Synthesis, *in-vitro* antimicrobial evaluation and docking studies of newly synthesized benzoxazole derivatives

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Abstract

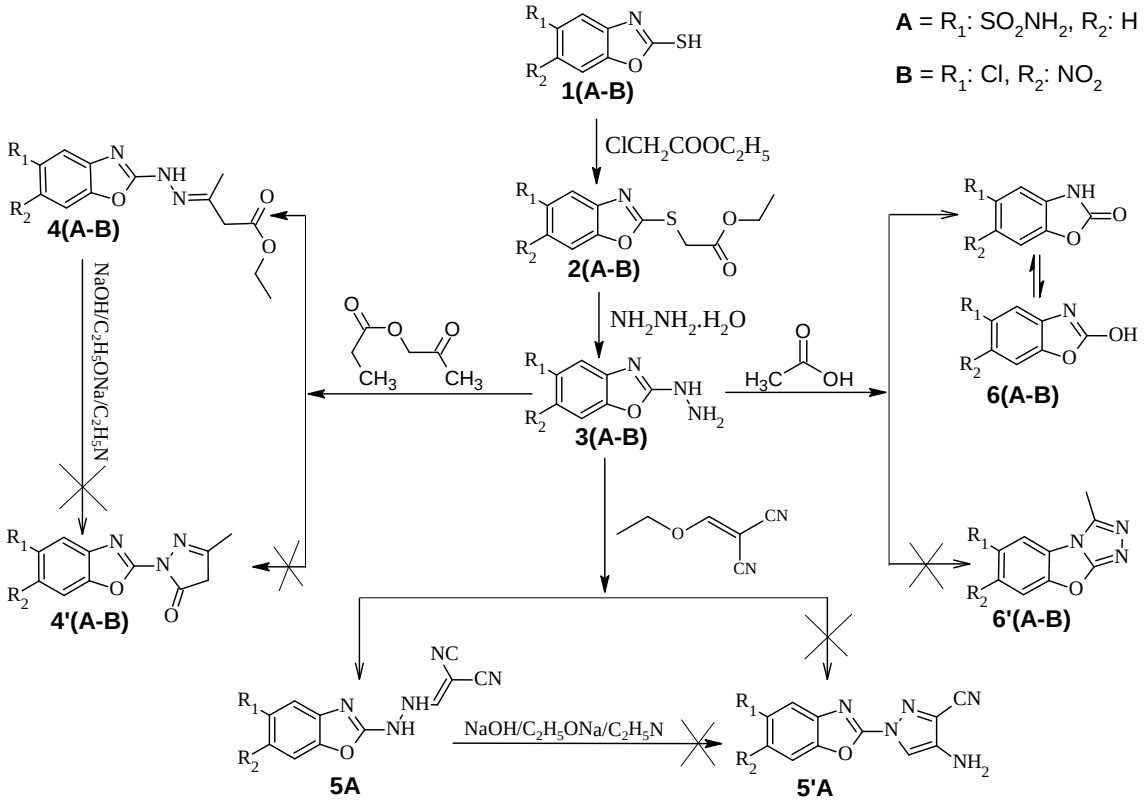
Different varieties of benzoxazole derivatives have been synthesized from ethyl aceto acetate, ethoxy methylene malanonitrile and with acetic acid/formic acid. Analytical tools like proton NMR (^1H), carbon NMR (^{13}C), infrared spectroscopy (IR) and LC-MS mass spectrometry were used for structural characterization. Influences on microbes were studied by in silico docking methods to explore the structural insights into the binding approach. The docking results against X-ray crystallographic structure of Staphylococcus aureus, UDP-N-acetylenolpyruvylglucosamine reductase (MurB) (PDB ID: 1HSK) protein have shown minimum binding energy of -7, -7, -8.4, -8.5, -7.5, -7.6, -8.3, -8.6, -8.7, -7.5 and -7.4 for 1A, 1B, 2A, 2B, 3A, 3B, 4A, 4B, 5A, 6A and 6B molecules respectively.

2B, 4B and 5A molecules have proved as effective antimicrobial agents from agar well diffusion method and minimum inhibition concentration techniques against fungi, gram-positive and gram-negative bacteria in the initial screening.

Keywords: Benzoxazole, ethoxy methylene malanonitrile, antimicrobial activity, molecular docking.

Introduction

Heterocyclic molecules are the most important class of organic chemistry being used as drugs in the field of pharmaceuticals. In which molecules like quinolines, benzoxazole, benzothiozole possess most remarkable and a wide range of biological activities.[1,2,11] Benzoxazole is common heterocycle in medicinal chemistry that possesses diverse chemotherapeutic activities including antimicrobial[15], antiviral[6], antioxidant[17] and anticancer[9] activities. Study has revealed that substituted benzoxazoles and related heterocycles are biologically active with lower toxicities[14] and also show *in vitro* inhibitory activity[7].



Scheme 1: Synthetic route for the preparation of benzoxazole derivatives

Some phenyl benzoxazole derivatives have been discovered as potent acetylcholinesterase inhibitors with antioxidant property to enhance learning and memory[20]. Recently 2-substituted benzoxazole derivatives have been studied for anti-proliferative activities and were found as new generation anti-breast cancer agents[13].

The considerable biological activities of benzoxazole compounds in drug discovery and their importance in medicinal field have stimulated the present investigation on the synthesis of derivatives of this ring system. Hence it was planned to synthesize fused benzoxazole derivatives, but the stability of the intermediate formed during the reactions are so high that the attempts to cyclize the molecule by cyclizing agents such as pyridine, C_2H_5ONa were not successful as depicted in scheme 1.

The molecules are small ligands and the literature survey has shown remarkable biological activity[18] for such molecules. The functionalities like sulphonamide[10], nitriles[19], halogens[12], nitro[16] groups as well as ester[8] derivatives of compounds have shown effective biological activities, accordingly synthesized molecules were exposed for *in vitro* antimicrobial investigation.

The synthesized molecules were screened for *in silico* antimicrobial studies against target *Staphylococcus aureus* UDP-N-acetylenolpyruvylglucosamine reductase. *In silico* studies of synthesized molecules play a key role in predicting the best conformation considering lowest binding energy and the number of hydrogen bonds. Based on docking studies, the molecules which show binding energy more than -7.5 kcal/mol molecules were subjected to *in vitro* antibacterial and antifungal investigation.

Material and Methods

Chemistry: TMS was used as internal standard for ^1H and ^{13}C NMR spectral analysis with delta values as ppm. NMR spectra were recorded on Bruker 400 MHz spectrometer MIT, MAHE, Manipal, Karnataka, India and IISc, Bangalore, Karnataka, India. LC-MS were recorded on LCMS 2010A, SHIMANDZU, JAPAN with C18 column and rate of flow 0.2 ml/min using ESI (electron spray ionization) method. Bruker Fourier transformed infrared (FT-IR) spectrophotometer is used for recording IR spectra by KBr pallet method. 230-400 mesh sized silica gel is used for column chromatography. For TLC analysis, silica gel 60 GF_{254} (Merck) plates were used and spots analysed by UV light of wavelength 254 nm.

5-sulfamoyl and 5-chloro-6-nitro substituted 1,3-benzoxazole-2-thiol (1A)/(1B): 0.1 mol KOH and 60 ml of methanol were taken in 100 ml round bottomed flask. To this, CS_2 (0.1 mol) was added dropwise in ice cold condition (0-5°C) with constant stirring for few minutes, followed by addition of opsamide/ 4-chloro-5-nitro-2-aminophenol (0.1 mol). The reaction mixture was refluxed for 3 hr 30 min and 5 hr 30 min respectively and poured to crushed ice, acidified with dilute acetic acid (pH 6.0) with stirring. The solid product obtained was immediately filtered, dried and recrystallized with ethanol.

5-sulfamoyl and 5-chloro-6-nitro substituted ethyl [(1,3-benzoxazol-2-yl)sulfanyl]acetate (2A)/(2B): 0.1 mol of marcapto substituted benzoxazole (1A/1B) was taken in a 100 ml round bottomed flask containing dry acetone (40 ml) and anhydrous potassium carbonate (3g) followed by addition of ethyl chloroacetate (0.1 mol). Reflux it for 30 min and pour to crushed ice with constant stirring. The solid product obtained was filtered, dried and recrystallized with methanol.

5-sulfamoyl and 5-chloro-6-nitro substituted 2-hydrazinyl-1,3-benzoxazole (3A)/(3B): 0.1 mol of ester derivatives of substituted benzoxazole (2A)/(2B) were taken in a 100 ml round bottomed flask containing methanol (40 ml) followed by addition of hydrazine hydrate (0.1 mol) and refluxed for 30 min and stirred for 30 min respectively. Reaction mixture was poured on to crushed ice with constant stirring. The solid product obtained was filtered, dried and recrystallized with DMF.

5-sulfamoyl and 5-chloro-6-nitro substituted ethyl-3-[2-(1,3-benzoxazol-2-yl)hydrazinylidene]butanoate (4A)/(4B): 0.1 mol of substituted 2-hydrazinyl-1,3-benzoxazole (3A)/(3B) was taken in 100 ml round bottomed flask containing ethanol (20 ml), then add 0.1 mol of ethylacetoacetate and refluxed for 4 hr and 3hr respectively. Reaction mixture was poured on to crushed ice with constant stirring. The solid product obtained was filtered, dried and recrystallized with ethanol.

2-[2-(2,2-dicyanoethenyl)hydrazinyl]-1,3-benzoxazole-5-sulfonamide (5A): 0.1 mol of 5-sulfamoyl substituted 2-hydrazinyl-1,3-benzoxazole **(3A)** and 0.1 mol of ethoxymethylene malononitrile in dimethylformamide (30 ml) were refluxed for 2 hr. The reaction mixture was allowed to stand for few minutes and then poured onto crushed ice, the solid product obtained was filtered, dried and recrystallized with ethanol.

5-sulfamoyl and 5-chloro-6-nitro substituted 1,3-benzoxazol-2(3H)-one (6A)/(6B): 0.1 mol of substituted 2-hydrazinyl-1,3-benzoxazole (3A)/(3B) was taken in 100 ml round bottomed flask, 10 ml of formic acid/acetic acid is added along with few drops of concentrated hydrochloric acid and refluxed for 3 hr. The reaction mixture was allowed to stand for few minutes and then poured onto crushed ice, the solid product obtained was filtered, dried and recrystallized with ethanol.

5-sulfamoyl-1,3-benzoxazole-2-thiol (1A): IR (ν_{max}) in cm^{-1}: 3150 (-SH), 1331 (S=O). ^1H NMR (DMSO-d_6, 400 MHz): δ 7.454 (s, 2H, NH_2 of sulfonamide, disappeared on D_2O exchange) 7.611 (dd, H, C-7), 7.653 (d, H, C-9), 7.709 (d, H, C-6), 14.159 (s, 1H, SH, disappeared on D_2O

exchange). ^{13}C NMR (DMSO-d_6, 400 MHz): δ 108.4, 110.7, 122.2, 132.0, 141.7, 150.3 (for benzene ring of benzoxazole) and 181.5 (for C-SH group). MS (LCMS): m/z 230 [M+].

5-chloro-6-nitro-1,3-benzoxazole-2-thiol (1B): IR (ν_{max}) in cm^{-1}: 661 (C-Cl), 1511 (N-O) For nitro compound, 1618 (C=N), 3050 (-SH). ^{1}H NMR (DMSO-d_6, 400 MHz): δ 8.14 (s, H, C-6), 7.42 (s, H, C-9). ^{13}C NMR (DMSO-d_6, 400 MHz): δ 115.1, 118.4, 124.9, 140.5, 145.7, 157.5 (for benzene ring of benzoxazole) and 180.2 (for C-SH group). MS (LCMS): m/z 231 [M+], 233 [M+2].

Ethyl [(5-sulfamoyl-1,3-benzoxazol-2-yl)sulfanyl]acetate (2A): IR (ν_{max}) in cm^{-1}: 1191 (C-O). 1327 (S=O), 1732 (C=O), ^{1}H NMR (DMSO-d_6, 400 MHz): δ 8.01 (d, H, C-6), 7.843 (s, H, C-9), 7.79 (dd, H, C-7), 4.325 (s, 2H, -S-CH_2 proton), 4.148 to 4.201 (q, 2H, J = 7.0983 Hz, CH_2 protons of ester), 1.185 to 1.221 (t, 3H, J = 7.0979 Hz, -CH_3), 7.424 (s, 2H, NH_2 of sulfonamide, disappeared on D_2O exchange). ^{13}C NMR (DMSO-d_6, 400 MHz): δ 14.2 (-CH_3), 35.1 (S-CH_2), 61.5 (-OCH_2-), 110.1, 115.5, 125.4, 138.6, 145.2, 155.2, 165.7, 170.7 (C=O). MS (LCMS): m/z 316 [M+].

Ethyl [(5-chloro-6-nitro-1,3-benzoxazol-2-yl)sulfanyl] acetate (2B): IR (ν_{max}) in cm^{-1}: 817 (C-Cl), 1311 (C-O), 1484 (N-O) for nitro compound, 1741 (C=O). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 8.02 (s, H, C-6), 7.70 (s, H, C-9), 4.11 (s, 2H, -S-CH_2 proton), 4.23 to 4.28 (q, 2H, J = 7.1352 Hz, CH_2 protons of ester), 1.28 to 1.31 (t, 3H, J = 7.1269 Hz, -CH_3). ^{13}C NMR (DMSO-d_6, 400 MHz): δ 15.1 (-CH_3), 36.4 (S-CH_2), 60.5 (-OCH_2-), 111.1, 114.7, 126.7, 139.2, 144.2, 156.1, 164.9, 172.8 (C=O). MS (LCMS): m/z 317 [M+], 319 [M+2].

2-hydrazinyl-5-sulfamoyl-1,3-benzoxazole (3A): IR (ν_{max}) in cm^{-1}: 1308 (S=O), 3000 (NH_2), 3400 (-NH). ^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.646 (d, H, C-6), 7.503 (s, H, C-9), 7.742 (dd, H, C-7), 9.102 (s, 1H, NH, disappeared on D_2O exchange), 4.615 (s, 2H, NH_2, disappeared on D_2O exchange), 7.260 (s, 2H, NH_2 of sulfonamide, disappeared on D_2O exchange). ^{13}C NMR (DMSO-d_6, 400 MHz): δ 110.5, 117.5, 120.3, 136.8, 142.1, 152.5, 167.7 (C-NH-). MS (LCMS): m/z 228 [M+].

2-hydrazinyl-5-chloro-6-nitro-1,3-benzoxazole (3B): IR (ν_{max}) in cm^{-1}: 622 (C-Cl), 1565 (N-O) for nitro compound, 1641 (C=N), 3250 (NH_2). ^{1}H NMR (DMSO-d_6, 200 MHz): δ 8.23 (s, H, C-9), 7.51 (s, H, C-6), 9.73 (s, 1H, NH, disappeared on D_2O exchange), 4.82 (s, 2H, NH_2, disappeared on D_2O exchange). ^{13}C NMR (DMSO-d_6, 400 MHz): δ 110.2, 118.6, 130.8, 142.5, 148.2, 159.6, 168.9 (C-NH-). MS (LCMS): m/z 229 [M+], 231 [M+2].

Ethyl-3-[2-5-sulfamoyl(1,3-benzoxazol-2-yl)hydrazinyli dene]butanoate (4A): IR (vmax) in cm^{-1}: 1312 (S=O), 1649 (C=O), 1239 (C-O). 1H NMR (DMSO, 400 MHz): δ 7.335, 7.761-7.838 (m, 3H, C-6, C-7, C-9 aromatic protons), 2.187 (s, 2H, -CH_2-C=O proton), 4.012 to 4.135 (q, 2H, J = 7.0983 Hz, CH_2 protons of ester), 2.014 to 2.107 (t, 3H, J = 7.0979 Hz, -CH_3), 1.239 (s, 3H, -CH_3 proton), 11.177 (s, 1H, NH, disappeared on D2O exchange) 7.450 (s, 2H, NH_2 of sulfonamide, disappeared on D2O exchange). 13C NMR (DMSO-d6, 400 MHz): δ 14.5 (-CH_3), 17.2 (-CH_3), 45.1, 62.0 (-OCH_2-), 110.8, 120.2, 124.7, 138.5, 145.6, 150.1, 152.4, 154.5, 172.2 (C=O). MS (LCMS): m/z 340 [M+].

Ethyl-3-[2-5-chloro-6-nitro(1,3-benzoxazol-2-yl)hydrazi nylidene]butanoate (4B): IR (ν_{max}) in cm^{-1}: 1529 (N-O) for nitro compound, 590 (C-Cl), 1658 (C=N), 1710 (C=O), 1251 (C-O). ^{1}H NMR (DMSO, 400 MHz): δ 8.378 (s, H, C-9), 7.677 (s, H, C-6), 3.421 (s, 2H, -CH_2-C=O proton), 4.096 to 4.149 (q, 2H, J = 7.131 Hz, CH_2 protons of ester), 1.195 to 1.230 (t, 3H, J = 7.124 Hz, -CH_3), 2.034 (s, 3H, -CH_3 proton), 11.632 (s, 1H, NH, disappeared on D_2O exchange) ^{13}C NMR (DMSO-d_6, 400 MHz): δ 14.9 (-CH_3), 17.4 (-CH_3), 43.7, 61.0 (-OCH_2-), 112.8, 116.6, 124.8, 140.5, 145.7, 152.1, 154.6, 157.5, 168.1 (C=O). MS (LCMS): m/z [M+] 341, [M+2] 343.

2-[2-(2,2-dicyanoethenyl)hydrazinyl]-1,3-benzoxazole-5-sulfonamide (5A): IR (ν_{max}) in cm^{-1}: 2221 (C≡N), 3241 (NH), 1324 (S=O). ^{1}H NMR (DMSO, 400 MHz): δ 7.88 (d, H, -CH), 8.01 (d, H, C-6), 8.12 to 8.22 (m, 4H, C-7, C-9, NH-NH Proton and 2H of NH-NH disappeared on D_2O exchange), 7.52 (s, 2H, NH_2 of sulfonamide, disappeared on D_2O exchange). ^{13}C NMR (DMSO-d_6, 400 MHz): δ 56.5 (-$C(CN)_2$), 111.2, 115.3 (-CN), 119.7, 123.5, 135.4, 141.2, 151.6, 165.4, 172.5. MS (LCMS): m/z 305 [M+].

5-sulfamoyl-1,3-benzoxazol-2(3H)-one (6A): IR (ν_{max}) in cm^{-1}: 3180 (NH), 1750 (C=O). ^{1}H NMR (DMSO, 400 MHz): δ 7.441 to 7.578 (m, 3H, C-6, C-7, C-9 aromatic protons), 12.01 (s, 1H, NH, disappeared on D_2O exchange), 7.38 (s, 2H, NH_2 of sulfonamide, disappeared on D_2O exchange). ^{13}C NMR (DMSO-d_6, 400 MHz: δ 121.35, 123.63, 124.69, 131.54, 133.11, 138.72 (for benzene ring of benzoxazole) and 141.45 (for C=O group). MS (LCMS): m/z 215 [M+].

5-chloro-6-nitro-1,3-benzoxazol-2(3H)-one (6B): IR (ν_{max}) in cm^{-1}: 3260 (NH), 2918 (OH), 1767 (C=O), 1564 (N-O), 813 (C-Cl). ^{1}H NMR (DMSO, 400 MHz): δ 7.422 to 8.288 (dd, 2H, C-6, C-9 aromatic protons), 11.40 (s, 1H, NH, disappeared on D_2O exchange). ^{13}C NMR (DMSO-d_6, 400 MHz: δ 120.31, 124.32, 126.48, 135.27, 144.11, 148.27 (for benzene ring of benzoxazole) and 155.54 (for C=O group). MS (LCMS): m/z 215 [M+], 217 [M+2].

Results and Discussion

***In silico* studies:** The newly synthesized benzoxazole nucleus derivatives (1A, 1B, 2A, 2B, 3A, 3B, 4A, 4B, 5A, 6A and 6B) were docked against target *Staphylococcus aureus* UDP-N-acetylenolpyruvylglucosamine reductase (MurB) (PDB ID: 1HSK)[4] using AutoDoc Vina (ADT) platform[3] and the binding energies of the same are tabulated in table 1. The study has been made to find the possible binding mode and interaction of molecules with amino acid

residues. Initial structure optimization and energy minimization of all ligands were carried out using OpenBabel 2.3.2[5] followed by the addition of Gasteiger charges along with polar hydrogen atoms.

An objective-based docking technique was utilized in this investigation to set up the grid maps framework. The lattice points of the grid box and grid centre were set at X = 24, Y = 24, Z = 34 and x = 179.207, y = 149.393, z = 163.677 respectively.

Docking simulation was performed with various modes set to 10 and exhaustiveness to 20. ADT scoring function used for the determination of ligand binding affinity and it was anticipated as negative Gibbs free energy (ΔG) (Kcal/mol). On the basis of the binding energy and the formation of hydrogen bonds, the nature of the interaction, whether strong, medium or low, has been determined. The posture with the highest negative value is regarded as the best conformation. Ligplot and PyMol have been used to perform post-docking analysis.

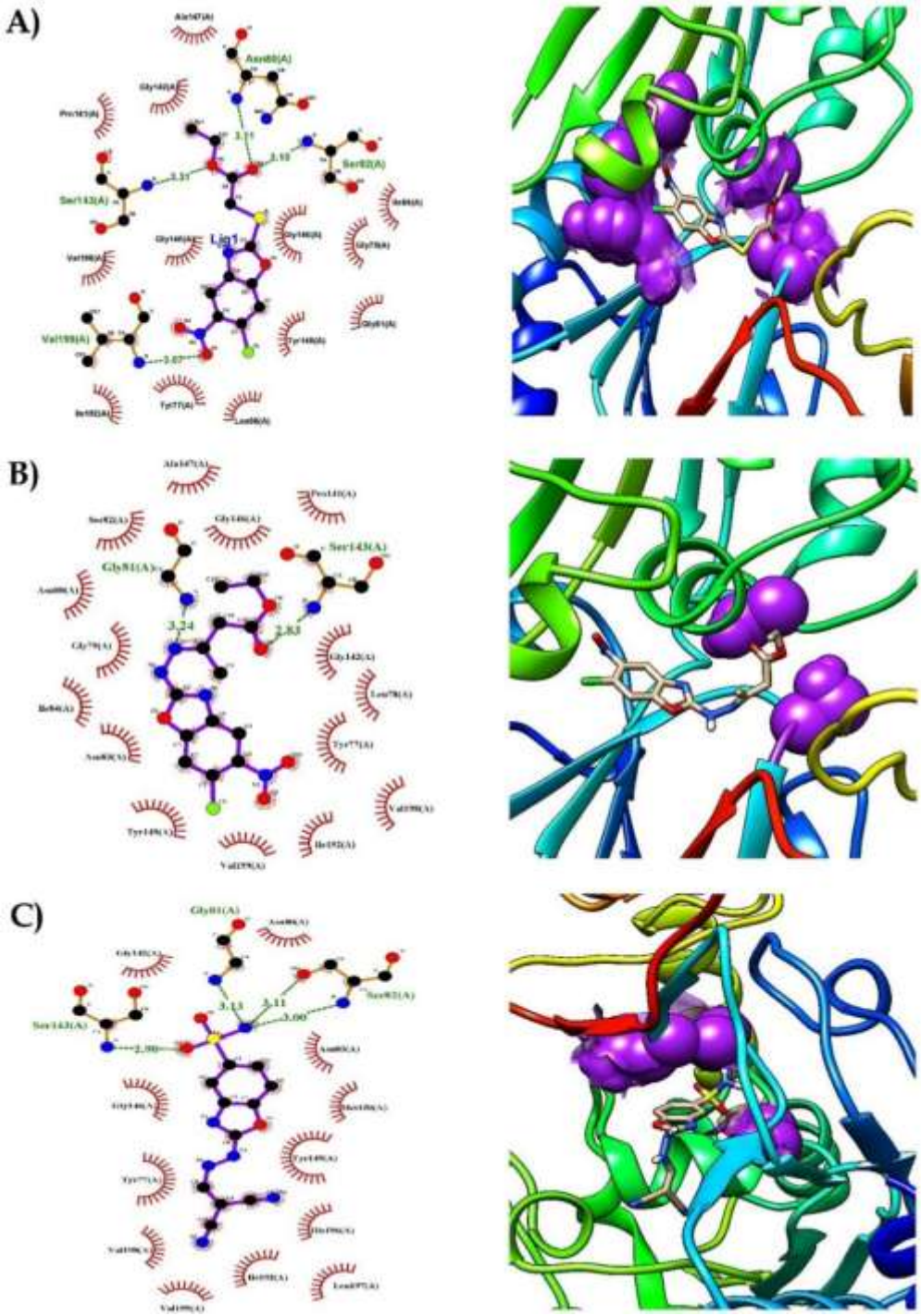


Figure 1: Molecular docking analysis of lead compounds 2B, 4B and 5A against *Staphylococcus aureus* UDP-N-acetylenolpyruvylglucosamine reductase (MurB) receptor.

Among the eleven molecules, it was found that the binding energy of molecules 2B, 4B and 5A was the lowest viz. -8.5, -8.6 and -8.7 by exhibiting four, two and four hydrogen bonds respectively (Table 2). In the active pocket amino acid residues, Ser143, Val199, Ser80, Asn80, Gly81, Ser82 were forming the hydrogen bonds with receptor MurB. The hydrogen and hydrophobic interaction profiles of molecules 2B, 4B and 5A with the receptor MurB and their distances are shown in figure. 1.

From the present study, it can be suggested that 2B, 4B and 5A ligand molecules can show better inhibition. In this context, those molecules which outperformed in the docking studies by showing binding energy more than -7.5 kcal/mol were further subjected to anti-microbial experimental validation to confirm these findings.

Biological studies: Among eleven synthesized compounds, eight were evaluated for their antibacterial and antifungal activity on the basis of promising binding energy in docking studies i.e. more than -7.5 kcal/mol. Antimicrobial activities were performed by agar well diffusion method. Before doing agar well assay, minimum inhibitory capacity of compound was analysed in nutrient broth with 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL and 3.125 µg/mL concentrations. 5ml of liquid broth media was taken in 6 test tubes inoculated with 10 µL. 100 µL of each concentration were added, incubated overnight separately for all eight compounds. *Escherichia coli, Salmonella typhi, Pseudomonas syringae, Staphylococcus aureus, Aspergillus terrus* and *Penicillium brocae* organisms taken for the study. Results are tabulated in table 3. Once recorded positive results, further 3 minimum concentrations were taken ahead for agar well assay.

***In vitro* Antibacterial Studies:** The antibacterial activity was performed against three gram-negative and one gram-positive bacterial strains such as *Escherichia coli* (MTCC-1599), *Salmonella typhi* (MTCC-734), *Pseudomonas syringae (*MTCC- 1604*)* and *Staphylococcus aureus* (MTCC-4734) by agar well diffusion method. The drug molecules were dissolved in DMSO and different dilutions were prepared (100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL and 3.125µg/mL). The derivatives were screened by taking Ciprofloxacin as positive control and DMSO served as negative control.

Table 1
Binding energies by Molecular docking studies to the compounds against *Staphylococcus aureus* UDP-N-acetylenolpyruvylglucosamine reductase (MurB) receptor

Compounds	1A	1B	2A	**2B**	3A	3B	4A	**4B**	**5A**	6A	6B
Binding energy (kcal/mol)	-7	-7	-8.4	-8.5	-7.5	-7.6	-8.3	-8.6	8.7	-7.5	-7.4

Table 2
Molecular docking results of lead compounds against *Staphylococcus aureus* UDP-N-acetylenolpyruvylglucosamine reductase (MurB) receptor

Lead compounds	**Binding energy (kcal/mol)**	**Protein-ligand interaction**		
		No. of H-bonds	**Amino acid residues**	**Distance (Å)**
2B	-8.5	4	Ser143; Val199; Ser80; Asn80	3.31; 3.07; 3.10; 3.11
4B	-8.6	2	Gly81; Ser143	3.24; 2.83
5A	-8.7	4	Ser143; Gly81; Ser82	2.90; 3.13; 3.11; 3.00

Table 3
Minimum Inhibitory concentration of bacterial and fungal strains

Compound	**MIC values in µg/mL**					
	Escherichia coli	***Salmonella typhi***	***Pseudomonas syringae***	***Staphylococcus aureus***	***Aspergillus terrus***	***Penicillium brocae***
2A	50	R	50	50	50	100
2B	25	R	25	50	25	50
3A	25	R	50	50	50	50
3B	25	R	100	50	50	100
4A	25	R	100	50	50	50
4B	25	R	50	50	25	50
5A	12.5	R	25	25	12.5	25
6A	25	R	100	50	50	100

A standardized suspension of the test bacteria was inoculated and incubated for 16-18 hrs at 37℃ to obtain fresh cultures. Sterilized Petri dishes were poured with 20 mL of LB agar media, 100 µL of fresh inoculum was smeared and allowed to dry for 10mins. Appropriate wells were made on these agar plates by using an agar punch and different concentrations of compounds (25 µg/mL, 12.5 µg/mL, 6.25 µg/mL) along with control standards were added to each of the labelled wells. The Petri dishes were prepared in triplicates and incubated at 37℃ for 16-18 hrs. The antibacterial activity of each compound was analysed by calculating the diameter of the inhibition zone and the potency of each compound was correlated with Ciprofloxacin. The results are tabulated in table 4.

Out of all the compounds tested, 5A showed good inhibitory capacity at 25 µg/mL against all the organisms except *Salmonella typhi*. 2B has inhibited *Escherichia coli* very efficiently followed by *Pseudomonas syringae* and *Staphylococcus aureus* as shown in table 4. Followed by 2B, 4B has shown good inhibition in *Escherichia coli* and moderate inhibition in both *Pseudomonas syringae* and *Staphylococcus aureus.* However, 5A has inhibited gram-positive organism *Staphylococcus aureus* effectively than that of other organisms taken for study.

***In vitro* Antifungal Studies:** The antifungal activity was performed by agar well diffusion method against two fungal strains, *Aspergillus terrus* and *Penicillium brocae* which are characterized at lab. The drug molecules were dissolved in DMSO and different dilutions were prepared (100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL and 3.125µg/mL). The derivatives were screened by taking fluconazole as positive control and DMSO served as negative control. A standardized suspension of the fungal strains was inoculated and incubated for 3-4 days at room temperature to obtain fresh cultures.

Table 4
***In vitro* Antibacterial study of compounds**

Compound	***Escherichia coli***			***Salmonella typhi***			***Pseudomonas syringae***			***Staphylococcus aureus***		
Conc.	**25 µg/ mL**	**12.5 µg/ mL**	**6.25 µg/ mL**	**25 µg/ mL**	**12.5 µg/ mL**	**6.25 µg/ mL**	**25 µg/ mL**	**12.5 µg/ mL**	**6.25 µg/ mL**	**25 µg/ mL**	**12.5 µg/ mL**	**6.25 µg/ mL**
2A	2.5	1.5	0.5	0.5	R	R	1	0.5	0.5	1.5	1	0.4
2B	4	2	1	0.5	0.5	R	2	1.5	1	2	1.5	1
3A	3	1.5	1	0.5	R	R	2	1	0.5	2	1.5	1
3B	3	1.5	1	0.5	R	R	1	1	0.5	1.5	1	0.5
4A	3.5	2	1	0.5	R	R	1	0.5	0.5	1.5	1	0.5
4B	4	2.5	1.5	1	0.5	0.5	1.5	1	1	1.5	1	1
5A	6	5.5	4	1.5	1	0.5	2.5	2	1	4	2.5	1.5
6A	3	1.5	1	0.5	R	R	1	0.5	0.5	1.5	1	0.5
Ciprofloxacin 3 µg/mL	10			3			7			8		

Inhibition zones are shown in mm

Table 5
***In vitro* antifungal study of compounds**

Compound	***Aspergillus terrus***			***Penicillium brocae***		
Conc.	**50 µg/ mL**	**25 µg/ mL**	**12.5 µg/ mL**	**50 µg/ mL**	**25 µg/ mL**	**12.5 µg/ mL**
2A	3	1	R	0.5	R	R
2B	4	2	1	2.5	1	0.5
3A	3	1	R	1	0.5	R
3B	3	0.5	R	0.5	R	R
4A	3.5	1.5	1	1.5	1	R
4B	4	2	1	1.5	1	R
5A	6	2.5	1	3	1.5	0.5
6A	3.5	1	0.5	1	0.5	R
Fluconazole 12.5 µg/mL	10			12		

Inhibition zones are shown in mm

Sterilized Petri dishes were poured with 20 mL of PDA media, 100 µl of fresh inoculum was smeared and allowed to dry for 10 mins. Appropriate wells were made on these agar plates by using an agar punch and different concentrations of compounds (50 µg/mL, 25 µg/mL, 12.5 µg/mL) along with control standards were added to each of the labelled wells. The Petri dishes were prepared in triplicate and incubated at room temperature for 3-4 days. The antifungal activity of each compound was analysed by calculating the diameter of the inhibition zone and the potency of each compound was correlated with fluconazole. The results are tabulated in table 5.

Out of all the compounds tested 5A showed good inhibitory capacity at 50 µg/mL against both the pathogenic fungal strains. Followed by 5A, 2B and 4B have shown good inhibition in *Aspergillus terrus* and *Penicillium brocae* whereas other compounds have not effectively inhibited the growth of both the organisms taken for study.

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Conclusion

Among the eleven synthesized compounds, 2B, 4B and 5A molecules have evolved as potent antimicrobial agents by *in silico* as well as by *in vitro* method. Against the molecules referred for the study, the presence of ester group along with nitro, chloro substitution for the benzoxazole moiety in 2B and 4B molecules triggered the antimicrobial activity. Also, the presence of sulphonamide with nitrile substitution in benzoxazole molecule 5A enhances the activity.

It can also be concluded that because of the small three-dimensional, stable structure and its ability to form hydrogen bonding, the molecules shows good activity towards antimicrobial. In progress to the current work, the potent molecules will be subjected to cytotoxicity and specific organ toxicity.

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