Original Research Article

Synthesis, antimicrobial activity, and docking study of some N^3, N^6-diphenylpyridazine-3,6-diamine derivatives as dihydrofolate reductase inhibitors

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Abstract

Objective: The present study focussed on the synthesis of pyridazine analogs to explore broad-spectrum antimicrobial study. Since pyridazine analogs are not conventionally found in nature, and hence, its analogs are studied later.

Materials and Methods: All the synthesized compounds were characterized by spectroscopic techniques, namely, UV, IR, ^{1}HNMR, and mass spectrometry. Antimicrobial activity was screened by serial dilution method and absorbance was recorded using ELISA reader, subsequently minimum inhibitory concentrations were determined. Docking study was done into the active site of dihydrofolate reductase using Auto Dock 4.2.

Results: The present investigation about synthesis, characterization, and biological studies of some new pyridazine analogs were carried out to obtain potent and pharmacologically active compounds. The free energy of binding was in the range of −5.12 to −8.97 kcal/mole. *In silico* study report was in good tune with laboratory experiments.

Conclusions: Most of the compounds were moderate-to-good toward the antimicrobial activity. Compound AJ27 was found to be most active. Results of anti-microbial activity establishes the importance of N^3, N^6-diphenylpyridazine-3,6-diamine as the basic skeleton required for the antimicrobial activity.

Keywords: Antimicrobial activity, biphenyl, dihydrofolate reductase inhibitors, docking study, pyridazine analogs

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INTRODUCTION

Antimicrobials are the broad classes of substances acting against microorganisms.[1,2] Resistance to antimicrobial agents has been encountered as a major hindrance in clinical therapy and cure of many diseases and deserves scientific intervention to bring about some control measures.[3,4] Several drug discovery strategies have evolved, including incremental improvements to existing antibiotics by chemical manipulation and the search for novel drug targets based on genomic approaches. There is a need to explore new chemical moieties for developing new drugs.[3,5] Pyridazine derivatives are new and important compound of heterocyclic nature, containing two nitrogen atoms at 1 and 2 positions in a six-membered ring. A substantial numbers of drug molecules used in medicine contain a

Access this article online	
Quick Response Code:	Website: www.jpionline.org
	DOI: 10.4103/jphi.JPHI_37_18

How to cite this article: Javed A, Sapra S, Alam A. Synthesis, antimicrobial activity, and docking study of some N^3, N^6-diphenylpyridazine-3,6-diamine derivatives as dihydrofolate reductase inhibitors. Int J Pharma Investig 2018;8:138-44.

phenyl ring. Replacing these phenyl rings with Pyridazine rings opens an access to several thousands of diaza analogs, presenting more interaction possibilities, lower Log *P* values, improved crystalline salts, and more hydrogen bonding possibilities.[6,7]

Pyridazines derivatives are class of heterocyclic compound that is of substantial interest because of the diverse range of their biological activity.[8] Pyridazine derivatives possess a wide range of activities such as antimicrobial,[9] antifungal,[10] antiviral,[11] anti-protozoan,[12] anti-inflammatory[13] diuretic,[14] antimalarial,[15] antitubercular,[16] and many others.[17] The pyridazine structure is found within some herbicides such as credazine, pyridafol, pyridate, and maleic hydrazide. Pyridazine is also found within the structure of several drugs, for instance, cefozopran, cadralazine, minaprine, hydralazine, and cilazapril.[18-23] Biphenyl is a neutral molecule, and it is neutral due to the lack of functional group. However, it is present in many natural and pharmacologically active products. The substituted biphenyl derivatives possess a wide range of activity such as anti-hypertensive, antimicrobial, diuretic, anti-diabetic, antipsychotic, anxiolytic, and anti-inflammatory.[24-27]

MATERIALS AND METHODS

Synthesis of hydrazine dihydrochloride

Concentration hydrochloric acid (0.110 moles) was added dropwise to cold hydrazine monohydrate (0.123 moles) with constant stirring. The solution was heated to evaporate water. The solution was brought back to room temperature and concentration HCl (0.113 moles) was again added under stirring in cold and precipitate formed was filtered and washed with methanol.[28]

Synthesis of 3,6-pyridaze diol (BM1)

A volume of 50 ml of water was added to 0.005 moles of hydrazine dihydrochloride. Then, 0.005 moles of maleic anhydride was added. The solution was kept in the boiling conditions for 3 h.[21]

Synthesis of 3,6-dichloropyridazine (BM2)

0.0446 moles of 3,6-pyridazine diol was refluxed with the excess of phosphorous oxychloride (3.2 moles) for 5 h. Excess reagent was removed by distillation. Then, it was put into cold water. Filters were used for filtration. Finally, the product was separated by removing water to obtain the pure product.[29]

General procedure for the synthesis of N^3, N^6-BIS Pyridazine-3,6-diamine analogs (AJ11-AJ30).

1×10^{-3} mole of 3,6-dichlopyridazine was mixed with 2×10^{-3} mole of variously substituted anilines. The reaction was refluxed for 8 h in toluene. The reaction mixture was assessed using thin-layer chromatography (TLC) and toluene was removed under negative pressure [Table 1]. The final product was recrystallized in methanol [Figure 1].

Antimicrobial activity

Microbial strains used in the present study

Gram-negative bacteria: *Staphylococcus aureus, Micrococcus luteus*, *Escherichia coli*. Gram-positive bacteria: *Pseudomonas aeruginosa, Pseudomonas aurantiaca*. Fungi: *Trichophyton rubrum, Cryptococcus laurentii*.

Test procedure

The nutrient broth was used for the anti-microbial activity. 96 well plates were used for microdilution assay to estimate the minimum inhibitory concentration. A volume of 100 µl double strength media was poured in the first row and in remaining well 100 µl single strength media was poured. In the first row, 100 µg/ml of concentrated solution was added and further serial dilutions were made till ninth well so the concentration is as follows: 1000 µg/ml, 500 µg/ml, 250 µg/ml, 125 µg/ml, 62.5 µg/ml, 31.25 µg/ml, 15.6 µg/ml, 7.8 µg/ml, and 3.9 µg/ml.

The experiment was performed in triplicates. Inoculums of wells were done with 50 µl of standard inoculums of the test organism. Positive and negative controls were prepared in triplicates in remaining columns.

The plates were incubated at 37°C for 24 h and plates were read in the ELISA plate reader at 595 nm. Standard drugs used were ciprofloxacin and ketoconazole. The optical density of growth in each well was observed and then MIC was calculated.

Preparation of inoculums

For the preparation of inoculums automated media, i.e., The nutrient broth was taken to laminar air flow bench, and microbiological test tubes were half filled with media and then standard microbial stains were inoculated in that media and incubated at 37°C for 24 h.

Docking study

A library of 20 N^3, N^6-diphenylpyridazine-3,6-diamine derivatives was prepared using ChemDraw ultra 8.0. The 3D crystal structure of the di hydrofolate reductase (DHFR) was obtained from Protein Data Bank (PDB codes: 7 DFR). The 3D structures of DHFR, (from 7DFR) were used for the virtual screening. The co-crystallized DHFR and water molecules were removed, and Auto Dock 4.2 was used for molecular docking studies. Docking parameters were set to default values on the basis of Lamarckian

genetic algorithm principle. The 20 compounds with the highest estimated free energy of binding were docked into the active site of DHFR [Table 2]. Synthesized compounds were biologically evaluated for antimicrobial study, and attempt was made to establish the structure-activity relationship of the compounds to explore new lead compounds [Figure 2].

RESULTS

All the compounds were synthesized as per the standard procedure of schemes. Log*P* value was determined using UV spectrophotometer, and the data are presented.

Spectral characterizations

Spectral data of synthesized compounds (AJ-11–AJ-30)

AJ11 IR (KBr, cm^{-1}): 3365 (NH), 3059 (C-H Stretch Aromatic), 1644 (C = N). ^{1}H NMR (400MHz, DMSO-d_6) δ, 6.80 (d, *J* = 11.2 Hz, 1H), 6.97 (m, 4H), 7.25 (d, *J* = 11.2 Hz, 1H), 7.39 (d, *J* = 8.2 Hz, 4H), 7.74 (m, 2H), 7.92 (s, 1H, NH), 13.7 (s, 1H, NH). Analytical calculated value for $C_{16}H_{14}N_4$: C, 73.26; H, 5.38; N, 21.36. Analytical Observed value for $C_{16}H_{14}N_4$: C, 73.29; H, 5.45; N, 21.39. m/z: 262.12.

AJ12 IR (KBr, cm^{-1}): 3413 (NH), 2921 (C-H Aromatic). ^{1}H NMR (400MHz, DMSO-d_6): δ 6.90 (d, *J* = 9.4 Hz, 1H), 7.38 (d, *J* = 9.4 Hz, 1H), 7.60 (s, 1H, NH), 7.88 (s, 4H), 13.14 (s, 1H, NH). Analytical Calculated value for $C_{16}H_{10}N_4Br_6$: C, 26.12; H, 1.10; N, 7.62, Br, 65.17. Analytical Observed value for$C_{16}H_{10}N_4Br_6$: C, 26.09; H, 1.06; N, 7.58, Br, 65.12. m/z: 730.

AJ13 IR (KBr, cm^{-1}): 3316 (NH), 1596 (C = N), 1555 and 1298 (C-NO_2). ^{1}H NMR (400MHz, $CDCl_3$):δ 6.87 (d, *J* = 8.1 Hz, 2H, 5'-H), 6.93 (d, *J* = 10.1 Hz, 1H), 7.27 (d, *J* = 10.1 Hz, 1H), 7.36 (m, 2H, 4'-H), 7.41 (s, 2H, 1'-H), 7.59 (d, *J* = 7.6 Hz, 2H, 3'-H), 8.3 (s, 1H, NH), 14.2 (s, 1H, NH). Analytical calculated value for $C_{16}H_{12}O_4N_6$: C, 54.55; H, 3.43; N, 23.85; O, 18.17. Analytical observed value for $C_{16}H_{12}O_4N_6$: C, 54.48; H, 3.39; N, 23.79; O, 18.22. m/z: 352.

AJ14 IR (KBr, cm^{-1}): 3984 (NH), 1588 and 1298 (C-NO_2). ^{1}H NMR (400MHz, DMSO-d_6): δ 6.62 (d, *J* = 7.2 Hz, 4H, 1'-H), 6.93(d, *J* = 9.1 Hz, 1H) 7.29 (d, *J* = 9.1 Hz, 1H), 7.94 (d, *J* = 8.2 Hz, 4H, 2'-H), 8.4 (s, 1H, 1H), 14.03 (s, 1H, 1H). Analytical calculated value for $C_{16}H_{12}O_4N_6$: C, 54.55;H, 3.43; N, 23.85; O, 18.17. Analytical observed value for$C_{16}H_{12}O_4N_6$: C, 54.49;H, 3.39; N, 23.89; O, 18.23 m/z: 352.

AJ15 IR (KBr, cm^{-1}): 3119 (NH), 2988 (C-H Aromatic), 1659 (C = N), 784 (C-Cl). ^{1}H NMR (400MHz, $CDCl_3$): δ 6.43 (d, *J* = 7.6 Hz, 2H, 5'-H), 6.60 (m, 2H, 4'-H), 6.86 (d, *J* = 9.4 Hz, 1H), 6.91 (m, 2H, 3'-H), 7.09 (d, *J* = 7.2 Hz, 2H, 2'-H), 7.26 (d, *J* = 9.4 Hz, 1H), 8.6 (s, 1H, NH), 12.6 (s, 1H, NH). Analytical calculated value for $C_{16}H_{12}N_4Cl_2$: C, 58.02; H, 3.65; N, 16.92; Cl, 21.41. Analytical observed value for $C_{16}H_{12}N_4Cl_2$: C, 58.09; H, 3.68; N, 16.86; Cl, 21.35. m/z: 330, 334.

AJ16 IR (KBr, cm^{-1}): 3109 (NH), 2994 (C-H), 1654 (C = N), 790 (C-Cl). ^{1}H NMR (400MHz, $CDCl_3$): δ 6.39 (d, *J* = 7.3 Hz, 2H, 5'-H), 6.52 (s, 2H, 1'-H), 6.72 (d, *J* = 7.9 Hz, 2H, 3'-H), 6.89 (d, *J* = 9.8 Hz, 1H), 7.04 (m, 2H, 4'-H), 7.27 (d, *J* = 9.8 Hz, 1H), 8.5 (s, 1H, NH), 11.4 (s, 1H, NH). Analytical calculated value for $C_{16}H_{12}N_4Cl_2$: C, 58.02; H, 3.65; N, 16.92; Cl, 21.41. Analytical observed

Table 1: Various substitutions at benzene ring

Compound code	R_1	R_2	R_3	R_4	R_5
AJ11	H	H	H	H	H
AJ12	Br	H	Br	H	Br
AJ13	H	NO_2	H	H	H
AJ14	H	H	NO_2	H	H
AJ15	Cl	H	H	H	H
AJ16	H	Cl	H	H	H
AJ17	H	H	Cl	H	H
AJ18	H	H	CF_3	H	H
AJ19	Cl	H	Cl	H	Cl
AJ20	Br	H	H	H	H
AJ21	H	Br	H	H	H
AJ22	H	H	Br	H	H
AJ23	Br	H	NO_2	H	NO_2
AJ24	Cl	H	Br	H	H
AJ25	I	H	H	H	H
AJ26	H	H	I	H	H
AJ27	COOH	H	OH	H	H
AJ28	$COOCH_3$	H	OCH_3	OCH_3	H
AJ29	C_2H_5	H	H	H	CH_3
AJ30	CH_3	H	OCH_3	H	H

Table 2: List of estimated free energy of binding of synthesized compounds

Compound code	Estimated free energy of binding (K cal/mol)
AJ-11	−5.72
AJ-12	−7.31
AJ-13	−5.81
AJ-14	−7.02
AJ-15	−6.01
AJ-16	−5.84
AJ-17	−6.89
AJ-18	−5.84
AJ-19	−6.26
AJ-20	−5.92
AJ-21	−6.91
AJ-22	−6.89
AJ-23	−8.97
AJ-24	−5.91
AJ-25	−5.12
AJ-26	−5.50
AJ-27	−8.46
AJ-28	−6.93
AJ-29	−5.34
AJ-30	−5.44

Docking score of synthesized compounds. DHFR: Dihydrofolate reductase

Table 3: Anti-microbial activity of all synthesized compounds

Compound code	MIC (µg/ml)						
	S. a.	*M. l.*	*E. coli*	*P. a.*	*P. aurantiaca*	*T. r.*	*C. l.*
AJ11	1000	NA	1000	1000	NA	NA	1000
AJ12	125	500	250	500	500	125	125
AJ13	500	250	250	500	500	250	500
AJ14	125	62.5	250	500	500	125	250
AJ15	500	250	125	500	1000	250	125
AJ16	500	250	500	250	1000	125	250
AJ17	125	62.5	250	1000	250	500	62.5
AJ18	250	125	500	250	500	500	250
AJ19	500	125	125	500	250	125	500
AJ20	500	62.5	250	1500	1000	125	500
AJ21	62.5	125	62.5	125	250	62.5	31.25
AJ22	125	250	62.5	1000	1000	62.5	625
AJ23	7.8	15.6	31.2	125	125	7.8	15.6
AJ24	250	125	125	500	125	500	250
AJ25	500	250	125	500	500	125	62.5
AJ26	125	62.5	62.5	125	250	62.5	62.5
AJ27	62.5	125	250	125	62.5	125	500
AJ28	1000	NA	NA	1000	NA	NA	1000
AJ29	NA	1000	1000	1000	500	1000	NA
AJ30	1000	NA	1000	1000	1000	NA	NA
CPF	7.8	7.8	7.8	7.8	7.8	-	-
KTZ	-	-	-	-	-	7.8	7.8

Gram-negative - *S. a.: Staphylococcus aureus, M. l.: Micrococcus luteus, E. coli: Escherichia coli.* Gram-positive - *P. a.: Pseudomonas aeruginosa, P. aurantiaca: Pseudomonas aurantiaca. Fungi - T. r.: Trichophytonrubrum, C. l.: Cryptococcus laurentii.* CPF: Ciprofloxacin, KTZ: Ketoconazole, NA: Not available, MIC: Minimal inhibitory concentration

value for $C_{16}H_{12}N_4Cl_2$:C, 58.01; H, 3.69; N, 16.86; Cl, 21.42. m/z: 330, 334.

AJ17 IR(KBr, cm^{-1}): 3361 (NH), 3156 (C-H Aromatic), 1611 (C = N), 784 (C-Cl). ^{1}H NMR (400MHz, DMSO-d_6):δ 6.50 (d, *J* = 7.2 Hz, 4H, 1'-H), 6.89 (d, *J* = 9.8 Hz, 1H), 7.12 (d, *J* = 7.8 Hz, 4H, 2'-H), 7.27 (d, *J* = 9.8 Hz, 1H), 8.3 (s, 1H, NH), 11.5 (s, 1H, NH). Analytical calculated value for $C_{16}H_{12}N_4Cl_2$: C, 58.02; H, 3.65; N, 16.92; Cl, 21.41. Analytical observed value for $C_{16}H_{12}N_4Cl_2$: C, 58.05; H, 3.62; N, 16.87; Cl, 21.38. m/z: 330, 334.

AJ18 IR(KBr, cm^{-1}): 3316 (NH), 2988 (C-H Aromatic), 1659 (C = N), 1115 (C-F). ^{1}H NMR (400MHz, $CDCl_3$): δ 6.41 (d, *J* = 7.3 Hz, 4H), 6.92 (d, *J* = 10.2 Hz, 1H), 7.31 (d, *J* = 10.2 Hz, 1H), 7.47 (d, *J* = 8.1 Hz, 4H), 8.1 (s, 1H, NH), 13.8 (s, 1H, NH). Analytical calculated value for $C_{18}H_{12}N_4F_6$: C, 54.28; H, 3.04; N, 14.07; F, 28.62. Analytical observed value for $C_{18}H_{12}N_4F_6$: C, 54.32; H, 3.07; N, 14.11; F, 28.56. m/z: 398.

AJ19 IR(KBr, cm^{-1}): 3428 (NH), 3082 (C-H Aromatic), 1614 (C = N), 788 (C-Cl). ^{1}H NMR (400MHz, $CDCl_3$): δ=6.89 (s, 4H, 2',4'-H), 6.95 (d, *J* = 9.8 Hz, 1H), 7.25 (d, *J* = 9.8 Hz, 1H), 8.4 (s, 1H, NH), 12.8 (s, 1H, NH). Analytical calculated value for $C_{16}H_8N_4Cl_6$: C, 40.98; H, 1.72; N, 11.95, Cl, 45.36. Analytical observed value for $C_{16}H_8N_4Cl_6$: C, 40.92; H, 1.66; N, 11.98, Cl, 45.39. m/z: 466.

AJ20 IR(KBr, cm^{-1}): 3355 (NH), 2996 (CH Aromatic), 1670 (C = N), 614 (C-Br). ^{1}H NMR (400MHz, $CDCl_3$): δ 6.39 (d, *J* = 7.5 Hz, 2H, 5'-H), 6.71 (m, 2H, 3'-H), 6.96 (d, *J* = 9.7 Hz, 1H), 7.03 (m, 2H, 4'-H), 7.25 (d, *J* = 2.5 Hz, 2H, 2'-H), 7.36 (d, *J* = 9.7 Hz, 1H), 7.9 (s, 1H, NH), 11.6 (s, 1H, NH). Analytical calculated value for $C_{16}H_{12}N_4Br_2$: C, 45.74; H, 2.88; N, 13.34; Br, 38.04. Analytical observed value for $C_{16}H_{12}N_4Br_2$: C, 45.68; H, 2.82; N, 13.29; Br, 38.10. m/z: 419.9.

AJ21 IR(KBr, cm^{-1}): 3371 (NH), 2992 (C-H Aromatic), 1670 (C = N), 616 (C-Br). ^{1}H NMR (400MHz, $CDCl_3$):δ 6.52 (d, *J* = 7.6 Hz, 2H, 5'-H), 6.73 (s, 2H, 1'-H), 6.86 (m, *J* = 7.8 Hz, 2H, 4'-H), 6.93 (d, *J* = 9.3 Hz, 1H), 6.99 (d, *J* = 2.6 Hz, 2H, 3'-H), 7.32 (d, *J* = 9.3 Hz, 1H), 8.1 (s, 1H, NH), 12.9 (s, 1H, NH).

Analytical calculated value for $C_{16}H_{12}N_4Br_2$: C, 45.74; H, 2.88; N, 13.34; Br, 38.04. Analytical observed value for $C_{16}H_{12}N_4Br_2$. C, 45.68; H, 2.89; N, 13.39; Br, 38.11. m/z: 418.

AJ22 IR(KBr, cm^{-1}): 3439 (NH), 2840 (C-H Aromatic), 1619 (C = N), 668 (C-Br). ^{1}H NMR (400MHz, DMSO-d_6): δ 6.45 (d, *J* = 7.4 Hz, 4H, 1'-H), 6.89 (d, *J* = 9.3 Hz, 1H), 7.25 (d, *J* = 2.7 Hz, 4H, 2'-H), 7.28 (d, *J* = 9.3 Hz, 1H), 8.5 (s, 1H, NH), 13.3 (s, 1H, NH). Analytical calculated value for $C_{16}H_{12}N_4Br_2$: C, 45.74; H, 2.88; N, 13.34; Br, 38.04. Analytical observed value for $C_{16}H_{12}N_4Br_2$: C, 45.78; H, 2.92; N, 13.37; Br, 38.08. m/z: 418.

AJ23 IR(KBr, cm^{-1}): 3447 (NH), 3070 (C-H Aromatic), 1681 (C = N), 1510 and 1339 (C-NO_2) ^{1}H NMR (400MHz, DMSO-d_6): δ 6.97 (d, *J* = 9.3 Hz, 1H), 7.36 (d, *J* = 9.3 Hz, 1H), 7.9 (s, 1H, NH), 8.55 (s, 2H, 4'-H), 8.85 (s, 2H, 2'-H), 12.4 (s, 1H, NH). Analytical calculated value for $C_{16}H_8O_8N_8Br_2$: C, 32.02; H, 1.34; N, 18.67; Br, 26.63; O, 21.33. Analytical observed value for $C_{16}H_8O_8N_8Br_2$: C, 32.08; H, 1.38; N, 18.73; Br, 26.69; O, 21.39 m/z: 598, 602.

AJ24 IR(KBr, cm^{-1}): 3455(NH), 3072(C-H Aromatic), 1603(C = N), 810(C-Cl), 652(C-Br). ^{1}H NMR (400MHz, $CDCl_3$): δ 6.64 (d, *J* = 8.6 Hz, 2H, 5'-H), 6.93 (d, *J* = 9.4 Hz, 1H), 7.19 (d, *J* = 2.5 Hz, 2H, 4'-H), 7.33 (d, *J* = 9.4 Hz, 1H), 7.37 (s, 2H, 2'-H), 7.8 (s, 1H, NH), 11.6 (s, 1H, NH). Analytical calculated value for $C_{16}H_{10}N_4Cl_2Br_2$: C, 39.3; H, 2.06; N, 11.46; Cl, 14.5; Br, 32.68. Analytical observed value for $C_{16}H_{10}N_4Cl_2Br_2$: C, 39.1; H, 2.11; N, 11.51; Cl, 14.2; Br, 32.73. m/z: 486, 494.

AJ25 IR(KBr, cm^{-1}): 3415 (NH), 2901 (C-H aromatic), 1619 (C = N), 418 (C-I). ^{1}H NMR (400MHz, $CDCl_3$): δ 6.41 (m, 2H, 3'-H), 6.59 (d, *J* = 8.0 Hz, 2H, 5'-H),

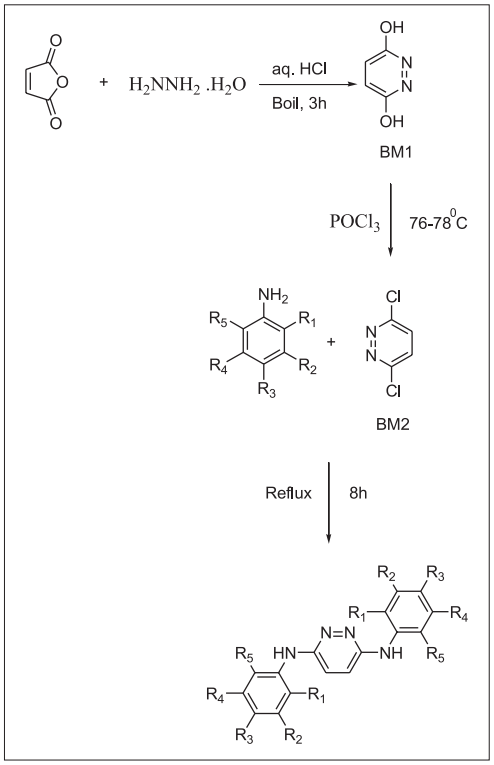

Figure 1: General synthetic scheme

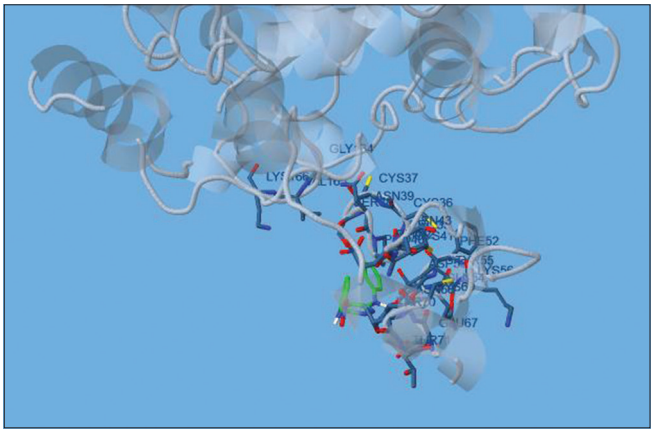

Figure 2: Stereo view of the complex formed by DHFR and the docked compound (AJ-23).The amino acids: GLY34, ASN39, CYS37, LYS 166, CYS36 AND CYS36 were involved in interaction with compounds

6.89 (d, *J* = 9.4 Hz, 1H), 7.15 (m, 2H, 4'-H), 7.28 (d, *J* = 9.4 Hz, 1H), 7.41 (d, *J* = 7.3 Hz, 2H, 2'-H), 8.0 (s, 1H, NH), 11.8 (s, 1H, NH). Analytical calculated value for $C_{16}H_{12}N_4I_2$: C, 37.38; H, 2.35; N, 10.9; I, 49.37. Analytical observed value for $C_{16}H_{12}N_4I_2$: C, 37.33; H, 2.38; N, 10.5; I, 49.34. m/z: 513.9.

AJ26 IR(KBr, cm^{-1}): 3410 (NH), 3042 (C-H Aromatic), 1659 (C = N). ^{1}H NMR (400MHz, DMSO-d_6):δ 6.62 (d, *J* = 8.0 Hz, 4H), 6.97 (d, *J* = 9.6 Hz, 1H), 7.28 (d, *J* = 9.6 Hz, 1H), 7.38 (d, *J* = 8.6 Hz, 4H), 8.3 (s, 1H, NH), 11.9 (s, 1H, NH). Analytical calculated value for $C_{16}H_{12}N_4I_2$: C, 37.38; H, 2.35; N, 10.9; I, 49.37. Analytical observed value for $C_{16}H_{12}N_4I_2$: C, 37.34; H, 2.31; N, 10.5; I, 49.33. m/z: 513.9.

AJ27 IR(KBr, cm^{-1}): 3400-3000 (OH Broad Peak), 1659 (C = O), 1581 (C = N). ^{1}H NMR (400MHz, DMSO-d_6): δ 5.4 (s, 2H, OH), 6.59 (d, *J* = 7.2 Hz, 2H, 5'-H), 6.88 (d, *J* = 7.7 Hz, 2H, 4'-H), 6.98 (d, *J* = 9.6 Hz, 1H), 7.32 (d, *J* = 9.6 Hz, 1H), 7.35 (s, 2H, 2'-H), 9.2 (s, 1H, NH), 10.45 (s, 2H, COOH), 14.4 (s, 1H, NH). Analytical calculated value for $C_{18}H_{14}O_6N_4$: C, 56.55; H, 3.69; N, 14.65; O, 25.11. Analytical observed value for $C_{18}H_{14}O_6N_4$: C, 56.51; H, 3.66; N, 14.68; O, 25.15. m/z: 382.3.

AJ28 IR(KBr, cm^{-1}): 3458 (NH), 2954 (OH Aromatic), 1674 (C = O ester), 1626 (C = N), 1283 (C-O ester). ^{1} H NMR (400MHz, $CDCl_3$): δ 3.81 (s, 6H, OCH_3),3.82 (s, 6H, OCH_3), 3.83 (s, 6H, $COOCH_3$), 6.13 (s, 2H, 5'-H) 7.91 (d, *J* = 9.8 Hz, 1H), 7.28 (s, 2H, 2'-H), 7.36 (d, *J* = 9.8 Hz, 1H), 8.9 (s, 1H, NH), 13.8 (s, 1H, NH). Analytical calculated value for $C_{24}H_{26}O_8N_4$: C, 57.83; H, 5.26; N, 11.24; O, 25.68. Analytical observed value for $C_{24}H_{26}O_8N_4$: C, 55.74; H, 5.56; N, 11.79; O, 26.92. m/z: 498.2.

AJ29 IR(KBr, cm^{-1}): 3416 (NH), 2961 (C-H Aromatic), 1616 (C = N). ^{1}H NMR (400MHz, $CDCl_3$): δ 1.32 (m, 6H, *CH_3), 2.35 (s, 6H, CH_3), 2.61 (m, 4H, *CH_2), 6.47 (m, 2H, 3'-H), 6.68 (d, *J* = 2.6 Hz, 2H, 2'-H), 6.93 (d, *J* = 9.8 Hz, 1H), 7.12 (d, *J* = 7.6 Hz, 2H, 4'-H), 7.30 (d, *J* = 9.8 Hz, 1H), 8.3 (s, 1H, NH), 13.8 (s, 1H, NH). Analytical calculated value for $C_{22}H_{26}N_4$: C, 76.27; H, 7.56; N, 16.17. Analytical observed value for $C_{22}H_{26}N_4$: C, 76.24; H, 7.59; N, 16.19. m/z: 346.4.

AJ30 IR(KBr, cm^{-1}): 3325 (NH), 3033 (C-H Aromatic), 2799 (C-H). ^{1}H NMR (400MHz, $CDCl_3$): δ 2.33 (s, 3H, CH_3), 3.72 (s, 6H, OCH_3), 6.62 (d, *J* = 8.6 Hz, 2H, 5'-H), 6.67 (d, *J* = 2.6 Hz, 2H, 4'-H), 6.90 (d, *J* = 10.1 Hz, 1H), 7.27 (d, *J* = 10.1 Hz, 1H), 7.39 (s, 2H, 2'-H), 8.6 (s, 1H, NH), 13.3 (s, 1H, NH). Analytical calculated value for $C_{20}H_{22}O_2N_4$: C, 68.55; H, 6.33; N, 15.99; O, 9.13. Analytical observed value for $C_{20}H_{22}O_2N_4$: C, 68.53; H, 6.37; N, 15.96; O, 9.17. m/z: 350.1.

Docking study

All the synthesized compounds were docked into active site of DHFR and the results are presented as estimated free energy of binding in Table 2.

Antimicrobial activity

All the synthesized compounds were subjected to antimicrobial activity using five bacterial and two fungal

stains. The MIC of all the synthesized compounds were calculated and reported in tabular form [Table 3]. Ciprofloxacin was used as a standard against bacterial stains, and ketoconazole was used as a standard against fungal stains.

DISCUSSION

Starting material for the synthesis was obtained by the reaction between 3,6-pyridazine diol with phosphorous oxychloride [Table 1]. Final compounds were designed by the reaction between 3,6-dichlopyridazine and various anilines. An attempt was made to synthesize diphenylpyridazine analogs. All the reactions were monitored throughout by TLC. All the structures of final compounds were confirmed by IR, NMR, and mass spectrometry. The synthesized pyridazine analogs were screened for their antibacterial activity using broth microdilution method against *S. aureus, M. luteus*, *E. coli, P. aeruginosa* and *P. aurantiaca*. Ciprofloxacin was used as standard drug for comparison. The title compounds were also evaluated for their antifungal activity against *T. rubrum* and *C. laurentii* using Ketoconazole standard drug. The results revealed that synthesized compounds showed excellent broad-spectrum antimicrobial activity against all bacterial as well as fungal stains and compound AJ23 was found to be most active compound. However, since Nitroaromatic compounds are actually toxic and mutagenic and many are suspected or established carcinogens and which can be removed at this point in time. However, compound bearing hydroxyl group and carboxyl group was found to be most active next most active compound, i.e., Compound AJ27. From the literature, it has been observed that the incorporation of electron withdrawing substituent on phenyl ring causes increase in activity and are important structural requirement to be a good antimicrobial agent.

A careful analysis of all the data for the antimicrobial activity of all pyridazine analogs demonstrated an interesting finding that incorporation of one or more NO_2, Cl, Br groups in the skeleton causes increase in activity. Incorporation of fluorine in methyl group instead of the main skeleton does not yields good results. Compounds having–OCH_3 are less active than other compounds of the series. Addition of–COOH group on the main skeleton, increases the activity. Similarly, compounds bearing 2-iodo compounds are less active than 4-iodo compounds. Same finding is continued for bromine as well as chlorine-containing compounds in the same series. In drug receptor interaction study ligands were ranked according to docking score/estimated free energy of binding. The free energy of binding of ligands was in the range between −5.12 to −8.97 Kcal/mole. Top-ranked compound (AJ-23) and (AJ-12) with −8.97 and −7.31 Kcal/mole free energy of binding, respectively, were in correlation with wet laboratory experiments. The protein-ligand analysis also has shown its strong interactions with target protein and had six hydrogen bond interactions in (AJ-23) and five hydrogen bond interactions in (AJ-12). The excellent interactions of DHFR with all top ranked compounds indicated a high degree of coherent relationship between *in silico* approach and *in vitro* studies. Large numbers of hydrogen bond interactions exist between different amino acids of the DHFR and NO_2/hydroxyl/methoxyl group present in ring A, B, and heterocyclic ring C. High anti-microbial activities of the compounds demand further *in vivo* and clinical studies, and these compounds might find an important place in the new array of molecules targeting DHFR-dependent biological functions. Keeping in view, the biological and pharmacological importance of the pyridazine derivatives and biphenyl derivatives, it is our endeavor, to bring two important moieties into the single molecular frame by appropriate synthetic routes. This will stand not only as a source for new biologically active compounds but also as a model for molecular conjunction in the design of new drugs. The *in silico* results of the study were in good tune with the laboratory work experiments, and nearly 80% of docking results were same as that of *in vitro* experiments. Ligand-protein interactions were profoundly found in the present study and gave an insight for further evaluation of study up to molecular level.

CONCLUSIONS

All the test compounds (AJ11-AJ30) were screened for antimicrobial activity against five bacterial and two fungal stains, namely, *S. aureus, M. luteus, E. coli, P. aeruginosa, P. aurantiaca, T. rubrum, C. laurentii*. Most of the compounds showed remarkably good broad-spectrum activity ranging from 7.8 to 500 ug/ml. The remaining compounds have shown moderate activity. N^3, N^6-diphenylpyridazine-3,6-diamine is established as structural skeleton required for the antimicrobial activity. Compound AJ27 is found to be the most active compound. Docking studies revealed that carbonyl oxygen, NO_2, OH, and OCH_3 of the selected derivatives were involved in hydrogen bonding with various amino acids of the receptor. This confirms our hypothesis that conjugation of two pharmacophores might improve the pharmacological profile of synthesized compounds

Acknowledgment

The authors are thankful to Vice-chancellor Shoolini University for providing laboratory facilities.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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