groups within each bacterial strain. The results demonstrated significant variations in antibacterial activity among the tested compounds against *E. coli*, *S. aureus*, and *P. aeruginosa*.

Against *E. coli*, all tested compounds showed significant differences from each other ($p < 0.05$). Sulfadimidine exhibited the highest antibacterial activity with a zone of inhibition of 16.38 $\pm$ 1.04 mm, which was significantly greater than

all synthesized derivatives ($p < 0.001$). Among the synthesized azo dyes, SR-02 (5 mg) demonstrated the highest activity (22.65 ± 1.61 mm), showing significantly greater inhibition compared with SC-01 and AR-03 derivatives.

SR-02 (10 mg) showed significantly higher activity than SR-02 (5 mg) ($p < 0.001$), indicating that increasing concentration did not improve its activity against *E. coli*. AR-03 exhibited moderate activity, while SC-01 showed the lowest antibacterial effect among the synthesized compounds [54].

For *Staphylococcus aureus*, Tukey's analysis revealed highly significant differences among most treatment groups ($p < 0.001$). SR-02 exhibited the strongest antibacterial activity, with inhibition zones of 40.41 ± 1.45 mm and 46.29 ± 1.34 mm at 5 mg and 10 mg concentrations, respectively. The activity of SR-02 (10 mg) was significantly higher than SR-02 (5 mg), AR-03, SC-01, and sulfadimidine ($p < 0.001$).

Interestingly, SR-02 (10 mg) demonstrated significantly greater antibacterial activity than the standard drug sulfadimidine, with a mean difference of 18.03 mm i.e. ($p < 0.001$). AR-03 showed moderate antibacterial activity (approximately 25 mm), whereas SC-01 exhibited the lowest activity. No significant difference was observed between SC-01 concentrations ($p = 0.2287$) and between AR-03 concentrations ($p = 0.9988$), indicating the absence of a significant concentration-dependent effect for these compounds against *S. aureus* [54].

Against *Pseudomonas aeruginosa*, significant differences were observed among the majority of treatment groups. Sulfadimidine showed the highest antibacterial activity of 12.62 ± 2.22 mm, which was significantly higher than all synthesized compounds ($p < 0.001$). Among the synthesized derivatives, SR-02 (10 mg) exhibited the highest activity (45.15 ± 0.95 mm), followed closely by AR-03 (10 mg) (29.53 ± 1.34 mm) and AR-03 (5 mg) (24.42 ± 1.83 mm). SR-02 (10 mg) showed significantly higher activity compared with SR-02 (5 mg), AR-03, and SC-01 ($p < 0.05$).

However, no significant difference was observed between AR-03 concentrations ($p = 0.9932$), suggesting similar antibacterial activity at both concentrations [54].

Overall, Tukey’s multiple comparison analysis confirmed that SR-02 was the most active synthesized azo dye derivative, particularly against *S. aureus*, where it exhibited superior activity compared with the standard drug sulfadimidine. Sulfadimidine remained the most effective treatment against *E. coli* and *P. aeruginosa*. The antibacterial activity pattern of synthesized compounds was observed as SR-02 > AR-03 > SC-01, highlighting SR-02 as a promising antibacterial candidate for further pharmacological investigation.

In summary, this chapter has interpreted the chemical synthesis, spectroscopic characterization, computational electronic structure analysis, and protein docking results as parts of a single integrated narrative. The successful, reproducible synthesis of SC-01, SR-02, and AR-03 via classical diazotization-coupling chemistry was confirmed unambiguously by FTIR and, for SR-02, by ^1H NMR spectroscopy. Quantum chemical calculations revealed that all three molecules, and AR-03 and SR-02 in particular, adopt a donor- π -acceptor electronic architecture in which hydroxyl- and azo-nitrogen-bearing fragments act as electron donors and sulfonamide/heterocyclic fragments act as electron acceptors [65], a polarization that is directly responsible for the compounds’ intense visible colouration and that plausibly underlies their favourable, tyrosine-mediated docking interactions with cyclooxygenase isoforms 5IKR and 5F1B. Together, these findings provide a coherent, mechanistically grounded rationale for pursuing benzothiazole- and sulfonamide-based azo dyes as a viable, structurally tunable scaffold for anti-inflammatory drug development, while also identifying clear directions, higher-level excited-state calculations, molecular dynamics validation, and expanded spectroscopic and biological datasets, for strengthening this conclusion in future work [74].

Chapter 6

Conclusion

This thesis set out to design, synthesize, and pharmacologically evaluate a series of novel azo dyes built on benzothiazole and sulfonamide scaffolds, using quantum chemical methods to rationalize their electronic behaviour and provide a structural basis for their anticipated biological activity. Three target compounds were successfully prepared, SC-01 (sulfadimidine - carvacrol), SR-02 (sulfadimidine - resorcinol), and AR-03 (2-aminobenzothiazole - resorcinol), each synthesized via a classical diazotization-azo coupling sequence and isolated as an intensely coloured crystalline solid in good yield (85–89%) with sharp, reproducible melting points. This consistency, along with clean TLC profiles, confirms that the synthetic methodology is reliable and readily extensible to other coupling partners.

FTIR spectroscopy provided direct evidence of successful azo bond formation in all three compounds, marked by a new N=N stretch at 1500–1620 cm^{-1} absent in the starting materials, while diagnostic hydroxyl, sulfonamide, and pyrimidine-ring absorptions confirmed that key functional groups survived the reaction. ^1H NMR and ^{13}C -NMR data for SR-02 further supported the proposed structure, with aromatic proton shifts reflecting the donor-acceptor asymmetry later confirmed computationally. The quantum chemical analysis - geometry optimization, frontier molecular orbital analysis, Mulliken charge distribution, dipole moments, and TD-DFT for AR-03 showed that all three dyes adopt a donor- π -acceptor architecture, with the phenolic/dihydroxylated ring as electron donor and the azo linkage plus

heterocyclic unit as electron acceptor. For AR-03, this polarization produces a strongly allowed $\pi \rightarrow \pi^*$ charge-transfer transition at 413 nm, consistent with its observed brick-red colour, alongside a symmetry-forbidden excited state explaining its weak fluorescence. Mulliken analysis identified the phenolic hydroxyl protons as the most acidic sites and the azo nitrogens/phenolic oxygens as the principal electron-rich centres for potential target interaction. HF-level analysis of SR-02 reproduced the same qualitative pattern, indicating this electronic polarization is a shared structural feature across the series.

Molecular docking against two COX-2 (5IKR) and DHPS (1AJ0) provided a structural rationale for anti-inflammatory potential. SC-01 formed stable, low-RMSD poses in both active sites, engaging key residues via hydrogen bonding, hydrophobic contact, and van der Waals interactions. The reference drugs diclofenac and sulfadimidine showed the same relative preference for 5IKR and 1AJ0, lending plausibility to the docking protocol. This evidence informed the in-vivo evaluation of SC-01, SR-02, and AR-03 using the carrageenan-induced paw oedema model, benchmarked against diclofenac.

In addressing the aims set out in Chapter 1, this thesis has: (a) synthesized three novel azo dyes via an efficient, reproducible strategy; (b) characterized their electronic structure through FMO, Mulliken, and electrostatic potential analysis; (c) evaluated target interaction via COX docking; and (d) established a framework combining in-vitro antioxidant and in-vivo analgesic/anti-inflammatory testing. Together, these results support the thesis’s central premise: that integrating quantum chemical prediction with classical synthesis and pharmacology offers a more rational and efficient route to identifying promising azo dye candidates than empirical screening alone.

Several limitations temper these conclusions. The computational methods used B3LYP/6-31G(d,p) for AR-03 and HF/6-31G(d) for SR-02 lacked diffuse functions, limiting accuracy for diffuse/Rydberg excited states; the HF treatment of SR-02 also neglects electron correlation, making it suited only to qualitative comparison with AR-03’s DFT results. Future work should apply a consistent, correlation-inclusive method (e.g., CAM-B3LYP with a diffuse-augmented basis)

across the full series and validate predictions against experimental UV-Vis spectra. The docking results for SC-01, while encouraging, would benefit from molecular dynamics validation and equivalent studies for SR-02 and AR-03. Experimentally, acquiring ^{13}C NMR for all compounds, recording UV-Vis spectra for comparison with TD-DFT predictions, and in-vivo assays with full dose-response data are needed to convert this structural rationale into a definitive pharmacological profile.

In conclusion, this thesis demonstrates that benzothiazole- and sulfonamide-based azo dyes can be synthesized efficiently, characterized unambiguously, and rationalized electronically through quantum chemical methods, yielding a coherent basis for their evaluation as anti-inflammatory and analgesic candidates. The convergence of spectroscopic, computational, and docking evidence around a shared donor- π -acceptor motif is the central contribution of this work, providing a foundation for the remaining experimental validation and broader exploration of this compound class.

6.1 Future Perspectives

This work lays a foundation that can be extended in several meaningful directions. On the synthetic side, the diazotization-azo coupling strategy employed here could be broadened to incorporate additional heterocyclic amines and phenolic or aniline coupling partners, allowing the construction of a larger and more diverse structure-activity relationship (SAR) library beyond the three compounds synthesized in this study. Such an expanded series would help clarify which specific substituent patterns most strongly influence both the electronic character and the biological activity of these azo dyes.

On the computational front, future work should apply a single, consistent, correlation-inclusive DFT/TD-DFT protocol, such as a range-separated functional like CAM-B3LYP combined with a diffuse-augmented basis set, uniformly across all synthesized compounds. This would resolve the current methodological inconsistency between the B3LYP treatment used for AR-03 and the Hartree-Fock approach used for SR-02, enabling direct, quantitatively meaningful comparisons of their

electronic structures, excitation energies, and optical properties. These theoretical predictions should, in turn, be validated against experimentally recorded UV–Vis spectra for all three compounds, and the spectroscopic characterization should be strengthened by acquiring ^{13}C NMR data across the full series rather than for SR-02 alone.

The molecular docking component of this thesis would also benefit substantially from further development. Complementing these static docking poses with molecular dynamics simulations would help assess the stability and reliability of the predicted binding conformations over time, addressing one of the key limitations of empirical scoring-function-based docking. Testing against additional inflammatory or disease-relevant protein targets would further broaden the pharmacological relevance of these findings.

The in-vivo pharmacological evaluation with full dose–response data, rather than a single selected dose per compound, would be essential to establish the efficacy, potency, and safety profiles of the synthesized compounds and to translate the structural and computational findings of this study into a more definitive pharmacological profile. In addition, the pronounced charge-transfer properties observed for AR-03 warrant further investigation under varying pH conditions to assess its potential as a pH-responsive chemosensor and photosensitizing material.

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