RESEARCH PAPER

"An Efficient One Pot Synthesis And Antioxidant Activity of 6-(Benzylthio)-2*H*-Pyrazolo[3,4-*d*]Pyrimidine-3,4-Diamine"

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Abstract
A series of novel 6-(benzylthio)-2*H*-pyrazolo[3,4-*d*]pyrimidine-3,4-diamine (5a-j) have been designed and synthesized by condensation of 4-amino-2-(benzylthio)-6-(methylthio)pyrimidine-5-carbonitrile (3) with various hydrazine derivatives (4a-j) by using catalyst as anhydrous potassium carbonate and DMF solvent. Compound (3) was synthesized by reaction of S-benzylthiuronium chloride (1) and 2-(bis(methylthio)methylene malononitrile (2) under similar reaction condition which is used for title compounds. The newly synthesized compounds were characterized by IR, ^{1}H-NMR, ^{13}C-NMR and Mass spectral analysis. Additionally, the antioxidant activity of these synthetic substances was examined. Antioxidant activity results indicate that the majority of the substances exhibited strong action. This procedure gives the advantages of mild reaction condition, easy work up, short reaction time and high yield.

Keywords: S-benzylthiuronium chloride, anhydrous potassium carbonate, antioxidant activity and 2-(bis(methylthio)methylene malononitrile.

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Introduction

Numerous biologically active natural products and synthesized medicinal products include nitrogen-containing heterocyclic nucleus, making nitrogen heterocyclic compounds one of the most important groups of molecules in both medical and pharmaceutical chemistry. Nitrogen atoms strengthen the ring system's electrical characteristics, polarity, capacity to form hydrogen bonds, and ability to interact with biological receptors, enzymes, and nucleic acids [1-5]. It has been found that heterocyclic compounds with pyrazolopyrimidine skeletons as pharmacophores are extremely adaptable and powerful in unfolding pharmacologically active lead molecules. There are a number of known isomers of pyrazolopyrimidine, the most significant of which are 1*H*-pyrazolo[3,4-*d]*, 1*H*-pyrazolo[4,3-*d*], and pyrazolo[1,5-*a*]. Most significantly, the basic structure of the adenine moiety in DNA and the pyrazolopyrimidine moiety are similar [6], which motivates researchers to construct other synthetic strategies and assess its biological potency. This molecule's distinctive quality is that all of its isoforms are pharmacologically active and sold as active pharmaceuticals. For instance, pyrazolopyrimidine are sold under the pharmacological class of sedative-hypnotics as Zaleplon and Indiplo, respectively, As well as Allopurinol, Oxypurinol, as an in vivo inhibitor of the two enzymes Xanthine oxidase, and it is also helpful for maintaining uric acid levels in the treatment of kidney stones, gout, and chemotherapy for cancer [7-8]. According to reports, pyrazolopyrimidines also have pharmacological potential as antiviral[9-11], anticoccidials[12-13], antimicrobial[14-17], antitumor[18-19], tuberculostatic[20], antileishmanial[21], anti-inflammatory[22], antioxidant[23], CNS agents[24], antidiabetic[25]. Our research group have reported synthesis and antimicrobial activity of fused benzo[4,5]thiazolo[3,2-*a*]pyrazolo[3,4-*d*]pyrimidine derivatives[26] and Synthesis and antioxidant study of substituted pyrazolo[3,4-*d*[pyrimidine derivatives[27]. According to the literature, the pharmacological properties of the pyrazolo[3,4-*d*]pyrimidine ring system are significantly altered when the 1*H* of the pyrazole is replaced by another bioactive group. In light of this, the goal of the current study is to synthesize novel pyrazolo[3,4-*d*]pyrimidine derivatives and assess their antimicrobial and antioxidant activity.

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Material and Methods

All compounds were purchased from Loba, SD-Fine and Spectrochem chemical companies and used without any additional purification. Melting points of synthesized compounds were recorded by Electrothermal IA 9000 SERIES digital melting point apparatus and were uncorrected. Purity of all the compounds were routinely checked by thin layer chromatography (TLC) on pre-coated sheets of silica gel-C plates of 0.25 mm thickness. FT-IR spectra were recorded in Nujol or as KBr pallets on infrared spectrophotometer. Brukner

advance spectrophotometer 400 MHz was used to record ^{1}H-NMR and ^{13}C-NMR spectra using tetramethylsilane (TMS) as internal standard, Mass spectra were recorded on FT-VC-7070 H Mass spectrometer using the EI technique at 70 eV.

General procedure:

Synthesis of 4-amino-2-(benzylthio)-6-(methylthio) pyrimidine-5-carbonitrile (3)

A mixture S-benzylthiuronium chloride (1) (0.01mol) and 2-(bis(methylthio)methylene malononitrile (2) (0.01mol) in 10 ml of DMF and anhydrous potassium carbonate (10mg) was refluxed for 3 hours. The reaction progress was monitored by thin layer chromatography (TLC) by using ethyl acetate:hexane (3:7) as solvent. After completion of reaction, the reaction mixture was allowed to cool at room temperature and transferred in to ice cold water. The separated solid product was filtered, washed with water and recrystallized from ethanol to give pure compound (3).

Synthesis of 6-(benzylthio)-2*H*-pyrazolo[3,4-*d*] pyrimidine-3,4-diamine (5a-j).

As per scheme-2, a mixture of 4-amino-2-(benzylthio)-6-(methylthio) pyrimidine-5-carbonitrile (3) (0.001mol) and various hydrazines derivatives (4a-j) (0.001mol) were separately refluxed in 10 ml of DMF and anhydrous K_2CO_3 (10mg) for 4-6 hours. After completion of reaction, the reaction mixture was allowed to cool at room temperature and transferred in to ice cold water. The separated solid product was filtered, washed with water and recrystallized from ethanol to give pure compound (5a-j).

Table 1. Physicochemical data.

Comp. No.	Molecular Formula	Mol. Wt.	Colour	Yield %	M.P °C
3	$C_{13}H_{12}N_4S_2$	288	Yellow	85.10	171-173
5a	$C_{12}H_{12}N_6$ S	272	Brown	75.05	183-185
5b	$C_{18}H_{16}N_6S$	348	Brown	79.12	167-170
5c	$C_{18}H_{14}N_8O_4S$	438	Brown	63.29	171-173
5d	$C_{13}H_{13}N_7S_2$	331	Yellow	67.75	219-221
5e	$C_{13}H_{13}N_7OS$	315	Brown	62.26	225-227
5f	$C_{19}H_{15}N_7S_2$	405	Brown	67.33	165-167
5g	$C_{19}H_{14}N_8O_2S_2$	450	Brown	58.08	154-156
5h	$C_{20}H_{17}N_7S_2$	419	Brown	64.44	231-233
5i	$C_{21}H_{19}N_7S_2$	433	Brown	68.00	198-200
5j	$C_{19}H_{18}N_6S$	362	Brown	71.82	214-213

Spectral Analysis

4-amino-2-(benzylthio)-6-(methylthio)pyrimidine-5-carbonitrile (3).

IR (KBr/cm^{-1}) 2216 (CN), 3154, 3355 (NH_2): ^{1}HNMR (400 MHz,DMSO-d6, ppm): δ = 2.54 (s, 3H, SCH_3), δ = 4.40 (s, 2H, -SCH_2-Ar) 7.80 (s, 2H, NH_2), 7.24-7.43 (m, 5H Ar-H): ^{13}C NMR (DMSO-d6,ppm): δ = 12.16 (SCH_3), δ= 34.13 (SCH_2), 80.59 (C-CN), 114.58 (CN), 161.46 (C-NH_2), 171.90 (C-SCH_3), 172.16 (-S-C=N), 137.64, 129.34, 128.86, 127.30, 127.09 (Ar-C) EI-MS(m/z: RA%): 287 (M-1).

6-(benzylthio)-2*H*-pyrazolo[3,4-*d*]pyrimidine-3,4-diamine (5a).

IR (KBr/cm^{-1}) 3356.27 and 3155.95 (NH_2): ^{1}HNMR (400 MHz,DMSO-d_6): δ 4.40 (s, 2H, -SCH_2-Ar) δ 6.49 (s, 2H, NH_2), 7.81 (s, 2H, NH_2), 7.26-7.43 (m, 5H, Ar-H), 12.60 (s, 1H, NH): EI-MS (m/z: RA%): 272 (M+).

6-(benzylthio)-2-phenyl-2*H*-pyrazolo[3,4-*d*]pyrimidine-3,4-diamine (5b).

IR (KBr/cm^{-1}) 3314.31 and 3024.07 (NH_2): ^{1}HNMR (400 MHz,DMSO-d6, ppm): δ 4.38 (s, 2H, -SCH_2-Ar) δ 7.08 (s, 2H, NH_2), 7.86 (s, 2H, NH_2), 7.24-7.54 (m, 10H, Ar-H): EI-MS (m/z: RA%): 347 (M-1).

6-(benzylthio)-2-(2,4-dinitrophenyl)-2*H*-pyrazolo[3,4-*d*]pyrimidine-3,4-diamine (5c).

IR (KBr/cm^{-1}) 3220.20 and 3348.43 (NH_2): ^{1}HNMR (400 MHz,DMSO-d6, ppm): δ 4.36 (s, 2H, -SCH_2-Ar), δ 6.27 (s, 2H, NH_2), 7.01 (s, 2H, NH_2), 7.25-8.81 (m, 8H, Ar-H): EI-MS (m/z: RA%): 438 (M^+).

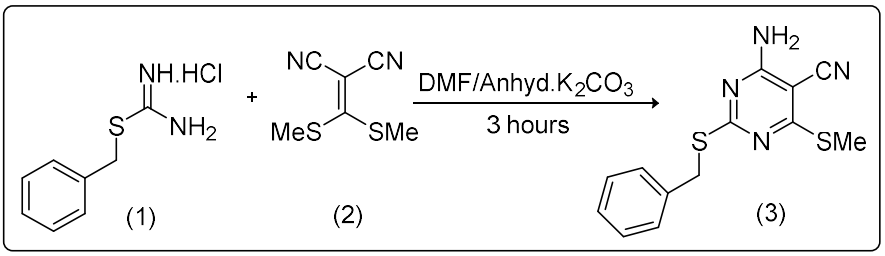
2-(benzo[d]thiazol-2-yl)-6-(benzylthio)-2*H*-pyrazolo[3,4-*d*]pyrimidine-3,4-diamine (5f).

IR (KBr/cm^{-1}) 3210.52 and 3383.64 (NH_2): ^{1}HNMR (400 MHz, DMSO-d_6 ppm): δ 4.54 (s, 2H, -SCH_2-Ar) δ 7.11 (s, 2H, NH_2), 8.14 (s, 2H, NH_2), 7.25-7.71 (m, 9H, Ar-H): EI-MS (m/z: RA%): 404 (M-1).

Result and Disscussion

We discovered that 4-amino-2-(benzylthio)-6-(methylthio) pyrimidine-5-carbonitrile (3) is a crucial step for the production of pyrazolopyrimidines when synthesizing a variety of heterocyclic compounds. Consequently, a series of 6-(benzylthio)-2*H*-pyrazolo[3,4-*d*] pyrimidine-3,4-diamine (5a-j) have been produced. S-benzylthiuronium chloride (1) and bis(methylthio)methylene malononitrile (2) were condensed in DMF and 7–10 mg of anhydrous K_2CO_3 to produce the crucial intermediate (3) Scheme-1. Spectral evidence was used to determine the structure of a molecule (3). Strong bands are seen in the IR spectrum at 2216 cm^{-1}, which is caused by –CN stretching, and 3355 and 3154 cm^{-1}, which are caused by symmetric and asymmetric stretching absorption. Due to the –SCH_3 group, the NMR spectrum displays a singlet at 2.54 ppm, a broad singlet at 7.8 ppm for -NH_2, and an aromatic

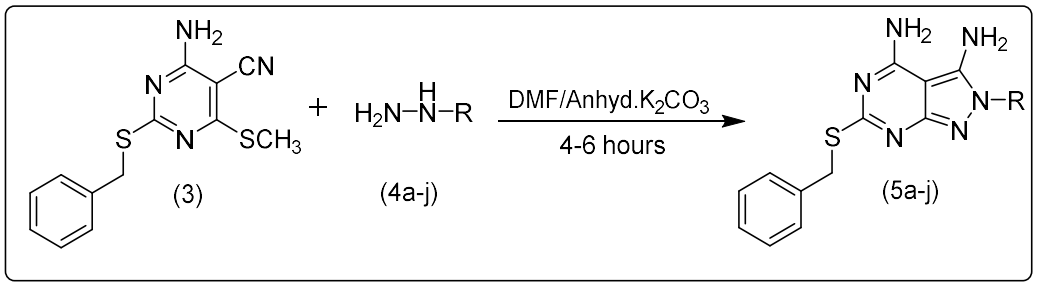
proton at 7.24–7.43 ppm. Ten peaks were appeared in C^{13} NMR spectrum which is corresponding to ten equivalent set carbon atoms in compound (3). Mass spectrum shows the molecular ion peak at 287 (M-1) which in agreement to molecular weight of compound (3).



Scheme-1. Formation of 4-amino-2-(benzylthio)-6-(methylthio) pyrimidine-5-carbonitrile (3).

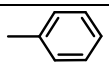
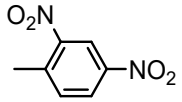
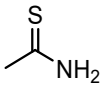
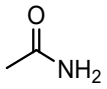
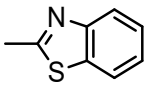
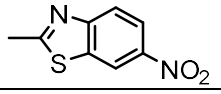
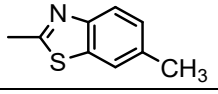
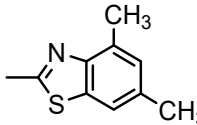
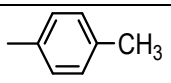
The molecule (3) has an electron-withdrawing cyano group at position five and an interchangeable active thiomethyl group at position six. Because compound (3) has an electrophilic center cyano group and a good leaving thiomethyl group, it is susceptible to nucleophilic substitution-cyclization. Under comparable experimental conditions, compound (3) independently condensed with different hydrazine derivatives (4a-j) to get 6-(benzylthio)-2*H*-pyrazolo[3,4-*d*] pyrimidine-3,4-diamine(5a-j).

Scheme-2



Scheme-2. Formation of 6-(benzylthio)-2*H*-pyrazolo[3,4-*d*]pyrimidine-3,4-diamine derivatives (5a-j).

Table 2. Compound numbers and various substituents.

Comp. No.	R	Comp. No.	R
5a	—H	**5b**	
5c		**5d**	
5e		**5f**	
5g		**5h**	
5i		**5j**	

Physical and spectral (IR, ^{1}H-NMR, and MS) data were used to describe all of the produced compounds (5a-j). These compounds' spectral analyses match the provided structures.

Antioxidant Activity

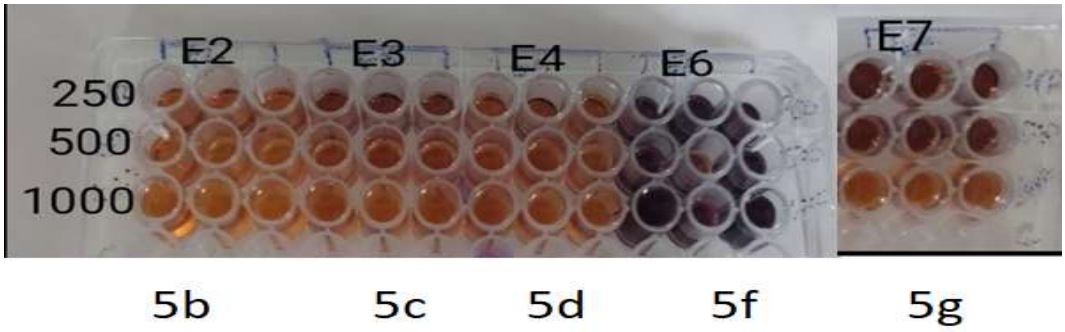
DPPH radical scavenging assay :

DPPH (2,2-diphenyl-1-1picrylhydrazyl) is a stable free radical with a deep violet color, Antioxidants reduce DPPH, causing a color change from violet to yellow, which can be measured spectrophotometrically at 517 nm.

Procedure:
Add 100 µL of each test sample or standard into wells in triplicates. Add 100 µL of freshly prepared 0.1 mM DPPH solution to each well (Except Blank). Add 100 µL methanol to blank wells (no DPPH). Cover plate with aluminium foil and incubate in the dark for 30 minutes at room temperature. Read absorbance at 517 nm using microplate reader. The result of DPPH reduction is summarized in table-3.

Table 3. Antioxidant activity of selected compounds

Sr No.	Sample Code	Conc µg/ml	OD			Mean	Percent inhibition
1	Control	-	1.456	1.564	1.485	1.502	--
2	**Standard (Ascorbic Acid)**	250	0.339	0.334	0.332	0.335	77.69
		500	0.311	0.299	0.298	0.303	79.84
		1000	0.208	0.208	0.211	0.209	**86.08**
3	**5b**	250	0.456	0.444	0.451	0.450	70.01
		500	0.421	0.411	0.412	0.415	72.36
		1000	0.389	0.388	0.387	0.237	**74.20**
4	**5c**	250	0.452	0.438	0.432	0.441	70.65
		500	0.356	0.347	0.344	0.349	76.75
		1000	0.269	0.267	0.266	0.267	**82.19**
5	**5d**	250	0.567	0.574	0.574	0.572	61.93
		500	0.564	0.545	0.554	0.554	63.08
		1000	0.523	0.521	0.518	0.521	**65.32**
6	**5f**	250	0.689	0.699	0.688	0.692	53.91
		500	0.652	0.657	0.674	0.661	55.98
		1000	0.589	0.597	0.588	0.591	**60.62**
7	**5g**	250	0.689	0.699	0.688	0.692	53.91
		500	0.652	0.657	0.674	0.661	55.98
		1000	0.589	0.597	0.588	0.591	**60.62**



Discussion:
The present study evaluated the antioxidant potential of different fractions of given sample extract using the DPPH free radical scavenging assay in a 96-well plate method. The results clearly demonstrate a concentration dependent increase in antioxidant activity across all tested samples, indicating the presence of bioactive phytoconstituents capable of donating hydrogen or electrons to neutralize DPPH radicals.
The standard antioxidant, ascorbic acid, showed strong radical scavenging activity ranging from 77.69% at 250 µg/ml to 86.08% at 1000 µg/ml, confirming the validity of the assay and serving as a benchmark for comparison.

Among the tested extracts:

- 5c (SBTR-II-E3) exhibited the highest antioxidant activity among all samples, with inhibition increasing from 70.65% (250 µg/ml) to 82.19% (1000 µg/ml). This suggests that this fraction contains a higher concentration of active antioxidant compounds such as phenolics and flavonoids.
- 5g (SBTR-II-E7) showed better activity from70.94% (250 µg/ml) to 75.91% (1000 µg/ml), which may indicate sample variability, extraction inconsistency, or fraction heterogeneity.
- 5b (SBTR-II-E2) demonstrated moderate activity (70.01% to 74.20%), suggesting the presence of antioxidant constituents but at relatively lower concentrations or potency.
- 5d (SBTR-II-E4) and 5f (SBTR-II-E6) showed comparatively lower antioxidant activity, with maximum inhibition values of 65.32% and 60.62% respectively, indicating weaker radical scavenging capacity.

Overall, the antioxidant activity trend among the samples can be summarized as:

Ascorbic Acid > 5c (SBTR-II-E3) > 5g (SBTR-II-E7)> 5b (SBTR-II-E2) > 5d (SBTR-II-E4) > 5f (SBTR II-E6)

The observed antioxidant activity is likely attributed to the presence of phytochemicals such as flavonoids, phenolic acids, tannins, and other secondary metabolites, which are known for their redox properties and ability to scavenge free radicals.

Antioxidant Conclusion:

The DPPH radical scavenging assay revealed that all tested fractions of 6-(benzylthio)-2*H*-pyrazolo[3,4-*d*]pyrimidine-3,4-diamine derivatives possess antioxidant activity, though with varying potency. Among them, 5c (SBTR-II-E3) exhibited the most significant antioxidant potential, showing activity comparable to the standard ascorbic acid at higher concentrations. This suggests that 5c (SBTR-II-E3) is the most promising fraction for further phytochemical isolation and characterization of active antioxidant compounds.

Antimicrobial Activity

The antimicrobial activity is estimated by comparing the inhibition of growth of sensitive micro- organisms produced by known concentration of the isolated substance or extract or synthetic compound to be examined against a reference substance.

Methods of Analysis:

Two general methods usually employed; One is the cup-plate method [Agar well diffusion method)-The agar cup plate method depends upon diffusion of the antibiotic from a vertical agar [well] Cylinder through a solidified agar layer on a Petri dish. Sterile Agar is inoculated by suspension of the microbial inoculum. Then well with diameter of 6 to 8 mm is punched aseptically, and then of the antimicrobial solution at desired concentration is introduced into the well. Then, agar plates are incubated under suitable conditions depending upon the test microorganism. The antimicrobial agent diffuses in the agar medium and inhibits the growth of the microbial strain entirely in a zone around the cylinder containing a solution of the substance to be tested.

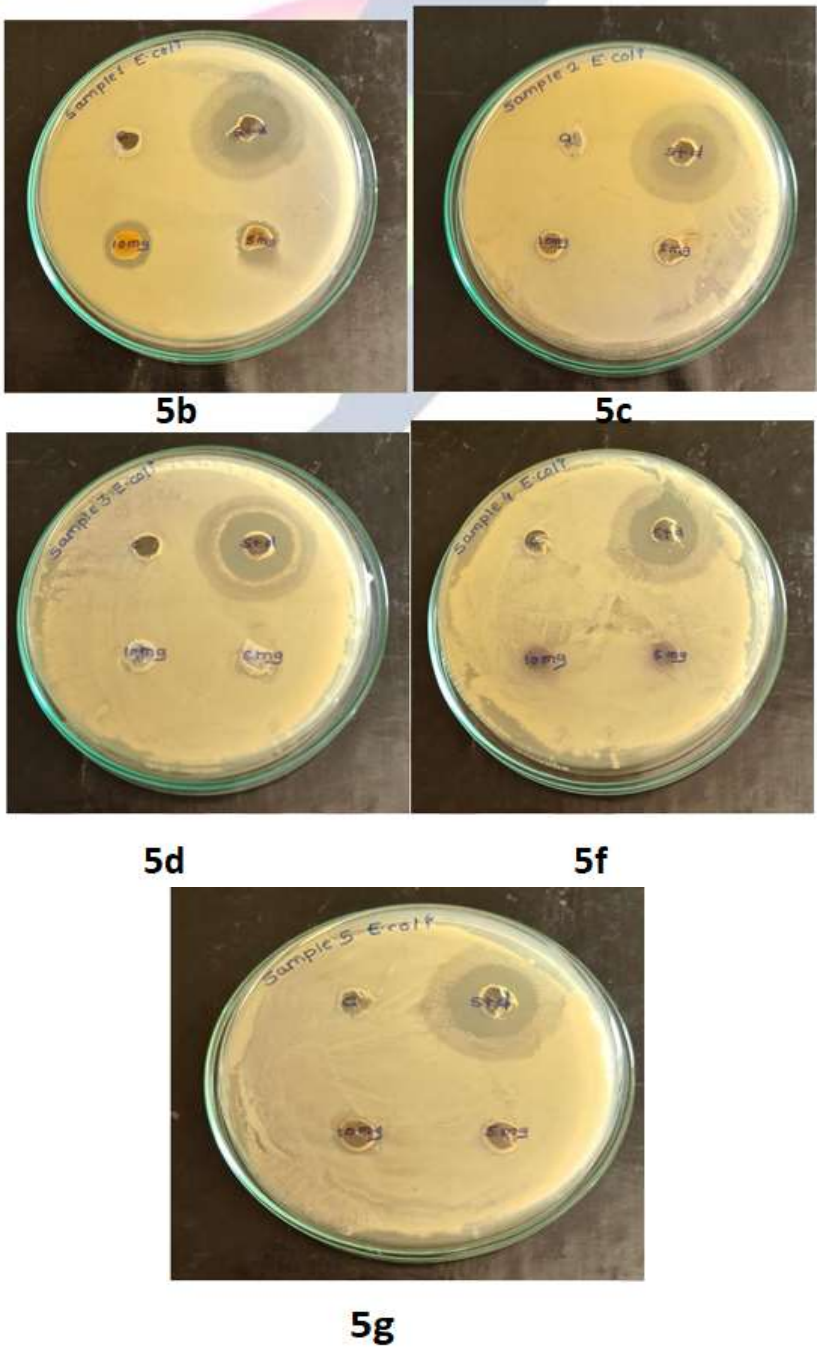
Table 4: Antimicrobial Activity

Sr No.	Sample	Concentration	Zone of Inhibition (mm) S. Aureus (ATCC no-6538)	Zone of Inhibition (nm) E. Coli (ATCC no-8739)
	Control	**--**	**--**	**--**
	Standard Streptomycin	**01 mg/ml**	**35**	**32**
1	**5b** (SBTR-II E2)	**05 mg/ml**	**12**	**06**
		10 mg/ml	**18**	**10**
2	**5c** (SBTR-II E3)	**05 mg/ml**	**02**	**01**
		10 mg/ml	**04**	**02**
3	**5d** (SBTR-II E4)	**05 mg/ml**	**01**	**01**
		10 mg/ml	**04**	**02**
4	**5f** (SBTR-II E6)	**05 mg/ml**	**01**	**01**
		10 mg/ml	**02**	**02**
5	**5g** (SBTR-II E7)	**05 mg/ml**	**01**	**01**
		10 mg/ml	**03**	**03**

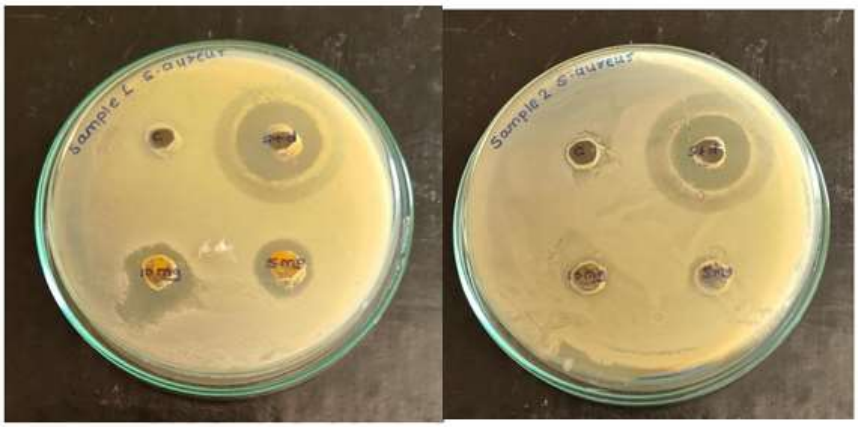
Antimicrobial Conclusion:

The antibacterial activity study demonstrated that all tested samples exhibited varying degrees of inhibitory effects against Staphylococcal Infection and Escherichia coli Infection. The standard antibiotic Streptomycin showed the highest antibacterial activity, producing zones of inhibition of 35 mm against S. aureus and 32 mm against E. coli, thereby validating the experimental method. Among the tested samples, antibacterial activity generally increased with increasing concentration from 5 mg/ml to 10 mg/ml, indicating concentration-dependent antimicrobial efficacy. Samples 5b, 5c, 5d, 5f and 5g demonstrated comparatively moderate or low antibacterial activity. In particular, sample-1(5b-SBTR-II E2) exhibited the highest activity among all test samples, showing zones of inhibition of 18 mm against S. aureus and 10 mm against E. coli at 10 mg/ml. Samples 5c, 5d, 5f and 5g also showed low antibacterial effects, with inhibition zones ranging between 02–04 mm for both bacterial strains at higher concentration. Overall, the findings indicate that several of the tested formulations possess promising moderate or low antibacterial activity against both Gram-positive and Gram-negative bacteria.

Escherichia Coli ATCC no-8739

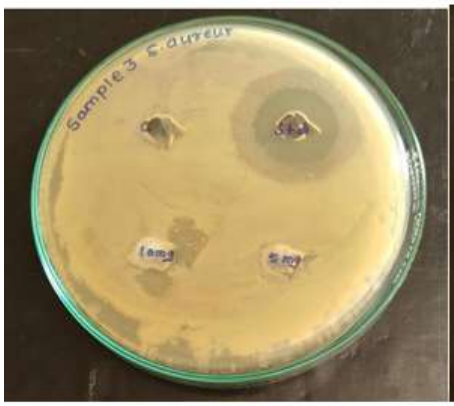


Staphylococcus aureus ATCC no-6538



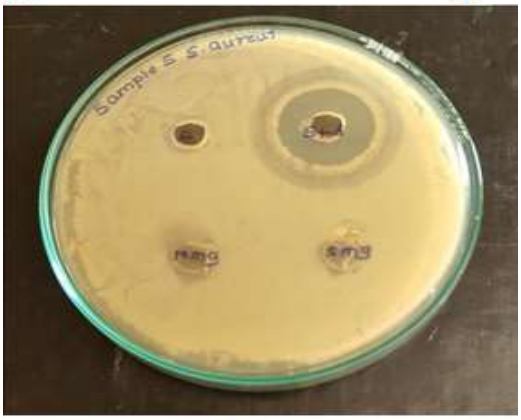
5b

5c



5d

5f



5g

Conclusion

In conclusion, we have proven the synthesis, characterisation, and antioxidant activity of novel heterocyclic compounds such 6-(benzylthio)-2*H*-pyrazolo[3,4-*d*]pyrimidine-3,4-diamine (5a-j) in order to make a significant contribution to heterocyclic chemistry innovation, were acquired using an easy method with a high yield of product. 6-(benzylthio)-2*H*-pyrazolo[3,4-*d*]pyrimidine-3,4-diamine up, mild reaction conditions, and product purity

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and Simson life Sciences Pvt. Ltd., Hyderabad, for providing spectra.

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