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Original Article

Chemical and Pharmacological Assessment of Substituted 1,2,4-Triazole Fused Pyrimidines as Anti-microbial Agent

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ABSTRACT

Objectives: To synthesize a novel series of 3-(Aryl/Heteroaryl)-5-Methyl-7-Phenyl [1,2,4]triazolo[4,3a]Pyrimidines (5a-i). **Materials and Methods:** Synthesis was carried out though the microwave assisted synthesis, Spectroscopic approaches such as IR,1HNMR and MASS spectroscopy were used to characterize the produced substances. Antimicrobial screening was done by Cup Plate Method for the antibiotic zone determination and Minimum Inhibitory Concentration was determined by the tube dilution method. Experimental Work: The oxidation of substituted pyrimidinylhydrazones in dichloromethane with iodobenzene diacetate was used in the synthesis. Substituted pyrimidinylhydrazones was obtained by the condensation of 1-(1,4-dihydro-6-methyl-4-phenylpyrimidine-2-yl)-hydrazine containing a variety of aromatic/heteroaromatic aldehydes. 3-(Aryl/Heteroaryl)-5-Methy l-7-Phenyl-[1,2,4]triazolo[4,3a]Pyrimidines (5a-i) were investigated *in vitro* for antibacterial activity against gram-positive bacteria such as *Bacillus subtilis* and *Staphylococcus aureus*, as well as gram-negative bacteria such as *Escherichia coli* and *Pseudomonas putida*. **Results:** 3-(4-fluorophenyl)-5-Methyl-7-Phenyl-[1,2,4]Triazolo[4,3a]Pyrimidine (5a) and 3-(4-Nitrophenyl)-5-Methyl-7-Phenyl-[1,2,4]Triazolo[4,3a]Pyrimidine (5e) found effective when compared to known marketed drugs Streptomycin and Chloramphenicol as both compounds are having the substitution of most electronegative groups which shows strong interaction with targeted DNA Gyrase enzyme of the bacteria. **Conclusion:** Microwave found to be an eco-friendly method for cyclization of pyrimidinylhydrazone to triazolopyrimidine. This method was found to be clean, safe and operationally simple which give triazolopyrimidine products with high yield.

Keywords: Triazolopyrimidines, Pyrimidinylhydrazones, Iodobenzene diacetate, Antibacterial activity.

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INTRODUCTION

The triazolopyrimidine derivatives are resembled to purine bases[1] and act as a valuable pharmacophores bearing diverse group of biological activities such as antiproliferation caused by arresting different stages of mitosis and cellular apoptosis.[2] inhibitors of *Aspergillus fumigatus* as a potent herbicide,[3] Vipan Kumar *et al.*, have reported a multitargeted approach for *in vitro* anti-plasmodial efficacy of Triazolopyrimidine and 4-Aminoquinoline based hybrids against Chloroquine sensitive (3D7) and resistant (W2) *P. falciparum* strains[4] Lavinia L. Ruta *et al.*, have reported a synthesis of a new series of copper (II) ligand complexes of 2,2′-bipyridine or 1,10-phenanthroline and 5,7-dimethyl-1,2,4-triazolo[1,5-a] pyrimidine and investigated their mode of action as anti-cancer and anti-bacterial agents.[5]

In recent years advances and innovative techniques are introducing in the green chemistry. One of the most prevalent and efficient methods is the microwave based synthesis, which is preferred by chemists due to increased yield, reduced time, and environmental friendliness.[6] Mohamed Youssef and Mahmoud A Amin *et al.*, have reported the microwave irradiation of thiazolopyrimidine, thiazolodipyrimidine, and thiazolopyrimidopyrimidine derivatives with potential antioxidant and antibacterial action has piqued their attention.[7] 1,2,4-triazoles and Pyrimidine are very remarkable heterocyclic moiety as those moieties have demonstrated their function as chemotherapeutics. In current decades, data in the literature is constituted with nitrogen bearing heterocyclic ring from synthetic as well as natural products become a good therapeutic agent in the field of medicine.[8] As a result, we shed further light on the synthesis of triazolopyrimidine derivatives through microwave assisted synthesis which might be suitable as anti-bacterial agents.

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In organic synthesis, the application of hypervalent iodine contain moiety as a moderate oxidizing agent has gaining a lot of interest.[9] The current study describes the chemical and pharmacological estimation of fused 1,2,4-Triazolopyridines.

MATERIALS AND METHODS

Melting points were recorded by using a silicon oil bath and microprocessor-based melting point apparatus made by VEEGO and melting point were uncorrected. On columns, chromatographic separation was carried out using neutral alumina, activity grade I, and silica gel with a mesh size of 100–200. Chromatographic separation was carried out on silica gel columns 100-200 mesh and neutral alumina, activity grade I. TLC was carried on 2x5 cm pre-coated silica gel 60 F_{254} (Merck) plates of thickness of 225 µm the chromatograms were observed using UV (254 nm) and/or iodine vapor exposure. The anhydrous sodium sulphate was used to remove the moisture from the chemicals All reagents used were of analytical reagents grade, obtained from SD fine chemicals, Spectrochem and Qualige. All the solvents were purified by column chromatographic technique. All the glassware's were dried in hot air oven prior to use. Using potassium bromide discs, IR spectra (wave numbers in cm^{-1}) were captured using a BRUKER ALPHA FT-IR spectrophotometer. Using a BRUKER AVANCE II 500 MHz equipment and TMS as an internal standard, NMR spectra were captured. Chemical shift values are given in ppm, a unit of measurement. Mass spectra was captured by using Schimatzu LC MS 2010 spectrophotometer.

Experimental work

Chemical Work

Synthesis of 3,4-Dihydro-6-Methyl-4-Phenylpyrimidine-2(1-H)-Thione[2](Figure 1)

Sodium metal (2 g) was reacted with ethanol (50 mL) and further refluxed for 20 min. Then thiourea (0.624 g, 8.2 mmols) and 4-phenylbut-3-en-2-one [1] (Figure 1) (1 g, 6.8 mmol) were added to the reacting mixture and microwaved for 10 min. Product was obtained by cooling the reaction mixture at room temperature and by neutralizing by water followed by hydrochloric acid to get the crude product. Methanol was used to recrystallize the crude product, resulting in the product 3,4-dihydro-6-methyl-4-p henylpyrimidine-2(1H)-thione [2] (Figure 1).

Synthesis of 1-(1,4-Dihydro-6-Methyl-4-Phen ylpyrimidine-2-yl)-Hydrazine [3] (Figure 1)

3,4-Dihydro-6-Methyl-4-Phenylpyrimidine-2(1H)-thione [2] (1 g. 4.9 mmole) and hydrazine hydrate (2 mL) was swirled in a microwave at 150 watts and further irradiated in a solution of ethanolic potassium hydroxide (30 mL, 2%) for 10 min. Product was obtained by cooling the reaction mixture at room temperature, the precipitated residue was isolated by filtration, sundried, and crystallized from purified methanol to produce 1-(1,4-dihydro-6-methyl-4-phenylpyrimidine-2-yl)-hydrazine [3].

Synthesis of 2-[(2-Aryl/Heteroaryl) hydrazinyl]-6-methyl-4-phenyl-1,4-dihydropyrimidine[4] (Figure 1)

In ethanol, 1-(1,4-Dihydro-6-methyl-4-phenylpyrimidine-2-yl)-hydrazine was dissolved, and aryl/heteroaryl aldehydes were added respectively. The contents were irradiated in the microwave for 15 min and then left for the cooling to room temperature. The resulting crystalline product was filtered followed by washing with ethanol, and sundried to yield substituted pyrimidinylhydrazones.[4]

Synthesis of 3-Aryl/Heteroaryl-5-methyl-7-phenyl-[1,2,4]triazolo[4,3a]pyrimidines [5a-i]

Accurately weighed of substituted pyrimidinylhydrazones [4] (0.01 mmol) dissolved in 25 ml dichloromethane at room temperature, Iodobenzene diacetate (0.011 mmol) was mixed in portion wise with 10 min duration. A steam bath was used to evaporate the solvent. The residual mass contains crude product containing iodobenzene. This residual mass was triturated with petroleum ether to get solid product. Recrystallization of the product from methanol yielded pure substituted triazolopyrimidines.[10] [5a-i]. (Figure 1).

Biological work

Antibacterial Activity

Anti-microbial action of the synthesized compound was estimated by the cup-plate technique against gram-positive bacteria such as *Bacillus subtilis* and *Staphylococcus aureus*, as well as gram-negative bacteria such as *Escherichia coli* and *Pseudomonas putida*.[11]

Working standards

Test and standard stock solutions were produced in DMSO at a conc. of 1000 µg/mL. The further dilution has been conducted to reach conc. of 10 µg/mL, 50 µg/mL, 100 µg/mL.

Media Composition

1. Agar 15%.
2. Peptic Digest of Animal Tissue 5%.
3. Sodium chloride 5%.
4. Bees extract 1.5%.
5. Yeast extract 1.5%.
6. Final pH (After Sterilization) 7.5 0.2.
7. Distilled water up to 1000 mL.

Media was prepared as per the given composition and mixed with water. This solution was heated for 1.5 hr. in a water bath until it turned transparent. This nutritional media was sterilized by autoclaving at 121°C for 15 min at 15 psi.

Culture

Bacillus subtilis and *Staphylococcus aureus* were employed in this investigation as gram +ve bacteria as well gram-negative bacteria such as *Escherichia coli and Pseudomonas putida* were employed. The culture was grown on the nutrient media's agar slant and refrigerated.

Preparation of Inoculum

A tiny amount of culture was transferred from the working culture in an aseptic setting to roughly 10-15 mL of sterile normal saline. (0.9% NaCl solution), mixed well before being utilized for the estimation of antibacterial effect. About 0.5 mL of inoculums was added to the uncontaminated petridish, along with media, mixed and allowed to harden. The test and reference solutions were injected into the wells that had been created in the agar plate at a consistent volume. All petridish were incubate at 37°C for 24 hr.[12]

Procedure for Minimum Inhibitory Concentration (MIC)

Sterilized test tubes were used for the MIC study, added 4.0 mL nutrient broth, 0.5 mL of the test fluid and 0.5 mL of the microbial culture in the earliest test tube and mixed well then 0.5 mL of the solution was transferred in the subsequent test tube and volume was made up to 5 mL by nutrient broth and mixed well to get serial dilution. All test tubes were incubated at 37°C for 24 hr. After 24 hr., the test turbity in test tubes was examined using a photo colorimeter at 470 nm, and the MIC was computed.[13]

RESULTS

It was observed that Iodobenzene diacetate-mediated oxidative approach work satisfactorily gives desired products with excellent yields and purity.

There is a discussion of the triazolopyrimidines' physicochemical properties in Table 1.

Spectral Studies

The spectral characterization of the produced substances by IR, MASS, and ^{1}HNMR Spectroscopy served as confirmation.

3-(4-Flurophenyl)-5-Methyl-7-Phenyl-[1,2,4Ttriazole[4,3a] Pyrimidine. (5a):

IR (KBr) cm^{-1}: 1601 (C=N strh.), 1401 (C-H bend.), 1080 (C-F strh.); ^{1}H NMR ($CDCl_3$): 2.01 (*s, 3H, CH_3*), 7.27-7.78 (*m, 8H, Ar-H*), 7.91-7.96 (*d, 2H, Ar-H*); *m/z*: 304.9 (M^+), Micro analysis: C, 71.04; H, 4.31; F, 6.24; N, 18.41.

3-(4-Chlorophenyl)-5-Methyl-7-Phenyl-[1,2,4]Triazole[4,3a] Pyrimidine. (5b):

IR (KBr) cm^{-1}: 1574 (C=N), 1401 (C-H bend.), 740 (C-Cl); ^{1}H NMR ($CDCl_3$): 2.05 (*s, 3H, CH_3*), 7.28-7.68 (*m, 8H, Ar-H*), 7.82-7.86 (*d, 2H, Ar-H*); *m/z*: 320.7 (M^+), Micro analysis: C, 67.40; H, 4.08; Cl, 11.05; N, 17.47.

3-(3-Chlorophenyl)-5-Methyl-7-Ph enyl-[1,2,4]Triazole[4,3a]Pyrimidine. (5c):

IR (KBr) cm^{-1}: 1580 (C=N), 1401 (C-H bend.), 674 (C-Cl); ^{1}H NMR ($CDCl_3$): 2.05 (*s, 3H, CH_3*), 7.20-7.48 (*m, 9H, Ar-H*), 7.78 (*s, 1H, Ar-H*); *m/z*: 320.9 (M^+) Micro analysis: C, 67.40; H, 4.08; Cl, 11.05; N, 17.47.

Table 1: Physicochemical Properties of Synthesized Compounds.

Compound code	Mol. Formula	Mol. Weight	m.p.(°C)	R_f* R_f Value
5a	$C_{18}H_{13}N_4F$	304	177-178	0.36
5b	$C_{18}H_{13}N_4Cl$	320.5	198-201	0.38
5c	$C_{18}H_{13}N_4Cl$	320.5	202-205	0.41
5d	$C_{20}H_{19}N_5$	329	108-110	0.34
5e	$C_{18}H_{13}N_5O_2$	331	152-154	0.42
5f	$C_{18}H_{13}N_5O_2$	331	202-204	0.40
5g	$C_{17}H_{13}N_5$	287	210-212	0.48
5h	$C_{17}H_{13}N_5$	287	208-210	0.46
5i	$C_{16}H_{12}N_4S$	292	175-177	0.42

*Solvent system: Chloroform: Methanol (8:2)

Table 2: Antimicrobial Screening of Synthesized compounds by the Cup Plate Method.

Compound	Conc. (µg/mL)	Inhibition Zone Diameter (mm)			
		E. coli	*P. putida*	*B. subtilis*	*S. aureus*
5a	10	37.33	29.33	30.66	29.66
	50	63.66	49.66	47.66	48.33
	100	70.33	67.66	67.33	66.33
5b	10	11.29	09.67	13.66	10.11
	50	14.28	15.32	22.26	23.33
	100	26.44	23.22	41.72	41.81
5c	10	23.33	28.33	27.66	26.66
	50	31.66	48.66	40.66	41.66
	100	47.33	66.66	59.33	60.66
5d	10	-	-	-	-
	50	-	08.33	08.34	-
	100	11.33	12.66	13.33	10.66
5e	10	29.33	32.66	27.33	30.66
	50	46.33	49.33	42.66	46.66
	100	66.66	70.33	61.66	66.33
5f	10	29.33	25.33	25.66	36.66
	50	46.33	39.66	39.33	59.33
	100	65.66	56.33	57.33	69.66
5g	10	12.33	11.66	27.33	12.33
	50	21.33	20.33	39.66	20.66
	100	40.66	40.66	59.66	41.33
5h	10	-	-	-	-
	50	08.33	12.33	09.66	10.33
	100	12.33	19.66	14.33	16.67
5i	10	-	-	-	-
	50	10.33	08.33	08.34	-
	100	18.66	12.66	13.66	08.33
Chloramphenicol	10	30.33	29.33	36.33	35.33
	50	50.40	53.40	57.20	50.40
	100	75.40	71.24	74.20	75.40
Streptomycin	10	26.40	38.20	36.40	33.20
	50	49.40	48.20	57.42	49.40
	100	72.24	72.24	73.24	69.40

3-(4-Dimethylaminophenyl)-5-Methyl-7-Phenyl-[1,2,4]Triazole[4,3a]Pyrimidine. (5d):

IR (KBr) cm^{-1}: 1645, 1576 (C=N), 1400 (C-H bend.), 1293(C-N); ^1H NMR ($CDCl_3$): 2.14 (s, 3H, CH_3), 3.1(s, 6H), 7.28-7.48 (m, 8H, Ar-H), 6.6 (d, 2H, Ar-H); *m/z*: 330.2 (M+1), Micro analysis: C, 72.93; H, 5.81; N, 21.26.

3-(4-Nitrophenyl)-5-Methyl-7-Phenyl-[1,2,4]Triazole[4,3a]Pyrimidine. (5e):

IR (KBr) cm^{-1}: 1574 (C=N), 1521 (NO_2 asymmetric) and 1344 (-NO_2 symmetric); ^1H NMR ($CDCl_3$): 2.01 (*s, 3H,* CH_3), 7.27 (s, heteroaromatic H), 7.35-7.38 (t, 2H), 7.50-7.52 (d, 1H), 7.60-7.61 (d, 2H), 7.84-7.86 (d, 2H), 8.16-8.17 (d, 2H); m/z: 331.9 (M^+), Micro analysis: C, 65.25; H, 3.95; N, 21.14; O, 9.66.

Table 3: **Minimum Inhibitory Concentration (μg/mL) of Substituted 1,2,4-triazolo fused pyrimidines.**

Compound	*E. coli*	*P. putida*	*B. subtilis*	*S. aureus*
5a	06	08	04	04
5b	07	16	08	08
5c	32	32	16	16
5d	255	265	290	300
5e	04	02	04	08
5f	10	12	08	10
5g	16	16	08	16
5h	250	260	220	210
5i	275	290	310	320
Chloramphenicol	02	04	02	02
Streptomycin	04	04	02	02

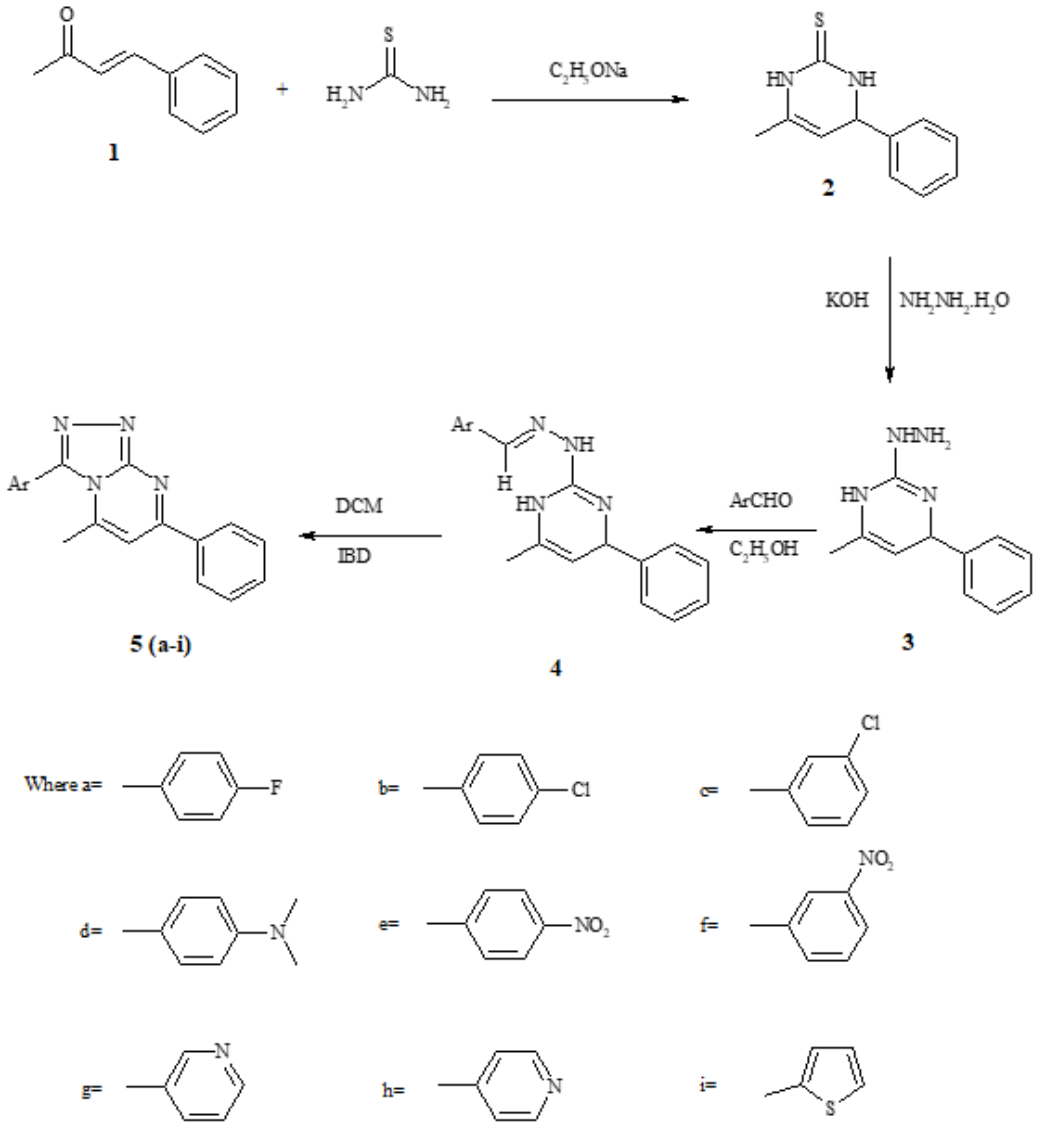

Figure 1: Scheme of synthesis of *1,2,4-Triazole* fused *Pyrimidine* derivatives.

3-(3-Nitrophenyl)-5-Methyl-7-Ph enyl-[1,2,4]Triazole[4,3a]Pyrimidine. (5f):

IR (KBr) cm^{-1}: 1600 (C=N), 1545 (-NO_2 asymmetric) and 1350 (-NO_2 symmetric); ^{1}H NMR ($CDCl_3$): 2.17 (s, *3H,* CH_3), 7.10-7.69 (*m, 9H, Ar-H*), 8.10 (*s, 1H, Ar-H*); m/z: 331.3 (M^+) Micro analysis: C, 65.25; H, 3.95; N, 21.14; O, 9.66.

3-(3-Pyridino)-5-Methyl-7-Ph enyl-[1,2,4]Triazole[4,3a]Pyrimidine. (5g):

IR (KBr) cm^{-1}: 1599, 1491 (C=N); ^{1}H NMR ($CDCl_3$): 2.07 (*s, 3H,* CH_3), 7.12-7.71 (*m, 6H, Ar-H*), 7.6-8.39 (*d, 4H of pyridine ring*); m/z: 287.1 (M^+) and 288.1 (M+1). Micro analysis: C, 71.06; H, 4.56; N, 24.37.

3-(4-Pyridino)-5-Methyl-7Phenyl-[1,2,4]Triazole[4,3a]Pyrimidine (5h):

IR (KBr) cm^{-1}: 1585, 1490 (C=N); *m/z*: 287.1 (M^+), 288.4 (M+1) and 289.1 (M+2), Micro analysis: C, 71.06; H, 4.56; N, 24.37.

Biological Activity

All derivatives were estimated for the *in vitro* antibacterial activity at varying concentrations (10, 50, and 100µ g/mL), against two gram-positive bacteria, *Bacillus subtilis* and *Staphylococcus aureus*, and gram-negative bacteria, *Escherichia coli* and *Pseudomonas putida* by measuring the diameter of the zone of inhibition, which is given in Table 2 additionally, the results of Minimum Inhibitory Concentration study (MIC) was demonstrated by tube dilution method shown in Table 3. Compounds 5a, 5c, 5e, and 5f demonstrated effective antibacterial activity.

DISCUSSION

Rationale of this work was to synthesize 1,2,4-triazole fused pyrimidine from pyrimidinylhydrazone under mild conditions with the use of Iodobenzene dichloride as an excellent green oxidizing agent. In place of typical cyclizing agents such acetic acid hydrochloric acid, sulphuric acid, formic acid, and acetic anhydride, iodobenzene diacetate were applied because cyclization with conventional agents requires drastic condition with minimum of 5-6 hr reflux, whereas cyclization with iodobenzene requires mild conditions. The use of iodobenzene is an eco-friendly method.[14]

Chemical Studies

To synthesize 3-(aryl/heteroaryl)-5-Methyl-7-Phenyl [1,2, 4]Triazolo[4,3a], oxidizing agent iodobenzene diacetate was used with the pyrimidines (5a-i) in Figure 1. 4-Phenylbut-3-en-2-one (1) (Shown in Figure 1) was designed in accordance with the described process.[15] Thioxopyrimidine (2) (Figure 1) was synthesized by condensation of 4-phenylbut-3-en-2-one (1) with thiourea in alkaline medium. Condensation of thioxopyrimidine (2) with hydrazine hydrate using ethanolic potassium hydroxide to yield 1-(1,4-Dihydro-6-Methyl-4-Phenylpyrimidine-2-yl)-hydrazine (3). Author synthesized a series of newly substituted pyrimidinylhydrazones (4) by the condensation of compound (3) with different aromatic/heteroaromatic aldehydes under mild condition with good yields and purity. Finally, oxidation of substituted pyrimidinylhydrazones (4) was carried out with 1.1 equivalent of Iodobenzene dichloride as mild oxidative agent in Dichloromethane (DCM) to afford desired products as 3-(aryl/heteroaryl)-5-Methyl-7-phenyl-[1,2,4]triazolo[4,3a]pyrimidines (5a-i). The newly synthesized compounds 5a (Fluoro Substituted) and 5e (Nitro substituted) (Shown in Figure 1) as fluro and nitro groups are electronegative, they show strong interaction with targeted enzyme of the bacteria, both compounds were found to be most effective bacterial growth inhibitors when compared to known compounds.

CONCLUSION

Anti-bacterial activities of all derivatives were screened. Bacterial growth was shown to be strongly inhibited by the chemicals 5a and 5e. This might be caused by the aromatic ring's para position being replaced by electron-withdrawing F (5a) and NO_2 (5e) groups. Less activity has been observed in compounds with heterocyclic rings substituted. This outcome might encourage future pyrimidine structure development for improved pharmacotherapeutic outcomes.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

NMR: Nuclear magnetic resonance; **LC-MS:** Liquid chromatography-Mass spectrometry; **FT-IR:** Fourier Transform infrared spectroscopy; **MIC:** Minimum inhibitory concentration; **TMS:** Tetramethylselane; **TLC:** Thin layer chromatography.

SUMMARY

Thioxopyrimidine (2) was synthesized by condensation of 4-phenylbut-3-en-2-one (1) with thiourea in alkaline medium bacterial growth was shown to be strongly inhibited by the compounds 5a (Fluoro substituted) and 5e (nitro substituted). This outcome might encourage future pyrimidine structure development for improved pharmacotherapeutic outcomes.

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