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Synthesis, Characterization, Molecular Docking, Cell viability and Biological Activity of New Metronidazole Analogues against Cellulitis Causing Pathogens

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Development of microbial resistance against commercial imidazoles intended present study to develop some new metronidazole analogues against cellulitis causing pathogens. In present study, *N*-(4-substituted benzylidene)-2-(2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethoxy)-acetohydrazide (**2a-c**) were synthesized by treating hydrazide derivative of metronidazole (**1**) with various aromatic aldehydes. The structures of synthesized compounds **2a-c** were characterized using FT-IR, ^{1}H NMR, ^{13}C NMR and mass spectrometric data. The synthesized compounds **2a-c** were evaluated for their inhibitory potential against cellulitis triggering bacteria *S. aureus*, *E. coli* and cell viability profile using MTT assay. Among compounds **2a-c**, compound **2c** incorporated with high electronegative group, exhibited maximum inhibitory potential against cellulitis triggering pathogens. This potential was also supported by the docking data of compounds **2c** against glucosamine-6-phosphate (2VF5). The significant antibacterial potential of compounds **2a-c** against *S. aureus* and *E. coli*, high cell viability against HEK 293 cells (more than 75%) and high docking score of compounds with 2VF5 supports their potential application in cellulitis treatment. However, the synthesized compounds should be further evaluated for their *in vivo* preclinical significance.

Keywords: Metronidazole analogues, Hydrazide, Cellulitis, Docking studies.

INTRODUCTION

Nitrogen containing heterocycles plays a crucial role in medicinal chemistry [1]. For several decades, imidazoles have gained significant attention from researchers attributed to their diverse therapeutic properties, particularly for their remarkable antimicrobial activities [2]. The ability of imidazole to establish hydrogen bonds with biological targets enhances its importance as a scaffold for drug development [3]. The imidazole heterocycles are known for their antibacterial potential against *S. aureus* and *E. coli* [4], the key causative bacteria responsible for cellulitis [5,6]. Cellulitis a localized soft tissue infection that results from bacterial entry through a disrupted skin barrier, has an annual incidence of 200 cases per 100,000 individuals [7].

In current era, emergence of imidazoles resistance in *S. aureus* and *E. coli* has become a serious concern, attributed to the rising incidence of multidrug-resistant strains of these pathogens [8]. Hence, the development of new imidazole derivatives is the need of the current era. The computational molecular docking is an effective tool *in silico* drug discovery and development, that evaluates binding affinity between ligands and target receptors [9]. Metronidazole is the preferred drug for treatment of a wide variety of bacterial infections, however the associated resistance of metronidazole limits its therapeutic application [10]. Facts suggest that incorporation of imino in different therapeutic organic moieties enhances their biological potential [11,12]. Therefore, based on the problem of cellulitis, the associated resistance and high activity of imines and imidiazoles, the current investigation study was designed to perform

synthesis, molecular docking and biological activity of new metronidazole analogues against cellulitis causing bacteria *S. aureus* and *E. coli*.

EXPERIMENTAL

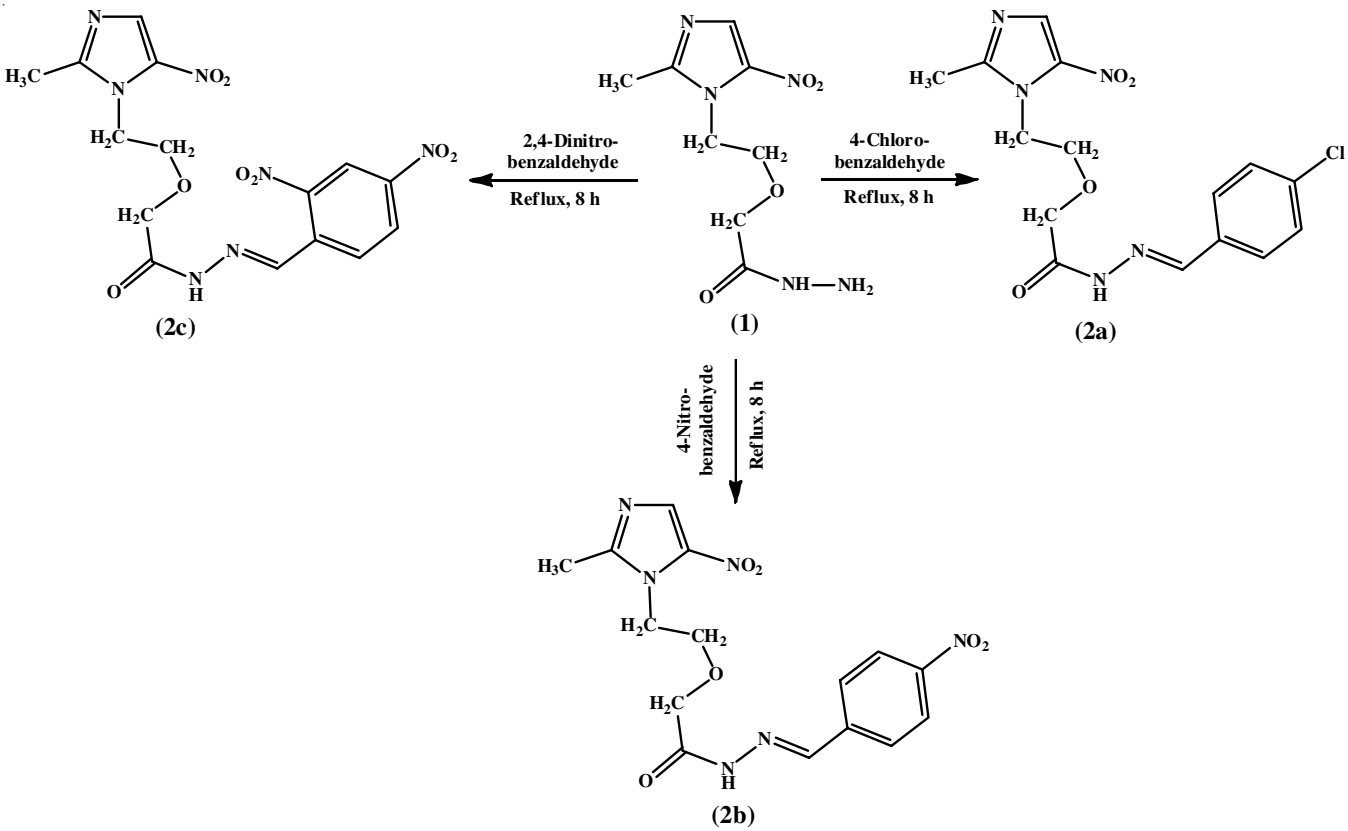
The chemicals and reagents for the synthesis and biological activity of *N*-(4-substituted benzylidene)-2-(2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)-ethoxy)acetohydrazide (**2a-c**) were acquired from Merck KGa, Sigma-Aldrich, HmbG® and Qrec-Chemicals. The purityof the synthesized was determined using an open-capillary method in SMP11 analogue instrument and are uncorrected. The recording of spectra for NMR was done at 700 MHz using ASCENDTM spectrometer on δ value scale; IR on ATR-FTIR Shimadzu spectrometer and Mass on Direct Infusion Ion Trap MS.

Synthesis of *N*-(4-substituted benzylidene)-2-(2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethoxy)acetohydrazide (2a-c): Compound **2a** was synthesized as per standard experimental protocol with some minor modifications [13,14]. Briefly, the mixture of 0.01 M of compound **1**, hydrazinated derivative of metronidazole ester, was refluxed for 8 h with 4-chlorobenzaldehyde in 0.01 M concentration. The reaction was initiated using absolute ethanol in sufficient quantity, which was distilled off after the reaction completion. To the resultant mixture, ice cold water was added with continuous stirring to offer the crude solid product, which was purified using methanol to offer pure crystals of compound **2a** (**Scheme-I**). Similarly, using similar experimental protocol, other compounds **2b-c** was also synthesized by treating with 2-nitrobenzaldehyde and 2,4-dinitrobenzaldehyde, respectively. During reaction anhydrous conditions were maintained, the reaction was catalyzed using a drop of H_2SO_4 acid and reaction progress was determined using TLC.

***N′*-(4-Chlorobenzylidene)-2-(2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethoxy)acetohydrazide (2a):** White crystals (yield: 91%, m.p.: 194 ºC); ATR-IR (cm^{-1}): 3348 (-N-H), 3062 (=C-H), 2926 (-C-H), 1702 (-C=O), 1589 (-C=N); ^{1}H NMR (DMSO-d_6, 700 MHz) δ ppm: 2.49 (3H, s, CH_3), 3.62 (2H, t, J = 5.9, CH_2), 3.86 (2H, t, J = 5.9 Hz, CH_2), 4.28 (2H, s, O-CH_2), 8.01 (1H, s, NH), 8.22 (1H, s, N=CH), 7.32-7.68 (4H, m, Ar-H); ^{13}C NMR (DMSO-d_6, 700 MHz) δ ppm: 12.1 (CH_3), 38.2 (CH_2), 71.8 (O-CH_2), 73.6 (CH_2-C=O), 129.2, 130.8, 132.4, 138.6 (Ar-C), 128.1, 141.3, 152.6 (Ar-C), 143.5 (C=N); MS: *m/z*: 365.

***N′*-(4-Nitrobenzylidene)-2-(2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethoxy)acetohydrazide (2b):** Yellow crystals (yield: 88%, m.p.: 184 ºC); ATR-IR (cm^{-1}): 3342 (-N-H), 3058 (=C-H), 2928 (-C-H), 1699 (-C=O), 1587 (-C=N); ^{1}H NMR (DMSO, ppm) δ: 2.46 (3H, s, CH_3), 3.65 (2H, t, J = 5.9, CH_2), 3.82 (2H, t, J = 5.9 Hz, CH_2), 4.31 (2H, s, O-CH_2), 7.99 (1H, s, NH), 8.25 (1H, s, N=CH), 7.94-8.34 (4H, m, Ar-H); ^{13}C NMR (DMSO-d_6, 700 MHz) δ ppm: 12.4 (CH_3), 38.1 (CH_2), 71.6 (O-CH_2), 73.4 (CH_2-C=O), 122.3, 131.4, 138.9, 150.6 (Ar-C), 127.9, 140.9, 152.8 (Ar-C), 144.6 (C=N); MS: *m/z*: 376.

***N′*-(2,4-Dinitrobenzylidene)-2-(2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethoxy)acetohydrazide (2c):** White crystals (yield: 82%, m.p.: 194 ºC); ATR-IR (cm^{-1}): 3348 (-N-H), 3062 (=C-H), 2926 (-C-H), 1702 (-C=O), 1586 (-C=N); ^{1}H NMR (DMSO-d_6, 700 MHz) δ ppm: 2.49 (3H, s, CH_3), 3.62



Scheme-I: Synthesis of novel metronidazole analogues **2a-c**

(2H, t, J = 5.9 Hz, CH_2), 3.86 (2H, t, J = 5.9, CH_2), 4.28 (2H, s, O-CH_2), 8.01 (1H, s, NH), 8.24 (1H, s, N=CH), 8.14-9.26 (4H, m, Ar-H); ^{13}C NMR (DMSO-d_6, 700 MHz) δ ppm: 12.2 (CH_3), 38.2 (CH_2), 71.4 (O-CH_2), 73.2 (CH_2-C=O), 119.2, 128.4, 131.6, 133.5, 150.6, 152.2 (Ar-C), 128.3, 141.6, 152.2 (Ar-C), 144.2 (C=N); MS: *m/z*: 421.

Antibacterial activity: The antibacterial activity of the synthesized compounds **2a-c** was evaluated against *S. aureus* and *E. coli* using the disc diffusion method following NCCLS guidelines [15,16]. The bacterial strains were cultured in nutrient broth at 36.5 ºC for 18 h with shaking at 120 rpm and adjusted to approximately 12 × 10^8 CFU/mL using the 0.5 McFarland standard. Mueller-Hinton agar plates were prepared and 100 µL of the bacterial suspension was spread evenly using an L-shaped spreader. Sterile discs were dipped in compounds **2a-c** solutions at concentrations of 100, 50 and 10 µg/mL, while gentamicin (1 µg/mL) served as positive control and 0.5% DMSO as the negative control. The discs were placed on the inoculated agar surface and the plates were incubated at 37 ºC for 24 h. Zones of inhibition were measured in millimeters and all experiments were conducted in triplicate to ensure reproducibility.

Toxicity analysis (viability study): The synthesized compounds were further examined for cell viability study towards HEK293 cells using MTT assay [17]. Briefly, HEK293 cells were propogated at 37 ºC using Dulbecco's modified eagle medium (DMEM) in 95% relative humidity, followed by proliferation in 96 well plate with per well density of 1×10^4 cells density and overnight incubation for cell attachment. For viability studies, the test compounds diluted in DMEM were added to plate wells (in concentration ranging from 6.5-100 µg/mL), followed by incubation for 24 h (at 37 ºC and 5% CO_2), re-incubation in dark for 4 h (after addition of 10 µL MTT solution), removal of contents from plate wells, addition of DMSO (100 µL) and finally absorbance measurement at 570 nm using microplate reader to calculate % cell-viability using following expression:

$$\text{Cell viability (\%)} = \frac{\text{Sample absorbance}}{\text{Control absorbance}} \times 100$$

Molecular docking: The molecular docking experiment was executed to evaluate the compounds **2a-c** interaction with target enzyme glucosamine-6-phosphate (PDB ID: 2VF5) using AutoDock Vina on an Intel i7 system with 16 GB RAM as per the standard protocol [18-20]. Ligand structures were drawn in ChemDraw, converted to 3D and energy minimized using Chem3D. The minimized ligand files were then converted to PDB format using Discovery Studio Visualizer and prepared as PDBQT files in AutoDock Tools by adding polar hydrogens and assigning Gasteiger charges. The target protein (2VF5) was downloaded from the RCSB Protein Data Bank and prepared by removing water molecules, checking for missing atoms, adding polar hydrogens and assigning Kollman charges using AutoDock Tools. Grid parameters were defined around the active site to generate the grid configuration. Molecular docking was executed using AutoDock Vina and the docking results were analyzed for binding interactions and docking scores using Discovery Studio Visualizer.

RESULTS AND DISCUSSION

Evidence over the problem of cellulitis, the antimicrobial resistance with imidazoles and high potential of imines inspired the investigators of this study to perform synthesis, characterization, molecular docking and biological activity of new metronidazole analogues against *S. aureus* and *E. coli* (cellulitis causing bacteria).

Treatment of hydrazide derivative of metronidazole (**1**) with 4-chlorobenzaldehyde, 4-nitrobenzaldehyde and 2,4-nitrobenzaldehyde offered imino derivatives of metronidazole (**2a-c**) *via* Schiff reaction in anhydrous condition. The synthesized compounds **2a-c** were purified by recrystallization and purity was further confirmed based on sharp melting points and single spot in TLC experiment. The chemical structures of compounds **2a-c** were elucidated by IR, NMR and mass analysis data. The structures of compounds **2a-c** were found to be in agreement with their spectral data. Characteristically, presence of IR band at 1689-1686 cm^{-1} (C=N), ^{1}H NMR peak at δ value between 8.22-8.25 (N=CH) and ^{13}C NMR peak at δ 143.5-144.6 (C=N) confirmed the structures of compounds **2a-c**. The characterization data of compounds **2a-c** was also supported with the literary facts [10-12], further validating the findings.

Molecular docking: The molecular docking experiment was executed to assess the affinity of synthesized compounds **2a-c** to binding site of glucosamine-6-phosphate (PDB ID: 2VF5). The docking scores of the synthesized compounds **2a-c** are presented in Table-1.

TABLE-1
DOCKING SCORES OF NOVEL METRONIDAZOLE ANALOGUES **2a-c**

Ligand	D-score
1	-2.4
Metronidazole	-3.4
2b	-3.4
2a	-5.2
2c	-5.5

Based on the docking scores data of compound **2c** with 2VF5 (Fig. 1a), it is revealed that 2D structure of compound **2c** underwent significant interactions with 2VF5. Specifically, the conventional hydrogen bonds are formed between the oxygen atom of nitro group of benzene and imidazole ring compound **2c** and the glutamine (GLN) residue at position 275 and arginine at position 421 in chain X of the protein 2VF5 (Fig. 1a). Hydrogen bonds of compound **2c** with glutamine/arginine protein residue stabilized complex and enhanced the affinity [6]. The hydrogen bond 2D pose (Fig. 1a) demonstrates the compound **2c** orientation at 2VF5 binding site, which highlights interaction between compounds **2a-c** and protein residues. 3D docking pose of compound **2c** & 2VF5 complex further proves such interaction and supports fitting of compound **2c** on the 2VF5 active site (Fig. 1b).

Antibacterial activity: Compounds **2a-c** were evaluated for their *in vitro* antibacterial potential against *S. aureus* and *E. coli* using disc diffusion method as per NCCLS guidelines [15,16]. Table-2 presents the zone of inhibition of compounds

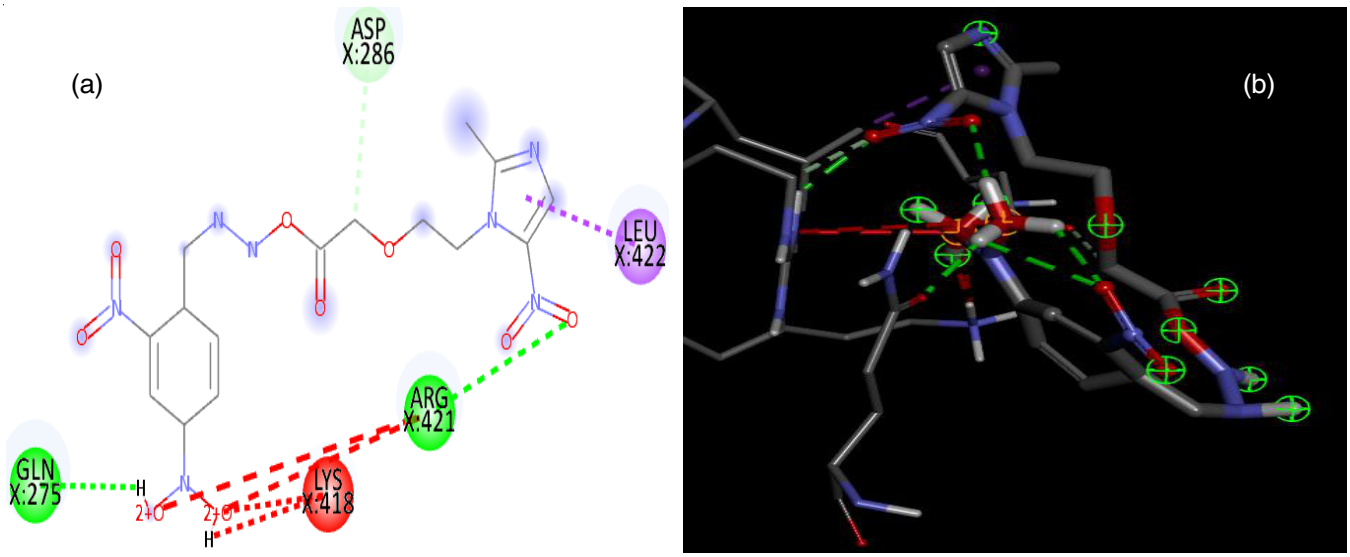


Fig. 1. 2D ligand interaction diagram (a) & 3D pose of novel metronidazole analogues **2c** with 2VF5 (b)

TABLE-2
DIAMETER OF ZOI WITH DIFFERENT CONCENTRATION OF NOVEL METRONIDAZOLE ANALOGUES **2a-c**

Compd.	Concentration (µg/mL)	Diameter of inhibition zone (mm)	
		S. aureus	*E. coli*
1	10	4	3
	50	12	7
	100	21	19
2a	10	6	4
	50	13	11
	100	22	19
2b	10	9	6
	50	16	13
	100	22	20
2c	10	18	16
	50	21	19
	100	23	21
Gentamicin	1	23	22

2a-c at different concentrations, which indicates that all the synthesized compounds **2a-c** inhibit the growth of both Gram-negative and positive bacteria. Among the synthesized compounds **2a-c**, compound **2c** exhibits maximum ZOI and also as concentration of compounds **2a-c** increases the ZOI against *S. aureus* and *E. coli* also increases. This study revealed that compound **2c** incorporated with two high electronegative group, exhibited the maximum inhibitory potential against cellulitis triggering pathogens. The results of the present study were also in agreement with other the results of other investigations [16,21].

Toxicity studies: As safety of drug is an important concern in drug development process, so current study involved assessment of all the synthesized compounds **2a-c** towards HEK 293 cells using MTT assay [16]. Study revealed that compound **2c** exhibited highest cell viability raging from 93.49 to 83.45% at a concentration ranging from 6.5 to 100 µg/mL when compared with the standard ampicillin (Table-3) and the reason is attributed due to the presence of two high electronegative groups. Whereas other compounds **2b** and **2c** also exhibited cell viability more than 75% when tested against HEK 293 cells at a concentration ranging from 6.5 to 100 µg/mL that supports their safety profile against normal cells.

Conclusion

Present study successfully synthesized new metronidazole analogues (**2a-c**) whose structures were in agreement with spectrometric data of NMR, IR and mass analysis. The synthesized compounds to exhibit not only the significant antibacterial activity against *E. coli* and *S. aureus*, the cellulitis triggering pathogens, but also offers high safety profile over normal health cells. The synthesized compounds were also proven to exhibit antibacterial potential as exhibited good interaction with glucosamine-6-phosphate (PDB ID: 2VF5) during *in silico*

TABLE-3
CELL VIABILITY OF NOVEL METRONIDAZOLE ANALOGUES **2a-c**

Concentration (µg/mL)	Cell viability (%)			
	2a	**2b**	**2c**	Ampicillin
6.5	83.69 ± 1.92	85.78 ± 4.58	93.49 ± 3.81*	98.86 ± 1.78
12.5	81.83 ± 2.67*	83.07 ± 6.79	90.42 ± 2.17*	96.43 ± 1.23
25	82.54 ± 4.78	81.17 ± 5.23	88.75 ± 1.45	94.34 ± 1.92
50	79.86 ± 1.27*	78.93 ± 1.77*	85.84 ± 2.16	92.71 ± 1.89
100	76.98 ± 3.98*	75.68 ± 2.86*	83.45 ± 1.39*	89.65 ± 0.83

Note: Data presented as mean ± standard error with each experiment were performed in triplicate. Mean values having superscript '*' statistically indicates by $*p < 0.05$.

molecular docking experiment. Current study recommends that the preclinical examination must be done to further support the metronidazole analogues efficacy in cellulitis.

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CONFLICT OF INTEREST

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

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