Received: 2 April 2020 | Revised: 11 August 2020 | Accepted: 13 August 2020

DOI: 10.1002/ardp.202000103

FULL PAPER

DPhG ARCH PHARM Archiv der Pharmazie

Novel pyrazole-clubbed thiophene derivatives via Gewald synthesis as antibacterial and anti-inflammatory agents

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Abstract

The aim of this study was to synthesize newer potent Schiff bases by condensing 2-amino-5-(2,4-dichlorophenyl)thiophene-3-carbonitrile and 1,3-disubstituted-1*H*-pyrazole-4-carbaldehydes, and to investigate their biological activity. The compounds were synthesized via Gewald synthesis and characterized by spectral data and elemental analyses. They were screened for their in vitro antibacterial and anti-inflammatory activities. The synthesized compounds were also evaluated for in vitro antitubercular activity against *Mycobacterium tuberculosis* H37Rv using the microplate Alamar Blue assay. Compounds **8b**, **8c**, **8f**, **8g**, **8k**, **8n**, and **8o** showed promising antibacterial activity. The interactions between the substituted pyrazoles and bovine protein showed promising anti-inflammatory activity. The experimental results revealed compound **8a** as a promising antitubercular agent. Hemolytic assays confirmed that the compounds are nontoxic, with percentage hemolysis ranging from 3.6 to 20.1, at a concentration of 1 mg/ml. The results suggest that the pyrazole ring and the substitution pattern on the heterocyclic moiety have an effect on the bioactivity.

KEYWORDS

anti-inflammatory, antimicrobial, antitubercular, Gewald reaction, Schiff bases

1 | INTRODUCTION

Over recent decades, much effort has been dedicated to the development of new antibacterial and anti-inflammatory agents. Specifically, there is a significant amount of publications concerning the synthesis and evaluation of Schiff base, thiophene, and pyrazole with a potential bacteria inhibitory activity. Nevertheless, although a great number of synthesized compounds were found to be potent as antibacterial agents, only a portion of them revealed a safer pharmacological profile, compared with marketed antibacterial drugs.

A Schiff base is a privileged scaffold, which is found in a great number of medicines and drug candidates including best-selling drugs. Schiff bases or imines are the product of the condensation of carbonyl compounds (aldehydes or ketones) with primary amines. These are important molecules that are extensively studied due to their broad range of industrial and biomedical applications.[1] These are the foremost widely used organic compounds in medicinal chemistry due to their imine or azomethine (–C═N–) functional group. They are regularly used as pigments, dyes, catalysts, and intermediates in organic synthesis, and as polymer stabilizers.[2] Their importance is highly growing due to their diverse pharmacological properties like antibacterial, antifungal, antimalarial, anti-inflammatory, and antiviral properties.[3–5] These derivatives are also extensively studied due to their antitubercular[6–10] activity. As this functionality has shown a relatively well response as an antitubercular agent, it has become a topic of interest in the field of research. Furthermore, as there are few antitubercular drugs available and there is an ever-increasing fear of drug resistance, it is becoming more and more essential for the researcher to synthesize some more drugs with higher potency and low toxicity. In medicinal chemistry, thiophene derivatives have been familiar with their therapeutic applications. There are a variety of reasons why thiophene derivatives are of interest to the pharmaceutical chemists. One reason is based on the concept of bioisosterism. Thiophene with its three pairs of electrons aromaticity and being sterically similar to

benzene derivatives may well exhibit similar activities. At the same time, the presence of the heteroatom in thiophene may alter its metabolic fate; thus, thiophene derivatives may have less toxic effects and a better therapeutic profile.[11] Pyrazoles have proven to be a remarkable scaffold for the synthesis of biologically active compounds.[12–14] Their wide range of medical applications mainly includes antimicrobial,[15–17] antifungal,[13,17,18] anti-inflammatory,[12] analgesic, and anxiolytic activities.[18,19] Some pyrazole derivatives have also shown antitumor activities toward some cancer cell lines, including leukemia (K562), HeLa cervix adenocarcinoma, and Fem-x melanoma.[20] Consequently, in recent years, pyrazole-based compounds have attracted great interest. The various biological and pharmaceutical properties possessed by this important structural motif have attracted the researchers for the development of new drug molecules. Literature reports of some bioactive 1,3-disubstituted-1*H*-pyrazole derivatives are presented in Figure 1.

Recently, there has been an increase in microbial infections dramatically, because pathogens are becoming resistant to antibiotics, which has led to serious health hazards. The clinical limitations of currently used antimicrobial drugs, such as drug resistance, toxicity, and side effects, lead us for the development of novel, safer, and more effective antimicrobial agents. In this context, considerable attention has been focused on fused heterocycles. Taking into account the broad biological activities described for the Schiff base,

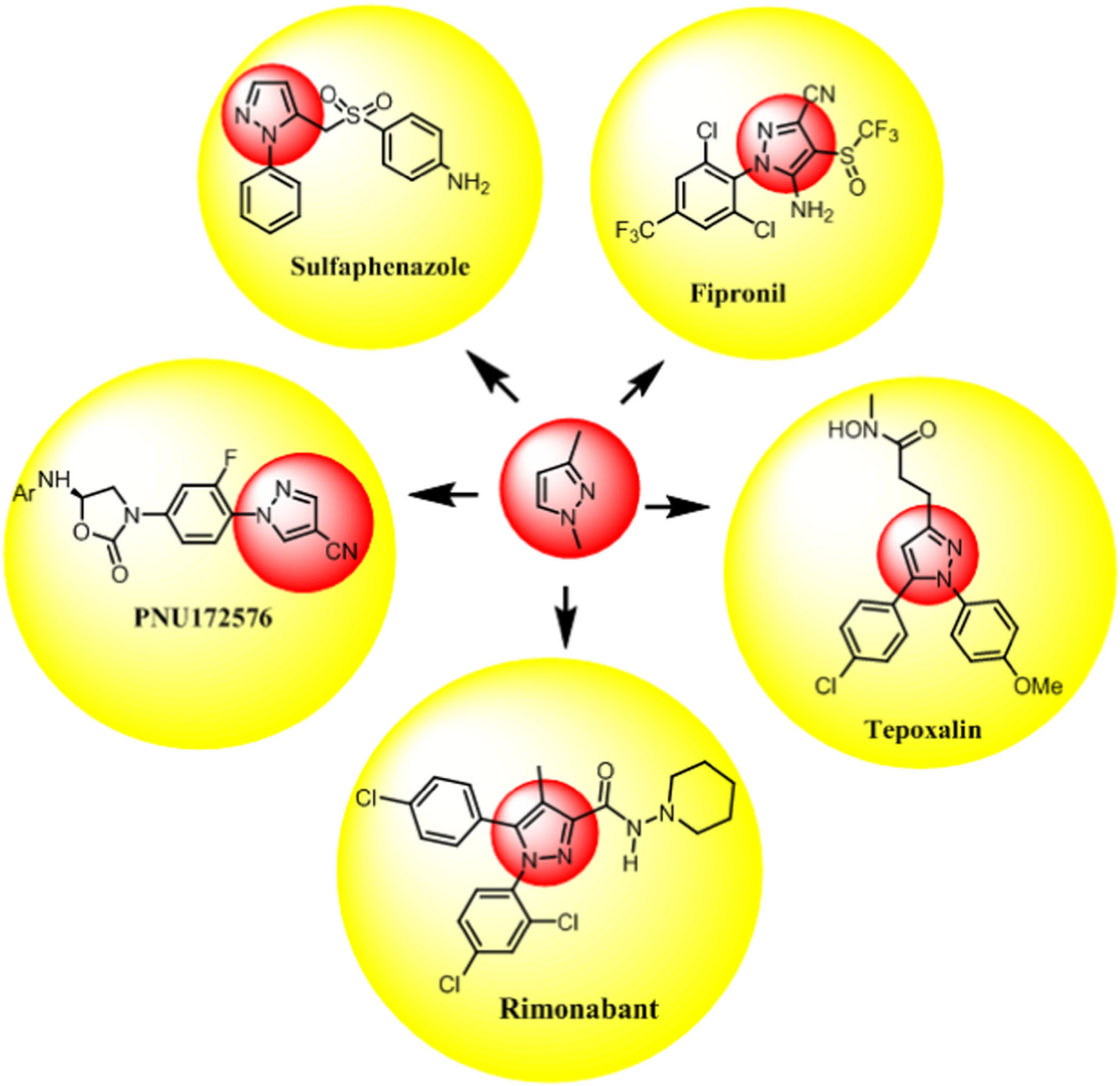


FIGURE 1 Literature reports of active 1,3-disubstituted-1*H*-pyrazole derivatives[20,21]

thiophene, and pyrazole derivatives including their therapeutic potential, we planned to synthesize thiophene and pyrazole-clubbed Schiff bases, and to evaluate their potential bioactivity by measuring the antimicrobial, anti-inflammatory, and antitubercular activity.

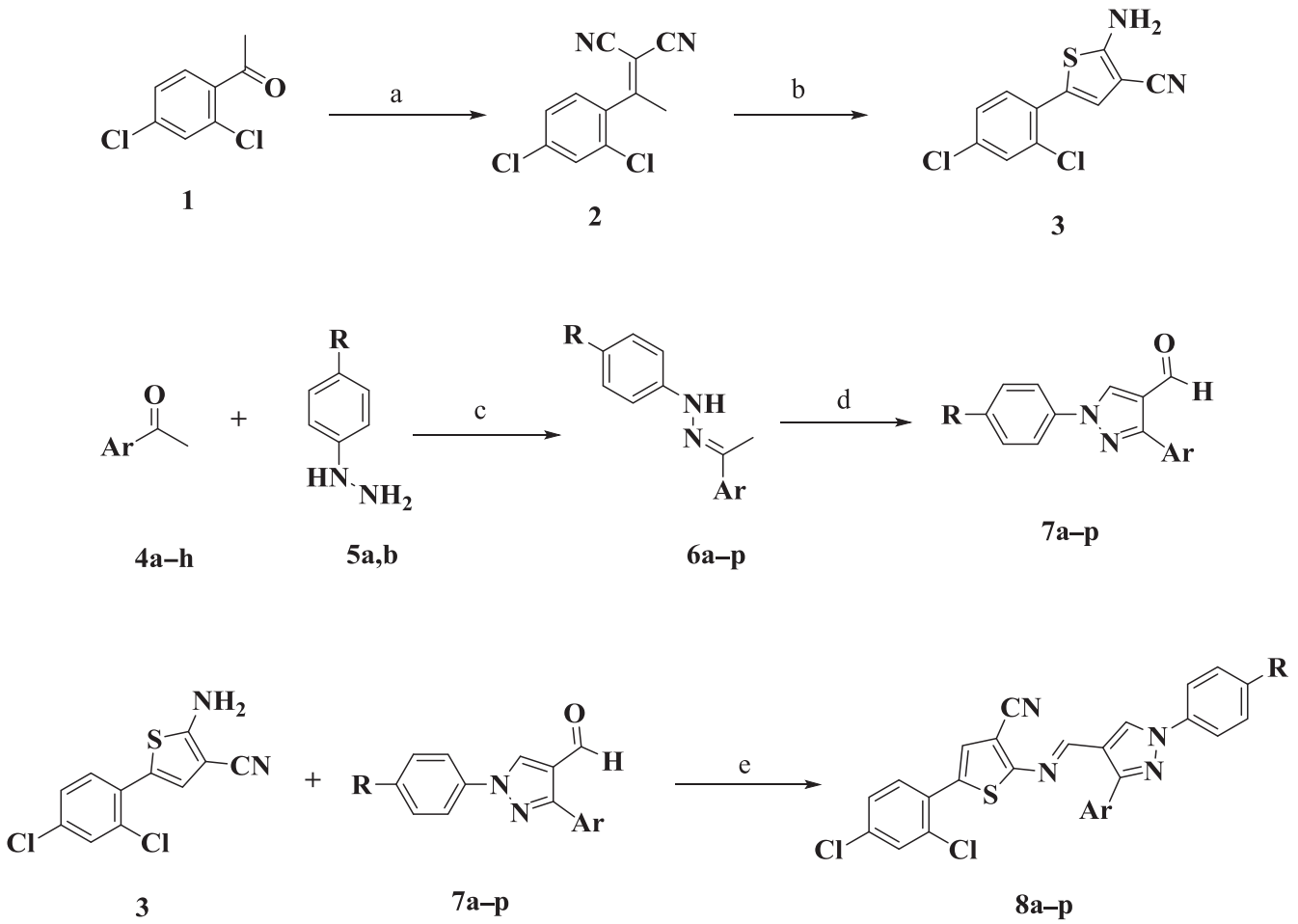
2 | RESULTS AND DISCUSSION

2.1 | Chemistry

Our strategy to synthesize new heterocyclic Schiff bases involves the use of 1,3-disubstituted-1*H*-pyrazole-4-carbaldehydes **7a**–**p** that exhibit good pharmacological properties. The reaction sequence for the synthesis of title compounds, **8a**–**p**, is depicted in Scheme 1. Commercially available 2,4-dichloroacetophenone **1** upon reaction with malononitrile, ammonium acetate in toluene, and a catalytic amount of acetic acid under reflux condition afforded 2-[1-(2,4-dichlorophenyl)ethylidene]malononitrile **2**. Then it was converted into 2-amino-5-(2,4-dichlorophenyl)thiophene-3-carbonitrile **3** in the presence of sulfur powder and sodium bicarbonate in tetrahydrofuran (THF) via Gewald synthesis.

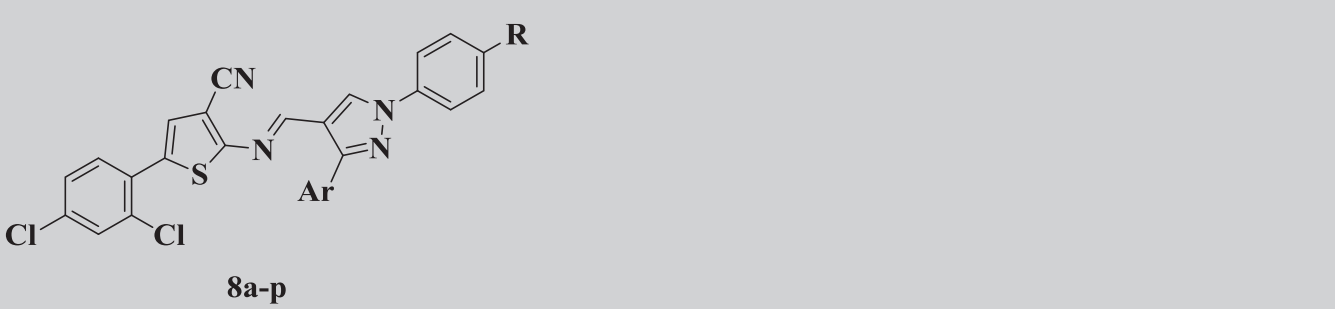
1,3-Disubstituted-1*H*-pyrazole-4-carbaldehydes **7a**–**p** were synthesized by Vilsmeier–Haack reaction in good yields. Finally, preparation of 2-{[(substituted phenyl-1*H*-pyrazol-4-yl)methylene]amino}-5-(2,4-dichlorophenyl)thiophene-3-carbonitriles **8a**–**p** was done by treating the intermediate 2-amino-5-(2,4-dichlorophenyl)thiophene-3-carbonitrile **3** with 1,3-disubstituted-1*H*-pyrazole-4-carbaldehydes **7a**–**p** in the presence of catalytic amount glacial acetic acid in ethanol with a reasonably good yield. The formation of substituted 2-{[(phenyl-1*H*-pyrazol-4-yl)methylene]amino}-5-(2,4-dichlorophenyl)thiophene-3-carbonitriles **8a**–**p** (Table 1) is evidenced by their elemental analysis and spectral data.

The formation of target compounds **8a**–**p** from corresponding 1,3-disubstituted-1*H*-pyrazole-4-carbaldehydes was confirmed by spectral data (infrared [IR], ^{1}H nuclear magnetic resonance [NMR], ^{13}C NMR, mass spectroscopy [MS]).



SCHEME 1 An outline for the synthesis. Reagents and conditions: (a) malononitrile, ammonium acetate, acetic acid, toluene, reflux: (b) tetrahydrofuran, sulfur powder, sodium bicarbonate, water, room temperature (rt), 24 hr; (c) AcOH, MeOH, reflux, 4 hr; (d) DMF/$POCl_3$, 0–5°C, rt, 2 hr; (e) **7a**–**p**, ethanol, catalytic amount of acetic acid

TABLE 1 Physical data of the newly synthesized substituted 2-{[(phenyl-1*H*-pyrazol-4-yl)methylene]amino}-5-(2,4-dichlorophenyl)thiophene-3-carbonitriles (**8a–p**)



8a-p

Compound no.	Ar	R	Chemical formula	Molecular weight (g/mol)	Yield	Melting point (°C)
8a	C_6H_5	4-H	$C_{27}H_{16}Cl_2N_4S$	499.41	73	248–250
8b	4-Cl-C_6H_4	4-H	$C_{27}H_{15}Cl_3N_4S$	533.86	76	216–218
8c	4-F-C_6H_4	4-H	$C_{27}H_{15}Cl_2FN_4S$	517.40	71	202–204
8d	4-Br-C_6H_4	4-H	$C_{27}H_{15}Cl_2BrN_4S$	578.31	75	196–198
8e	3-Br-C_6H_4	4-H	$C_{27}H_{15}Cl_2BrN_4S$	578.31	73	214–216
8f	4-OCH_3-C_6H_4	4-H	$C_{28}H_{18}Cl_2N_4OS$	529.44	77	204–206
8g	4-CH_3-C_6H_4	4-H	$C_{28}H_{18}Cl_2N_4S$	513.44	77	212–214
8h	3-Thienyl	4-H	$C_{25}H_{14}Cl_2N_4S_2$	505.44	73	246–248
8i	C_6H_5	4-Cl	$C_{27}H_{15}Cl_3N_4S$	533.86	74	211–213
8j	4-Cl-C_6H_4	4-Cl	$C_{27}H_{14}Cl_4N_4S$	568.30	71	242–244
8k	4-F-C_6H_4	4-Cl	$C_{27}H_{14}Cl_3FN_4S$	551.85	70	232–234
8l	4-Br-C_6H_4	4-Cl	$C_{27}H_{14}Cl_3BrN_4S$	612.75	72	220–222
8m	3-Br-C_6H_4	4-Cl	$C_{27}H_{14}Cl_3BrN_4S$	612.75	73	238–240
8n	4-OCH_3-C_6H_4	4-Cl	$C_{28}H_{17}Cl_3N_4OS$	563.88	75	246–248
8o	4-CH_3-C_6H_4	4-Cl	$C_{28}H_{17}Cl_3N_4S$	547.88	72	210–212
8p	3-Thienyl	4-Cl	$C_{25}H_{13}Cl_3N_4S_2$	539.88	74	235–237

2.2 | Biological activity

2.2.1 | Antibacterial activity

It is well known that pyrazole derivatives and also Schiff bases have unveiled a promising antimicrobial activity. Therefore, antibacterial potency of newly synthesized derivatives (**8a–p**) was evaluated by determining their antibacterial activity using the broth dilution method.[22] Two Gram-positive bacteria, *Staphylococcus aureus* and *Enterococcus faecalis*, and two Gram-negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa*, were selected to investigate the antimicrobial activity. Ciprofloxacin was used as a reference drug. The minimum inhibitory concentration (MIC) was defined as the lowest concentration without visible growth. The results of the preliminary antibacterial activity of compounds **8a–p** are presented in Table 2. The results obtained revealed that almost all the newly synthesized compounds showed excellent antibacterial activity against Gram-negative bacterial strains (*E. coli* and *P. aeruginosa*) as compared with the standard drug (ciprofloxacin). However, in the case of Gram-positive bacterial strains (*S. aureus* and *E. faecalis*), the synthesized compounds showed a moderate activity. From the screening results, it could be seen that the majority of the tested compounds displayed a moderate-to-good antibacterial activity (MIC values of 1.56–12.5 μg/ml) as compared with the standard drug (ciprofloxacin). The antibacterial screening of compounds **8a–p** revealed that halogen-, methoxy-, and methyl-substituted derivatives are highly active.

The antibacterial bioscreening data given in Table 2 displayed that most of the Schiff bases were potent. Among all the synthesized compounds, **8b** and **8j**, with a 4-chlorophenyl ring; **8c** and **8k**, bonding to 4-fluorophenyl ring; **8f** and **8n**, possessing 4-methoxyphenyl ring; and **8g** and **8o**, bearing 4-methylphenyl ring, derivatives showed a good activity as compared with the standard drug (ciprofloxacin).

Compound **8b** (Ar = 4-Cl-C_6H_4 and R = 4-H) showed an MIC value of 1.56 μg/ml against *E. faecalis* (Gram-positive) and *P. aeruginosa* (Gram-negative). But derivative **8b** (Ar = 4-Cl-C_6H_4 and R = 4-H) did not show a good activity against *S. aureus* and *E. coli*. One more

TABLE 2 Antibacterial activity of synthesized compounds, **8a–p**, by the broth dilution method (incubation time 24 hr at 37°C)

Antibacterial activity data of the target compounds (8a–p) in terms of minimum inhibitory concentration in μg/ml (MIC) (incubation time 24 hr at 37°C)						
			Gram-positive		Gram-negative	
Entry	Ar	R	*Staphylococcus aureus*	*Enterococcus faecalis*	*Escherichia coli*	*Pseudomonas aeruginosa*
8a	C_6H_5	4-H	6.25	3.125	3.125	**1.56**
8b	4-Cl-C_6H_4	4-H	12.5	**1.56**	12.5	**1.56**
8c	4-F-C_6H_4	4-H	**1.56**	12.5	12.5	**1.56**
8d	4-Br-C_6H_4	4-H	3.125	25	**1.56**	25
8e	3-Br-C_6H_4	4-H	12.5	**1.56**	3.125	3.125
8f	4-OCH_3-C_6H_4	4-H	**1.56**	12.5	**1.56**	12.5
8g	4-CH_3-C_6H_4	4-H	50	25	**1.56**	**1.56**
8h	3-Thienyl	4-H	50	25	50	25
8i	C_6H_4	4-Cl	12.5	3.125	6.25	25
8j	4-Cl-C_6H_4	4-Cl	25	12.5	**1.56**	3.125
8k	4-F-C_6H_4	4-Cl	3.125	**1.56**	3.125	**1.56**
8l	4-Br-C_6H_4	4-Cl	12.5	3.125	**1.56**	3.125
8m	3-Br-C_6H_4	4-Cl	3.125	3.125	3.125	**1.56**
8n	4-OCH_3-C_6H_4	4-Cl	12.5	**1.56**	**1.56**	3.125
8o	4-CH_3-C_6H_4	4-Cl	**1.56**	3.125	3.125	**1.56**
8p	3-Thienyl	4-Cl	50	25	25	25
Std	Ciprofloxacin		**6.25**	**6.25**	**3.125**	**6.25**

Note: Significance of bold face at lower value of concentration the derivative are active.
Abbreviation: MIC, minimum inhibitory concentration.

chloro-substituted derivative, **8j** (Ar = 4-Cl-C_6H_4 and R = 4-Cl), was found to be highly active against *E. coli* at a lower concentration (1.56 mg/ml). When chloro is replaced by fluoro compound, **8c** (Ar = 4-F-C_6H_4 and R = 4-H) showed a good MIC value of 1.56 μg/ml against two bacterial strains, namely *S. aureus* and *P. aeruginosa*, but failed to show activity against *E. faecalis* and *E. coli* bacterial strains. However, compound **8k** (Ar = 4-F-C_6H_4 and R = 4-Cl) showed a good activity against *E. faecalis* and *P. aeruginosa*. Halogen derivatives showed good activity results, which are summarized in Table 2.

Schiff bases possessing 4-methoxyphenyl ring were highly active. Among these, compound **8f** (Ar = 4-OMe-C_6H_4 and R = 4-H) showed an activity at a lower concentration (1.56 mg/ml) against *S. aureus* and *E. coli*, and it was least active against *E. faecalis* and *P. aeruginosa*. In a similar way, compound **8f** (Ar = 4-OMe-C_6H_4 and R = 4-Cl) was active against *E. faecalis* and *E. coli*. Methyl-substituted derivatives also showed good activity results. Compounds **8g** (Ar = 4-Me-C_6H_4 and R = 4-H) and **8o** (Ar = 4-Me-C_6H_4 and R = 4-Cl) were found to be active against *S. aureus*, *E. coli*, and *P. aeruginosa* bacterial strains. Remaining derivatives were moderately active against the tested bacterial strains as compared with the standard drug (ciprofloxacin). However, compounds **8a–p** were less active against Gram-positive bacteria (*S. aureus* and *E. faecalis*) and active against Gram-negative bacteria (*E. coli* and *P. aeruginosa*) as compared with the standard drug (ciprofloxacin). The results obtained are presented in Table 2.

2.2.2 | Anti-inflammatory activity

Inflammation is a biological response of the body to the harmful pathogens, and alteration of neighboring protein tissue is one of the causes of inflammatory condition. There are complications in using animal models in in vivo research, such as lack of groundwork, lack of maintenance, lack of animal risk management, and ethical disputes for their use. Inflammation is associated with pain, and it involves an increase in protein denaturation. In vivo denaturation of proteins leads to the synthesis of autoantigens in some arthritic diseases,[23] and therefore the compounds that inhibit denaturation are desirable for anti-inflammatory discovery. The worthwhile condition for anti-inflammatory drug discovery is to stop the protein denaturation by the formation of complexes. Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used for the management of inflammatory conditions. Heat-induced protein denaturation prevention by synthesized compounds was analyzed, using diclofenac sodium as a standard drug. One of the properties noticed in several NSAIDs is to

TABLE 3 Anti-inflammatory activity results of substituted 2-{[(phenyl-1*H*-pyrazol-4-yl)methylene]amino}-5-(2,4-dichlorophenyl)-thiophene-3-carbonitrile derivatives **8a–p**

Compounds	Inhibition of protein denaturation (%)					
Conc. (μg/ml)	20	40	60	80	100	IC_{50} (μg/ml)
Standard	35.4	58.1	71.2	82.6	93.2	31.4
8a	**27.2**	**43.1**	**65.8**	**74.1**	**86**	**40.3**
8b	**34.1**	**50.5**	**69.9**	**79**	**87**	**34.1**
8c	13.9	32.8	47.5	69	82.3	60.9
8d	**33.1**	**50.5**	**67.3**	**82.1**	**92.6**	**34.3**
8e	**26**	**33**	**55**	**68**	**86**	**46.3**
8f	**28.3**	**46.5**	**57.1**	**69.8**	**88.3**	**41.0**
8g	2.9	18.8	25.2	34.6	48.9	104.5
8h	**34.3**	**42.8**	**54.6**	**71.2**	**83.9**	**40.9**
8i	**28.6**	**42.9**	**59.5**	**78.2**	**87.1**	**40.3**
8j	19.7	38.3	42.6	50.1	70.9	65.4
8k	19.6	28.3	34.6	51.3	78.6	66.1
8l	16.2	28.8	32.1	65.1	78.2	64.8
8m	**25.3**	**32.5**	**41.6**	**68.9**	**89.3**	**49.0**
8n	24.3	34.1	42.5	56.9	76.5	58.5
8o	15.3	22.8	31.5	48.6	59.3	90.0
8p	**30.2**	**42.5**	**59.6**	**68.9**	**79.9**	**42.8**

Note: Significance of bold face at lower value of concentration the derivative are active.

alleviate heat-exposed albumin denaturation at a physiological pH range of 6.2–6.5.[24] However, these drugs have several adverse side effects, especially gastric irritation, leading to the formation of gastric ulcers. Therefore, the search for natural sources and phytochemicals with anti-inflammatory activity has greatly increased in recent years.

As a part of the investigation on the mechanism of the anti-inflammatory activity, the ability of the synthesized derivatives, **8a–p**, to inhibit protein denaturation was studied using diclofenac sodium as a standard drug. IC_{50} values showed the concentration-dependent inhibition of protein denaturation in the range of 20–100 mg/ml and are summarized in Table 3. A graphical representation of anti-inflammatory results of synthesized compounds is presented in Figure 2. From the results, it was found that most of the compounds show an excellent anti-inflammatory activity with IC_{50} values close to the standard drug. Electron-withdrawing halogen-substituted compounds **8b** (Ar = 4-Cl-C_6H_4 and R = 4-H), **8d** (Ar = 4-Br-C_6H_4 and R = 4-H), **8e** (Ar = 3-Br-C_6H_4 and R = 4-H), **8i** (Ar = C_6H_5 and R = 4-Cl), and **8m** (Ar = 3-Br-C_6H_4 and R = 4-Cl) showed a noticeable anti-inflammatory activity with IC_{50} values of 34.1, 34.3, 46.3, 40.3, and 49 μg/ml, respectively, when comparing with the standard drug. Electron-withdrawing functional groups in the *para* position of the phenyl group enhance the anti-inflammatory activity. In the same way, electron-donating –H in **8a** (Ar = 4-H-C_6H_4 and R = 4-H), –OCH_3 in **8f** (Ar = 4-OCH_3-C_6H_4 and R = 4-H), and -3-thiophene in **8h** (Ar = 3-thienyl and R = 4-H) and **8p** (Ar = 3-thienyl and R = 4-Cl) groups of phenyl ring also showed a more potent inhibitory action by obtaining IC_{50} values of 40.3, 41, 40.9, and 42.8 μg/ml, respectively, when compared with the IC_{50} value, 31.4, of diclofenac sodium (control). Compounds **8g** and **8o** showed the least activity with IC_{50} values of 104.5 and 90 μg/ml, respectively, and remaining compounds (**8c**, **8j**, **8k**, **8l**, and **8n**) showed a moderate activity against the protein denaturation of bovine albumin. Overall, the compound **8b** showed a remarkable anti-inflammatory activity, and remaining compounds (**8a**, **8d**, **8e**, **8f**, **8h**, **8i**, **8m**, and **8p**) having electron-withdrawing and -donating substitutions at *para* position of phenyl ring showed a good anti-inflammatory activity. By observing these results, we conclude that the activity result enhances on the basis of the substitution on the phenyl ring.

2.2.3 | Antitubercular activity

The antitubercular properties of all prepared compounds were assessed against *Mycobacterium tuberculosis* at different concentrations ranging from 0.2 to 100 μg/ml using streptomycin, ciprofloxacin, and pyrazinamide as standard drugs. The results of in vitro antitubercular activity of the synthesized compounds against *M. tuberculosis* H37Rv are listed in Table 4. Few compounds were found active against *M. tuberculosis*, as shown in Figure 3. *M. tuberculosis* is unique due to thick and waxy surrounding cell walls, owing to which effective

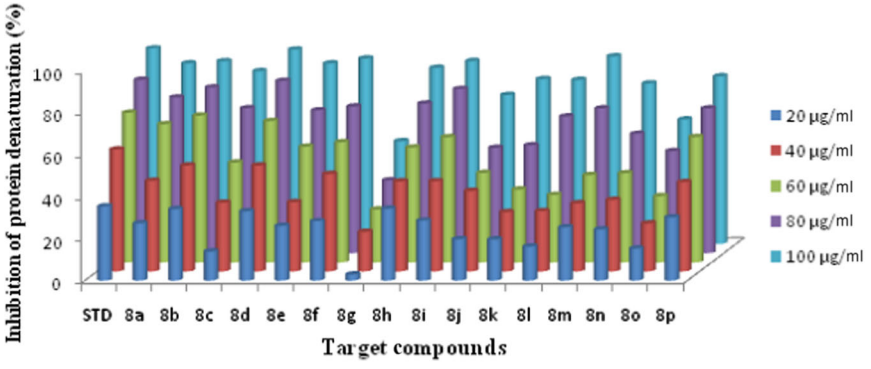


FIGURE 2 A graphical representation of in vitro protein denaturation of Schiff base analogs **8a–p** with the standard drug

TABLE 4 Antitubercular activity of compounds by the microplate Alamar Blue assay method against *Mycobacterium tuberculosis*

Compound no.	Ar	R	100 μg/ml	50 μg/ml	25 μg/ml	12.5 μg/ml	6.25 μg/ml	3.125 μg/ml	1.6 μg/ml	0.8 μg/ml
8b	4-Cl-C_6H_4	4-H	R	R	R	R	R	R	R	R
8j	4-Cl-C_6H_4	4-Cl	R	R	R	R	R	R	R	R
8k	4-F-C_6H_4	4-Cl	R	R	R	R	R	R	R	R
8l	4-Br-C_6H_4		R	R	R	R	R	R	R	R
8n	4-OCH_3-C_6H_4	4-Cl	S	R	R	R	R	R	R	R
8o	4-CH_3-C_6H_4	4-Cl	S	R	R	R	R	R	R	R
8p	3-Thienyl	4-Cl	R	R	R	R	R	R	R	R
8a	C_6H_5	4-H	S	S	R	R	R	R	R	R

Abbreviations: R, resistant; S, sensitive.

antitubercular drug should be reasonably lipophilic to penetrate the bacterial cell wall.[25] The MIC was defined as the lowest drug concentration that prevented the color change from blue to pink. The lowest concentration where the fragment shows a blue color is the MIC value. In Table 4, we have highlighted the compounds in terms of sensitivity and resistivity based on their activeness. The term resistance indicated that the compound is not active at a particular concentration. The term sensitive refers to the color change and that the compound is active at a particular concentration.

Among the tested compounds, the compound that contains only the phenyl group on the aryl part showed inhibition at 50 μg/ml concentration, which means the compound is sensitive; in other words, in the next concentration, it changed color from blue to pink. Among the tested compounds that contain methyl and chlorine substitution on the aryl part also showed the color change from blue to pink at a concentration of 100 μg/ml. The remaining compounds were resistant at the lower concentration. Further studies for the higher concentration for the compound can be done.

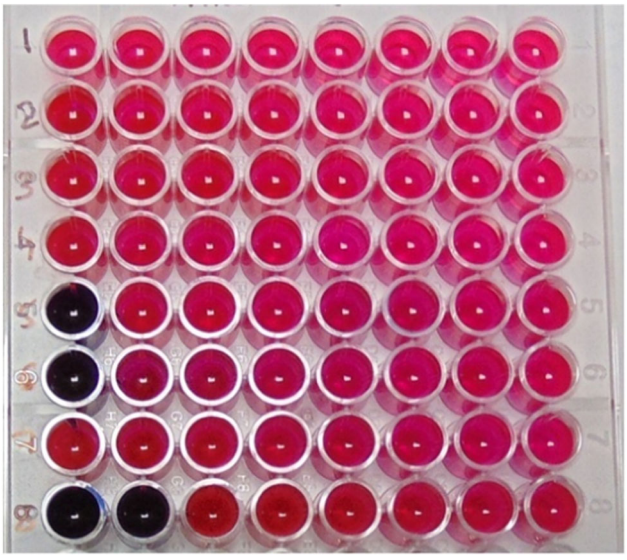


FIGURE 3 The antitubercular activity of the synthesized compounds

2.2.4 | Hemolytic assay

The safety of the products is one of the major questions to address during the drug development process. Many of the biologically active compounds may pose toxicity issues in terms of hemolysis, characterized by the rupture of red blood cells (erythrocytes) to release hemoglobin. The free hemoglobin in plasma may cause damage to

TABLE 5 The toxicity assay of the compounds in terms of percentage hemolysis at a concentration of 1 mg/ml

Compound	% Hemolysis
8a	**4.81**
8b	**6.07**
8c	**5.35**
8d	**6.02**
8e	**8.98**
8f	20.1
8g	**10.64**
8h	**3.6**
8i	**5.04**
8j	**8.25**
8k	12.54
8l	**6.91**
8m	13.1
8n	**6**
8o	5.25
8p	18.26

Note: Significance of bold face at lower value of concentration the derivative are active.

vital organs, such as heart, kidney, and liver.[26,27] Hence, it is essential to investigate the hemolytic activity of the biologically active compounds. One of the simplest, inexpensive, and quick methods for preliminary toxicity evaluation is the hemolytic assay, as it can be evident from several reports that the results obtained by this method complement those obtained by the cytotoxicity assay carried out against normal cell lines.[28,29] To test the preliminary toxicity, the compounds were treated with human erythrocytes (red blood cells) for hemolysis; the results are presented in Table 5. Most of the compounds were nontoxic, so they were found to be safe even at higher concentrations.

3 | CONCLUSION

A library of new pyrazole-clubbed thiophene derivatives was synthesized and evaluated for their antibacterial, anti-inflammatory, and antitubercular studies. Compounds **8a**, **8b**, **8d**, **8e**, **8f**, **8g**, **8h**, **8i**, **8k**, **8m**, **8n**, **8o**, and **8p** showed high potency toward both antimicrobial and anti-inflammatory activitites when compared with standard drugs ciprofloxacin and diclofenac sodium, respectively. Compound **8a** exhibited a potent activity against *M. tuberculosis* strain. These results suggested that it is a new and potential route in the discovery of drug against antibacterial, anti-inflammatory, and antitubercular activities.

4 | EXPERIMENTAL

4.1 | Chemistry

4.1.1 | General

All the required chemicals and reagents for this study were obtained from Merck, S.D. Fine, and Sigma-Aldrich. The reagents and solvents purchased were of analytical grade and were used without purification. The melting points of the synthesized compounds were determined in open glass capillaries and were uncorrected. FT(ATR)-IR absorption spectra were recorded on Thermo Nicolet, Avatar 370 in range 4,000–400 cm^{-1}. ^{1}H NMR spectra and ^{13}C NMR spectra were recorded on Bruker Avance III, 500 MHz, and chemical shift values were expressed in δ ppm. Mass spectra were recorded on Waters Synapt G2 high detection mass spectrometer and were uncorrected. Elemental analysis was carried out using CHNS Elementar Vario EL III. All reactions were monitored by thin-layer chromatography (TLC) analyses and were performed on silica plates; spots were visualized either by UV light or on exposure to iodine vapors. Hexane/ethyl acetate (9:1) was adopted as a solvent system. Compounds were prepared according to reported procedures.

The original spectra of the investigated compounds are provided as Supporting Information Data. Their InChI codes, together with the biological activity data, are also provided as Supporting Information Data.

4.1.2 | General synthetic procedure for 2-[1-(2,4-dichlorophenyl)ethylidene]malononitrile (2)[30]

Malononitrile (1.1 mmol), ammonium acetate (0.1 mmol), and acetic acid (0.2 mmol) were added to the solution of 1-(2,4-dichlorophenyl) ethan-1-one (**1**; 1 mmol) in dry toluene. The reaction mixture was maintained under reflux condition for 24–28 hr using a Dean–Stark water separator. Completion of the reaction was monitored by TLC. Upon cooling the reaction mixture, the solid product was separated. The crude product was recrystallized from ethyl acetate and toluene mixture to afford the title compound in a 75% yield.

4.1.3 | General synthetic procedure for 2-amino-5-(2,4-dichlorophenyl)thiophene-3-carbonitrile (3)[30]

A mixture of 2-[1-(2,4-dichlorophenyl)ethylidene]malononitrile (**2**; 1 mmol) and sulfur powder (1.3 mmol) was suspended in THF (25 ml) and warmed to an internal temperature of 35°C. A solution of sodium bicarbonate (1.4 mmol) in 5 ml of water was added over a period of 1 hr. The mixture was stirred for 24 hr. After completion of the reaction (TLC), the reaction mass was cooled and quenched to ice-cold water then filtered and washed with water. The crude product was recrystallized from toluene and ethyl acetate mixture to afford compound **3** in a 75% yield.

4.1.4 | General procedure for synthesis of 1-(aryl)-2-[1-(aryl)ethylidene]hydrazines 6a–p[30]

To a solution of substituted acetophenones **4a**–**h** (5 g, 19 mmol) in methanol (40 ml), substituted phenyl hydrazine **5a**, **5b** (2.1 g, 19 mmol) was added, followed by glacial acetic acid (15 ml), at room temperature. The resulting reaction mixture was heated to reflux for 4 hr. After the reaction solvent was evaporated under reduced pressure, and the resulting mass was stirred for 15 min with ice water (25 ml) at 10°C. The precipitated solid was filtered washed with ice water (20 ml), followed by *n*-pentane (20 ml) and dried under vacuum to afford 1-(1-(substituted phenyl)ethylidene)-2-phenylhydrazines **6a**–**p**.

4.1.5 | General procedure for the synthesis of 1,3-diaryl-1*H*-pyrazole-4-carbaldehydes 7a–p[30]

Phosphorus oxychloride (6.2 ml, 66 mmol) was added to *N*,*N*-dimethylformamide (DMF; 26.5 ml, 339 mmol) at −5°C to 0°C dropwise over a period of 10 min and stirred at the same temperature for 30 min, followed by the addition of **6a**–**p** (6.6 g, 22 mmol) dissolved in DMF (10 ml) dropwise at −5°C over a period of 15 min. The reaction mixture was allowed to attain room temperature and stirred for 1.5 hr. After completion (TLC), the reaction mixture was slowly added to ice-cold water and basified with saturated $NaHCO_3$ solution to

adjust the pH at 7–8. The mixture was then stirred for 1 hr at room temperature and the precipitated solid was filtered, washed with water, and dried under vacuum to get the crude compound (**7a**–**p**). The crude compound was washed with methanol, filtered, and dried under vacuum.

4.1.6 | General procedure for 2-{[(aryl-1*H*-pyrazol-4-yl)methylene]amino}-5-(2,4-dichlorophenyl)-thiophene-3-carbonitrile derivatives 8a–p

2-Amino-5-(2,4-dichlorophenyl)thiophene-3-carbonitrile **3** (1 mmol) and 1,3-disubstituted-1*H*-pyrazole-4-carbaldehydes **8a**–**p** (1 mmol) were dissolved in 20 ml of warm ethanol containing a catalytic amount of glacial acetic acid. The reaction mixture was refluxed for 5–7 hr. The major part of the product was precipitated when the reaction mixture was hot. The solid formed on cooling was filtered, washed with hot ethanol, and dried under vacuum to give respective Schiff bases **8a**–**p**. The crude material was purified with ethyl acetate recrystallization.

*5-(2,4-Dichlorophenyl)-2-{[(1,3-diphenyl-1H-pyrazol-4-yl)methylene]-amino}thiophene-3-carbonitrile (**8a**)*

Yield: 73%; m.p.: 248–250°C; IR (KBr, ν_{max} in cm^{-1}): 3,143 (C–H), 2,411 (C≡N), 1,623 (C═N), 1,456 (N═N), 1,312 (C═C); ^{1}H (500 MHz, dimethyl sulfoxide [DMSO]-d_6, δ ppm): 7.30 (1H, s, Ar-H), 7.41–7.54 (7H, m, Ar-H), 7.65–7.70 (6H, m, Ar-H), 8.44 (1H, s, Py-H), 8.46 (1H, s, –N═CH); ^{13}C (100 MHz, DMSO, δ ppm): 82.8, 113.0, 115.3, 119.9, 125.5, 126.2, 127.4, 127.5, 128.7, 129.2, 129.3, 130.3, 130.9, 133, 133.6, 135, 135.7, 139.7, 141.7, 150.4, 160.0, 160.3; electron ionization mass spectroscopy (EI–MS): *m/z* [M+], 498, [M+2+H]+, 500; anal. calcd. for $C_{27}H_{16}Cl_2N_4S$ (499.41) C, 64.94; H, 3.23; N, 11.22. Found: C, 64.96; H, 3.25; N, 11.23%.

*2-({[3-(4-Chlorophenyl)-1-phenyl-1H-pyrazol-4-yl]methylene}amino)-5-(2,4-dichlorophenyl)thiophene-3-carbonitrile (**8b**)*

Yield: 76%; m.p.: 216–218°C; IR (KBr, ν_{max} in cm^{-1}): 2,229 (C≡N), 1,593 (C═N), 754 (C–Cl); ^{1}H (500 MHz, DMSO-d_6, δ ppm): 7.45 (1H, s, Ar-H), 7.54–7.60 (7H, m, Ar-H), 7.84 (1H, d, Ar-H, J = 1.5 Hz), 8.02 (2H, d, Ar-H, J = 8 Hz), 8.12 (2H, d, Ar-H, J = 8.5 Hz), 8.75 (1H, s, Py-H), 9.29 (1H, s, –N═CH); ^{13}C (100 MHz, DMSO, δ ppm): 84.23, 105.5, 114.7, 119.1, 119.7, 121.9, 128.2, 129.0, 129.8, 130.2, 130.9, 131.3, 131.9, 133.4, 133.8, 133.9, 134.2, 134.9, 137.1, 139.0, 152.1, 155.6, 164.8; EI–MS: *m/z* [M+], 532, [M+2+H]+, 534, [M+4+H]+, 536; anal. calcd. for $C_{27}H_{15}Cl_3N_4S$ (533.86) C, 60.75; H, 2.83; N, 10.49. Found: C, 60.74; H, 2.85; N, 10.48%.

*5-(2,4-Dichlorophenyl)-2-({[3-(4-fluorophenyl)-1-phenyl-1H-pyrazol-4-yl]methylene}amino)thiophene-3-carbonitrile (**8c**)*

Yield: 71%; m.p.: 202–204°C; IR (KBr, ν_{max} in cm^{-1}): 3,122 (C–H), 2,383 (C≡N), 1,659 (C═N), 1,429 (N═N), 1,343 (C═C); ^{1}H (500 MHz, DMSO-d_6, δ ppm): 7.30–7.52 (7H, m, Ar-H), 7.65–7.70 (4H, m, Ar-H), 7.86 (2H, d, J = 8 Hz), 8.44 (1H, s, Py-H), 9.32 (1H, s, –N═CH); ^{13}C (100 MHz, DMSO, δ ppm): 82.8, 113.0, 115.3, 119.9, 123.1, 125.5, 126.2, 127.4, 128.3, 129.3, 130.3, 130.9, 132.0, 132.1, 133.6, 135.0, 135.7, 139.7, 141.7, 150.4, 160, 160.3; EI–MS: *m/z* [M+], 516, [M+2+H], 518; anal. calcd. for $C_{27}H_{15}Cl_2FN_4S$ (517.40) C, 62.68; H, 2.92; N, 10.83. Found: C, 62.67; H, 2.93; N, 10.84%.

*2-({[3-(4-Bromophenyl)-1-phenyl-1H-pyrazol-4-yl]methylene}amino)-5-(2,4-dichlorophenyl)thiophene-3-carbonitrile (**8d**)*

Yield: 75%; m.p.: 196–198°C; IR (KBr, ν_{max} in cm^{-1}): 3,354 (C–H), 2,215 (C≡N), 1,605 (C═N), 1,435 (N═N), 1,257 (C═C); ^{1}H (500 MHz, DMSO-d_6, δ ppm): 7.30 (1H, s, Ar-H), 7.41–7.55 (6H, m, Ar-H), 7.65–7.70 (4H, m, Ar-H), 7.78 (2H, d, J = 8 Hz), 8.44 (1H, s, Py-H), 8.46 (1H, s, –N═CH); ^{13}C (100 MHz, DMSO, δ ppm): 82.8, 113.0, 115.3, 119.9, 123.1, 125.5, 126.2, 127.4, 128.3, 129.3, 130.3, 130.9, 132.0, 132.1, 133.6, 135, 135.7, 139.7, 141.7, 150.4, 160, 160.3; EI–MS: *m/z* [M+], 576, [M+2+H], 578; anal. calcd. for $C_{27}H_{15}Cl_2BrN_4S$ (578.31) C, 56.08; H, 2.61; N, 9.69. Found: C, 56.07; H, 2.62; N, 9.69%.

*2-({[3-(3-Bromophenyl)-1-phenyl-1H-pyrazol-4-yl]methylene}amino)-5-(2,4-dichlorophenyl)thiophene-3-carbonitrile (**8e**)*

Yield: 73%; m.p.: 214–216°C; IR (KBr, ν_{max} in cm^{-1}): 3,354 (C–H), 2,215 (C≡N), 1,605 (C═N), 1,435 (N═N), 1,257 (C═C); ^{1}H (500 MHz, DMSO-d_6, δ ppm): 7.30 (1H, s, Ar-H), 7.41–7.55 (6H, m, Ar-H), 7.65–7.70 (4H, m, Ar-H), 7.78 (2H, d, J = 8 Hz), 8.44 (1H, s, Py-H), 8.46 (1H, s, –N═CH); ^{13}C (100 MHz, DMSO, δ ppm): 82.8, 113.0, 115.3, 119.9, 122.2, 125.5, 126.2, 127.4, 128.3, 129.3, 130.3, 130.9, 132.0, 132.1, 133.6, 135, 135.7, 139.7, 141.7, 150.4, 160, 160.3; EI–MS: *m/z* [M+], 576, [M+2+H], 578; anal. calcd. for $C_{27}H_{15}Cl_2BrN_4S$ (578.31) C, 56.08; H, 2.61; N, 9.69. Found: C, 56.09; H, 2.62; N, 9.68%.

*5-(2,4-Dichlorophenyl)-2-({[3-(4-methoxyphenyl)-1-phenyl-1H-pyrazol-4-yl]methylene}amino)thiophene-3-carbonitrile (**8f**)*

Yield: 77%; m.p.: 204–206°C; IR (KBr, ν_{max} in cm^{-1}): 2,224 (C≡N), 1,594 (C═N), 1,484 (C═C), 754 (C–Cl); ^{1}H (500 MHz, DMSO-d_6, δ ppm): 3.83 (3H, s, –OCH_3), 7.06–7.09 (2H, m, Ar-H), 7.43 (1H, t, Ar-H), 7.54–7.60 (5H, m, Ar-H), 7.84 (1H, d, J = 2 Hz), 7.96–7.98 (2H, m, Ar-H), 8.03 (2H, d, J = 8.75 Hz), 8.71 (1H, s, Py-H), 9.25 (1H, s, –N═CH); ^{13}C (100 MHz, DMSO, δ ppm): 55.7, 105.1, 114.4, 114.5, 114.8, 118.8, 119.6, 121.7, 124.3, 128.0, 128.2, 129.8, 130.2, 130.9, 132, 132.8, 133.4, 133.8, 134.9, 137.2, 139.1, 153.6, 156.0, 160.4, 165.1; EI–MS: *m/z* [M+], 528.00, [M+2+H]+, 530.00; anal. calcd. for $C_{28}H_{18}Cl_2N_4OS$ (529.44) C, 63.52; H, 3.43; N, 10.58. Found: C, 63.53; H, 3.44; N, 10.58%.

*5-(2,4-Dichlorophenyl)-2-({[1-phenyl-3-(p-tolyl)-1H-pyrazol-4-yl]-methylene}amino)thiophene-3-carbonitrile (**8g**)*

Yield: 77%; m.p.: 212–214°C; IR (KBr, ν_{max} in cm^{-1}): 3,117 (C–H), 2,262 (C≡N), 1,631 (C═N), 1,497 (N═N), 1,320 (C═C); ^{1}H (500 MHz, DMSO-d_6, δ ppm): 2.34 (3H, s, –CH_3), 7.15 (2H, d, J = 8 Hz), 7.30 (1H, s, Ar-H), 7.41–7.70 (10H, m, Ar-H), 8.44 (1H, s, Py-H), 9.32 (1H, s, –N═CH); ^{13}C (100 MHz, DMSO, δ ppm): 21.3, 82.8, 113.0, 114.8, 115.3, 119.9, 125.3, 125.5, 126.2, 127.4, 128.5, 129.5, 130.3, 130.9, 133.6, 135, 135.7, 139.7, 141.7, 150.4, 160.0, 160.3; EI–MS: *m/z*

[M+], 512, [M+2+H]+, 514; anal. calcd. for $C_{28}H_{18}Cl_2N_4S$ (513.44) C, 65.50; H, 3.53; N, 10.91. Found: C, 65.51; H, 3.53; N, 10.92%.

*5-(2,4-Dichlorophenyl)-2-({[1-phenyl-3-(thiophen-3-yl)-1H-pyrazol-4-yl]methylene}amino)thiophene-3-carbonitrile (**8h**)*

Yield: 73%; m.p.: 246–248°C; IR (KBr, ν_{max} in cm^{-1}): 3,182 (C–H), 2,462 (C≡N), 1,679 (C=N), 1,497 (N=N), 1,300 (C=C); ^1H (500 MHz, DMSO-d_6, δ ppm): 7.30 (1H, s, Ar-H), 7.38–7.52 (5H, m, Ar-H), 7.65–7.70 (4H, m, Ar-H), 7.89 (1H, d, J = 8 Hz), 7.98 (1H, s, Ar-H), 8.46 (1H, s, Py-H), 8.47 (1H, s, –N=CH); ^{13}C (100 MHz, DMSO, δ ppm): 82.8, 114.0, 115.3, 119.9, 121.7, 125.5, 126.2, 127.4, 128.2, 128.3, 129.3, 130.3, 130.9, 133.3, 133.6, 135.0, 135.7, 139.7, 141.7, 142.3, 160.0, 160.3; EI–MS: *m/z* [M+], 504, [M+2+H]+, 506; anal. calcd. for $C_{25}H_{14}Cl_2N_4S_2$ (505.44) C, 59.41; H, 2.79; N, 10.09. Found: C, 59.42; H, 2.78; N, 10.09%.

*2-({[1-(4-Chlorophenyl)-3-phenyl-1H-pyrazol-4-yl]methylene}amino)-5-(2,4-dichlorophenyl)thiophene-3-carbonitrile (**8i**)*

Yield: 74%; m.p.: 211–213°C; IR (KBr, ν_{max} in cm^{-1}): 3,119 (C–H), 2,222 (C≡N), 1,591 (C=N), 1,482 (N=N); ^1H (500 MHz, DMSO-d_6, δ ppm): 7.16–7.18 (1H, m, Ar-H), 7.56–7.71 (7H, m, Ar-H), 7.84 (1H, d, J = 1.5 Hz), 7.97–8.00 (3H, m, Ar-H), 8.45 (1H, d, J = 3.5 Hz), 8.84 (1H, s, Py-H), 9.29 (1H, s, –N=CH); ^{13}C (100 MHz, DMSO, δ ppm): 105.7, 114.8, 118.7, 121.1, 121.2, 122.1, 128.2, 128.5, 129.6, 129.8, 130.2, 131.9, 132.3, 132.5, 133.4, 133.7, 133.8, 133.9, 134.7, 134.9, 137.1, 137.2, 137.6, 147.7, 155.2, 164.9; EI–MS: *m/z* [M+], 532, [M+2+H]+, 534, [M+4+H]+, 536; anal. calcd. for $C_{27}H_{15}Cl_3N_4S$ (533.86) C, 60.75; H, 2.83; N, 10.49. Found: C, 60.76; H, 2.84; N, 10.48%.

*2-({[1,3-Bis(4-chlorophenyl)-1H-pyrazol-4-yl]methylene}amino)-5-(2,4-dichlorophenyl)thiophene-3-carbonitrile (**8j**)*

Yield: 71%; m.p.: 242–244°C; IR (KBr, ν_{max} in cm^{-1}): 3,182 (C–H), 2,124 (C≡N), 1,582 (C=N), 1,404 (N=N), 1,217 (C=C), 723 (C–Cl); ^1H (500 MHz, DMSO-d_6, δ ppm): 7.30 (1H, s, Ar-H), 7.35–7.41 (3H, m, Ar-H), 7.52–7.55 (4H, m, Ar-H), 7.65–7.70 (2H, m, Ar-H), 7.98 (2H, d, J = 8 Hz), 8.44 (1H, s, Py-H), 8.46 (1H, s, –N=CH); ^{13}C (100 MHz, DMSO, δ ppm): 82.8, 113, 115.3, 119.8, 125.5, 127.4, 128.9, 129.3, 129.4, 130.3, 130.9, 131.1, 131.8, 133.6, 134.3, 135, 135.7, 137.8, 141.7, 150.4, 160.0, 160.3; EI–MS: *m/z* [M+], 566, [M+2+H]+, 568, [M+4+H], 570; anal. calcd. for $C_{27}H_{14}Cl_4N_4S$ (568.30) C, 57.06; H, 2.48; N, 9.86. Found: C, 57.07; H, 2.49; N, 9.85%.

*2-({[1-(4-Chlorophenyl)-3-(4-fluorophenyl)-1H-pyrazol-4-yl]-methylene}amino)-5-(2,4-dichlorophenyl)thiophene-3-carbonitrile (**8k**)*

Yield: 70%; m.p.: 232–234°C; IR (KBr, ν_{max} in cm^{-1}): 3,429 (C–H), 2,212 (C≡N), 1,504 (C=N), 1,440 (N=N); ^1H (500 MHz, DMSO-d_6, δ ppm): 7.30–7.41 (6H, m, Ar-H), 7.55 (2H, d, J = 8 Hz), 7.65–7.70 (2H, m, Ar-H), 7.86 (2H, d, J = 8 Hz), 8.44 (1H, s, Py-H), 8.46 (1H, s, –N=CH); ^{13}C (100 MHz, DMSO, δ ppm): 82.8, 113, 115.3, 116, 119.8, 125.5, 127.4, 128.6, 129.4, 130.3, 130.6, 130.9, 131.8, 133.6, 135, 135.7, 137.8, 141.7, 150.4, 160.0, 160.3; EI–MS: *m/z* [M+], 550, [M+2+H]+, 552, [M+4+H]+, 554; anal. calcd. for $C_{27}H_{14}Cl_3FN_4S$ (551.85) C, 58.77; H, 2.56; N, 10.15. Found: C, 58.77; H, 2.58; N, 10.16%.

*2-({[3-(4-Bromophenyl)-1-(4-chlorophenyl)-1H-pyrazol-4-yl]-methylene}amino)-5-(2,4-dichlorophenyl)thiophene-3-carbonitrile (**8l**)*

Yield: 72%; m.p.: 220–222°C; IR (KBr, ν_{max} in cm^{-1}): 3,162 (C–H), 2,347 (C≡N), 1,601 (C=N), 1,403 (N=N), 1,320 (C=C); ^1H (500 MHz, DMSO-d_6, δ ppm): 7.30–7.41 (4H, m, Ar-H), 7.55 (4H, d, J = 8 Hz), 7.65–7.70 (2H, m, Ar-H), 7.78 (2H, d, J = 8 Hz), 8.44 (1H, s, Py-H), 8.46 (1H, s, –N=CH); ^{13}C (100 MHz, DMSO, δ ppm): 82.8, 113, 115.3, 116, 119.8, 123.1, 125.5, 127.4, 128.3, 129.4, 130.3, 132, 132.1, 133.6, 135, 135.7, 137.8, 141.7, 150.4, 160.0, 160.3; EI–MS: *m/z* [M+], 610, [M+2+H]+, 612, [M+4+H]+, 614; anal. calcd. for $C_{27}H_{14}Cl_3BrN_4S$ (612.75) C, 52.92; H, 2.30; N, 9.14. Found: C, 52.93; H, 2.31; N, 9.15%.

*2-({[3-(3-Bromophenyl)-1-(4-chlorophenyl)-1H-pyrazol-4-yl]-methylene}amino)-5-(2,4-dichlorophenyl)thiophene-3-carbonitrile (**8m**)*

Yield: 73%; m.p.: 238–240°C; IR (KBr, ν_{max} in cm^{-1}): 3,287 (C–H), 2,242 (C≡N), 1,622 (C=N), 1,441 (N=N), 1,241 (C=C), 731 (C–Cl); ^1H (500 MHz, DMSO-d_6, δ ppm): 7.30–7.45 (6H, m, Ar-H), 7.55 (2H, d, J = 8 Hz), 7.65–7.78 (4H, m, Ar-H), 8.44 (1H, s, Py-H), 8.46 (1H, s, –N=CH); ^{13}C (100 MHz, DMSO, δ ppm): 82.8, 113, 115.3, 116, 119.8, 123.1, 125.5, 127.4, 128.3, 129.4, 130.3, 132, 132.1, 133.6, 135, 135.7, 137.8, 141.7, 150.4, 160.0, 160.3; EI–MS: *m/z* [M+], 610, [M+2+H]+, 612, [M+4+H]+, 614; anal. calcd. for $C_{27}H_{14}Cl_3BrN_4S$ (612.75) C, 52.92; H, 2.30; N, 9.14. Found: C, 52.93; H, 2.31; N, 9.15%.

*2-({[1-(4-Chlorophenyl)-3-(4-methoxyphenyl)-1H-pyrazol-4-yl]-methylene}amino)-5-(2,4-dichlorophenyl)thiophene-3-carbonitrile (**8n**)*

Yield: 75%; m.p.: 246–248°C; IR (KBr, ν_{max} in cm^{-1}): 3,387 (C–H), 2,222 (C≡N), 1,602 (C=N), 1,444 (N=N), 1,249 (C=C); ^1H (500 MHz, DMSO-d_6, δ ppm): 3.81 (3H, s, –OCH_3), 7.03 (2H, d, J = 8 Hz), 7.30–7.41 (4H, m, Ar-H), 7.55 (4H, d, J = 8 Hz), 7.65–7.70 (2H, m, Ar-H), 8.44 (1H, s, Py-H), 8.46 (1H, s, –N=CH); ^{13}C (100 MHz, DMSO, δ ppm): 55.8, 82.8, 113, 114.8, 115.3, 119.8, 125.3, 125.5, 127.4, 128.5, 129.4, 130.3, 130.9, 131.8, 133.6, 135, 135.7, 137.8, 141.7, 150.4, 160.0, 160.3; EI–MS: *m/z* [M+], 562, [M+2+H]+, 564, [M+4+H]+, 566; anal. calcd. for $C_{28}H_{17}Cl_3N_4OS$ (562.02) C, 59.64; H, 3.04; N, 9.94. Found: C, 59.65; H, 3.05; N, 9.95%.

*2-({[1-(4-Chlorophenyl)-3-(p-tolyl)-1H-pyrazol-4-yl]methylene}-amino)-5-(2,4-dichlorophenyl)thiophene-3-carbonitrile (**8o**)*

Yield: 72%; m.p.: 210–212°C; IR (KBr, ν_{max} in cm^{-1}): 2,224 (C≡N), 1,594 (C=N), 1,455 (N=N), 770 (C–Cl); ^1H (500 MHz, DMSO-d_6, δ ppm): 2.47 (3H, m, –CH_3), 7.36 (2H, d, Ar-H, J = 8 Hz), 7.54–7.61 (3H, m, Ar-H), 7.65 (2H, d, Ar-H, J = 8.5 Hz), 7.85 (1H, d, Ar-H, J = 2 Hz), 7.88 (2H, d, Ar-H, J = 8 Hz), 8.09 (2H, d, Ar-H, J = 9 Hz), 8.69 (1H, s, Py-H), 9.30 (1H, s, –N=CH); ^{13}C (100 MHz, DMSO, δ ppm): 23.37, 114.5, 114.5, 114.6, 115.3, 115.5, 118.5, 119.1, 121.3, 122.6, 124.1, 124.3, 130.1, 130.6, 130.9, 132.1, 132.3, 135.7, 137.9, 155.6, 160.5, 160.6, 185.07; EI–MS: *m/z* [M+], 546, [M+2+H]+, 548, [M+4+H]+,

550; anal. calcd. for $C_{28}H_{17}Cl_3N_4S$ (547.89) C, 61.38; H, 3.13; N, 10.23. Found: C, 61.37; H, 3.14; N, 10.23%.

*2-({[1-(4-Chlorophenyl)-3-(thiophen-3-yl)-1H-pyrazol-4-yl]-methylene}amino)-5-(2,4-dichlorophenyl)thiophene-3-carbonitrile (**8p**)*

Yield: 74%; m.p.: 235–237°C; IR (KBr, ν_{max} in cm^{-1}): 3,427 (C–H), 2,400 (C≡N), 1,496 (C═N), 1,438 (N═N), 1,408 (C═C); ^1H (500 MHz, DMSO-d_6, δ ppm): 7.30–7.41 (5H, m, Ar-H), 7.55 (2H, d, Ar-H, J = 8 Hz), 7.65–7.70 (2H, m, Ar-H), 7.89–7.98 (2H, m, Ar-H), 8.47 (1H, s, Py-H), 9.32 (1H, s, –N═CH); ^{13}C (100 MHz, DMSO, δ ppm): 82.8, 114, 115.3, 119.8, 121.7, 125.5, 127.4, 128.2, 128.3, 129.2, 129.4, 130.3, 130.9, 131.8, 133.3, 133.6, 135, 135.7, 137.8, 141.7, 142.3, 160, 160.3; EI–MS: *m/z* [M+], 538, [M+2+H]+, 540, [M+4+H]+, 542; anal. calcd. for $C_{25}H_{13}Cl_3N_4S_2$ (539.89) C, 55.62; H, 2.43; N, 10.38. Found: C, 55.63; H, 2.42; N, 10.39%.

4.2 | Biological studies

4.2.1 | Antibacterial assay

The susceptibility of the test organisms to synthetic compounds was assessed using broth dilution assay, as MIC. Triplicates were performed for each of the standard strains.

Culture media

Brain Heart Infusion (BHI) broth test organisms. Four microorganisms were selected for the study: *S. aureus* Microbial Type Culture Collection MTCC 12598, *E. faecalis* MTCC 35550, *E. coli* MTCC 443, and *P. aeruginosa* MTCC 25668. All microorganisms were previously subcultured in appropriate media and under gaseous conditions to confirm their purity at 35°C for 48 hr before testing of the vehicles.

Inoculum preparation

The growth method or the log phase method was performed as follows. At least three to five well-isolated colonies of the same morphological type were selected from an agar culture plate. The top of each colony was scooped with a loop, and they were transferred into a tube containing 4–5 ml of BHI broth. The broth culture was incubated at 35°C for 2–6 hr until it achieved the turbidity of the 0.5 McFarland standard. The turbidity of actively growing broth culture was adjusted with the broth to obtain a final turbidity, optically comparable to that of the 0.5 McFarland standard, which was done visually by comparing the inoculum tube and the standard against a white card with contrasting black lines.

Broth dilution method

A total of 10 tubes were taken and nine dilutions of the vehicle were done with BHI for MIC. In the initial tube, only 200 ml of the vehicle was added. For further dilutions, 200 ml of BHI broth was added to the next nine tubes separately. In the second tube, 200 ml of the vehicle was added which already contained 200 ml of BHI broth. This was considered as 10 dilutions. From the 10 diluted tubes, 200 ml was transferred to the second tube to make 10 dilutions. The serial dilution was repeated up to 10 dilutions for each vehicle. From the maintained stock cultures of the required microorganisms, 5 ml was taken and added to 2 ml of BHI broth. In each serially diluted tube, 200 ml of the above culture suspension was added. The last tube contained only the media and the culture suspension, that is, the negative control. The tubes were kept for incubation for 24 hr at 37°C in a bacteriological incubator and observed for turbidity.

4.2.2 | Anti-inflammatory activity

The anti-inflammatory activity of the compounds was studied by using inhibition of albumin denaturation technique, which was studied according to Govindappa et al.[31] and Sakat et al.[32] The reaction mixture consisted of test compounds and 1% aqueous solution of bovine albumin fraction; the pH of the reaction mixture was adjusted using a small amount of 1 N HCl. The sample extracts were incubated at 37°C for 20 min and then heated to 51°C for 20 min. After cooling the samples, the turbidity was measured at 660 nm (UV–Visible spectrophotometer). The experiment was performed in triplicate. Diclofenac sodium was used as the standard compound.

The anti-inflammatory activity of the compounds was estimated on the basis of the percentage of inhibition of albumin denaturation as the following equation:

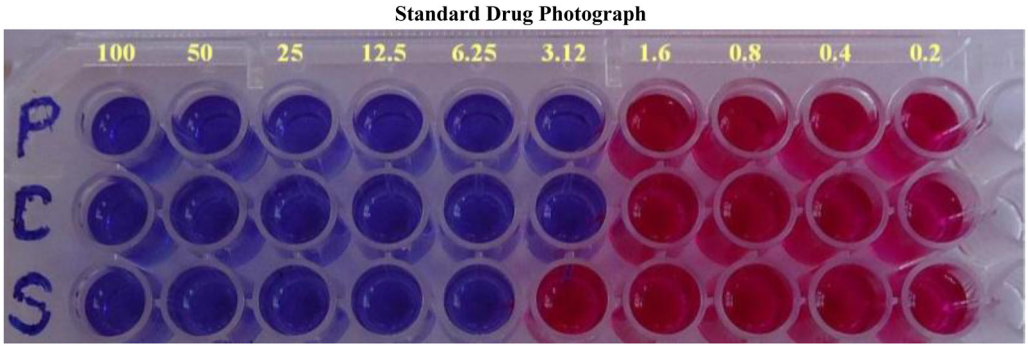
$$\%\,\text{Inhibition} = \frac{\text{Control absorbance} - \text{Sample absorbance}}{\text{Control absorbance}} \times 100.$$

4.2.3 | Antitubercular activity

The antitubercular activity of compounds was assessed against *M. tuberculosis* (ATTC 2729415) using the microplate Alamar Blue assay.[33] The methodology is nontoxic, which uses a thermally stable reagent and shows a good correlation with proportional and BACTEC radiometric methods. Briefly, 200 µl of sterile deionized water was added to all outer perimeter wells of sterile 96-well plates (Falcon 3072; Becton Dickinson, Lincoln Park, NJ) to minimize evaporation of the medium in the test wells during incubation. The 96 plates received 100 µl of the Middlebrook 7H9 Broth (Difco Laboratories, Detroit, MI), and a serial dilution of the compounds was performed directly on the plate. The final drug concentrations tested were 100–0.2 µg/ml. Plates were covered and sealed with a parafilm and incubated at 37°C for 5 days. After this time, 25 µl of a freshly prepared 1:1 mixture of Alamar Blue (AccuMed International, Westlake, OH) reagent and 10% Tween-80 was added to the plate and incubated for 24 hr. Blue color in the well indicated no bacterial growth and pink color indicated growth. MIC was defined as the lowest drug concentration, which prevented a color change from blue to pink.

Standard values for the anti-Tb test that was performed are as follows:

Pyrazinamide (P): 3.125 µg/ml
Ciprofloxacin (C): 3.125 µg/ml
Streptomycin (S): 6.25 µg/ml



4.2.4 | Hemolytic assay

The hemolytic assay was carried out as per the literature report.[34] The human blood was collected in a vacutainer containing EDTA (2 mg/ml). The resulting suspension was centrifuged at 800*g* for a period of 10 min to separate buffy coat and plasma. Successively, the settled erythrocytes were suspended in saline to acquire a suspension of 5% erythrocytes after washing three times with normal saline (0.9%). The cells were later incubated in the presence of test compounds (1 mg/ml or 1,000 µg/ml) for 2 hr at 37°C. After incubation, the suspensions were subjected to centrifugation at 800*g* for 10 min, followed by measurement of the absorbance of the supernatant solutions in a UV spectrophotometer at 540 nm. The 2% Triton X-100 (Sigma-Aldrich, MO) served as a positive control. The percentage of hemolysis is expressed as a percentage of Triton X-100-induced hemolysis and is calculated by the following formula:

$$\%\,\text{Hemolysis} = \frac{(\text{Absorbance of the sample}) - (\text{Absorbance of blank})}{\text{Highest absorbace of positive control}} \times 100.$$

ACKNOWLEDGMENTS

We are thankful to DST-PURSE, Mangalore University, and SAIF Punjab for providing elemental and spectral analysis of newly synthesized compounds. We are grateful to Dr. Prabhakar Kore Basic Science Research Centre, KLE Academy of Higher Education and Research, Belgaum, and Maratha Mandal's NGH Institute of Dental Sciences and Research Centre for their help in carrying out the biological studies.

CONFLICTS OF INTERESTS

The authors declare that there are no conflicts of interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Nayak SG, Poojary B, Kamat V. Novel pyrazole-clubbed thiophene derivatives via Gewald synthesis as antibacterial and anti-inflammatory agents. *Arch Pharm.* 2020;e2000103. https://doi.org/10.1002/ardp.202000103