Design, Synthesis, and Characterization of Quinoxaline Derivatives as a Potent Antimicrobial Agent

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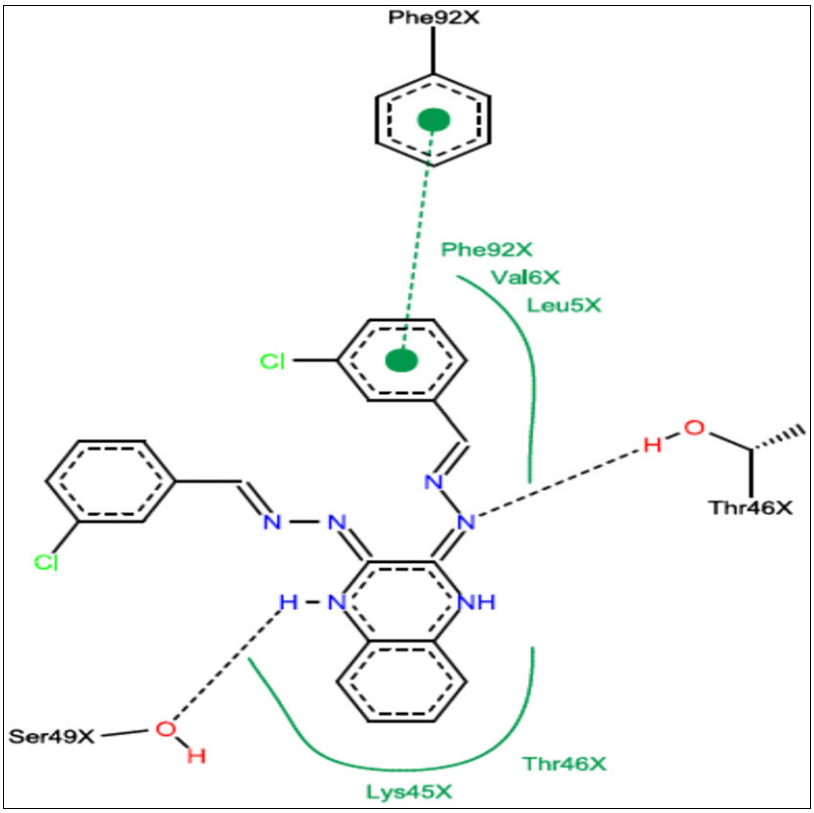


A series of quinoxalinone derivatives were synthesized by the reaction of o-phenylenediamine with oxalic acid to yield 1, 4-dihydro quinoxaline-2, 3-dione (**1**) and then treated with thionyl chloride to yield 2, 3 dichloro quinoxaline (**2**). This was further reacted with hydrazine hydrate to produce 2, 3-dihydrazinyl quinoxaline (**3**). This was finally reacted with substituted aromatic aldehydes to produce 2,3-*bis*[2-(sustituted benzylidine) hydrazinyl] quinoxalines (**4**). These quinoxalinone derivatives were characterized by infrared spectroscopy and nuclear magnetic resonance spectroscopy and MASS spectral data. All the synthesized compounds were evaluated for their antimicrobial activity. The results of the antimicrobial study revealed that compounds **4c**, **4d**, and **4i** were active and exhibited better inhibitory activities as compared to standard drug ciprofloxacin. The results were further checked with protein legend interaction by using docking studies, and all the compounds exhibited good docking scores between −8.72 and −11.29 kcal/mol against dihydrofolate reductase protein fragment from *Staphylococcus aureus* (PDB ID-4XE6). Among all compound, **4c** has shown maximum docking score and found in agreement to *in vitro* studies.

INTRODUCTION

Antimicrobial therapy nowadays is widely used for the treatment and prevention of the most of the microbial infections. Unfortunately, the long-term use and misuse of these agents contribute to severe adverse effect and development of resistance. The failure of recently available antimicrobial therapies and development of resistance, increased a load of adverse effects. It suggest us to develop new therapeutic agents with minimal adverse effect and also enhanced antimicrobial spectrum which could decrease the acquaintance of resistance in microorganisms [1–5].

Echinomycin, levomycin, and actinoleutins antibiotics are the drugs containing quinoxaline as basic nucleus and are widely used in the clinical practice. Echinomycin inhibits microbial RNA synthesis by intercalation in double-stranded DNA through nucleotide sequence selection [6,7].

Quinoxaline derivatives are extensively spattered bioactive class of heterocyclic compound and act as a bioisostere of naphthalene, benzothiophene, and quinoline which are known leads for many antibiotics [8–11].

Quinoxaline and its derivatives exhibited diverse pharmacological properties like antibacterial [12–14], antitubercular [15,16], antiviral [17], antifungal [18,19], anticancer [20,21], antimalarial [22,23], and anti-inflammatory [24]. Various reports have shown that quinoxaline analogs were used as potential dihydrofolate reductase (DHFR) inhibitors [25]. DHFR is a lead enzyme in the biosynthesis of folic acid and nucleic acids through catalysis of the 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate. Moreover, it is actively involved in the production of purine and pyrimidine base. Hence, DHFR has become a crucial and established potential target in antimicrobial therapy [26].

In the present study, new quinoxaline derivatives were synthesized, and their structures were confirmed by physicochemical and spectroscopic data. The minimal inhibitory concentration (MIC) of derivatives was assessed on two gram-positive strains *S. aureus* and *S. pyogenes* and two gram-negative *Escherichia coli* and *Pseudomonas aeruginosa* by disk diffusion method using ciprofloxacin as a standard drug. Furthermore, the derivatives were subjected to docking study to understand the protein–ligand interaction.

EXPERIMENTAL

An open capillary method was adopted to determine the melting points, and the purity of compounds was checked by thin-layer chromatography (TLC). Fourier transform infrared (FTIR) spectra (KBR, cm^{-1}) were recorded on Perkins Elmer Infrared-283 FTIR, nuclear magnetic resonance spectroscopy (^{1}H-NMR) were on a Bruker 500MHz spectrometer using tetramethylsilane as an internal reference, and the mass spectroscopy (MS) were recorded on Aapi 3000 LC-MS.

The synthesized compounds **4(a–l)** were examined for their *in vitro* antibacterial activities against two gram-negative bacteria [*E. coli* (MTCC 443) and *P. aeruginosa* (MTCC 424)] and two gram-positive bacteria [*S. aureus* (MTCC 96) and *S. pyogenes* (MTCC 442)]. Zone of inhibition of all derivatives was determined and compared with standard ciprofloxacin which was used as reference drug. Finally, biological studies were correlates with docking study.

General method of synthesis of 1, 4-dihydro quinoxaline-2, 3-dione (1). An aqueous solution of oxalic acid dihydrate (0.238 mol, 15 g) and 4.5 mL of concentrated HCl (4N) was heated to 100°C followed by the addition of o-phenylenediamine (0.204 mol, 11 g) with continuous stirring. The reaction mixture was refluxed at 100°C for 1 h. The precipitate was obtained after addition of reaction mixture to the crushed ice and was filtered and washed with water; compound was recrystallized from the precipitate and was obtained after addition of reaction mixture to the crushed ice [27].

Synthesis of 2, 3 dichloro quinoxaline (2). A mixture of quinoxaline-2, 3-dione (0.1 mol, 8.1 g) and freshly distilled thionyl chloride ($SOCl_2$, 60 mL) was refluxed with *N*,*N*-dimethylformamide (5 mL) for 2 h. Resulting reaction mixture was left for 30 min. The reaction mixture was slowly poured into ice water with stirring. The resulting reddish solid was filtered and washed with water. The precipitate was recrystallized from a mixture of chloroform and n-hexane [28,29].

Synthesis of 2, 3-dihydrazinyl quinoxaline (3). A mixture of 2, 3 dichloro quinoxaline (4.6 g, 0.01 mol) and hydrazine hydrate (0.64 g, 0.01 mol) was dissolved in absolute ethanol and refluxed for 16 h on a water bath. After completion of the reaction, the reaction mixture was cooled and poured onto the crushed ice. The solid product so obtained was separated and crystallized to yield 2, 3-dihydrazinyl quinoxaline [30,31].

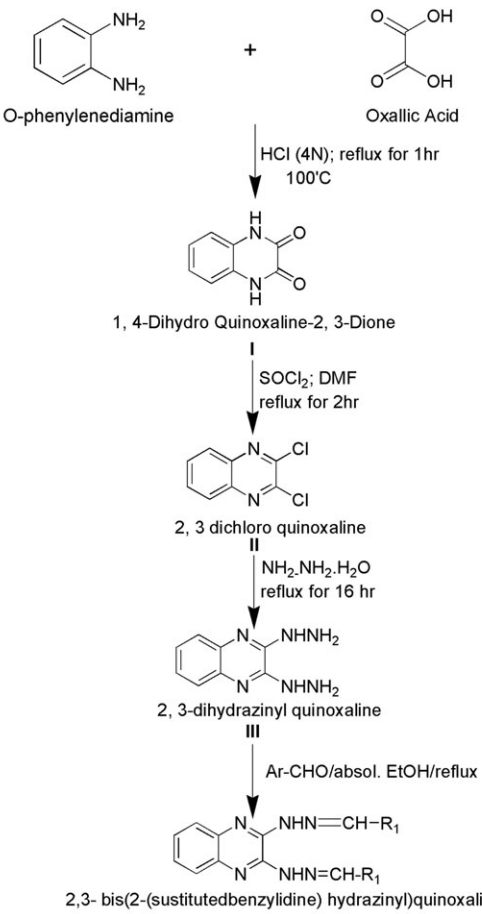
Synthesis of 2,3-*bis*(2-(sustituted benzylidine) hydrazinyl) quinoxalines (4). Equimolar quantities of 2, 3-dihydrazinyl quinoxaline and appropriate substituted aromatic aldehydes were refluxed on a water bath for 10 h using absolute ethanol as a solvent. After completion of reaction, the mixture was poured onto crushed ice, and the solid product so obtained was recrystallized to give 2,3-*bis*(2-(substituted benzylidine) hydrazinyl) quinoxalines [32].

IN VITRO ANTIBACTERIAL SCREENING OF SYNTHESIZED COMPOUNDS

The *in vitro* antimicrobial studies of all the synthesized compounds were performed against the gram-positive

bacteria [*S. aureus* (MTCC 96) and *S. pyogenes* (MTCC 442)] and gram-negative bacteria [*E. coli* (MTCC 443) and *P. aeruginosa* (MTCC 424)] by using the cup-plate/disc diffusion method. Ciprofloxacin was used as standard for antimicrobial studies. Nutrient agar (peptone 10 g, beef extract 10 g, agar 20 g, sodium chloride 5 g, and purified water 1000 mL) was used as culture media for the studies. The ingredients were dissolved in water, and pH is adjusted to 7.2 to 7.4 by using dilute acid/alkali. The resulting solution was autoclave at 120°C for 20 min; 30–35 mL of nutrient agar was transferred to the petridish; 1000 and 500 µg/disc concentrations of the test compounds are prepared; and dimethylformamide was used as vehicle. Aseptically nutrient agar plates were prepared to get a thickness of 5–6 mm and allowed them to solidify and invert the plate to prevent condensate falling on the agar surface. The plates were dried at 37°C just before inoculation. The standard inoculums were inoculated in the plates prepared earlier aseptically by dipping a sterile swab in the inoculums, removing the excess of inoculums by pressing and rotating the swab firmly against the sides of the culture tube above the level of the liquid, and finally streaking the swab all over the surface of sterile culture media. The sterilized discs for the test drugs were placed in the Petri dishes aseptically. Incubate the Petri dish at 37°C ± 0.2°C for about 18–24 h, after placing them in the refrigerator for 1 h to facilitate uniform diffusion. The average zone diameter of the plates was measured and recorded. All synthesized compounds were tested for antibacterial activity [33].

Minimal inhibitory concentration of the test compounds was performed for microorganisms used in the primary screening by using the micro-dilution susceptibility method in Muller–Hinton broth. Dimethyl sulphoxide was used as a solvent to dissolve the standard antibiotic and test compounds at 64 mg/mL concentration. The



4a;R=4FC$_6$H$_4$	4c;R=3ClC$_6$H$_4$	4e;R=4OCH$_3$C$_6$H$_4$	4g;R=3,4,5-(OCH$_3$)$_3$ C$_6$H$_2$	4i;R=4-NO$_2$ C$_6$H$_4$	4k;R=4-OH-3-OCH$_3$-C$_6$H$_3$
4b;R=2ClC$_6$H$_4$	4d;R=4ClC$_6$H$_4$	4f;R=3,4(OCH$_3$)$_2$ C$_6$H$_3$	4h;R=2-NO$_2$ C$_6$H$_4$	4j;R=-C$_6$H$_5$	4l;R=2-OH-C$_6$H$_4$

Figure 1. Representation of the series of reaction for the synthesis of different quinoxaline derivatives.

Table 1

Characterization data of synthesized quinoxaline derivatives.

Derivatives	R_1	Molecular formula	Molecular weight	Melting point (°C)	Appearance	Retention factor	Solubility	% yield (w/w)	λ max (nm)	Chemical name
4a	4F-C_6H_4	$C_{22}H_{16}F_2N_6$	402	278–280	White	0.64	Ethanol	73.73	282	2-{(2*E*)-2-[(4-fluorophenyl)methylidene] hydrazinyl}-3-{(2*Z*)-2-[(4-fluorophenyl) methylidene]hydrazinyl}quinoxaline
4b	2Cl-C_6H_4	$C_{22}H_{16}C_{l2}N_6$	435	317–319	Creamy white	0.73	DMF	66.82	315	2-{(2*E*)-2-[(2-chlorophenyl)methylidene] hydrazinyl}-3-{(2*Z*)-2-[(2-chlorophenyl) methylidene]hydrazinyl}quinoxaline
4c	3Cl-C_6H_4	$C_{22}H_{16}C_{l2}N_6$	435	318–320	Light brown	0.73	Ethanol	70	317	2-{(2*E*)-2-[(3-chlorophenyl)methylidene] hydrazinyl}-3-{(2*Z*)-2-[(3-chlorophenyl) methylidene]hydrazinyl}quinoxaline
4d	4Cl-C_6H_4	$C_{22}H_{16}C_{l2}N_6$	435	320–322	Brown	0.72	Ethanol	73	316	2-{(2*E*)-2-[(4-chlorophenyl)methylidene] hydrazinyl}-3-{(2*Z*)-2-[(4-chlorophenyl) methylidene]hydrazinyl}quinoxaline
4e	4OCH_3-C_6H_4	$C_{24}H_{22}N_6O_2$	426	300–302	Yellow	0.68	Ethanol	66.19	310	2-{(2*E*)-2-[(4-methoxyphenyl)methylidene] hydrazinyl}-3-{(2*Z*)-2-[(4-methoxy phenyl) methylidene]hydrazinyl}quinoxaline
4f	3,4(OCH_3)$_2$-C_6H_3	$C_{26}H_{26}N_6O_4$	486	360–363	White	0.82	Water	62.19	334	2-{(2*E*)-2-[(3,4-dimethoxyphenyl) methylidene]hydrazinyl}-3-{(2*Z*)-2-[(3,4-dimethoxy phenyl)methylidene]hydrazinyl} quinoxaline
4g	3,4,5(OCH_3)$_2$-C_6H_2	$C_{28}H_{30}N_6O_6$	546	376–378	Brown	0.84	Water	70.39	352	2-{(2*E*)-2-[(3,4,5-trimethoxyphenyl) methylidene]hydrazinyl}-3-{(2*Z*)-2-[(3,4,5-trimethoxy phenyl)methylidene]hydrazinyl} quinoxaline
4h	2-NO_2-C_6H_4	$C_{22}H_{16}N_8O_4$	457	328–330	Yellow	0.77	DMF	59.90	321	2,3-bis{(2*Z*)-2-[(2-nitrophenyl)methylidene] hydrazinyl}quinoxaline
4i	4-NO_2-C_6H_4	$C_{22}H_{16}N_8O_4$	457	332–334	Dark brown	0.77	Ethanol	63.73	320	2,3-bis{(2*Z*)-2-[(4-nitrophenyl)methylidene] hydrazinyl}quinoxaline
4j	-C_6H_5	$C_{22}H_{18}N_6$	366	221–223	White	0.54	Water	62.19	243	2,3-bis[(2*Z*)-2-benzylidenehydrazinyl] quinoxaline
4k	4-OH-3-OCH_3-C_6H_3	$C_{24}H_{22}N_6O_4$	458	345–347	Golden	0.79	Ethanol	63.39	324	2-{(2*E*)-2-[(3-dimethoxy-4-hydroxyphenyl) methylidene]hydrazinyl}-3-{(2*Z*)-2-[(3-dimethoxy-4-hydroxyphenyl)methylidene] hydrazinyl}quinoxaline
4l	2-OH-C_6H_5	$C_{22}H_{18}N_6O_2$	398	231–233	Golden	0.57	Ethanol	75.87	263	2,2'-(quinoxaline-2,3-diylbis{[(1*Z*)hydrazin-2-yl-1-ylidene](*Z*)methanylylidene}) diphenol

Table 2

Spectral data of synthesized quinoxaline derivatives.

Derivatives	IR (KBr cm^{-1})	^{1}H-NMR δ (ppm) (DMSO-d_6)	MS
4a	3112.22 (Ar-C-H str.), 1591.46 (Ar-C=C str.), 1232.60 (Ar-C-C str.), 1491.10 (C=N str.), 1231.10 (-C-N- str.), 1372.90 (C-F str.)	6.94-7.65 (m 8H, Ar-H), 4.1 (s 2H, -hydrazine), 7.60–8.20 (d 4H, quinoxaline ring), 8.14 (s 2H, -imine)	401^+
4b	3088.68 (Ar-C-H str.), 1545.28 (C=C str.), 1639.71 (C=N str.), 1177.44 (Ar-C-C str.), 1126.44 (C-N str.), 722.78 (C-Cl str.)	7.14-7.85 (m 8H, Ar-H), 4.0 (s 2H, -hydrazine), 7.68-8.07 (d 4H, quinoxaline ring), 8.20 (s 2H, -imine)	434^+
4c	3133.51 (Ar-C-H str.), 1522.43 (C=C str.), 1622.43 (C=N str.), 1116.44 (C-N str.), 1117.44 (Ar-C-C str.), 742.70 (C-Cl str.)	7.11-7.88 (m 8H, Ar-H), 4.2 (s 2H, -hydrazine), 7.50-8.15 (d 4H, quinoxaline ring), 8.10 (s 2H, -imine)	434^+
4d	3077.89 (Ar-C-H str.), 1667.89 (Ar-C=C str.), 1257.78 (Ar-C-C str.), 1557.89 (C=N str.), 1180.56 (-C-N- str.), 752.70 (C-Cl str.)	7.30-7.75 (m 8H, Ar-H), 4.0 (s 2H, -hydrazine), 7.68-8.27 (d 4H, quinoxaline ring), 8.10 (s 2H, -imine)	434^+
4e	2922.56 (Ar-C-H str.), 1633.56 (Ar-C=C str), 1111.67 (Ar-C-C str.), 1531.12 (C=N str.), 1223.87 (-C-N- str.), 1022.65 (C-O-C str. OCH_3)	6.65-7.75 (m 8H, Ar-H), 4.2 (s 2H, -hydrazine), 7.68-8.20 (d 4H, quinoxaline ring), 8.12 (s 2H, -imine), 3.45 (s 6H,-OCH_3)	425^+
4f	2880.45 (Ar-C-H str.), 1621.76 (Ar-C=C str.), 1118.45 (Ar-C-C str.), 1531.10 (C=N str.), 1266.45 (-C-N- str.), 1082.60 (C-O-C str. OCH_3)	6.71-7.25 (m 6H, Ar-H), 4.1 (s 2H, -hydrazine), 7.58-8.28 (d 4H, quinoxaline ring), 8.15 (s 2H, -imine), 3.75 (s 12H,-OCH_3)	485^+
4g	3134.78 (Ar-C-H str.), 1662.89 (Ar-C=C str.), 1136.89 (Ar-C-C str.), 1521.21 (C=N str.), 1254.76 (-C-N- str.), 1132.30 (C-O-C str. OCH_3)	6.65-7.31 (m 4H, Ar-H), 4.5 (s 2H, -hydrazine), 7.68-8.22 (d 4H, quinoxaline ring), 8.13 (s 2H, -imine), 3.73 (s 18H,-OCH_3)	545^+
4h	3068.45 (Ar-C-H str.), 1642.67 (Ar-C=C str.), 1115.78 (Ar-C-C str.), 1510.90 (C=N str.), 1220.43 (-C-N- str.), 1287.12 (NO_2 str.)	7.11-7.88 (m 8H, Ar-H), 4.0 (s 2H, -hydrazine), 7.68-8.04(d 4H, quinoxaline ring), 8.00 (s 2H, -imine)	456^+
4i	2920.56 (Ar-C-H str.), 1633.56 (Ar-C=C str.), 1131.67 (Ar-C-C str.), 1541.12 (C=N str.), 1220.87 (-C-N- str.), 1237.12 (NO_2 str.)	7.30-7.60 (m 8H, Ar-H), 4.0 (s 2H, -hydrazine), 7.60-8.10 (d 4H, quinoxaline ring), 8.15 (s 2H, -imine)	456^+
4j	2912.12 (Ar-C-H str.), 1625.45 (Ar-C=C str.), 1225.60 (Ar-C-C str.), 1525.10 (C=N str.), 1165.10 (-C-N- str.)	6.70-7.45 (m 10H, Ar-H), 4.25 (s 2H, -hydrazine), 7.65-8.25 (d 4H, quinoxaline ring), 8.15 (s 2H, -imine)	365^+
4k	3123.20 (Ar-C-H str.), 1580.45 (Ar-C=C str.), 1240.60 (Ar-C-C str.), 1452.10 (C=N str.), 1225.10 (-C-N- str.), 1018.65 (C-O-C str. OCH_3), 3443.89 (O-H str.)	6.70-7.70 (m 6H, Ar-H), 4.2 (s 2H, -hydrazine), 7.68-8.20 (d 4H, quinoxaline ring), 8.10 (s 2H, -imine), 3.75 (s 6H,-OCH_3), 5.1 (s 2H,-OH)	457^+
4l	3057.89 (Ar-C-H str.), 1647.89 (Ar-C=C str.), 1237.78 (Ar-C-C str.), 1556.89 (C=N str.), 1150.56 (-C-N- str.), 3393.89 (O-H str.)	6.80-7.40 (m 6H, Ar-H), 4.10 (s 2H, -hydrazine), 7.68-8.20 (d 4H, quinoxaline ring), 8.12 (s 2H, -imine), 5.0 (s 2H,-OH)	397^+

Table 3

Combustion analysis of quinoxaline derivatives.

	Combustion analysis	
Derivatives	Theoretical value	Observed values
4a	C (65.66%) H (4.01%) Cl (9.44%) N (20.88)	C (66.97%) H (4.09%) Cl (9.26%) N (21.29%)
4b	C (60.70%) H (3.70%) Cl (16.29%) N (19.31%)	C (59.49%) H (3.63%) Cl (16.61%) N (19.69%)
4c	C (60.70%) H (3.70%) Cl (16.29%) N (19.31)	C (61.91%) H (3.63%) Cl (15.96%) N (19.31)
4d	C (60.70%) H (3.70%) Cl (16.29%) N (19.31)	C (60.70%) H (3.70%) Cl (16.29%) N (18.92)
4e	C (67.59%) H (5.20%) O (7.50%) N (19.71%)	C (66.23%) H (5.30%) O (7.65%) N (19.31%)
4f	C (64.19%) H (5.39%) O (13.15%) N (17.27%)	C (65.48%) H (5.47%) O (13.15%) N (16.92%)
4g	C (61.53%) H (5.53%) O (17.56%) N (15.38%)	C (60.27%) H (5.63%) O (17.90%) N (15.07%)
4h	C (57.89%) H (3.53%) O (14.02%) N (24.55%)	C (59.04%) H (3.43%) O (13.73) N (25.04%)
4i	C (72.11%) H (4.95%) N (22.94%)	C (73.41%) H (4.909%) N (23.24%)
4j	C (62.87%) H (4.84%) O (13.96%) N (18.33%)	C (63.87%) H (4.74%) O (14.23%) N (18.01%)
4k	C (66.32%) H (4.55%) O (8.03%) N (21.09%)	C (66.46%) H (4.53%) O (7.97%) N (20.66%)
4l	C (65.66%) H (4.01%) F (9.44%) N (20.88%)	C (65.47%) H (4.07%) F (9.31%) N (20.67%)

solution was twofold diluted (64 to 0.5 mg/mL). A prepared suspension of the standard microorganisms was added to each dilution in a 1:1 ratio. Growth (or its lack) of microorganisms was determined visually after incubation for 24 h at 37°C. The lowest concentration at which there was no visible growth (turbidity) was taken as the MIC [34].

MOLECULAR DOCKING STUDY

Molecular docking study was performed using free available docking software Argus Lab 4.0 [35] to observe the way of the corresponding synergy of the test compound with the potential target. From the protein data bank, corresponding protein structure of DHFR from *S. aureus* (PDB ID-4XE6) was obtained. Chem draw ultra 10.0 was used for 3D molecule development, and the structure was saved as.pdb format. Genetic Algorithm dock and Argus dock (shape-based search algorithm) were used for docking simulation. In the present study, Argus dock was selected in docking engine to perform the docking simulation, and for calculations, "Dock" option was chosen as the calculation type. The protein–ligand interaction was determined by using Pymol 1.3 software [36].

Table 4

Antibacterial activity of synthesized quinolaxine derivatives using plate-hole diffusion method.

	Zone of inhibition (mm)							
	Gram-positive bacteria				Gram-negative bacteria			
	S. aureus		*S. pyogenes*		*E. coli*		*P. aeruginosa*	
Compounds	500 μg/mL	1000 μg/mL	500 μg/mL	1000 μg/mL	500 μg/mL	1000 μg/mL	500 μg/mL	1000 μg/mL
4a	11	21	11	19	13	20	12	19
4b	12	22	13	20	14	22	13	20
4c	14	23	14	23	16	23	14	22
4d	13	23	14	23	15	23	13	22
4e	12	17	10	17	12	17	--	--
4f	12	20	13	20	12	21	--	--
4g	09	16	10	18	--	--	10	14
4h	12	22	12	20	11	22	12	20
4i	13	23	13	22	15	22	13	21
4j	13	22	15	22	14	22	13	21
4k	11	20	12	20	14	21	10	19
4l	12	22	14	21	11	22	14	22
Ciprofloxacin	13	23	13	22	15	24	14	23
DMF (control)	--	--	--	--	--	--	--	--

Table 5

Antimicrobial activity of the synthesized compounds expressed as MIC (mg/mL).

	Gram-positive bacteria		Gram-negative bacteria	
Compounds	*S. pyogenes*	*S. aureus*	*E. coli*	*P. aeruginosa*
4a	1.10	1.28	1.89	1.97
4b	1.09	1.12	1.37	1.84
4c	0.97	0.92	0.93	0.98
4d	1.01	1.03	0.96	1.02
4e	2.87	3.28	3.17	3.14
4f	2.97	3.31	2.97	3.48
4g	3.03	3.43	3.23	3.17
4h	2.14	1.98	1.87	2.73
4i	1.04	1.06	1.10	1.07
4j	2.89	2.91	2.76	3.01
4k	1.87	2.12	2.28	1.98
4l	1.97	2.08	1.83	1.79
Ciprofloxacin	0.97	0.89	0.93	0.96

RESULTS AND DISCUSSION

The series of reactions are given in Scheme 1 of Figure 1. Quinoxaline derivatives were synthesized by the reaction of o-phenylenediamine with oxalic acid to yield 1, 4-dihydro quinoxaline-2, 3-dione and then treated with thionyl chloride to yield 2, 3 dichloro quinoxaline. This was further reacted with hydrazine hydrate to produce 2, 3-dihydrazinyl quinoxaline. This was finally reacted with the substituted aromatic aldehyde to produce 2,3-*bis*(2-(substituted benzylidine) hydrazinyl) quinoxaline. The purity of the derivatives was confirmed by TLC and melting point. Structure of these derivatives was set up by determining infrared spectroscopy (IR), ^{1}H-NMR, and MS. In addition to this, the synthesized derivatives were screened for their antimicrobial activities.

The synthetic way used to produce different quinoxaline derivatives is outlined in Scheme 1. In total, 12 different quinoxaline derivatives were prepared by treating 2, 3-dihydrazinyl quinoxaline with different aromatic aldehydes. Chemical structure, melting point, and other physical data were mentioned in Table 1. Formation of different 2,3-*bis*(2-(sustituted benzylidine) hydrazinyl) quinoxalines derivatives was established by recording there IR, ^{1}H-NMR, and MS.

The characterization of structure was performed by the FTIR, ^{1}H-NMR, MS, and elemental analysis. The IR spectra helped to confirm the structure by assuring the presence of different functional molecule. The IR spectra of compound III (intermediate) showed the appearance of peak around 1482 and 1260 cm^{-1} and were suggested the presence of C=N str. and C-N str. group, respectively, in compound as closed heterocyclic ring (quinoxaline). The support for quinoxaline heterocycle was made by the appearance of IR around 3025 cm^{-1} for C-H str. which is of fused aromatic ring. This interpretation was complemented by the appearance of NMR multiplet peak around δ 7.2–7.8 for Ar-C-H. Furthermore, the IR spectra

Table 6

Docking parameters used in Argus Lab 4.0.1.

Compound ID(s)	Constant term	vdW coefficient	H-bond coefficient neutral–neutral	Rotors coefficient	Hydrophobic coefficient
4m	2.783	−0.00096	0.38	−0.1	0.0373
4n	2.783	−0.00096	0.38	−0.1	0.0373
4o	2.783	−0.00096	0.38	−0.1	0.0373
4q	2.783	−0.00096	0.38	−0.1	0.0373
4q	2.783	−0.00096	0.38	−0.1	0.0373
4r	2.783	−0.00096	0.38	−0.1	0.0373
4s	2.783	−0.00096	0.38	−0.1	0.0373
4t	2.783	−0.00096	0.38	−0.1	0.0373
4u	2.783	−0.00096	0.38	−0.1	0.0373
4v	2.783	−0.00096	0.38	−0.1	0.0373
4w	2.783	−0.00096	0.38	−0.1	0.0373
4x	2.783	−0.00096	0.38	−0.1	0.0373

Grid parameters: spacing 0.375 Å and grid sizes 80X Å, 80Y Å, and 80Z Å.

Table 7

Properties of ligands on the basis of their molecular structure.

	Molecular parameters*							
Compound ID(s)	Strech	Bend	Strech–bend	Torsion	Non-1,4 VDW	1,4 VDW	Dipole/dipole	Total energy (kcal/mol)
4a	0.77	3.36	0.03	−20.06	−2.76	16.21	1.48	−0.95
4b	1.03	3.79	0.09	−17.67	−1.90	17.59	1.52	4.45
4c	0.82	3.36	0.06	−20.18	−2.98	17.11	1.51	−029
4d	0.81	3.37	0.05	−19.64	−2.92	17.05	1.49	0.22
4e	1.34	8.06	0.12	−17.70	−2.03	21.55	1.43	12.78
4f	1.93	8.48	−0.04	−2.17	−9.08	27.04	2.80	28.97
4g	2.85	12.35	−0.11	0.86	−10.57	32.40	3.30	41.10
4h	85.88	83.18	−237.30	−29.30	−4.77	8.66	−0.55	−94.13
4i	0.93	3.36	0.09	−18.64	−2.92	18.05	1.46	0.23
4j	0.73	5.68	0.05	−12.72	−3.21	15.64	1.38	7.56
4k	1.38	7.74	−0.04	−12.51	−6.45	21.86	1.00	12.98
4l	0.96	3.98	−0.04	−8.91	−3.75	16.40	2.50	11.14

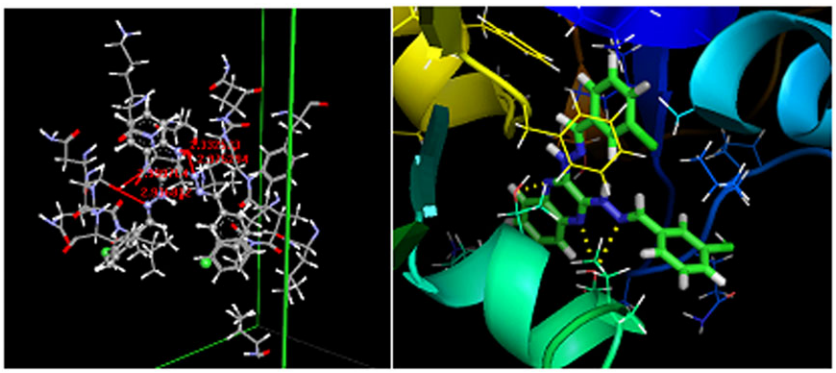
*Data generated using ChemBioDraw 13 software after MM2 energy minimization.

Table 8

Chemical properties of ligands

Compound ID(s)	Molecular formula	Molecular mass (g/mol)	CLogP values*	CMR	Gibbs energy: (kJ/mol)
4a	$C_{22}H_{16}F_2N_6$	402	6.55	11.66	302.11
4b	$C_{22}H_{16}C_{l2}N_6$	435	6.49	12.61	662.85
4c	$C_{22}H_{16}C_{l2}N_6$	435	7.69	12.61	662.85
4d	$C_{22}H_{16}C_{l2}N_6$	435	7.69	12.61	662.84
4e	$C_{24}H_{22}N_6O_2$	426	6.69	12.86	388.61
4f	$C_{26}H_{26}N_6O_4$	486	6.12	14.1	59.95
4g	$C_{28}H_{30}N_6O_6$	546	4.50	15.33	−268.71
4h	$C_{22}H_{16}N_8O_4$	457	5.04	12.85	--
4i	$C_{22}H_{16}N_8O_4$	457	5.04	12.85	--
4j	$C_{22}H_{18}N_6$	366	6.24	11.63	717.27
4k	$C_{24}H_{22}N_6O_4$	458	5.82	13.17	33.99
4l	$C_{22}H_{18}N_6O_2$	398	7.44	11.93	362.65

*Data generated using ChemBioDraw 13 software.



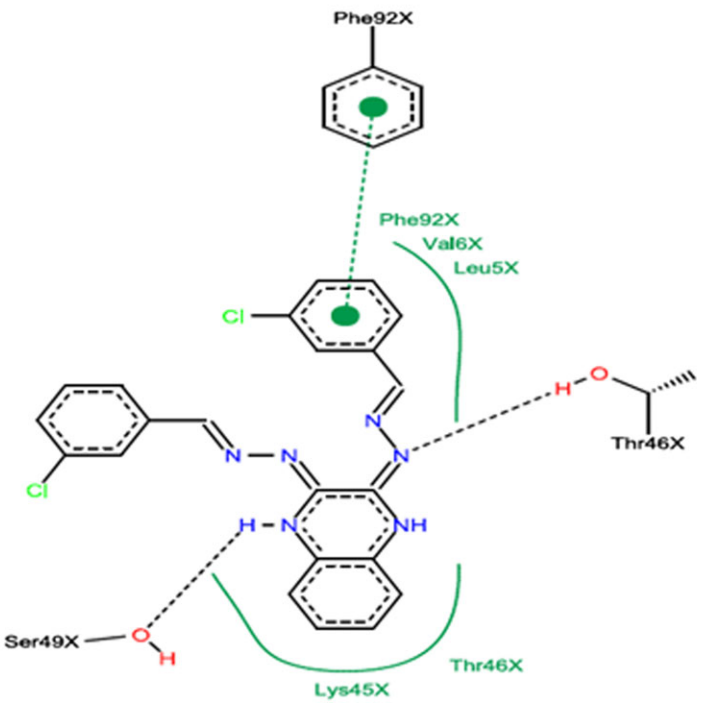


Figure 2. Interaction study and pose view of compound **4c** with binding domain of 4XE6 protein-hydrogen binding of analog **4e** with amino acids, amine group of hydrazine, and ring nitrogen of quinoxaline interact with 46THR and 49SER residues and bond distance 2.3 Å and 2.9 Å. [Color figure can be viewed at wileyonlinelibrary.com]

Table 9

Docking results of ligands and standard drug against DHFR (4XE6).

Compound ID (s)	Binding energy (kcal/mol)	No. of hydrogen bonds	Bond length of H-bonds in Å	H-bond with receptor residue	Enzyme's binding site residue
4a	−9.74	1	2.77	28LEU	57ARG(beta strand), 50ILE(coil), 20LEU(coil), 28LEU(alpha helix), 54LEU(beta strand), 29LYS(alpha helix), 32LYS(alpha helix), 52LYS(alpha helix), 92PHE(coil), 55PRO(beta strand), 46THR(alpha helix), 31VAL(alpha helix)
4b	−10.21	1	2.92	28LEU	57ARG(beta strand), 30HIS(alpha helix), 50ILE(coil), 20LEU(coil), 28LEU(alpha helix), 54LEU(beta strand), 40LEU(beta strand), 29LYS(alpha helix), 32LYS(alpha helix), 33LYS(alpha helix), 52LYS(alpha helix), 42MET(beta strand), 92PHE(coil), 55PRO(beta strand), 331VAL(alpha helix)
4c	−11.29	2	2.30 2.90	46THR 49SER	7ALA(beta strand), 18ASN(coil), 19GLN(coil), 15GLY(coil), 93GLY(coil), 14ILE(coil), 50ILE(coil), 5LEU(beta strand), 20LEU(coil), 28LEU(alpha helix), 54LEU(beta strand), 92PHE(coil), 98PHE(alpha helix), 123PHE(beta strand), 49SER(coil), 121THR(beta strand), 6VAL(beta strand), 31VAL(alpha helix)
4d	−11.17	2	2.60 2.80	46THR 49SER	57ARG(beta strand), 27ASP(alpha helix), 50ILE(coil), 20LEU(coil), 28LEU(alpha helix), 54LEU(beta strand), 29LYS(alpha helix), 32LYS(alpha helix), 45LYS(alpha helix), 92PHE(coil), 53PRO(beta strand), 55PRO(beta strand), 46THR(alpha helix), 31VAL(alpha helix)
4e	−9.70	2	2.30 2.80	52LYS 28LEU	57ARG(beta strand), 30HIS(alpha helix), 50ILE(coil), 20LEU(coil), 28LEU(alpha helix), 54LEU(beta strand), 29LYS(alpha helix), 32LYS(alpha helix), 33LYS(alpha helix), 52LYS(beta strand), 92 PHE (coil), 53PRO(beta strand), 55PRO(beta strand), 49SER(coil), 46THR(alpha helix), 31VAL(alpha helix)
4f	−9.78	3	2.63 2.66 2.69	7ALA 20LEU 49SER	7ALA(beta strand), 57ARG(beta strand), 18ASN(coil), 27ASP(alpha helix), 19GLN(coil), 15GLY(coil), 14ILE(coil), 50ILE(coil), 113ILE(beta strand), 5LEU(beta strand), 20LEU(coil), 24LEU(coil), 28LEU(coil), 40LEU(beta strand), 54EU(beta strand), 32LYS(alpha helix), 16PHE(coil), 92PHE(coil), 35SER(coil), 49SER(coil), 46THR(alpha helix), 22TRP(coil), 6VAL(beta strand), 31VAL(alpha helix)
4g	−8.72	4	2.34 2.40 2.72 2.99	12ARG 10LEU 12ARG 12ARG	12ARG(coil), 9ASP(coil), 11GLN(coil), 114GLU(coil), 129GLU(coil), 8HIS(beta strand), 10LEU(coil), 123PHE(beta strand), 128PHE(coil), 124PRO(beta strand), 125PRO(beta strand), 127THR(beta strand), 126TYR(beta strand), 112VAL(beta strand)
4k	−10.05	3	2.13 2.40 2.82	20LEU 22TRP 49SER	18ASN(coil), 27ASP(alpha helix), 19GLN(coil), 23HIS(coil), 50ILE(coil), 20LEU(coil), 24LEU(coil), 28LEU(alpha helix), 92PHE(coil), 49SER(coil), 22TRP(coil), 31VAL(alpha helix)
4i	−10.85	5	2.15 2.39 2.57 2.65 2.99	20LEU 18ASN 121THR 49SER 49SER	7ALA(beta strand), 18ASN(coil), 19GLN(coil), 17GLU(coil), 15GLY(coil), 93GLY(coil), 94GLY(coil), 14ILE(coil), 50ILE(coil), 5LEU(beta strand), 20LEU(coil), 45LYS(alpha helix), 16PHE(coil), 92PHE(coil), 98PHE(alpha helix), 49SER(coil), 46THR(alpha helix), 121THR(beta strand), 6VAL(beta strand)

(*Continues*)

Table 9

(*Continued*)

Compound ID (s)	Binding energy (kcal/mol)	No. of hydrogen bonds	Bond length of H-bonds in Å	H-bond with receptor residue	Enzyme's binding site residue
4j	−10.77	0	-	-	7ALA(beta strand), 27ASP(alpha helix), 93GLY(coil), 94GLY(coil), 23HIS(coil), 15ILE(coil), 50ILE(coil), 5LEU(beta strand), 20LEU(coil), 24LEU(coil), 28LEU(alpha helix), 54LEU(beta strand), 32LYS(alpha helix), 92PHE(coil), 98PHE(alpha helix), 22TRP(coil), 6VAL(beta strand), 31VAL(alpha helix)
4k	−10.08	4	2.49 2.74 2.80 2.99	49SER 50ILE 7ALA 7ALA	7ALA(beta strand), 57ARG(beta strand), 18ASN(coil), 27ASP(alpha helix), 19GLN(coil), 51GLY(coil), 93GLY(coil), 14ILE(coil), 50ILE(coil), 5LEU(beta strand), 20LEU(coil), 28LEU(alpha helix), 40LEU(beta strand), 54LEU(beta strand), 32LYS(alpha helix), 92PHE (coil), 35SER(coil), 49SER(coil), 46THR(coil), 22TRP(coil), 6VAL(beta strand), 31VAL(alpha helix)
4l	−10.60	4	2.42 2.65 2.68 2.83	27ASP 27ASP 22TRP 24LEU	7ALA(beta strand), 27ASP(alpha helix), 23HIS(coil), 30HIS(alpha helix), 50ILE(coil), 5LEU(beta strand, 20LEU(coil), 24LEU(coil), 28LEU(coil), 40LEU(beta strand), 54LEU(beta strand), 92PHE(coil), 25PRO(alpha helix), 111THR(beta strand), 22TRP(coil), 6VAL(beta strand), 31VAL(alpha helix)
Ciprofloxacin	−7.57	3	2.71 2.93 2.94	6VAL 7ALA 121THR	7ALA(beta strand), 18ASN(coil), 29GLN(coil), 95GLN(alpha helix), 15GLY(coil), 93GLY(coil), 94GLY(coil), 14ILE(coil), 50ILE(coil), 5LEU(beta strand), 20LEU(coil), 54LEU(beta strand), 45LYS(alpha helix), 16PHE(coil), 92PHE(coil), 98PHE(alpha helix), 49SER(coil), 46THR(alpha helix), 121THR(beta strand), 6VAL(beta strand), 31VAL(alpha helix)

Grid parameters (Argus Lab.): spacing 0.375 Å and grid sizes 80X Å, 80Y Å, and 80Z Å.

containing a peak at 3424 cm^{-1} for primary and absorption in the region 1120–1165 cm^{-1} for N-N str. confirm the presence of attached hydrazine group. The hydrazine group in also confirmed by the NMR absorption at δ 4.2 due to secondary amine (-NH-) and a doublet peak around δ 2.2 for primary amine ($-NH_2$) group. The formation of compound **4a–l** was assured due to the manifestation of NMR peak for secondary amine at around 4.3 and the absence of NMR peak for primary amine due to its conversion to tertiary amine after the attachment of benzal group with the formation of imine bond (-N=CH-Ar). The attachment of substituted aromatic ring in different derivatives (**4a–l**) was well supported by NMR spectra as on the presence of cluster of multiplet peaks around δ 7.4–8.6 for fused aromatic ring of quinoxaline and δ 6.2–7.3 for substituted aromatic as benzal. The presence of substituted ring was further confirmed by the observation of IR peak around 3393 and 3443 cm^{-1} for -OH group in compounds **4l** and **4k**, respectively, and 1022 and 1083 cm^{-1} C-O-C str. ($-OCH_3$) for compounds **4e** and **4f**, respectively. The presence of these substituent groups, namely, -OH and $-OCH_3$ groups in synthesized derivatives, were cross confirmed by NMR spectra around δ 5.00 and 3.45, respectively. The mass spectra of quinoxaline derivative (**4a**) showed a molecular ion peak at *m/z* 401 which is in conformity with the molecular formula $C_{22}H_{16}F_2N_6$. In the same way, spectral data of remaining derivatives are given in Tables 2 and 3.

The synthesized compounds were tested for antibacterial activity *in vitro* against microorganisms such as *S. pyogenes* and *S. aureus* (gram-positive) and *P. aeruginosa* and *E. coli* (gram-negative) using cup-plate/disc diffusion method. Ciprofloxacin was used as standard drug. The obtained results revealed that the tested compounds indicated varying extent of activity against the tested microorganisms Tables 4 and 5. In the given results, compounds (**4c**, **4d**, and **4i**) were highly active against both gram-positive and gram-negative bacterial strain. The compounds **4c** (23mm), **4d** (23mm), and **4i** (22mm) were significantly active against *E. coli* and *S. aureus* when compared to standard Ciprofloxacin (23 mm). Compounds **4e** and **4f** showed no activity against *P. aeruginosa*, and

compound **4g** was insensitive against *E. coli*. The rest of estimated derivatives were exhibited good to moderate activity against the selected strains of bacteria. The MIC study was performed for synthesized compounds for MIC determination micro-dilution susceptibility method was used. The results were shown in Tables 5. The gram-positive and gram-negative bacteria are sensitive to most of the tested compounds, and some compounds particularly **4c**, **4d**, and 4i have shown significant activity against both type of microbial strains than others. Antimicrobial activity of derivatives may be attributed to the presence of electron withdrawing groups. The presence of methoxy groups on the phenyl side chain at the quinoxaline ring does not exhibit the biological activity like standard ciprofloxacin.

The docking study gives an idea about an interaction between the test compound and the potential target protein as shown in Tables 6, 7, and 8. So to rationalize the potency of the derivatives as an antimicrobial agent, all synthesized derivatives were docked against DHFR *S. aureus* (PBD ID-4XE6) obtained from protein data bank (RCSB). It is well known that available docking software has good success in generating active pose of the ligands but that software are not generating docking scores in such a good rate [37,38]. But in the current study, docking scores seem to be in good agreement with the experimental findings. The binding energy of drug-receptor complex and standard drug are shown in Table 6. All synthesized molecules interacted with the active sites of enzyme DHFR from *S. aureus* (PBD ID-4XE6) through various bonds like hydrogen, van der Waals, p-cation, p-anion, p-alkyl, p-donor hydrogen, p-sulphur, carbon-hydrogen bond, and p-sigma. Test compound showed the different modes of binding with amino acids located at an active site of DHFR (Fig. 2). The nitrogen atom of hydrazine group and quinoxaline ring nitrogen heteroatom was forming the hydrogen bond with amino groups of protein extracted as PDB with specific bond distance. The ring nitrogen of quinoxaline and the nitrogen atom of hydrazine group in compound **4c** (49 SER 2.9A°, 46 THR, 2.3A°) and the nitrogen atom of quinoxaline of compound **4d** (49 SER 2.8A°, 46THR 2.6A°) make the hydrogen bond with an amino group of serine and threonine (Table 9). These amino acids make a cascade to facilitate binding and holding of the test compound in the active site of DHFR protein. The binding energies of the all the derivatives showed a good score of docking between −8.72 and −11.29 kcal/mol and were better as compared to standard drug ciprofloxacin. The compounds **4c** (−11.29 kcal/mol), **4d** (−11.17 kcal/mol), and **4i** (−10.85 kcal/mol) represent the promising docking score as compared with the standard DHFR inhibitor ciprofloxacin (−7.57 kcal/mol) (Table 9). The derivatives attributed the electron withdrawing group having the better docking score than other substituents at the same positions. Out of 21 amino acids of residue of DHFR which is responsible for the formation of bonds

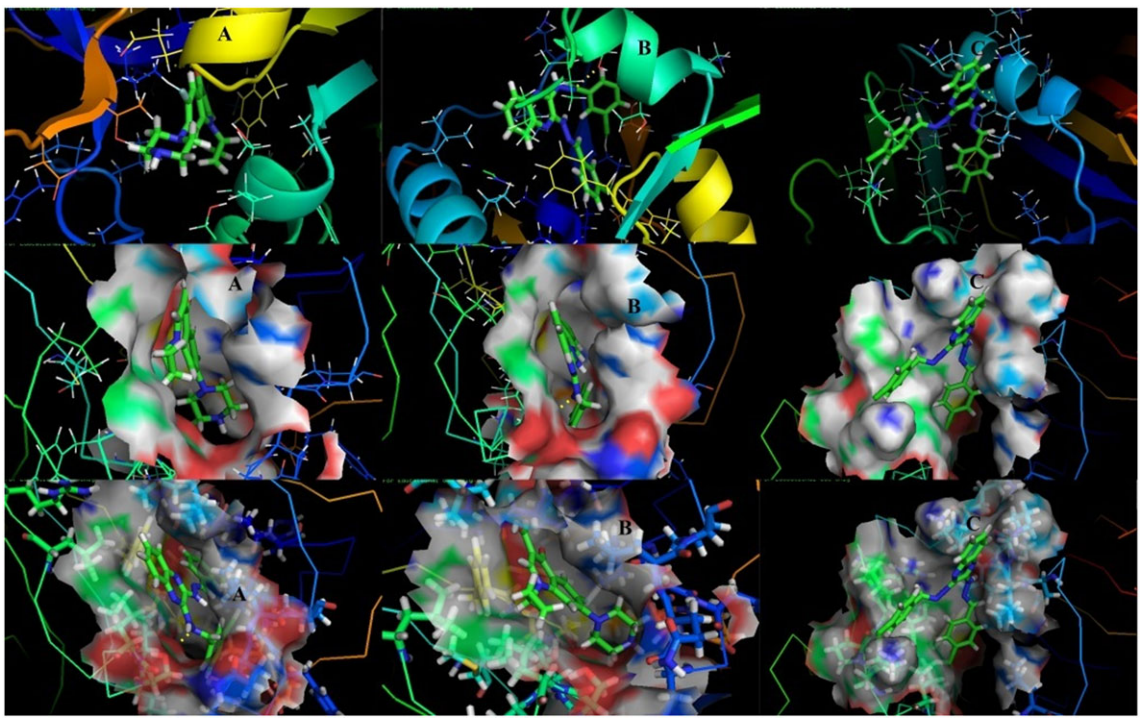


Figure 3. The figures A, B, C, represent 3D model of docking zone of ciprofloxacin, compounds **4c** and **4d** on the enzyme dihydrofolate reductase (4XE6) in cartoon (1), solid surface (2), and transparent (3), respectively. [Color figure can be viewed at wileyonlinelibrary.com]

with ciprofloxacin, 15 were found to be similar in compounds like **4c** [*viz*. 7ALA(beta strand), 18ASN(coil), 15GLY(coil), 93GLY(coil), 14ILE(coil), 50ILE(coil), 5LEU(beta strand), 20LEU(coil), 54LEU(beta strand), 92PHE(coil), 98PHE(alpha helix), 49SER(coil), 121THR(beta strand), 6VAL(beta strand), and 31VAL(alpha helix)] and 6 were found to be similar in compounds like **4d** [*viz*. 50ILE(coil), 20LEU(coil), 54LEU(beta strand45LYS(alpha helix), 92PHE(coil), 46THR(alpha helix), and 31VAL(alpha helix)]. The compound **4c** showed binding site alignment pattern resemblance to that of ciprofloxacin with amino acid chain of DHFR (4XE6) as shown in Figure 3.

STRUCTURE ACTIVITY RELATIONSHIP

From the results of *in vivo* antimicrobial activity of newly prepared quinoxaline derivatives, the following structural activity relationship was derived. The tested derivatives **4c**, **4d**, and **4i** were found to be promising active against the tested strain of microbes. Almost all the analogs showed good activity against all strains of bacteria (Table 4 and 5). The antimicrobial activity of synthesized compounds was compared to ciprofloxacin. The structure–activity relationship study recommended that electron withdrawing group substitutes were exhibiting better activity against almost all the bacteria. Compounds **4c**, **4d**, and **4i** were constituted with electron withdrawing substituents at the meta and para position of phenyl ring and had contemplated as lead compounds and exhibited significant activity. Whereas the compound substituted with an electron donating group or unsubstituted the phenyl ring offered a low to moderate activity against selected strains. Derivatives having an attachment of OCH_3 groups as a substituent on phenyl ring undergo the least effect on selected bacterial strains. Derivatives with unsubstituted phenyl ring have a moderate response on used strains as compared to standard.

CONCLUSION

A new series of different 2,3-*bis*(2-(sustituted benzylidine) hydrazinyl) quinoxalines derivatives were prepared by treating 2, 3-dihydrazinyl quinoxaline with different aromatic aldehydes, by a simple, suitable, and well-organized synthetic route. Physical and analytical parameters of the newly synthesized quinoxaline derivatives were confirmed by TLC, IR, ^{1}H-NMR, and MS. Subsequently, in biological screening, the compounds were considered as promising lead compounds for the further development of a new antimicrobial drug. From the *in vitro* studies, it is considered that compounds **4c**, **4d**, and **4i** exhibited optimistic antimicrobial activity; furthermore, docking studies suggested that compounds **4c** and **4d** were interacted with protein more efficiently, and hence, these derivatives could be further studied for their mechanisms of action in depth and could be developed as effective antimicrobial agents.

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