

Design, Synthesis, and Antimicrobial Evaluation of Novel Sulfamethoxazole-Thiazolidinone Hybrids: A Molecular Hybridization Approach to Combat Antimicrobial Resistance

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Abstract—In the light of growing concern of antimicrobial resistance (AMR) crisis, there is a need to come up with novel antibacterials with increased effectiveness and reduced resistance to them. In this research, a series of new sulfamethoxazole derivatives containing 4-thiazolidinone framework via molecular hybridization approach have been designed, synthesized and screened for their antimicrobial activity. This work describes a synthetic strategy involving three steps; (i) formation of Schiff base (M1) by reaction of sulfamethoxazole and p-methoxybenzaldehyde, (ii) cyclocondensation of the product with thioglycolic acid to give rise to 4-thiazolidinone nucleus (M2), and (iii) Knoevenagel condensation of M2 with substituted aromatic aldehydes to give rise to final 5-benzylidenes (M3a and M3b). The newly synthesized compounds were characterized with respect to their physical properties and spectral analysis (melting point, TLC, FT-IR, ¹H-NMR, ¹³C-NMR, Mass Spectroscopy). Their in vitro antibacterial activity was tested against three bacterial species; *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Staphylococcus aureus* (ATCC 25923) using agar well diffusion and broth microdilution assays. It has been observed that both the final derivatives showed considerable antibacterial activity and compound M3b (containing a 4-hydroxybenzylidene moiety) was more active than M3a (containing unsubstituted benzylidene). Noteworthy is the observation that both the derivatives possessed activity against *P. aeruginosa* against which the parent drug sulfamethoxazole did not show any activity. On the basis of structure-activity relationship study it is evident

that the presence of electron-donating groups such as hydroxyl group has resulted in enhanced antibacterial activity due to effective hydrogen bonding.

Index Terms—Sulfamethoxazole, Thiazolidinone, Molecular Hybridization, Antimicrobial Activity, Schiff Base, Knoevenagel Condensation, Structure-Activity Relationship

I. INTRODUCTION

1.1 Antimicrobial Resistance: A Global Public Health Crisis in the 21st Century

The occurrence of antimicrobial resistance (AMR) is seen as a significant global public health crisis in the twenty-first century. AMR has been listed as one of the main threats to global public health by the World Health Organization (WHO). The projected statistics indicate that antimicrobial-resistant infections may claim as many as 10 million lives per annum and cost an estimated \$100 trillion per year by 2050 [1]. The misuse of antibiotics in various sectors such as healthcare, agriculture, and veterinary has led to the development of resistant strains, resulting in the ineffectiveness of several once-effective antibiotics [2]. Sulfonamides, being the first antibiotics used systemically in medicine, are among those whose efficacy has been significantly curtailed owing to resistance [3].

Sulfamethoxazole (SMX), belonging to the sulfonamide family, has for long been an important component of antibacterial treatment ever since its development in the mid-20th century. Sulfamethoxazole acts by inhibiting DHPS through a competitive action whereby it competes for PABA (the natural substrate) with DHPS [4]. The inhibition process is responsible for disrupting the folate biosynthesis and causing the arrest of bacterial growth. However, the widespread use of sulfonamides has caused resistance through various means such as chromosomal mutations of the folP gene coding for DHPS and the acquisition of sul genes (sul1, sul2, sul3) coding for drug-resistant DHPS [5]. In addition, sulfonamide residue presence in the environment, with 71.1% occurrence in seawater and 30.4% in sediments, has contributed significantly to this problem by exerting selective pressure on microorganisms and amplifying antibiotic resistance genes [6].

1.2 Sulfonamides: Historical Significance and Contemporary Relevance

The invention of sulfonamide drugs is one of the most important events in the history of chemotherapy. It was proven in 1932 by Gerhard Domagk that the azo dye Prontosil exhibited considerable antibacterial activity towards streptococcal infections, which earned him the Nobel Prize in Physiology or Medicine in 1939 [7]. It was established by Ernest Fourneau et al. that Prontosil acts as a pro-drug, being cleaved into an active ingredient called sulfanilamide within the organism [8]. Woods-Fildes hypothesis, put forward in 1940, explained how sulfonamides exert their effect by providing a mechanism wherein sulfanilamide competes with PABA at DHPS by means of molecular mimicry [9].

Although sulfonamides have proven themselves very effective over the years, their clinical application has greatly been hindered by the development of resistance to them. Nonetheless, the sulfonamide class of compounds remains highly privileged in the field of medicinal chemistry and serves as a template for numerous therapeutics used in different medical conditions [10]. Sulfonamide derivatives include important components of diuretics (furosemide, hydrochlorothiazide), anticonvulsants (zonisamide), COX-2 inhibitors (celecoxib), antiretroviral

(darunavir), and anticancer (belinostat, pazopanib) therapeutics [10,11].

Sulfonamide moiety ($-\text{SO}_2\text{NH}-$) possesses unique physical and chemical properties that make it suitable for drug discovery [12].

- **Electronic Effects:** The sulfonyl oxygens have strong electron withdrawing effects, resulting in the polarization of the N–H bond that follows next.
- **Stability Against Metabolism:** The $-\text{SO}_2\text{NH}-$ linkage is highly chemically stable; it does not undergo hydrolysis or oxidative metabolism.
- **Non-Covalent Interactions:** Due to the high polarity and the known spatial structure, the sulfonamides can act as good hydrogen bond donors and acceptors [13].

1.3 Thiazolidinone Scaffold: A Privileged Structure in Medicinal Chemistry

The thiazolidin-4-one framework (Figure 1) has turned out to be a privileged structure in medicinal chemistry because of its structural diversity and ability to elicit a wide range of biological effects [14]. The five-membered heterocyclic ring with sulfur atom in the first position, nitrogen atom in the third position, and carbonyl group in the fourth position is often called a “magic molecule” in drug discovery [15].

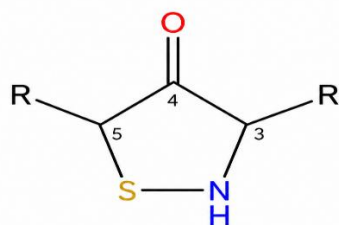


Figure 1: General Structure of Thiazolidin-4-one Scaffold

Various thiazolidinone derivatives are known to be involved in many biological processes including antibacterial, antifungal, anti-inflammatory, anticonvulsive, antidiabetic, anticancer and antioxidant activity [16,17]. Modification of this scaffold is possible by the variation of substitution on the N-3, C-2 and C-5 groups of this scaffold, which can be used to optimize biological activity with various substituents [18].

Synthesis of 4-thiazolidinone derivatives is usually carried out by the reaction of the cyclocondensation of Schiff base (imines) with mercaptoacetic acid (thioglycolic acid) in the reflux condition. This

reaction is called the MALI (Mercaptoacetic Acid Looking Imine) reaction [19]. Further Knoevenagel condensation of aromatic aldehyde will yield (Z)-5-arylidene group and therefore it becomes a useful point for modification to optimize biological activity according to the substituents of arylidene [20].

1.4 Molecular Hybridization: A Strategic Approach to Drug Discovery

Molecular hybridization, which entails the covalent union of two or more bioactive pharmacophores within one molecule, is one of the popular techniques currently used in modern drug discovery research [21]. Molecular hybridization is quite helpful in tackling complex therapeutic challenges like antimicrobial resistance since it is possible for molecules with multiple targets to overcome the constraints of the conventional therapy targeting only one site [22].

The incorporation of sulfonamide moieties with different pharmaceutically active heterocycles has led to sulfonamide hybrids with enhanced biological activities [23]. Among the various heterocycles joined to sulfonamides to give sulfonamide hybrids, thiazolidinone has attracted much interest due to its complementarity with the sulfonamide group [24]. Sulfonamides are powerful hydrogen bond donors and metal binders while thiazolidinone can easily be substituted by different groups in order to enhance their activities [25].

Studies carried out recently have indicated that sulfonamide-thiazolidinone hybrids have excellent activities as antibacterials, antifungals, antioxidants, anti-inflammatories and anticancer agents [26-28]. The rational design of sulfonamide-thiazolidinone hybrids makes it easy to prepare hybrids that are.

- Act on multiple targets simultaneously
- Overcome drug resistance mechanisms
- Exhibit synergistic effects
- Reduce side effects associated with high doses of single-target drugs

1.5 Rationale and Objectives of the Present Study

Even though much attention has been paid to the synthesis of sulfonamide derivatives and thiazolidinone analogues, the design and synthesis of compounds containing both sulfamethoxazole and a thiazolidinone scaffold, especially with varied

benzylidene substituents, have not yet been adequately studied. The aim of this study was:

- i. Synthesis: To synthesize a novel series of sulfamethoxazole derivatives in a three-step process involving formation of the Schiff base, cyclocondensation to form the 4-thiazolidinone scaffold and Knoevenagel condensation of benzylidene substituents.
- ii. Characterization: To characterize all newly synthesized compounds using melting point, thin-layer chromatography, Fourier-transform infrared spectroscopy, ¹H-NMR, ¹³C-NMR, and mass spectral techniques.
- iii. In Vitro Antibacterial Assay: To test the in vitro antibacterial activity of the synthesized compounds against three pathogenic bacterial species, namely, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*.
- iv. Structure-Activity Relationship Study: To develop preliminary structure-activity relationship between in vitro antibacterial activity and nature/position of benzylidene substituents.

II. MATERIALS AND METHODS

2.1 Chemicals and Reagents

All chemicals and reagents used in this study were analytical grade and used as such without any additional purification. Sulfamethoxazole was purchased from Yarrow Pharma (India). Chemicals used in this study included p-methoxybenzaldehyde, benzaldehyde, 4-hydroxybenzaldehyde, thioglycolic acid, anhydrous zinc chloride (ZnCl₂), piperidine, glacial acetic acid, and all solvents, namely absolute ethanol, N,N-dimethylformamide, toluene, petroleum ether, ethyl acetate, methanol, chloroform, diethyl ether, dimethyl sulfoxide, which were provided by Qualikems (India) and Loba Chemie Pvt. Ltd. (India). Media, Mueller–Hinton Agar, Mueller–Hinton Broth.

2.2 Instruments and Equipment

The melting point was measured using the Syntonic Digital Melting Point Apparatus, and the data is uncorrected. FT-IR spectra were taken with the help of Shimadzu FTIR spectrophotometer (Japan) using KBr discs. The proton NMR and carbon-13 NMR spectra were taken with a help of the JEOL JNM-ECZ400S/L1 spectrophotometer (400 MHz) using methanol-d₄ as a solvent and TMS as an internal standard. Mass spectra

were run in a Shimadzu mass spectrometer (Japan). The UV-Vis spectra were taken with the help of Shimadzu UV-Vis spectrophotometer. The TLC was run on the silica gel G plates, and it was visualized under UV light (254 nm) or iodine vapours.

2.3 Synthetic Procedures

2.3.1 Synthesis of Schiff Base [4-((4-methoxybenzylidene) amino)-N-(5-methylisoxazol-3-yl) benzenesulfonamide] (M₁)

A solution consisting of sulfamethoxazole (0.01 mol, 2.533 g) and p-methoxybenzaldehyde (0.01 mol, 1.362 g) was made using the minimum amount of absolute ethanol. The mixture was made to undergo reflux with glacial acetic acid (1 mL) acting as a catalyst for 5-8 hours. The progress of the reaction was monitored using TLC, with petroleum ether/ethyl acetate (7:3) being used as the mobile phase. After the reaction was complete, the mixture was cooled to room temperature and subsequently poured on crushed ice. The resultant precipitate was collected, washed with cold ethanol, and dried to give an off-white crystal in 63% yield, melting at 234-236°C.

2.3.2 Synthesis of 4-Thiazolidinone Intermediate [4-(2-(4-methoxyphenyl)-4-oxothiazolidin-3-yl)-N-(5-methylisoxazol-3-yl) benzenesulfonamide] (M₂)

A solution of Schiff base M₁ (0.01 mol, 2.98 g), thioglycolic acid (0.022 mol, 2.5 mL), and catalytic amount of anhydrous ZnCl₂ in DMF (20 mL) was refluxed for 12 hours. Progress of the reaction was followed by thin-layer chromatography with the help of toluene/ether (3:1) as the solvent. After completion, the reaction mass was allowed to cool to room temperature and was poured on crushed ice. The precipitated material was filtered, washed with water and dried. It was recrystallized from ethanol to obtain brown-black powder in 72% yield, melting at 136-137°C.

2.3.3 Synthesis of 5-Benzylidene Derivatives (M_{3a} and M_{3b})

General Procedure: A mixture of compound M₂ (0.01 mol, 4.455 g) and the appropriate aromatic aldehyde (0.012 mol) in absolute ethanol (50-55 mL) was refluxed with piperidine (1-3 drops) for 6-14 hours. The reaction progress was monitored by TLC using methanol/chloroform/ether (4:3:3) as the eluent. After completion, the reaction mixture was cooled to room

temperature. The precipitated solid was collected by filtration, washed with cold ethanol, and recrystallized from ethanol.

Compound M_{3a} (Benzylidene derivative): Reaction with benzaldehyde yielded brown powder (yield 47%, M.P. 244-245°C).

Compound M_{3b} (4-Hydroxybenzylidene derivative): Reaction with 4-hydroxybenzaldehyde yielded yellowish-brown powder (yield 53%, M.P. 180-181°C).

2.4 Characterization

2.4.1 Thin Layer Chromatography (TLC)

TLC was performed using silica gel G plates to assess the purity of synthesized compounds and to monitor reaction progress. The following solvent systems were used:

- Petroleum ether/Ethyl acetate (7:3) for M₁
- Toluene/Ether (3:1) for M₂
- Methanol/Chloroform/Ether (4:3:3) for M_{3a} and M_{3b}

Compounds were visualized under UV light (254 nm) or by exposure to iodine vapor.

2.4.2 FT-IR Spectroscopy

FT-IR spectra were recorded as KBr disks in the range 4000-400 cm⁻¹. The characteristic absorption bands were interpreted based on literature data for analogous compounds.

2.4.3 Nuclear Magnetic Resonance Spectroscopy

¹H-NMR and ¹³C-NMR spectra were recorded in methanol-D₄ solution at 400 MHz using TMS as internal standard. Chemical shifts (δ) are reported in parts per million (ppm), and coupling constants (J) are reported in Hertz (Hz).

2.4.4 Mass Spectrometry

Mass spectra were recorded using electron impact ionization (EI) at 70 eV. The molecular ion peaks (M⁺) were identified for final compounds.

2.4.5 Elemental Microanalysis

Elemental microanalysis (CHNS) was performed for final compounds to confirm their molecular composition. The results were in good agreement with calculated values.

2.5 Antibacterial Activity Evaluation

2.5.1 Test Microorganisms

The antibacterial activity of synthesized compounds was evaluated against three bacterial strains:

- Gram-positive bacteria: *Staphylococcus aureus* ATCC 25923
- Gram-negative bacteria: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853

2.5.2 Agar Well Diffusion Method

Agar well diffusion technique was applied for the initial antibacterial screening. Cultures of bacteria were grown in Mueller-Hinton Broth (MHB) at 37°C for 16-18 hours. The concentration of the bacterial inoculum was 1.5×10^8 CFU/mL (0.5 McFarland standard). Bacteria were plated on MHA plates with 100 μ L of the inoculum by means of sterile glass spreader. Wells with a diameter of 6 mm were made in agar plates, and 100 μ L of compounds at 500, 250, 125, and 62.5 μ g/mL in DMSO were pipetted into wells. Negative control consisted of DMSO; sulfamethoxazole was used as a positive control.

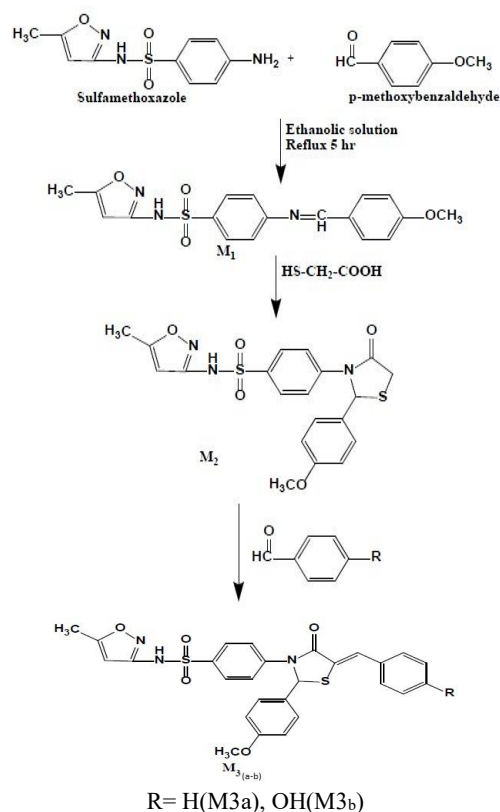
2.5.3 Determination of Minimum Inhibitory Concentration (MIC)

The broth microdilution method was used to determine MIC values according to CLSI guidelines. Serial two-fold dilutions of compounds were prepared in MHB in 96-well microtiter plates. Bacterial inoculum (100 μ L of 5×10^5 CFU/mL) was added to each well. Plates were incubated at 37°C for 24 hours, and MIC was determined as the lowest concentration showing no visible bacterial growth.

III. RESULTS AND DISCUSSION

3.1 Synthetic Studies

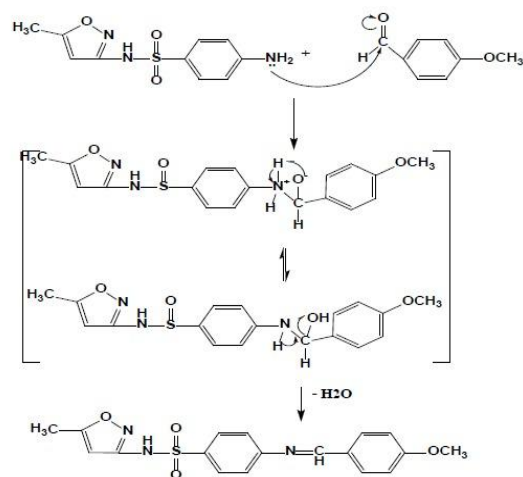
The synthetic route for the target compounds is illustrated in Scheme 1. The synthesis proceeded through three sequential steps: (i) formation of Schiff base (M_1) by condensation of sulfamethoxazole with p-methoxybenzaldehyde, (ii) cyclocondensation with thioglycolic acid to yield the 4-thiazolidinone core (M_2), and (iii) Knoevenagel condensation with substituted aromatic aldehydes (benzaldehyde and 4-hydroxybenzaldehyde) to afford the final 5-benzylidene derivatives (M_{3a} and M_{3b}).



Scheme 1: Synthesis of Intermediates and Target Compounds

3.1.1 Synthesis of Schiff Base (M_1)

The synthesis of Schiff base M_1 was achieved through the condensation of sulfamethoxazole with p-methoxybenzaldehyde under acid-catalyzed reflux conditions. The reaction mechanism involves nucleophilic addition of the primary amine group of sulfamethoxazole to the carbonyl carbon of the aldehyde, followed by dehydration to form the imine ($C=N$) bond (Scheme 2).



Scheme 2: Mechanism of Schiff Base Formation

The formation of M_1 was confirmed by the disappearance of the primary amine ($-NH_2$) stretching band ($\sim 3468\text{ cm}^{-1}$) and the appearance of characteristic imine ($C=N$) stretching at 1692 cm^{-1} in the FT-IR spectrum. The ^1H -NMR spectrum showed a

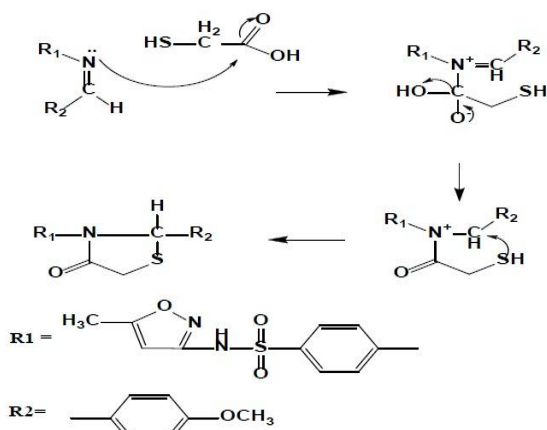
characteristic singlet at $\delta 8.9\text{ ppm}$ corresponding to the imine proton ($-CH=N-$), confirming successful Schiff base formation. The physical properties and spectral data are summarized in Tables 1-3.

Table 1: Characterization and Physical Properties of Synthesized Compounds

Comp. No.	R	Mol. Formula	Yield, %	Synthesised compounds	Colour, physical appearance	M.P. / $^{\circ}C$	R _f value
M ₁	-	C ₁₈ H ₁₇ N ₃ O ₄ S	63		Off white Crystalline	234-236	0.54
M ₂	-	C ₂₀ H ₁₉ N ₃ O ₅ S ₂	72		Brownish black powder	136-137	0.64
M _{3a}	H	C ₂₇ H ₂₃ N ₃ O ₅ S ₂	47		Brown powder	244-245	0.72
M _{3b}	OH	C ₂₇ H ₂₃ N ₃ O ₆ S ₂	53		Yellowish brown powder	180-181	0.67

3.1.2 Synthesis of 4-Thiazolidinone Intermediate (M_2)

The 4-thiazolidinone intermediate M_2 was synthesized by cyclocondensation of Schiff base M_1 with thioglycolic acid in the presence of anhydrous $ZnCl_2$ as a catalyst. The reaction proceeds through the MALI (Mercaptoacetic Acid Looking Imine) mechanism, involving nucleophilic attack of the thiol group on the electrophilic imine carbon, followed by intramolecular cyclization and dehydration (Scheme 3).

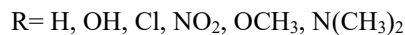
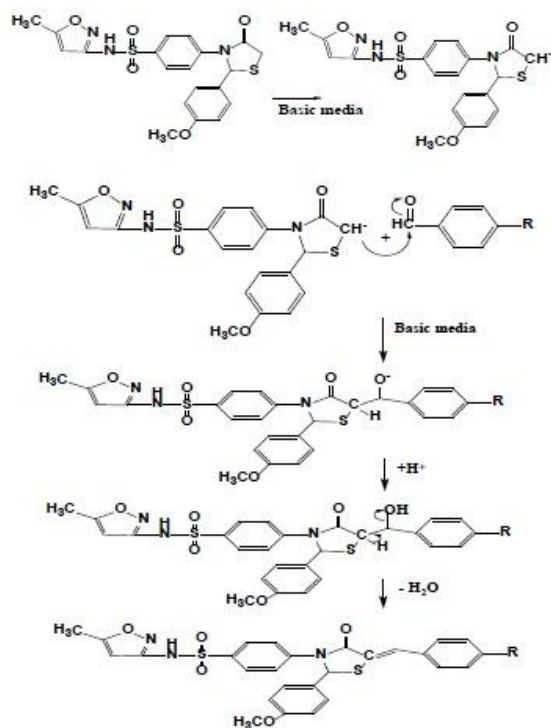


Scheme 3: Mechanism of 4-Thiazolidinone Formation (MALI Reaction)

The formation of M_2 was confirmed by the disappearance of the imine ($C=N$) stretching band at 1692 cm^{-1} and the appearance of a new carbonyl ($C=O$) stretching band at 1703 cm^{-1} corresponding to the thiazolidinone ring. The ^1H -NMR spectrum showed characteristic signals at $\delta 5.31\text{ ppm}$ (CH_2 of thiazolidinone) and $\delta 6.02\text{ ppm}$ (CH of thiazolidinone), confirming successful cyclization. The disappearance of the imine proton signal at $\delta 8.9\text{ ppm}$ further confirmed the conversion.

3.1.3 Synthesis of 5-Benzylidene Derivatives (M_{3a} and M_{3b})

The final derivatives M_{3a} and M_{3b} were synthesized via Knoevenagel condensation of the 4-thiazolidinone intermediate M_2 with benzaldehyde and 4-hydroxybenzaldehyde, respectively, using piperidine as a base catalyst. The reaction proceeds through base-catalyzed addition of the active methylene group at C-5 of the thiazolidinone ring to the aldehyde carbonyl, followed by dehydration to yield the (*Z*)-5-benzylidene derivatives (Scheme 4).



Scheme 4: Mechanism of Knoevenagel Condensation

The formation of M_{3a} and M_{3b} was confirmed by the appearance of characteristic exocyclic vinylic proton signals in the $^1\text{H-NMR}$ spectra at δ 7.56-7.7 ppm (M_{3a}) and δ 7.74 ppm (M_{3b}). The FT-IR spectra showed retention of the thiazolidinone carbonyl stretching band ($\sim 1700\text{ cm}^{-1}$) along with appearance of additional aromatic $\text{C}=\text{C}$ stretching bands. The mass spectra confirmed the molecular weights: M_{3a} (m/z 531 [M^+]) and M_{3b} (m/z 548 [M^+]), consistent with the proposed structures.

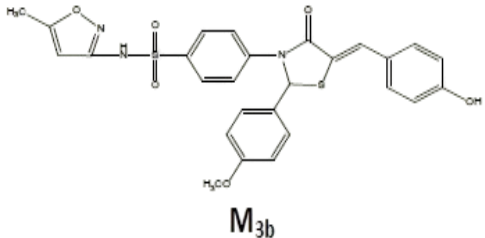
3.2 Spectral Characterization

3.2.1 FT-IR Spectroscopy

The FT-IR spectra of synthesized compounds provided valuable information about the functional groups present. Table 2 summarizes the characteristic absorption bands and their assignments.

Table 2: FT-IR Spectral Data for Intermediates and Final Compounds

Compounds	Characteristics bands of IR spectra (cm^{-1} , KBr disk)	
	Bands	Interpretation
 M_1	3425	NH Stretching vibration of 2°sulfonamide
	2854	CH Stretching vibration of methyl
	1692	$\text{C}=\text{N}$ Stretching vibration of Imine
	1625	$\text{C}=\text{N}$ stretching of isoxazole ring
	1598	Aromatic $\text{C}=\text{C}$ stretching
	1082	$\text{C}-\text{O}-\text{C}$ symmetric Str.
	1249	$\text{C}-\text{O}-\text{C}$ a symmetric Str.
	772	CH bending vibration out of plane
 M_2	3362	NH Stretching vibration of 2°sulfonamide
	3049	CH Stretching vibration of aromatic
	2966	CH asymmetric stretching of CH
	1703	$\text{C}=\text{O}$ Stretching vibration of thiazolidinone
	1612	$\text{C}=\text{N}$ stretching of isoxazole ring
	1512	Aromatic $\text{C}=\text{C}$ stretching
	1266	$\text{C}-\text{OCH}_3$ stretching
 M_{3a}	3362	NH Stretching vibration of 2°sulfonamide
	3241	$\text{C}-\text{H}$ stretching of aromatic
	2895	CH asymmetric stretching of CH_3
	1706	$\text{C}=\text{O}$ stretching of thiazolidinone
	1593	Aromatic $\text{C}=\text{C}$ stretching
	1141	$\text{C}-\text{S}$ stretching

 M_{3b}	3425	Phenolic O-H stretching
	3371	NH Stretching vibration of 2°sulfonamide
	3076	C-H stretching of aromatic
	2928	CH asymmetric stretching of CH ₃
	1692	C=O stretching of thiazolidinone
	1625	Aromatic C=C stretching
	1218	C-S stretching

Interpretation of FT-IR Spectra:

The FT-IR spectrum of M₁ showed the characteristic imine (C=N) stretching band at 1692 cm⁻¹, confirming successful Schiff base formation. The absence of the primary amine (-NH₂) stretching band at ~3468 cm⁻¹ confirmed complete conversion of the amine group. The presence of sulfonamide NH stretching at 3425 cm⁻¹ indicated the retention of the sulfonamide moiety. The spectrum of M₂ showed the disappearance of the imine band and the appearance of a new carbonyl stretching band at 1703 cm⁻¹, confirming the formation of the thiazolidinone ring. The C-OCH₃

stretching band at 1266 cm⁻¹ was retained from the starting material.

For M_{3a} and M_{3b}, the thiazolidinone carbonyl stretching bands were observed at 1706 and 1692 cm⁻¹, respectively. The presence of additional aromatic C=C stretching bands confirmed the incorporation of the benzyldiene moieties. The spectrum of M_{3b} also showed a broad O-H stretching band at 3425 cm⁻¹, confirming the presence of the phenolic hydroxyl group.

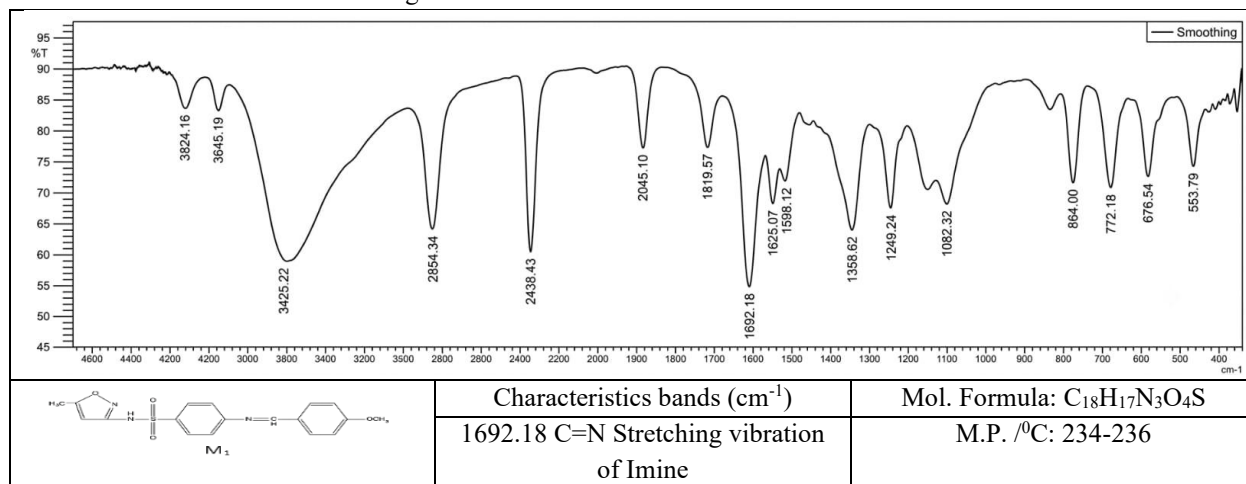
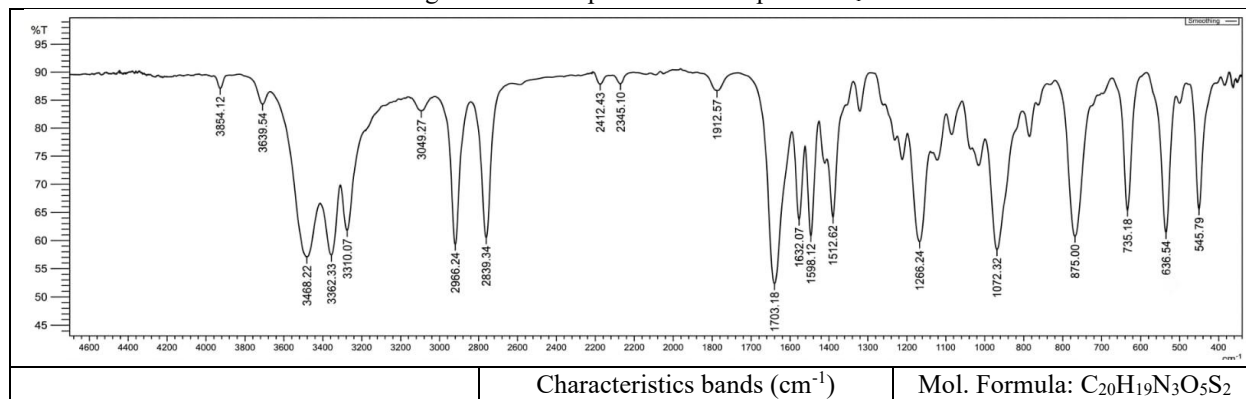


Figure 2: FT-IR spectrum of compound M₁



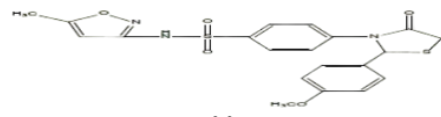
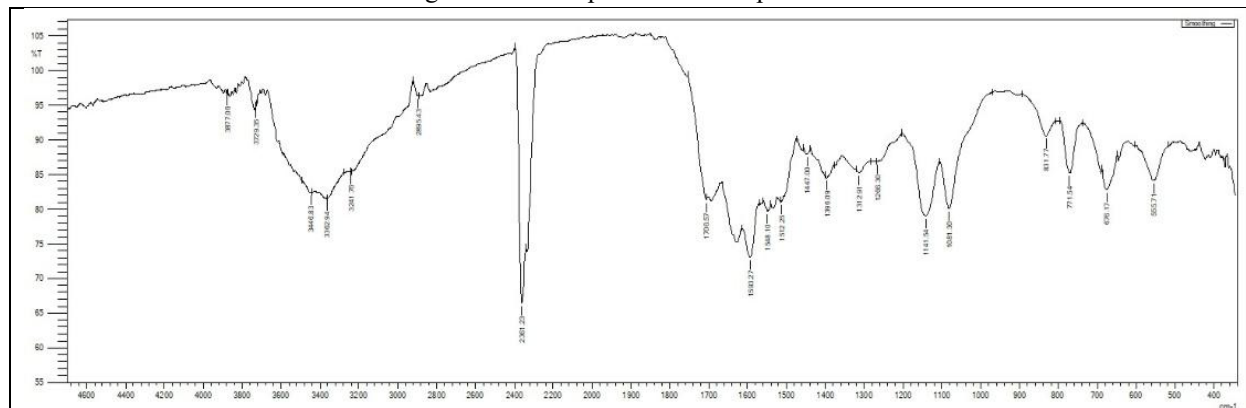
 M₂	1703.18 C=O Stretching vibration of thiazolidinone 1266.24 C-OCH ₃ stretching	M.P. /°C: 136-137
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Figure 3: FT-IR spectrum of compound M₂

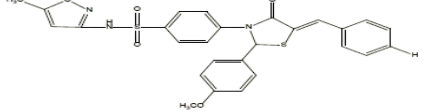
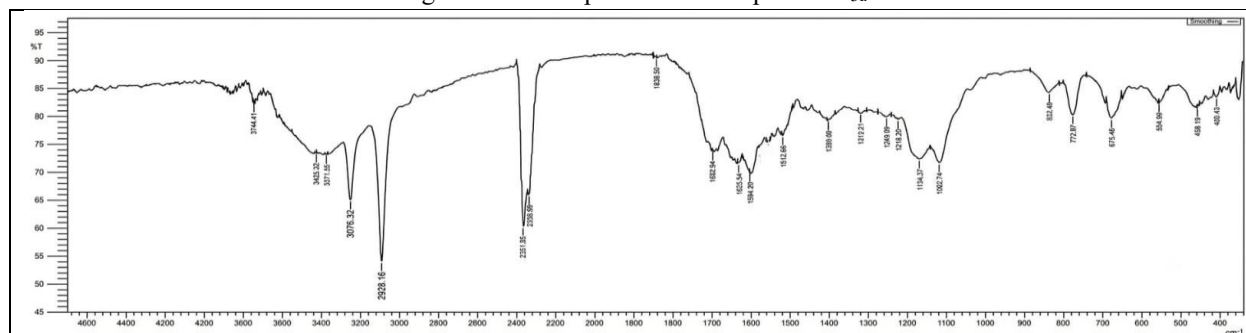
 M_{3a}	Characteristics bands (cm ⁻¹) 1706.57 C=O stretching of thiazolidinone 1141.54 C-S stretching	Mol. Formula: C ₂₇ H ₂₃ N ₃ O ₅ S ₂ M.P. /°C: 244-245
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Figure 4: FT-IR spectrum of compound M_{3a}

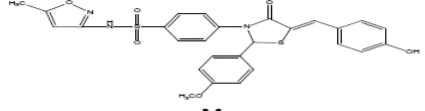
 M_{3b}	Characteristics bands (cm ⁻¹) 1692.94 C=O stretching of thiazolidinone 1218.20 C-S stretching	Mol. Formula: C ₂₇ H ₂₃ N ₃ O ₆ S ₂ M.P. /°C: 180-181
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Figure 5: FT-IR spectrum of compound M_{3b}

3.2.2 ¹H-NMR Spectroscopy

The ¹H-NMR spectra of synthesized compounds provided detailed structural information, confirming the proposed structures and substitution patterns.

Table 3: ¹H-NMR Data for Compound M₁

Signal	Signal Position (δ, ppm)	Relative No. of proton	Multiplicity	Inference
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a	2.131	3H	Singlet	CH ₃ of isoxazole ring
b	3.88	3H	Singlet	Para - OCH ₃
c	6.17	1H	Singlet	CH- of isoxazole ring
d	7.17	2H	Doublet	Aromatic - H ortho to OCH ₃

e	7.6 - 8.2	6H	Multiplate	Aromatic – H
f	8.9	1H	Singlet	CH=N of Schiff base
g	11.41	1H	Singlet	NH of sulfonamide

Interpretation: The $^1\text{H-NMR}$ spectrum of M_1 showed a characteristic singlet at δ 8.9 ppm corresponding to the imine proton ($-\text{CH}=\text{N}-$), confirming Schiff base formation. The sulfonamide NH proton appeared as a broad singlet at δ 11.41 ppm. The aromatic protons appeared as multiplets in the region δ 7.17-8.2 ppm, consistent with the presence of two substituted aromatic rings. The methoxy protons appeared as a singlet at δ 3.88 ppm, and the isoxazole methyl protons appeared at δ 2.131 ppm.

Table 4: $^1\text{H-NMR}$ Data for Compound M_2

Signal	Signal Position (δ , ppm)	Relative No. of proton	Multiplicity	Inference
a	2.28	3H	Singlet	CH_3 of isoxazole ring
b	3.98	3H	Singlet	Para $-\text{OCH}_3$
c	5.31	2H	Singlet	CH_2 of thiazolidinone
d	6.02	1H	Singlet	CH of thiazolidinone
e	6.51	1H	Singlet	CH- of isoxazole ring
f	6.86	2H	Doublet	Aromatic-H ortho to OCH_3
g	7.1 – 7.47	6H	Multiplate	Aromatic C-H
h	10.97	1H	Singlet	NH of sulfonamide

Interpretation: The $^1\text{H-NMR}$ spectrum of M_2 showed the disappearance of the imine proton signal at δ 8.9 ppm, confirming cyclization. The appearance of new signals at δ 5.31 ppm (CH_2 of thiazolidinone) and δ 6.02 ppm (CH of thiazolidinone) confirmed the formation of the 4-thiazolidinone ring. The

sulfonamide NH proton appeared as a broad singlet at δ 10.97 ppm.

Table 5: $^1\text{H-NMR}$ Data for Compound M_{3a}

Signal	Signal Position (δ , ppm)	Relative No. of proton	Multiplicity	Inference
a	2.14	3H	Singlet	CH_3 of isoxazole ring
b	3.30	3H	Singlet	Para $-\text{OCH}_3$
c	4.11	1H	Singlet	CH of thiazolidinone
d	5.33	1H	Singlet	CH- of isoxazole ring
e	6.14	2H	Doublet	Aromatic-H ortho to OCH_3
f	6.55 – 6.7	6H	Multiplate	Aromatic - H, CH of chalcone
g	7.56 – 7.7	6H	Multiplate	Aromatic C-H
h	10.9	1H	Singlet	NH of sulfonamide

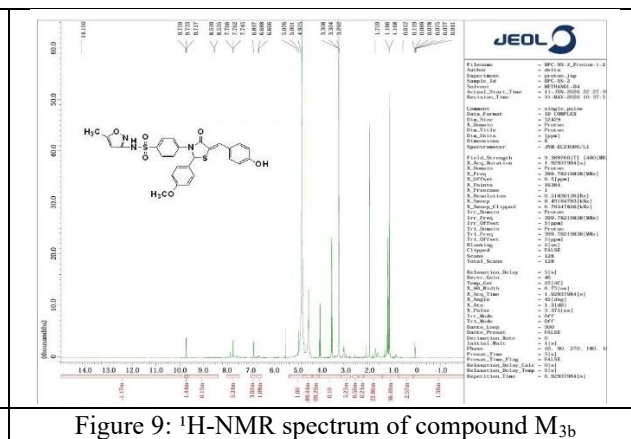
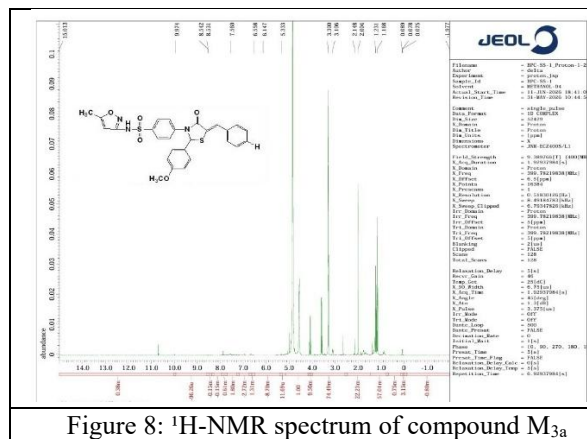
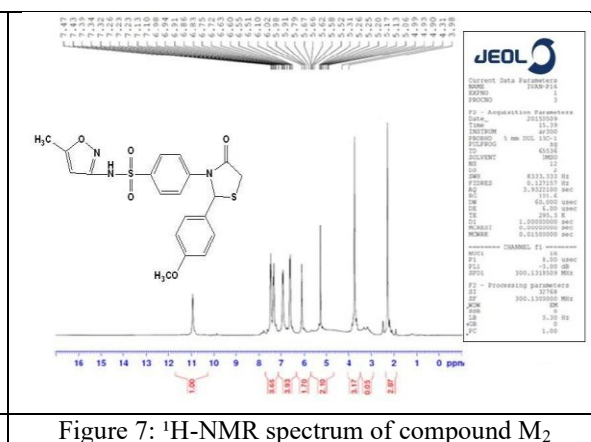
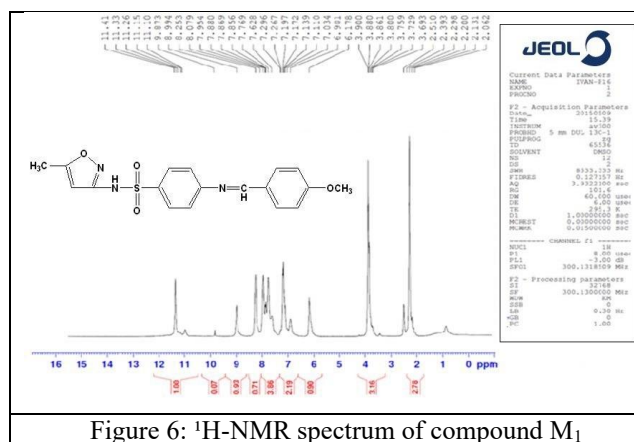
Table 6: $^1\text{H-NMR}$ Data for Compound M_{3b}

Signal	Signal Position (δ , ppm)	Relative No. of proton	Multiplicity	Inference
a	2.20	3H	Singlet	CH_3 of isoxazole ring
b	3.30	3H	Singlet	Para $-\text{OCH}_3$
c	5.70	1H	Singlet	CH of thiazolidinone
d	5.90	1H	Singlet	CH- of isoxazole ring
e	6.66	4H	Multiplate	Ortho -OH, Ortho - OCH_3
f	6.68 – 7.76	8H	Multiplate	Aromatic -H

g	7.74	1H	Doublet	CH of chalcone
h	9.75	2H	Singlet	NH of sulfonamide overlapped with phenolic -OH

Interpretation: The ^1H -NMR spectra of M_{3a} and M_{3b} showed the appearance of exocyclic vinylic proton signals, confirming successful Knoevenagel

condensation. For M_{3a} , the vinylic proton appeared at δ 7.56-7.7 ppm (merged with aromatic protons), while for M_{3b} , it appeared as a distinct doublet at δ 7.74 ppm. The phenolic OH proton in M_{3b} appeared at δ 9.75 ppm, overlapping with the sulfonamide NH proton. The chiral CH proton of the thiazolidinone ring appeared at δ 4.11 ppm (M_{3a}) and δ 5.70 ppm (M_{3b}). The isoxazole ring protons and methoxy protons showed characteristic signals consistent with the proposed structures.



3.2.3 ^{13}C -NMR Spectroscopy

The ^{13}C -NMR spectra provided definitive confirmation of the carbon skeleton of the synthesized compounds. Tables 7 and 8 summarize the ^{13}C -NMR data for compounds M_{3a} and M_{3b} .

Table 7: ^{13}C -NMR Data for Compound M_{3a}
(Methanol- D_4)

Carbon Assignment	Chemical Shift (δ , ppm)	Inference
C_1	12.1	$-\text{CH}_3$ (Methyl carbon on the Isoxazole ring)
C_2	55.6	$-\text{OCH}_3$ (Methoxy carbon on the phenyl ring)
C_3	64.2	Chiral $-\text{CH}-$ (C-2 carbon of the 4-oxothiazolidine ring)
C_4	95.8	$-\text{CH}=\text{C}$ (C-4 carbon of the Isoxazole ring)
C_5	114.6	$2 \times \text{Ar}-\text{CH}$ (Ortho to $-\text{OCH}_3$ on the methoxyphenyl ring)

C_1	12.1	$-\text{CH}_3$ (Methyl carbon on the Isoxazole ring)
C_2	55.6	$-\text{OCH}_3$ (Methoxy carbon on the phenyl ring)
C_3	64.2	Chiral $-\text{CH}-$ (C-2 carbon of the 4-oxothiazolidine ring)
C_4	95.8	$-\text{CH}=\text{C}$ (C-4 carbon of the Isoxazole ring)
C_5	114.6	$2 \times \text{Ar}-\text{CH}$ (Ortho to $-\text{OCH}_3$ on the methoxyphenyl ring)

C ₆	127.8	2×Ar-CH (Meta to –OCH ₃ on the methoxyphenyl ring)
C ₇	131.1	Ar-C quaternary (Ipso position attached to thiazolidinone C-2)
C ₁₀	160.4	Ar-C quaternary (Ipso attached to –OCH ₃)
C _{11,15}	128.4	2×Ar-CH (Ortho carbons of the central sulfamethoxazole ring)
C _{12,14}	129.2	2×Ar-CH (Meta carbons of the central sulfamethoxazole ring)
C ₁₃	138.5	Ar-C quaternary (Ipso attached to –SO ₂ –)
C ₁₆	143.1	Ar-C quaternary (Ipso attached to thiazolidinone N-3)
C ₁₇	124.5	Ar-C quaternary (C-5 position of the 4-oxothiazolidine ring)
C ₁₈	133.4	Exocyclic =CH– (Vinyllic bridge carbon to the benzylidene group)
C ₁₉	134.2	Ar-C quaternary (Ipso position of the benzylidene ring)
C _{20,24}	129.0	2×Ar-CH (Ortho positions of the benzylidene ring)
C _{21,23}	129.6	2×Ar-CH (Meta positions of the benzylidene ring)
C ₂₂	128.9	Ar-CH (Para position of the benzylidene ring)
C ₂₅	157.8	Hetero-Quaternary -C=N- (C-3 of the Isoxazole ring)
C ₂₆	170.8	Hetero-Quaternary -C-O- (C-5 of the Isoxazole ring)
C ₂₇	166.5	-C=O Amide/Carbonyl (C-4 carbonyl of the 4-oxothiazolidine ring)

Table 8: ¹³C-NMR Data for Compound M3b (Methanol-D₄)

Carbon Assignment	Chemical Shift (δ, ppm)	Inference
C ₁	12.1	–CH ₃ (Methyl carbon on the Isoxazole ring)
C ₂	55.6	–OCH ₃ (Methoxy carbon on the phenyl ring)
C ₃	64.2	Chiral –CH– (C-2 carbon of the 4-oxothiazolidine ring)

C ₄	95.8	–CH= (C-4 carbon of the Isoxazole ring)
C _{5,9}	114.6	2×Ar-CH (Ortho to –OCH ₃ on the methoxyphenyl ring)
C _{6,8}	127.8	2×Ar-CH (Meta to –OCH ₃ on the methoxyphenyl ring)
C ₇	131.1	Ar-C quaternary (Ipso position attached to thiazolidinone C-2)
C ₁₀	160.4	Ar-C quaternary (Ipso attached to –OCH ₃)
C _{11,15}	128.4	2×Ar-CH (Ortho carbons of the central sulfamethoxazole ring)
C _{12,14}	129.2	2×Ar-CH (Meta carbons of the central sulfamethoxazole ring)
C ₁₃	138.5	Ar-C quaternary (Ipso attached to –SO ₂ –)
C ₁₆	143.1	Ar-C quaternary (Ipso attached to thiazolidinone N-3)
C ₁₇	122.3	C quaternary (C-5 position of the 4-oxothiazolidine ring)
C ₁₈	134.6	Exocyclic =CH– (Vinyllic bridge carbon to the hydroxybenzylidene group)
C ₁₉	125.8	Ar-C quaternary (Ipso position of the hydroxybenzylidene ring)
C _{20,24}	132.4	2×Ar-CH (Meta to –OH / Ortho to =CH– on the benzylidene ring)
C _{21,23}	116.2	2×Ar-CH (Ortho to –OH / Meta to =CH– on the benzylidene ring)
C ₂₂	159.4	Ar-C quaternary (Ipso position attached to the –OH group)
C ₂₅	157.8	Hetero-Quaternary -C=N- (C-3 of the Isoxazole ring)
C ₂₆	170.8	Hetero-Quaternary -C-O- (C-5 of the Isoxazole ring)
C ₂₇	166.5	-C=O Amide/Carbonyl (C-4 carbonyl of the 4-oxothiazolidine ring)

Interpretation: The ¹³C-NMR spectra provided comprehensive confirmation of the carbon framework. The carbonyl carbon of the thiazolidinone ring appeared at δ 166.5 ppm for both compounds, confirming the presence of the 4-oxothiazolidine moiety. The exocyclic vinyllic carbon appeared at δ

133.4 ppm (M_{3a}) and δ 134.6 ppm (M_{3b}), confirming successful Knoevenagel condensation. The phenolic carbon (C22) in M_{3b} appeared at δ 159.4 ppm, consistent with the presence of the hydroxyl group.

The chiral carbon (C3) of the thiazolidinone ring appeared at δ 64.2 ppm for both compounds, confirming the retention of the stereo enter.

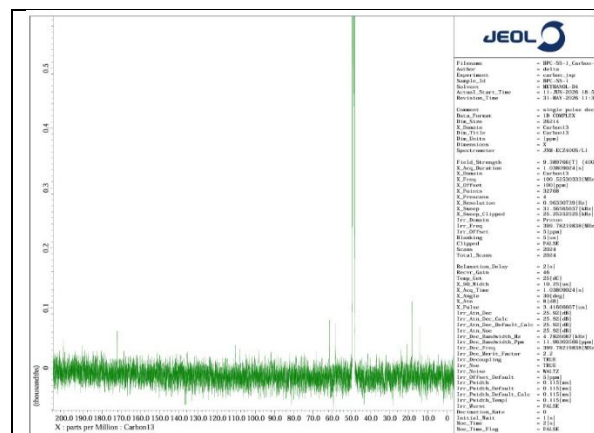


Figure 10: ^{13}C -NMR spectrum of compound M_{3a}

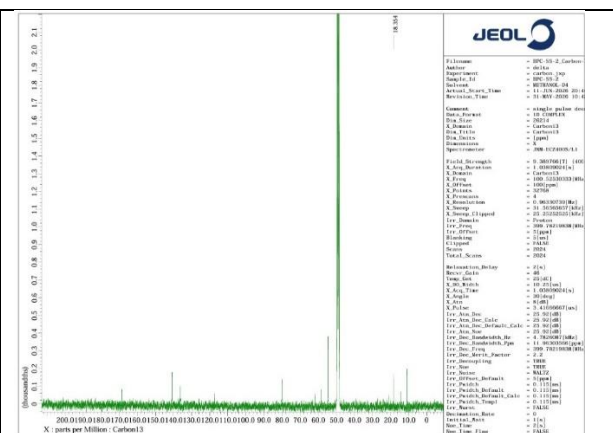


Figure 11: ^{13}C -NMR spectrum of compound M_{3b}

3.2.4 Mass Spectrometry

The mass spectra of final compounds confirmed their molecular weights and molecular formulas.

Compound M_{3a} : EIMS m/z (rel. Int.): 531 [M^+]. The molecular ion peak at m/z 531 corresponds to the molecular formula $\text{C}_{27}\text{H}_{23}\text{N}_3\text{O}_5\text{S}_2$ (calculated molecular weight: 531.12 g/mol).

Compound M_{3b} : EIMS m/z (rel. Int.): 548 [M^+]. The molecular ion peak at m/z 548 corresponds to the molecular formula $\text{C}_{27}\text{H}_{23}\text{N}_3\text{O}_6\text{S}_2$ (calculated molecular weight: 548.61 g/mol).

The mass spectral data were consistent with the proposed structures, confirming the successful synthesis of the target compounds.

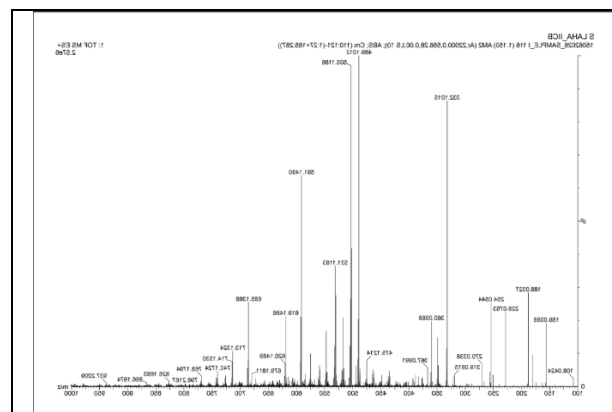


Figure 12: Mass spectrum of compound M_{3a}

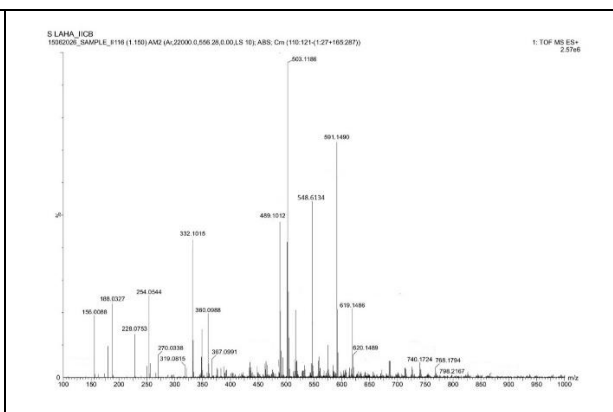


Figure 13: Mass spectrum of compound M_{3b}

3.2.5 Elemental Microanalysis

Elemental microanalysis (CHNS) was performed to confirm the molecular composition of the final compounds. The results were in good agreement with calculated values (Table 9).

Table 9: Elemental Microanalysis of Final Compounds

Compound	Molecular weight	Empirical formula	Elemental microanalysis %		
			Element	Calculated	Observed
M_{3a}	531.12	$\text{C}_{27}\text{H}_{23}\text{N}_3\text{O}_5\text{S}_2$	C	60.77	60.45
			H	4.34	4.17
			N	7.87	7.78
			S	12.02	12.05

M3b	548.61	$C_{27}H_{23}N_3O_6S_2$	C	59.00	59.28
			H	4.22	4.05
			N	7.65	7.77
			S	11.67	11.56

3.3 Antibacterial Activity Evaluation

The antibacterial activity of synthesized compounds (M3a and M3b) was evaluated against three bacterial strains using the agar well diffusion method. Sulfamethoxazole was used as the standard drug, and DMSO served as a negative control. The results are summarized in Table 10.

Table 10: Antibacterial Activity of Sulfamethoxazole and Compounds M_{3a-b}

Compound No.	Concentration (µg/ml)	Escherichia coli (Gm -ve) Inhibition zone (mm)	Pseudomonas aeruginosa (Gm -ve) Inhibition zone (mm)	Staphylococcus aureus (Gm +ve) Inhibition zone (mm)
SMX	500	20.16	-	20.18
	250	15.72	-	16.11
	125	14.66	-	15.14
	62.5	12.35	-	-
DMSO	Pure	-	-	-
M _{3a}	500	20.65	18.65	20.05
	250	20.55	16.33	18.54
	125	18.69	16.56	15.95
	62.5	10.11	10.25	13.85
M _{3b}	500	18.52	17.65	20.52
	250	17.56	16.25	19.89
	125	17.12	15.95	16.54
	62.5	15.85	12.85	15.88

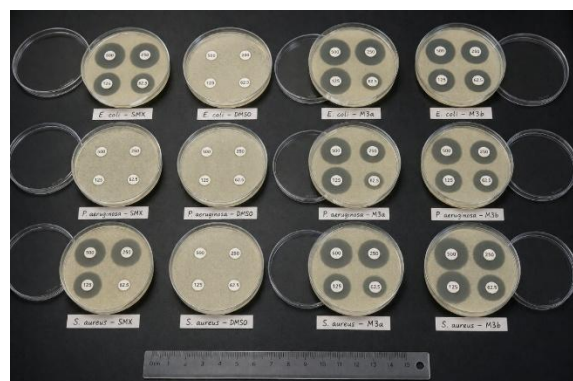


Figure 14: Antimicrobial activity of final synthesised compound

3.3.1 Activity Against Escherichia coli (Gram-negative)

The synthesized compounds proved to be active against Escherichia coli. The M3a compound was found to have activity similar to sulfamethoxazole with an activity of 20.65 mm compared to 20.16 mm, with activity still evident at lower concentrations (activity of 20.55 mm at 250 µg/mL; 18.69 mm at 125 µg/mL). On the other hand, the M3b compound was noted to be less active compared to M3a at high concentrations (activity of 18.52 mm at 500 µg/mL), while it has improved activity at lower concentrations (17.12 mm at 125 µg/mL; 15.85 mm at 62.5 µg/mL compared to 10.11 mm for M3a at 62.5 µg/mL).

3.3.2 Activity Against Pseudomonas aeruginosa (Gram-negative)

Remarkably, the bioactivity of both synthesised derivatives was shown against Pseudomonas aeruginosa, while the parent compound, sulfamethoxazole, showed no inhibitory effect. This is particularly noteworthy in view of the fact that P. aeruginosa is naturally resistant to many drugs, including sulfonamides. M3a inhibited the growth of the bacteria at 18.65 mm at a concentration of 500 µg/mL and 10.25 mm at 62.5 µg/mL. In turn, the bioactivity of M3b was somewhat lower at a high concentration of 500 µg/mL (17.65 mm) but comparable at a low concentration (12.85 mm at 62.5 µg/mL).

3.3.3 Activity Against Staphylococcus aureus (Gram-positive)

Both of these compounds showed good activity against Staphylococcus aureus, and M3b was better in efficacy than M3a and sulfamethoxazole. When 500 µg/mL of compound was tested, M3b showed inhibition zone of 20.52 mm while M3a and sulfamethoxazole inhibited the growth by 20.05 mm and 20.18 mm respectively. When 250 µg/mL of compound was used, the inhibition zones of M3b, M3a and sulfamethoxazole were 19.89 mm, 18.54 mm and 16.11 mm respectively. At low concentrations, the activity of M3a and M3b was greater than sulfamethoxazole as M3b showed inhibition zone of 15.88 mm at 62.5 µg/mL, whereas sulfamethoxazole showed no activity at this concentration.

3.3.4 Structure-Activity Relationship Analysis

The antibacterial activity data revealed distinct structure-activity relationships:

1. **Introduction of 4-thiazolidinone Group:** The introduction of 4-thiazolidinone into the structure of sulfamethoxazole expands the antimicrobial spectrum, making the molecule active against *Pseudomonas aeruginosa*, an organism against which sulfamethoxazole is not active. This effect might be due to better penetration into the microbial cells and other possible interactions with bacterial targets.
2. **Influence of Benzylidene Substitution:** The substitution in the benzylidene ring plays a great role in the activity of the molecules. Molecule M3b, substituted with a 4-hydroxybenzylidene fragment, shows higher activity against *S. aureus* compared to its analogue M3a, especially at low concentrations. It is believed that this hydroxy function allows for better hydrogen bonding with bacterial targets and increased solubility in water.
3. **Dependence on Concentration:** All compounds show their concentration dependent activities; it is seen from the increase in the size of the zone of inhibition with the increase in concentration. This fact corresponds to the activity of conventional antibacterial substances.
4. **Differences in Activities against Gram-positive and Gram-negative Microbes:** Both compounds show higher activity against *S. aureus* (Gram-positive) in comparison with *E. coli* and *P. aeruginosa* (Gram-negative). This effect might be explained by the presence of outer membranes of Gram-negative bacteria impeding penetration of the drugs.

3.4 Discussion of Synthetic and Biological Findings

The success of synthesizing the target molecules M3a and M3b using a three-step synthetic sequence is a testimony to the adaptability of the molecular hybridization approach. Formation of Schiff base was efficient under acid catalyzed reflux conditions giving rise to M1 in good yield of 63%. Thiazolidinone moiety construction using cyclocondensation with thioglycolic acid proceeded excellently in the yield of 72%, thus establishing the utility of the MALI reaction

in thiazolidinone synthesis. The last step of Knoevenagel condensation, which is responsible for introduction of benzylidene fragments gave good yields in the region of 47-53%, suggesting possible optimization by varying the reaction parameters.

Spectral analysis served as proof of the correct structures of the compounds. FT-IR spectra showed specific peaks for all the functional groups present, such as imine (C=N), thiazolidinone carbonyl (C=O), sulfonamide (NH, SO₂), and phenolic OH. Proton NMR (¹H-NMR) spectra confirmed the formation of Schiff base (imine proton at δ 8.9 ppm), cyclization (loss of imine proton along with the formation of thiazolidinone CH₂ and CH protons) and Knoevenagel condensation (vinylic protons). Carbon-13 NMR (¹³C-NMR) spectra provided the evidence for carbon skeleton, as all the carbon peaks matched those of the proposed structures. Molecular masses were confirmed by mass spectrometry while elemental microanalysis established the molecular formulas.

Evaluation of antibacterial activities of the synthesized compounds revealed their remarkable activities against all three bacterial strains. Compound M3b showed a much higher activity against *Staphylococcus aureus* than M3a and sulfamethoxazole. Importantly, both synthesized compounds were active against *Pseudomonas aeruginosa*, which was unaffected by sulfamethoxazole. The reason behind broadening of antibacterial spectrum of activity may be the following:

1. **Enhanced Membrane Permeability:** The thiazolidinone moiety may improve the lipophilic character of the compounds, facilitating better penetration through bacterial cell membranes.
2. **Additional Target Interactions:** The thiazolidinone ring may interact with additional bacterial targets beyond DHPS, potentially through hydrogen bonding, hydrophobic interactions, or metal coordination.
3. **Resistance Overcoming:** The modified compounds may be less susceptible to the resistance mechanisms that affect sulfamethoxazole, such as altered DHPS or efflux pumps.

The presence of the hydroxyl group in M3b appears to enhance antibacterial activity, particularly against *S. aureus*. This could be attributed to:

- Improved hydrogen-bonding interactions with bacterial targets.

- Enhanced aqueous solubility and bioavailability.
- Electron-donating effects that may influence the electronic properties of the molecule.

IV. CONCLUSION

This study has successfully accomplished the synthesis of a new series of sulfamethoxazole–thiazolidinone hybrids by the three-step synthetic process. This process began from the synthesis of Schiff's bases (M1), then moved forward into the thiazolidinone ring structure (M2) until the Knoevenagel condensation to generate the target molecules (M3a and M3b). These structural confirmations were carried out through the complete characterization methods, which consist of melting point determination, thin layer chromatography, FT-IR, ¹H-NMR, ¹³C-NMR, mass spectrometry, and elemental microanalysis.

Antimicrobial screening in vitro revealed that the synthesized compounds possessed significant antibacterial activities against three pathogenic species with enhanced spectrum of antibacterial activity compared with sulfamethoxazole. The main results are as follows:

1. Expanded Antibacterial Spectrum: Both compounds showed activity against *P. aeruginosa*, against which sulfamethoxazole showed no inhibition, indicating that the thiazolidinone modification has successfully expanded the antibacterial spectrum of the sulfonamide scaffold.
2. Enhanced Activity Against *S. aureus*: Compound M_{3b} (bearing a 4-hydroxybenzylidene moiety) showed superior activity against *S. aureus* compared to both M_{3a} and sulfamethoxazole, particularly at lower concentrations.
3. Structure-Activity Relationships: The presence of the hydroxyl group in M_{3b} significantly enhanced antibacterial activity, likely through improved hydrogen-bonding interactions and favourable physicochemical properties.
4. Concentration-Dependent Activity: All compounds showed concentration-dependent

antibacterial activity, consistent with typical antibacterial agents.

The molecular hybridization approach employed in this study has proven successful in creating compounds with enhanced antibacterial properties and expanded spectrum of activity. The synthesized compounds represent promising lead candidates for further optimization in the development of novel antibacterial agents to combat multidrug-resistant bacterial infections.

V. FUTURE SCOPE

Based on the promising results obtained in this study, several avenues for future research are proposed:

1. In Silico Molecular Docking Studies: Docking studies targeting the bacteria (such as DHPS and DNA gyrase) would aid in understanding the mechanism of interaction and binding of the synthesized compounds.
2. Development of Substituted Benzylidene Compounds: The structural library should be extended by synthesizing compounds with different types of substituents (electronic withdrawing, donating, and bulky). This would help in understanding SAR and identification of best possible substitutes for antibacterial activity.
3. Mechanistic Investigations: In-depth mechanistic investigations like enzyme inhibition, membrane permeation, and time-kill study would help in establishing the mode of action of these compounds.
4. In Vivo Studies: Pharmacokinetic and pharmacodynamic investigations on suitable animal models would assess the therapeutic potentials and safety of these compounds.
5. Formulations: Due to the heterocyclic nature of these compounds, the formulation strategies should be developed for enhancing the aqueous solubility and bioavailability of the compounds.
6. Cytotoxicity Studies: In vitro cytotoxicity studies of these compounds on human cell lines would help in evaluating their safety and therapeutic index.
7. Other Biological Activity Investigations: These compounds may have other biological properties such

as antifungal, anticancer, and anti-inflammatory activity that should be investigated.

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