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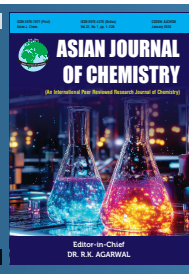

Synthesis, Characterization, Theoretical and Antimicrobial Studies of Substituted Isoxazoline Derivatives

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This research focuses on the design of new compounds and their biological applications to address various medical issues and pandemics caused by diverse microorganisms. In this work, two novel isoxazoline derivatives containing chlorine and methoxy groups at the *para* position were synthesized, characterized by various known techniques and QSAR properties were calculated using Osiris and docking against the protein targets such as 1HNJ, 1KZN and 1T15. The theoretical outcomes are compared with the experimental antimicrobial investigations on *B. subtilis*, *E. coli*, *S. aureus*, *K. pneumoniae*, *C. albicans* and *A. niger* using agar well-diffusion method. The derived derivatives exhibited good results and showed good coincidence with theoretical results. Out of two target compounds, activating methoxy group substituted isoxazoline compound showed better result against bacterial strains (12-16 mm), whereas deactivating chlorine group substituted compound revealed the good result (12-15 mm) against fungus strains.

Keywords: Isoxazolines, QSAR, Docking studies, Antimicrobial activity.

INTRODUCTION

Global development is encountering a number of economic challenges owing to the novel microbes and their effects on human health. Simultaneously, limited resistance against bacteria was seen due to the ongoing use of current antibiotic drugs, which diminished immunity [1,2]. The rising incidence of Gram-negative non-fermenters, which are resistant to all 'good' antibiotics, has created an immediate need for new antibiotics in clinical settings. Consequently, there has been a significant increase in funding for researches on the development of new antibiotics. Heterocyclic rings serve a vital purpose in medicinal chemistry and essential for advancing antibiotic development. The carbon backbone is only one component of heterocyclic molecules, which can also contain sulfur, nitrogen and other heteroatoms [3,4]. Several excipients and active medicinal ingredients contain heterocycles or other heteroatoms. The diverse pharmacological activities of heterocyclic compounds make them useful in the treatment of a extensive variety of diseases.

A heterocyclic ring is the main structural component of most current medical medications. Nitrogen in heterocyclic rings are unique among these compounds for several reasons, such as their abundance in nature, biological features and ease of synthesis [5,6]. Among several heterocycles, isoxazole and related heterocyclic compounds have a wide variety of biological effects, making them an important class of these compounds [7-9]. Clinical trials have shown that isoxazole is highly efficient against a wide range of microorganisms, inflammation, tuberculosis and cancer [10,11]. Owing to the structurally diverse nature of isoxazole ring, it can be used as building blocks to create new bioactive drugs with better efficacy and fewer side effects since they offer a wide range of pharmacological effects [12,13]. One potential site for the ring cleavage in isoxazole could be its aromaticity and poorer nitrogen-oxygen bonding, which are the structural properties. These compounds are essential building blocks in a wide variety of bioactive chemical syntheses due to the isoxazole ring system, which allows for facile modification of the ring structure. Consequently, chemists should constantly be aiming to create and research isoxazole-containing compounds that could have wider therapeutic uses [14,15].

The vast amount of literature on isoxazole implies that it would be useful to investigate multiple pathways that could result in the production of isoxazole analogues that possess various bioactive characteristics. Likely, theoretical computational techniques are used to identify the structural properties before of experimental biological studies. Recently, QSAR and docking methods are applied to the designed or derived structures for the preliminary structural activity predictions [16-18]. Hence, this study selected the isoxazole building block with two different functional groups at the *para*-position along with two secondary amide groups. The derived two compounds were carried for both theoretical QSAR, docking and experimental antibacterial activity investigations for future drug designs and applications.

EXPERIMENTAL

All chemicals were procured from Sigma-Aldrich, India and used as such. Solvents were received from Aura chemicals and carried without further purifications. An FTIR vibrational spectrum of the sample was acquired using the IRAffinity-1 model *via* the KBr pellet method. The Bruker 400 MHz instrument was used to record ^{1}H and ^{13}C NMR spectra in DMSO-d_6 solvent. The QSAR properties of synthesized compounds were retrieved from the Osiris offline java based software. Docking carried against *E. coli* protein 1KZN (*E. coli* 24kDa Domain) and 1HNJ (β-keto acyl-acp synthase III + malonyl-coa) to measure the inhibitory effect of the isoxazole derivatives on the growth of certain bacteria using autodock-vina and biovia visual-studio. The Mueller-Hinton agar (MHA-Hi medium, Mumbai) was ised for agar well diffusion antimicrobial testing. Clinical and Laboratory Standards Institute standard tables were used for the purpose of interpreting the results.

Synthesis of 2-(4-acetamidophenoxy)-*N*-(3-(5-(4-substituted phenyl)-4,5-dihydroisoxazole-3-yl)phenyl)acetamide (e, f): 3-Amino acetophenone (0.5 mmol) along with acetic acid (0.6 mmol) were stirred well at room temperature. After 5 min of stirring, chloroacetyl chloride (0.65 mmol) in acetic acid were added slowly over a period of 30 min and again stirred for 45 min. The reaction progress was monitored by TLC using 60:40 ethyl acetate and hexane mixture. The reaction was quenched by sodium acetate solution (0.6 M), the formed precipitate was allowed to cool in an ice bath for 5 min. Product **a** was filtered, washed with distilled water and then recrystallized with ethanol. Next, compound **a** (0.2 mmol) was added to 40 mL of DMF, stirred well followed by dropwise addition of K_2CO_3 (0.21 mmol) in 30 mL of DMF at 28 ºC. The reaction mixture was further stirred for 30 min and then N-(4-hydroxyphenyl)-acetamide was added over a period of 15 min. The whole mixture was refluxed for 4 h and then treated with crushed ice at the end of reaction. The solid was precipitated, washed with distilled water, filtered and dried under vacuum at 60 ºC. The dried product **b** was recrystallized from 90% ethanol and water. Compound **b** was isolated and carried for further steps using 4-chlorophenol and 4-methoxyphenol. Equal mole quantity of 2-(4-acetamidophenoxy)-*N*-(3-acetylphenyl)acetamide and the substituted aldehydes were mixed in ethanol. This mixture was treated with 20% NaOH solution was added slowly with constant stirring. It was refluxed for 6 h and stirred overnight. After 8 h, the reaction was quenched into crushed ice and slightly acidified by 10 % HCl. The obtained products such as 4-chlorophenol derivative (**c**) and 4-methoxyphenol derivative (**d**) were carried for further reaction after the recrystallization in ethanol. Derivatives (**c**, **d**, 0.1 mmol) and hydroxylamine hydrochloride (0.12 mmol) in ethanol were refluxed for 10 h along with sodium acetate (0.12 mmol) [19,20]. The product was separated and recrystallized in ethanol (**Scheme-I**). Compounds **e** and **f** were isolated and characterized by FT-IR, ^{1}H and ^{13}C NMR spectroscopies.

***N*-(3-Acetylphenyl)-2-chloroacetamide (a):** Yield: 60%, m.p.: 107 ºC, m.f.: $C_{10}H_{10}ClNO_2$. ^{1}H NMR (100 MHz, DMSO-d_6) δ ppm: 10.06 (s, 1H), 8.12 (t, *J* = 1.9 Hz, 1H), 7.93-7.87 (m, 1H), 7.72 (dt, *J* = 8.1, 1.4 Hz, 1H), 7.47 (t, *J* = 7.9 Hz, 1H), 4.32 (s, 2H), 2.49 (s, 3H).

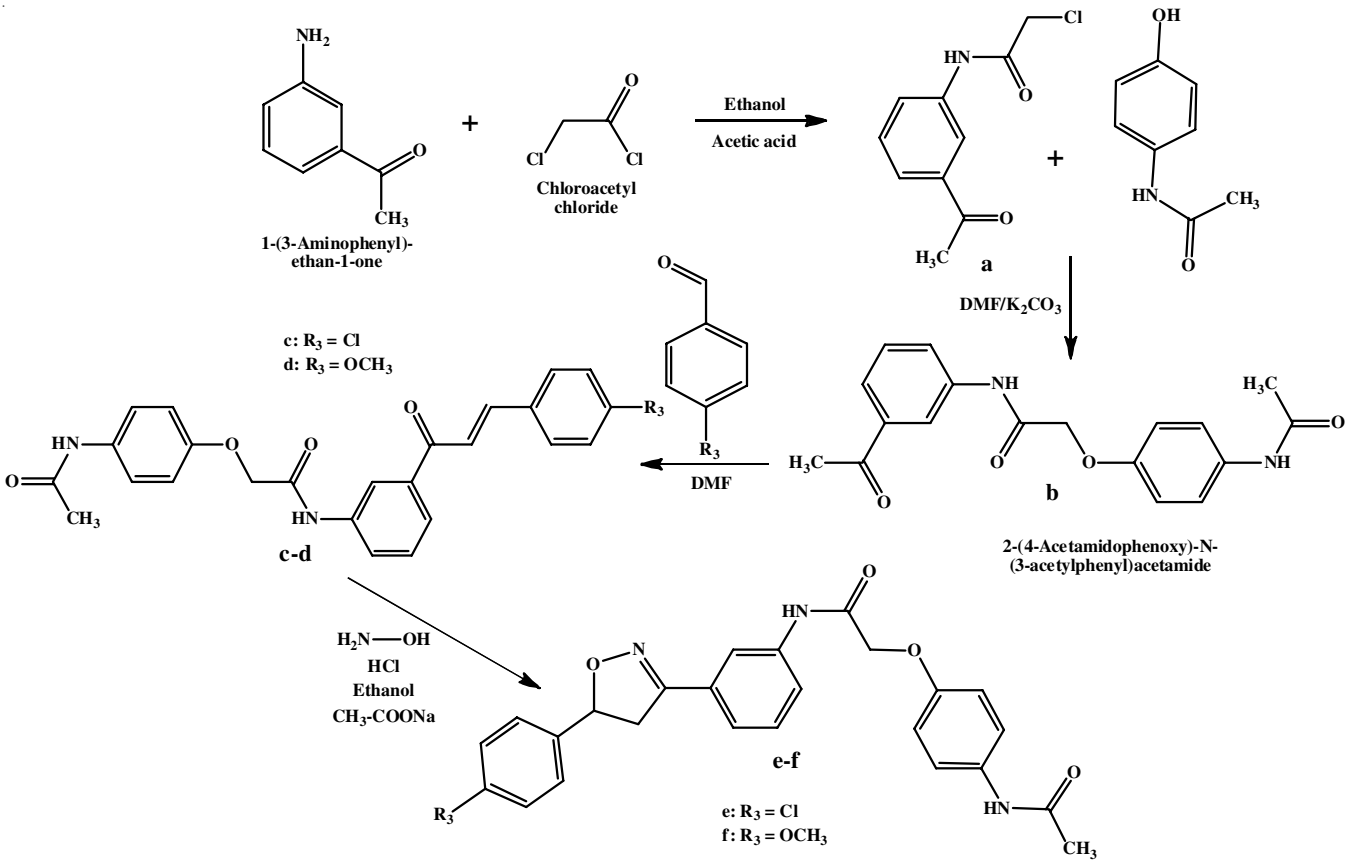
2-(4-Acetamidophenoxy)-*N*-(3-acetylphenyl)acetamide (b): Yield: 65%, m.p.: 116 ºC, m.f.: $C_{18}H_{18}N_2O_4$. ^{1}H NMR (100 MHz, DMSO-d_6) δ ppm: 10.05 (s, 1H), 9.83 (s, 1H), 8.12 (t, *J* = 1.9 Hz, 1H), 7.93-7.87 (m, 1H), 7.72 (dt, *J* = 8.1, 1.4 Hz, 1H), 7.50-7.43 (m, 3H), 7.02-6.96 (m, 2H), 4.67 (s, 1H). 2.49 (s, 3H), 2.03 (s, 3H)

2-(4-Acetamidophenoxy)-*N*-(3-(3-(4-chlorophenyl)-acryloyl)phenyl)acetamide (c): Yield: 55%, m.p.: 118 ºC, m.f.: $C_{18}H_{18}N_2O_4$. ^{1}H NMR (100 MHz, DMSO-d_6) δ ppm: 10.05 (s, 1H), 9.83 (s, 1H), 8.41 (t, *J* = 1.9 Hz, 1H), 7.84-7.78 (m, 1H), 7.78-7.72 (m, 3H), 7.69 (dt, *J* = 7.8, 1.4 Hz, 1H), 7.60-7.53 (m, 3H), 7.49-7.43 (m, 3H), 7.02-6.96 (m, 2H), 4.67 (s, 2H), 2.03 (s, 3H).

2-(4-Acetamidophenoxy)-*N*-(3-(3-(4-methoxyphenyl)-acryloyl)phenyl)acetamide (d): Yield: 55%, m.p.: 146 ºC, m.f.: $C_{26}H_{24}N_2O_5$. ^{1}H NMR (100 MHz, DMSO-d_6) δ ppm: 10.05 (s, 1H), 9.83 (s, 1H), 8.41 (t, *J* = 1.9 Hz, 1H), 8.05 (d, *J* = 15.9 Hz, 1H), 7.84-7.79 (m, 1H), 7.79-7.74 (m, 2H), 7.69 (dt, *J* = 7.8, 1.4 Hz, 1H), 7.57 (d, *J* = 15.9 Hz, 1H), 7.49-7.43 (m, 3H), 7.06-6.96 (m, 4H), 4.67 (s, 1H), 3.81 (s, 3H), 2.03 (s, 3H).

2-(4-Acetamidophenoxy)-*N*-(3-(5-(4-chlorophenyl)-4,5-dihydroisoxazol-3-yl)phenyl)acetamide (e): Yield: 54%, m.p.: 155 ºC, m.f.: $C_{25}H_{22}ClN_3O_4$. ^{1}H NMR (100 MHz, DMSO-d_6) δ ppm: 10.05 (s, 1H), 9.83 (s, 1H), 7.82-7.76 (m, 1H), 7.68 (dt, *J* = 8.1, 1.4 Hz, 1H), 7.49-7.43 (m, 2H), 7.40 (dd, *J* = 8.1, 7.1 Hz, 1H), 7.30 (t, *J* = 1.9 Hz, 1H), 7.20-7.14 (m, 2H), 7.02-6.95 (m, 4H), 5.93 (t, *J* = 5.4 Hz, 1H), 4.67 (s, 1H), 3.85 (dd, *J* = 15.7, 5.2 Hz, 1H), 3.60 (dd, *J* = 15.8, 5.4 Hz, 1H), 2.03 (s, 3H).^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 168.80, 168.36, 157.50, 156.89, 139.37, 138.12, 134.75, 133.75, 132.33, 129.25, 128.55, 128.31, 128.28, 124.48, 120.82, 117.29, 116.70, 74.92, 67.26, 37.25, 24.05.

2-(4-Acetamidophenoxy)-N-(3-(5-(4-methoxyphenyl)-4,5-dihydroisoxazol-3-yl)phenyl)acet amide (f): Yield: 54%, m.p.: 155 ºC, m.f.: $C_{26}H_{25}N_3O_5$, ^{1}H NMR (100 MHz, DMSO-d_6) δ ppm: 10.05 (s, 1H), 9.83 (s, 1H), 7.82-7.76 (m, 1H), 7.68 (dt, *J* = 8.1, 1.5 Hz, 1H), 7.49-7.43 (m, 2H), 7.40 (dd, *J* = 8.1, 7.1 Hz, 1H), 7.35-7.28 (m, 3H), 7.02-6.96 (m, 2H), 6.96-6.90 (m, 2H), 5.93 (t, *J* = 5.4 Hz, 1H), 4.67 (s, 1H), 3.85 (dd, *J* = 15.7, 5.2 Hz, 1H), 3.81 (s, 3H), 3.60 (dd, *J* = 15.8, 5.4 Hz, 1H), 2.03 (s, 2H). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm: 168.80, 168.36,



Scheme-I: Preparation of substituted isoxazole amide derivatives

159.36, 157.50, 156.89, 139.37, 135.52, 134.75, 132.33, 129.25, 128.28, 127.75, 124.48, 120.82, 117.29, 116.70, 114.06, 74.92, 67.26, 55.35, 37.25, 24.05.

Quantitative structure activity relationship (QSAR) and docking studies: Using the synthesized molecules, a QSAR calculation on the offline Osiris software using Chemsketch smiles notation were investigated. The notation copied and submitted to Osiris property explorer [21]. Using the auto dock software, the study examined the binding capabilities of the generated compounds against target proteins such as 1HNJ, 1KZN and 1T15. Initially, the Schiff base structure was drawn in mcule server and docking scores were recorded. The best pose was downloaded and was subjected along with target proteins in MOE software and docked using default settings. After the docking calculations, the binding affinity against the proteins was analyzed and the 2D interactions of the derived structure were calculated using Biovia discovery visual studio [22]. Molecular docking is an intriguing method for researching compound disease-causing protein interactions to create novel drugs. Binding sites that allow multiple ligand binding may aid medication design.

Antibacterial activity: The agar well-diffusion method was used to evaluate the antibacterial activity. Using bacterial strains according to the stated procedure, the antimicrobial determination was carried out [23,24]. The antibacterial activity of the synthesized compounds was evaluated using the agar well diffusion method in a Mueller-Hinton agar medium. To inoculate the agar plate, a certain amount of microbial inoculum was spread out across the whole surface of the agar. Afterwards, using a sterile corkborer or a tip, 8 mm diameter hole was aseptically punched. Next, 250 µg/mL of test sample was placed twice in a same plate along with gentamicin standard and DMSO placed as a negative control. After that, the test microbe dictates the parameters that the agar plates are cultured in. By dispersing throughout the agar media, the antimicrobial ingredient impedes the development of the tested microbial strain. Also, this work tested the minimum inhibition concentration by micro-dilution method.

Statistical analysis: All values were expressed mean ± SD. Statistical difference and linear regression analysis were performed using Graphpad prism 5 statistical software.

RESULTS AND DISCUSSION

The chloro and methoxy substituted 2-(4-acetamidophenoxy)-*N*-(3-(5-(4-phenyl)-1-isonicotinoyl-4,5-dihydro-1*H*-pyrazol-3-yl)phenyl)acetamide was synthesized for the biological applications. Compounds **e** and **f** were synthesized by sequence of chemical reactions as showed in **Scheme-I**. The required isoxazoline derivatives with good yield was prepared and characterized for further applications. The sequence of products **a** to **f** were characterized using UV, FTIR and NMR spectral techniques. The UV spectra of the compounds exposed the change in the excitation for the $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions based on the functional groups and the attached aromatic rings (Table-1). The absorption spectra of the derivatives are shown in Fig. 1.

TABLE-1
UV AND FTIR OUTCOMES OF THE DERIVATIVES **a-f**

Compd.	λ_{max} (UV)	Excitation	Frequency (FTIR)
a	222, 317	n→π*, π→π*	C=O (1650), NH (3325), C-Cl (fingerprint region)
b	265, 350	n→π*, π→π*	C=O (1660), C=C (1136), C-N (2280), NH (3300), C-H *str.* (2400)
c	285, 429	n→π*, π→π*	C=O (1687), C=C (1166), C-N (2358), NH (3466), C-H *str.* (2699)
d	285, 428n	n→π*, π→π*	C=O (1688), C=C (1169), C-N (2368), NH (3384), C-H *str.* (2898)
e	259, 343	n→π*, π→π*	C-H *str.* (3080), C=C *str.* (1508), C=O (1655), C=N *str.* (1600), NH (3387), C-H (2918)
f	254, 374	n→π*, π→π*	C-H *str.* (3057), C=C *str.* (1502), C=O (1629), C=N *str.* (1593), NH (3427), C-H (2935)

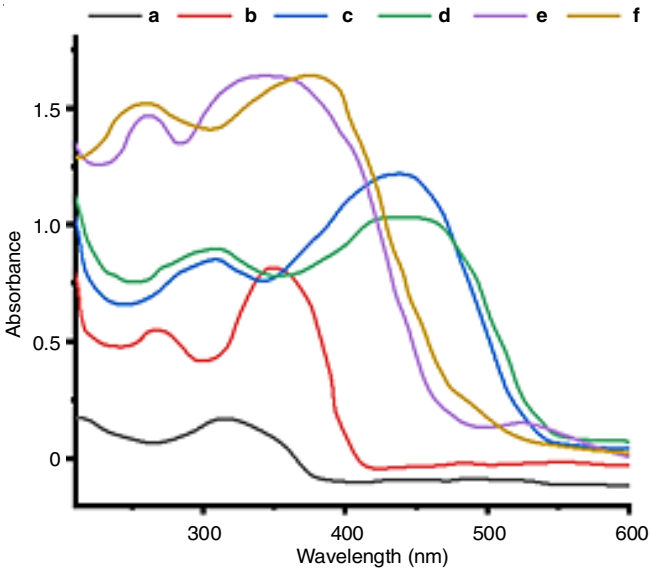


Fig. 1. Absorption spectra of the isoxazole derivatives **a-f**

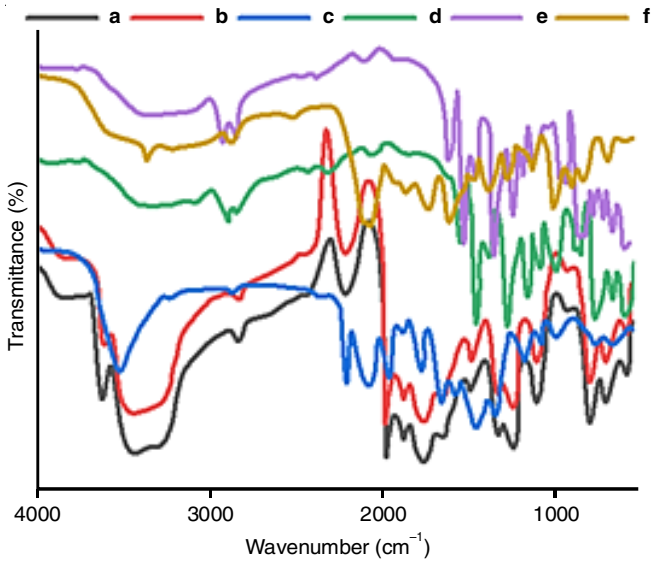


Fig. 2. Vibrational spectra of the isoxazole derivatives **a-f**

Infrared spectra of all compounds (Fig. 2) showed a sharp absorptions at 790-760 and 1170-1150 cm^{-1}, which are attributed to -Cl, -OCH_3 of the compounds **e**, **f** respectively. The IR spectra of synthesized products displayed the characteristic absorption bands at 1650-1500 cm^{-1} due to C=N of isoxazole. The compounds also exhibit bands at 3400-3250 cm^{-1} confirms the presence of –NH group. Synthesized compounds showed weak C-H stretching bands near 3100-2950 cm^{-1}, C=C skeletal vibrations near 1400-1100 cm^{-1} for aromatic rings. Likely, significant absorption observed around 3000 cm^{-1} for aliphatic nature of compounds. The IR spectra of compounds **e** and **f** displayed the characteristic absorption band at 1700-1650 cm^{-1} due to C=O of isoxazoline. These characterization results almost coincidence with the reported results [25,26]. After the vibrational spectra, the compounds were confirmed by NMR spectral technique.

The NMR data of compounds **e** and **f** exposed aromatic proton chemical shifts between δ 6 ppm and 8.5 ppm. Similarly, aliphatic protons were observed between δ 2 ppm and 4.5 ppm. The significant singlet peaks for -NH were observed at δ 9.44 ppm and another peak at 9.63 ppm for compound **c**. Compound **d** showed the NH chemical shifts at 9.66 ppm and 10.05 ppm respectively. Compounds **e** and **f** showed NH singlet protons chemical shift at δ 10.05 (s, 1H), 9.83 (s, 1H) respectively. Further, the compounds are confirmed by ^{13}C NMR. Carbon NMR showed the aromatic carbons between 114 and 160 ppm. Aliphatic carbons exhibit from 24 ppm to 75 ppm. Both compounds are differed at –C-Cl (155-160 ppm) and –C-OCH_3 (130-140 ppm). Simillarly, compounds **e** and **f** exposed the chemical shifts for C=N (151.8 ppm) and C=O (162.9, 167.1 ppm). All the characterization techniques have confirmed the prepared structures.

QSAR studies: After the characterization, the compounds were carried for the theoretical studies using Osiris Java software. The derived compounds structures were converted to smiles notation using Chemsketch and submitted in the software tool. The outcome results are presented in Table-2.

The results showed the good results for compounds **b**, **c**, **e** and **f**. The results exposed the non-toxic nature and good drug likeness score as per the following order such as **b** > **f** > **c** > **e** > **d**. Further the compounds were tested for their binding ability using docking software against the selected target proteins such as 1XCX and 3K0K. Some of the docked poses of the compounds are shown in Fig. 3. The outcome scores were compared with standard drugs streptomycin and fluconazole (Table-3). The synthesized derivatives are docked against the target proteins and a binding energy was calculated. Compounds **a-f** showed the docking score between -5.48 and -7.87 kcal/mol. When compared with the standards, this work observed the –2 kcal/mol energy difference. Compound **f** showed better results than compound **e** with the highest binding energy (-6.89 kcal/mol, -7.85 kcal/mol, -7.87 kcal/mol) against the selected targets such as 1HNJ, 1KZN and 1T15, respectively. When compared with the targets, compound **f** showed highest score against 1T15.

TABLE-2
THEORETICAL OSIRIS SOFTWARE QSAR PROPERTIES OF COMPOUNDS **a-f**

Properties	**a**	**b**	**c**	**d**	**e**	**f**
Molecular weight	211	326	448	444	463	459
Mutagenic	Toxic	Non toxic	Non toxic	Non toxic	Non toxic	Non toxic
Tumorigenic	Toxic	Non toxic	Non toxic	Non toxic	Non toxic	Non toxic
Irritant	Non toxic	Non toxic	Non toxic	Toxic	Non toxic	Non toxic
Reproductive effect	Toxic	Non toxic	Non toxic	Moderate	Non toxic	Non toxic
clogP	1.46	1.96	4.34	3.66	5.16	4.48
Solubility	-3.04	-3.93	-6.21	-5.49	-6.26	-5.54
TPSA	46.17	84.5	84.5	93.73	89.02	98.75
Drug likeness	-2.1	1.66	4.86	4.31	8.53	5.3
Drug score	0.11	0.74	0.42	0.24	0.36	0.45

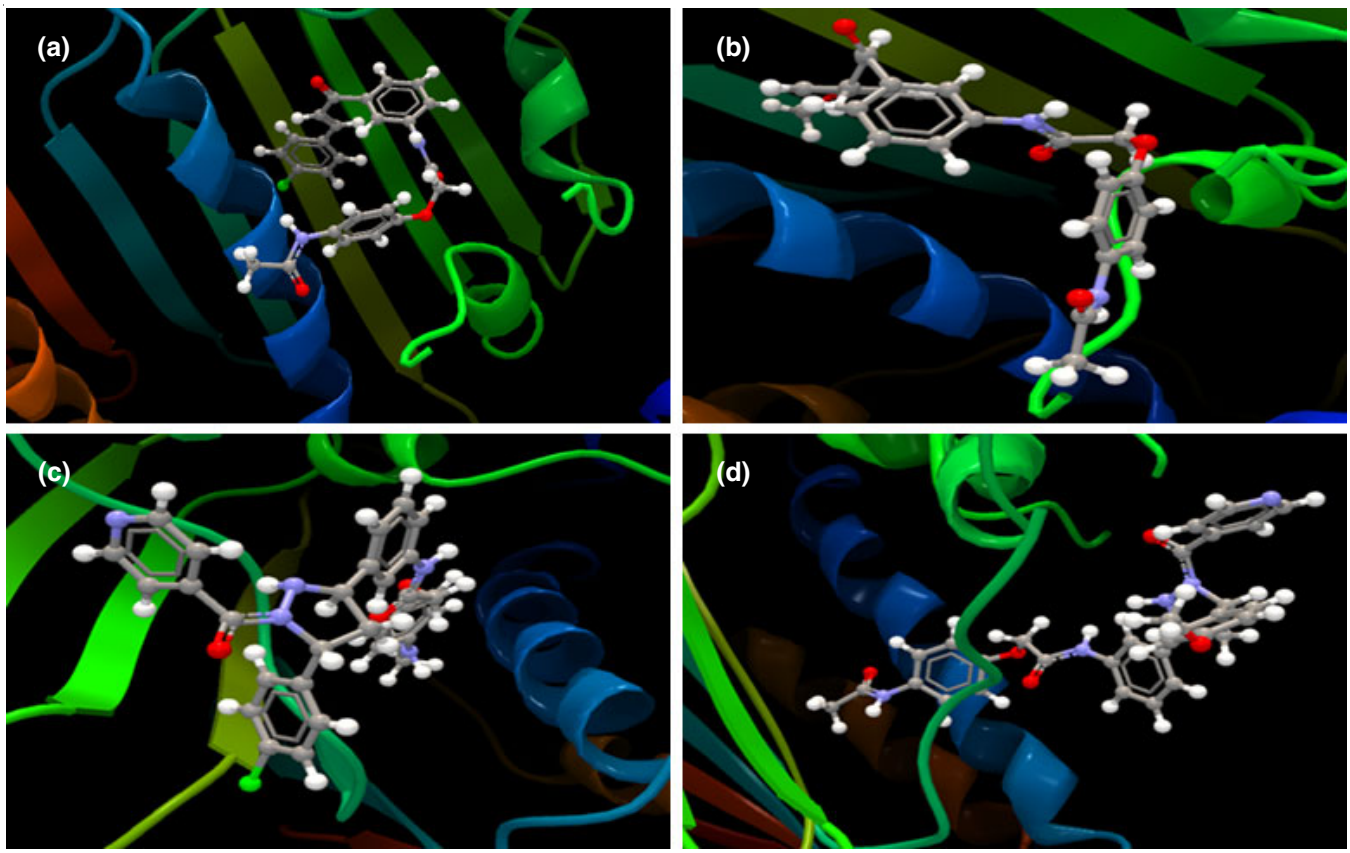


Fig. 3. Docked 3D pose of compound **f** against the target proteins such as (a) 1HNJ, (b) 1KZN, (c) 1T15 and (d) Pose of compound **e** and 1HNJ using CLC workbench-3

Standard drugs showed the score range of streptomycin (-8.24 to -9.12 kcal/mol) and fluconazole (-7.55 -8.44 kcal/mol), which is almost coincidence with the prepared compounds scores. 1T15 docked complex contained four hydrogen bonds with the distance between 2.38 Å and 3.26 Å. The hydrogen atoms of amino acid residues such as glu1698, lys1702, lys1690 and gln1779 established hydrogen bonds with the oxygen atoms of the ketonic and amide groups in the isoxazoline ring. The remaining compounds analyzed exhibited a variety of interactions beyond hydrogen bonding, including hydrophobic, alkyl-pi, van der Waal and polar/electrostatic interactions [27,28]. Activating group (-OMe) attached in compound **f** showed good binding energy when compared with deactivating group attached compound **e**. All the newly synthesized four isoxazoline derivatives incorporated with –Cl and –OCH_3 were examined for antibacterial and antifungal activities using an *in vitro* model [29,30].

TABLE-3
AUTODOCK VINA DOCKING SCORES OF
COMPOUNDS **a-f** AGAINST THE SELECTED TARGETS

Compound	Docking score against proteins (kj/mol)		
	1HNJ	1KZN	1T15
a	-5.72	-5.48	-5.64
b	-5.82	-5.98	-5.84
c	-6.73	-6.98	-7.84
d	-6.79	-6.89	-7.24
e	-6.57	-6.88	-7.36
f	-6.89	-7.85	-7.87
Streptomycin	-8.24	-8.77	-9.12
Fluconazole	-7.85	-7.55	-8.44

TABLE-4
ANTIMICROBIAL ACTIVITY DATA OF COMPOUNDS **a-f** AT 250 µg/mL

Bacteria	Zone of inhibition (mm) in diameter						
	Streptomycin	**a**	**b**	**c**	**d**	**e**	**f**
B. subtilis	16	7	8	12	10	14	12
E. coli	20	6	10	10	12	16	16
S. aureus	22	9	7	10	12	15	14
K. pneumoniae	15	–	10	8	11	12	15
Fungal	Fluconazole						
C. albicans	22	–	–	10	11	15	14
A. niger	27	–	–	8	8	12	11

Screening at the preliminary level was taken out for all the compounds against selected two Gram-positive, Gram-negative strains and two fungus strains by disk diffusion method at a concentration of 250 µg/mL. The zone of inhibition (mm) of each derivative was ascertained and compared with streptomycin and fluconazole taken as a standard drug for bacteria and fungi, respectively. DMSO was used to prepare stock solutions of tested derivatives. The findings of antimicrobial evaluation presented that most of the compounds have comparable activity against the bacterial and fungal strains (Table-4). Among the tested compounds, compound **f** (12-16 mm/250 µg/mL) was found to exhibit good antifungal activity in comparison to standard drug fluconazole. The outcome of antimicrobial activity evaluation of the synthesized compounds revealed that these compounds possessed antibacterial and antifungal activities [31,32]. From antimicrobial activity data, compounds (**e**, **f**) displayed better antibacterial activity (16 mm) against *E. coli* at 250 µg/mL and good antifungal activity (14 mm) against *C. albicans*.

From the microbial results, this work observed that the compounds without isoxazoline ring exposed the lower inhibition and the inhibition zones were increased after the ring introduced in the molecule. Also, this study examined activating (-OCH_3) and deactivating groups (-Cl) substituted compounds antimicrobial activity nature. The results revealed that the deactivating group substituted compound **e** showed good result against fungus strains when compared with compound **f**. Based on the results, this work revealed the antibacterial and antifungal suitability of the isoxazoline derivatives.

Conclusion

This work successfully synthesized the two isoxazoline derivatives with activating methoxy group and deactivating chlorine substituted compounds. The prepared compounds were characterized using UV, FTIR and NMR techniques. The structures theoretical QSAR properties were calculated by java based Osiris tool. After the confirmation of non-toxic character, the compounds were carried for docking studies and revealed the good binding score against the targets. The theoretical results were confirmed by antimicrobial activities. The experimental antibacterial activity results matched theoretical results and showed good results.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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