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Docking, synthesis and NDM-1 inhibitory activity of some novel cinnamic acid derivatives

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Abstract
A series of cinnamic acid derivatives (TA1–TA12) were docked, synthesized and tested for NDM-1 inhibitor activity. Azetreonam anitibiotic is taken as standard for docking and NDM inhibitory activity as azetreonam is the trial antibiotic used to treat the resistant infections caused by NDM-1 producing bacteria. This study found that out of 12 synthesized cinnamic acid derivatives, two compounds TA7 and TA9 show better activity than the standard. Results further revealed that compound TA8 shows equal activity as that of the standard. During the docking studies, compounds TA7 and TA9 have shown better binding affinity to NDM-1 protein while compound TA8 shows equal binding affinity to the standard antibiotic Azetreonam. Thus we can say that our docking results are in concurrence with the NDM-1 inhibitory activity.

Keywords: Cinnamic acid, NDM-1 inhibitors, Docking.

1. Introduction
Development of antibacterial resistance is observed since the first case of methicillin-resistant *Staphylococcus aureus* (MRSA) was identified, in the United Kingdom in 1962. Much of the antibacterial resistance problem stems from the misuse or excessive use of antibiotics. Though antibacterial resistance is not a recent phenomenon, it is a critical health issue today because of mutations in the microorganisms leading to development of newer enzymes. One of such enzymes is NDM-1which stands for *New Delhi metallo-beta-lactamase* enzyme. This enzyme destroys beta-lactam antibiotics including the penicillins, cephalosporins, and carbapenems. Majorly the strains of *Klebsiella pneumoniae*, *Escherichia coli*, and *Acinetobacter baumannii* genera of bacteria are known to possess the gene for NDM-1. The NDM-1 gene is also found to be present in *Morganella morganii, E. cloacae and Enterobacteriaceae* strains. The present study was undertaken to synthesize novel compounds effective against the NDM-1 producing microorganisms. After devising the structures and synthesizing them, docking study was performed. [1, 2, 3]

2. Experimental
2.1 General
All reagents were obtained from Sigma Aldrich and LobaChem Ltd. [India]. All the solvents used in this study were dried and distilled before use. Sineo, Microwave Chemistry Instrument, (Shanghai Sineo Microwave Chemistry Tech. Co. Ltd., China) was used to perform the microwave assisted reaction. Melting point [m.p.]: was determined using Veego VMP-PM digital melting point apparatus. Thin layer chromatography (TLC) was used for monitoring the progress of the reactions and purity was checked by TLC single spot study onuniform silica gel (silica gel-G) layer. KBr pellet method was used for recording infrared (IR) spectra on Shimadzu FTIR spectrophotometer. Bruker Avance II 300 MHz NMR Spectrophotometer was used for recording of ^{1}H-NMR spectra using appropriate deuterated DMSO (Dimethyl sulfoxide) as solvent and reported as chemical shift in Parts per million (δ, ppm).

2.2 Synthesis
The cinnamic acid derivatives were synthesized by a multistep synthetic route as presented in Scheme 1, 2 and 3.

Scheme 1: Synthesis of aliphatic carboxylic acid derivatives.

Reagents and conditions
(i) Br_2, PCl_3, 4 hours.
(ii) NH_2OH, 30 min.
R= -Br (A-1), $-CH_2Br$ (A-2), $-CH_2CH_2Br$ (A-3)

Scheme 2: Synthesis of caffeoyl amides.

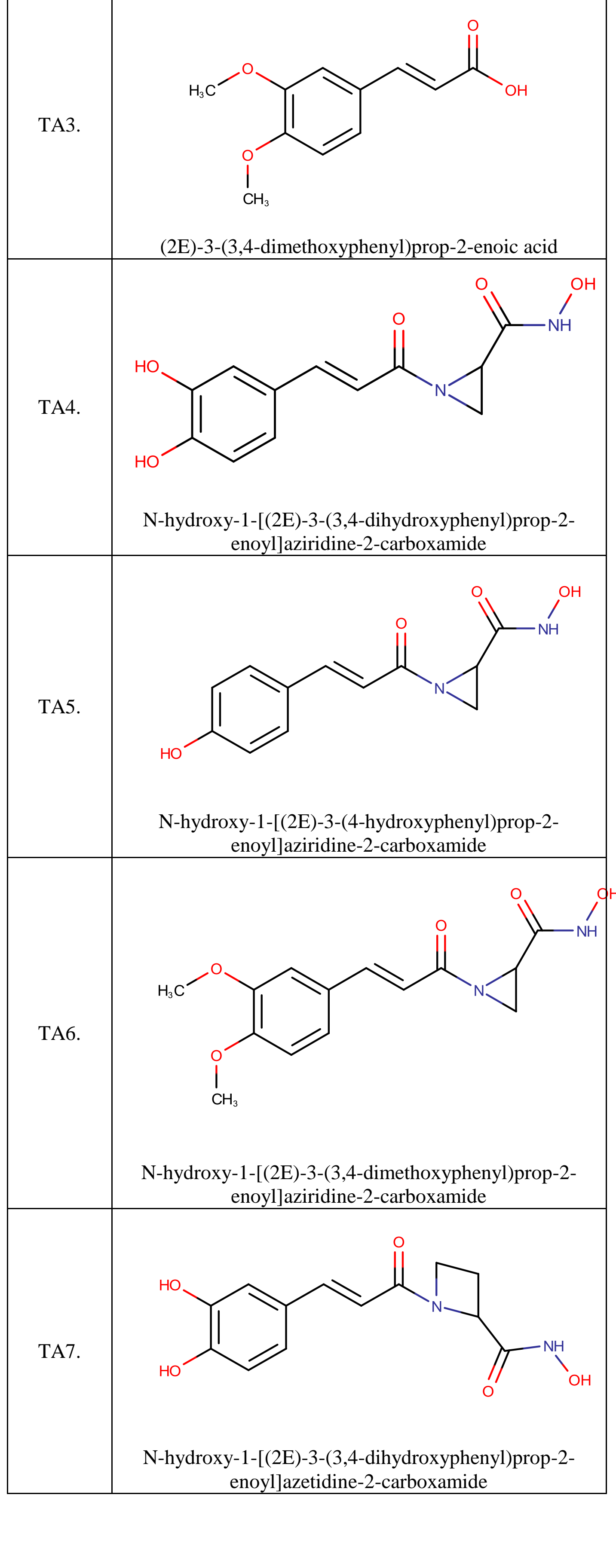
Reagents and conditions
(iii) Pyridine, aniline, 55^{0}C, 3 hours.
(iv) $SOCl_2$, 4 hours
(v) NH_3, 3 hours.
R_1= -OH, R_2= -OH (C-1), R_1= -H, R_2= -OH (C-2), R1= -OCH_3, R_2= -OCH_3 (C-3).

Scheme 3: Synthesis of caffeic acid derivatives.

Reagents and conditions: **(z)** 120 ^{0}C, K_3CO_3, 90 Watts, Microwave, 20 min.

2.3 Compounds Synthesised

Compound	Structure & IUPAC Name
TA1.	(2E)-3-(3,4-dihydroxyphenyl)prop-2-enoic acid
TA2.	(2E)-3-(4-hydroxyphenyl)prop-2-enoic acid



Compound	Structure & IUPAC Name
TA3.	(2E)-3-(3,4-dimethoxyphenyl)prop-2-enoic acid
TA4.	N-hydroxy-1-[(2E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]aziridine-2-carboxamide
TA5.	N-hydroxy-1-[(2E)-3-(4-hydroxyphenyl)prop-2-enoyl]aziridine-2-carboxamide
TA6.	N-hydroxy-1-[(2E)-3-(3,4-dimethoxyphenyl)prop-2-enoyl]aziridine-2-carboxamide
TA7.	N-hydroxy-1-[(2E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]azetidine-2-carboxamide

TA8.	N-hydroxy-1-[(2E)-3-(4-hydroxyphenyl)prop-2-enoyl]azetidine-2-carboxamide
TA9.	N-hydroxy-1-[(2E)-3-(3,4-dimethoxyphenyl)prop-2-enoyl]azetidine-2-carboxamide
TA10.	N-hydroxy-1-[(2E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]pyrrolidine-2-carboxamide
TA11.	N-hydroxy-1-[(2E)-3-(4-hydroxyphenyl)prop-2-enoyl]pyrrolidine-2-carboxamide
TA12.	N-hydroxy-1-[(2E)-3-(3,4-dimethoxyphenyl)prop-2-enoyl]pyrrolidine-2-carboxamide

2.4 General procedure for synthesis of phenyl butyric acid derivatives

Different acids were (0.01mol) kept in flask with molecular bromine (0.011mol) and when temperature reached at 65 ^{0}C, phosphorous trichloride (catalyst) was added. Then temperature was raised to 100^{0}C and mixture was refluxed for 4 hours to get (b). Then hydroxylamine (0.03mol) was added, toluene was used as solvent and system was refluxed for 30 minutes. In the end, toluene was evaporated using Schott Biotec Rotary evaporator and respective [X] was collected [4].

2.5 General procedure for preparation of caffeoyl amide derivatives

3, 4-dihydroxybenzaldehyde**(c)** (0.1mol) and malonic acid **(d)** (0.22mol) was added to 50 ml of dry pyridine, containing 1.4g of aniline, to form a solution. This solution was allowed to stand overnight, followed by heating for 3 hours at 55 ^{0}C in order to remove carbon dioxide. Reaction mixture was then poured into the mixture of 60 ml of concentrated hydrochloric acid and 100g of chopped ice. The acid precipitated immediately which was allowed to stand for few minutes for complete separation. The filtration was done. The product was washed using 10 ml of 5% hydrochloric acid and then with two portions of 10 ml water. At the end, drying of residue was carried out at room temperature. Substituted cinnamic acid **(e)** (0.1 mol) was refluxed with $SOCl_2$ (0.11 mol) for 4 hours. Thereafter, ammonia (1.0 mol) was added to the mixture containing (f) and system was further refluxed for 3 hours. The collection of caffeoyl amide derivatives was carried out. [5]

2.6 General procedure to synthesize final products

Respective [X] (1.1mmol) was mixed with [Y] (1.0 mmol). Potassium carbonate (1.1 mmol) was added as a catalyst. This step was performed by using microwave. The temperature was set at 120 ^{0}C, power was maintained at 90 watts and duration of reaction was set as 20 minutes. At the end, solvent was removed using Schott Biotec Rotary evaporator [6].

2.7 Physicochemical and Spectral Characterization

TA1)
Yield: 62%; mp: 211-213°C; IR ν cm^{-1}: 3433, 3420, 3232, 3026, 2954, 2864, 1695, 1654. ^{1}HNMR (δppm, DMSO):7.55 (1 H, d, CH), 7.07 (1 H, d, ArH), 6.95 (1 H, dd, ArH), 6.81 (1 H, d, ArH), 6.24 (1 H, d, CH), 5.35 (2H, s, OH), 11 (1H, s, OH).
TA2)
Yield: 67%; mp: 213-215°C; IR ν cm^{-1}: 3436, 3239, 30267 2944, 2872, 1672, 1634.
^{1}HNMR (δppm, DMSO):7.45 (1 H, d, CH), 7.56 (2H, d, ArH), 6.65 (2 H, d, J = 8.2, 2.0 Hz, ArH), 6.33 (1 H, d, J = 15.9 Hz, CH), 5.42 (1H, s, OH), 11.06 (1H, s, OH).
TA3)
Yield:65%; mp:181-183°C; IR ν cm^{-1}: 3439, 3242, 3022, 2964, 2853, 1690, 1647^{1}HNMR (δppm, DMSO):7.63 (1 H, s, CH), 7.22 (1 H, d, ArH), 6.89 (1 H, dd, ArH), 7.18 (1 H, d, ArH), 6.45 (1 H, d, CH), 3.83 (6H, s, CH_3), 10.94 (1H, s, OH).
TA4)
Yield: 56%;mp: 164-166°C;IR ν cm^{-1}: 3432, 3422, 3234, 3028, 2956, 2862, 1693, 1656, ^{1}HNMR (δppm, DMSO):7.32 (1 H, s, CH), 7.17 (1 H, d, ArH), 6.93 (1 H, dd, ArH), 6.79 (1 H, d, ArH), 7.03 (1 H, d, CH), 5.32 (2H, s, OH), 1.98-1.73 (2H, d, CH_2), 3.15 (1H, m, CH), 8.13 (1H, s, NH), 2.1 (1H, s,

OH).

TA5)

Yield: 62%; mp: 156-158°C;IR ν cm^{-1}: 3431, 3418, 3230, 3028, 2952, 2866, 1697, 1659, ^{1}HNMR (δppm, DMSO):7.28 (1 H, d, CH), 7.61 (2 H, d, ArH), 6.73 (2 H, dd, ArH), 7.11 (1 H, d, CH), 5.45 (1H, s, OH), 1.78-1.88 (2H, d, CH_2), 3.23 (1H, m, CH), 8.06 (1H, s, NH), 1.98 (1H, s, OH).

TA6)

Yield: 45%;mp:161-163°C;IR ν cm^{-1}: 3439, 3426, 3238, 3032, 2960, 2871, 1701, 1655^{1}HNMR (δppm, DMSO):7.28 (1 H, d, CH), 7.25 (1 H, s, ArH), 6.97 (1 H, d, ArH), 7.16 (1 H, d, ArH), 7.08 (1 H, d, CH), 2.02,1.81 (2H, s, CH_2) 3.22 (1H, m, CH), 3.82 (6H, s, CH_3), 8.04 (1H, s, NH), 2.06 (1H, s, OH).

TA7)

Yield: 61%;mp: 155-157°C;IR ν cm^{-1}: 3427, 3414, 3226, 3020, 2948, 2858, 1689, 1648^{1}HNMR (δppm, DMSO):: 7.29 (1 H, d, CH), 7.15 (1 H, s, ArH), 6.88 (1 H, d, ArH), 6.76(1 H, d, ArH), 7.08 (1 H, d, CH), 5.35 (2H, s, OH), 3.59,3.49 (2H, m, CH_2), 2.60,2.35 (2H, m, CH_2) 5.08 (1H, m, CH), 8.11 (1H, s, NH), 2.1 (1H, s, OH).

TA8)

Yield: 68%; mp: 165-167°C;IR ν cm^{-1}: 3433, 3420, 3232, 3026, 2954, 2864, 1695, 1654^{1}HNMR (δppm, DMSO):7.36 (1 H, d, CH), 7.61 (2 H, d, ArH), 6.78 (2 H, dd, ArH), 7.05 (1 H, d, CH), 5.40 (1H, s, OH), 3.57,3.46 (2H, m, CH_2), 2.57,2.33 (2H, m, CH_2) 5.23 (1H, m, CH), 8.03 (1H, s, NH), 2.4 (1H, s, OH).

TA9)

Yield: 52%; mp: 145-147°C;IR ν cm^{-1}: 3443, 3430, 3242, 3036, 2964, 2874, 1685, 1664^{1}HNMR (δppm, DMSO):7.37 (1 H, d, CH), 7.22 (1 H, s, ArH), 6.94 (1 H, d, ArH), 7.18 (1 H, d, ArH), 7.04 (1 H, d, CH), 3.52,3.46 (2H, m, CH_2), 2.61,2.30 (2H, m, CH_2) 5.12 (1H, m, CH),3.83 (6H, s, CH_3), 8.16 (1H, s, NH), 2.13 (1H, s, OH).

TA10)

Yield: 54%;mp: 153-155°C;IR ν cm^{-1}: 3438, 3425, 3237, 3031, 2959, 2868, 1699, 1651^{1}HNMR (δppm, DMSO):7.42(1 H, d, CH), 7.18 (1 H,s, ArH), 6.94 (1 H, d, ArH), 6.80 (1 H, d, ArH), 7.04 (1 H, d, CH), 3.41,3.32 (2H, m, CH_2), 1.99,1.68 (2H, m, CH_2), 1.60, 1.55 (2H, m, CH_2), 4.31 (1H, m, CH), 5.29 (2H, s, OH), 8.12 (1H, s, NH), 2.02 (1H, s, OH).

TA11)

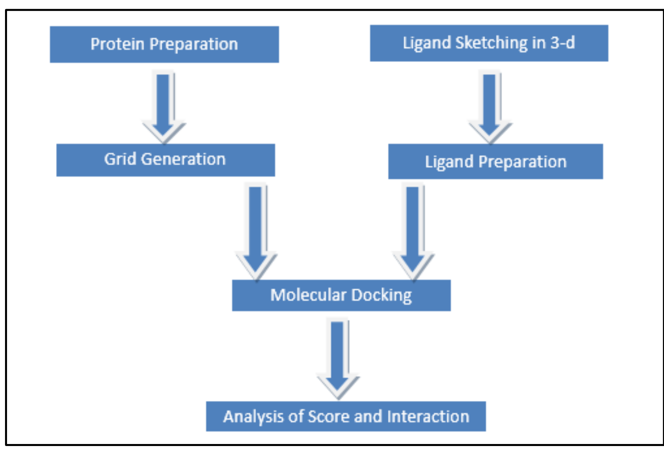
Yield: 59%;mp: 157-159°C; IR ν cm^{-1}: 3434, 3424, 3235, 3029, 2961, 2852, 1688, 1633^{1}HNMR (δppm, DMSO):7.32(1 H, d, CH), 7.56 (2 H,d, ArH), 6.67 (2 H, d, ArH), 7.03 (1 H, d, CH), 3.38,3.30 (2H, m, CH_2), 1.95,1.75 (2H, m, CH_2), 1.66, 1.52 (2H, m, CH_2), 4.35 (1H, m, CH),5.27 (1H, s, OH), 8.01 (1H, s, NH), 2.11 (1H, s, OH).

TA12)

Yield: 64%; mp: 149-151°C;IR ν cm^{-1}: ×3456, 3411, 3250, 3091, 2918, 2832, 1681, 1643^{1}HNMR (δppm, DMSO):738(1 H, d, CH), 7.19 (1 H,s, ArH), 6.97 (1 H, d, ArH), 7.20 (1 H, d, ArH), 7.07 (1 H, d, CH), 3.40,3.30 (2H, m, CH_2), 1.96,1.71 (2H, m, CH_2), 1.64, 1.54 (2H, m, CH_2), 4.29 (1H, m, CH),3.72 (6H, s, CH_3), 8.12 (1H, s, NH), 2.01 (1H, s, OH).

2.8 Docking Studies

Docking studies were performed for the synthesized molecules in the binding site of NDM-1protein (PDB entry:4RAW) using Auto Dock Vina 1.1.2 and graphical user interface, Autodock Tools 1.5.6 installed on a windows 7 ultimate (64 bit) computer to study the binding pattern of molecules. The procedure of docking protocol is as follows:



2.8.1. Ligand preparation: Marvin sketch 5.10.2 (Chem Axon Ltd.) was used to draw the 2D structures of the ligands and then conversion to 3D was done by means of Open Babel 2.3.2. The ligands were then prepared for docking by using AutoDock tools.

2.8.2. Protein preparation: The X- ray crystallographic information of NDM-1 protein was obtained from RCSB protein data bank (http://www.rcsb.org/pdb). After the complete evaluation of number of entries, the best protein (PDB entry: 3blm) was chosen by analyzing the protein with the highest resolution i.e. 2.0A^0. The PDB file of Beta lactamase protein was edited; all water molecules and all known interacting ions were removed. This fine tuning in crystal structure was executed with the help of PyMOL. The PDBQT files from orthodox PDB files were generated by using AutoDocK tools. The addition of all polar hydrogen atoms to the macromolecule was done.

2.8.3. Preparation of grid parameter file (config.txt file): The calculations of grid parameters were performed by using the Grid in AutoDock tools. The grid parameter file possessing all the information about the size of grid (40×40×40), protein, ligand and geometry of search space was prepared and saved as Conf.txt for all the ligands separately.

2.8.4. Docking methodology: Prepared ligand and prepared protein were then subjected to docking using Autodock software. This program predicted the binding energies and scored them. Then ranking list was generated as per energy affinity for all the compounds. All 12 compounds were extracted and compared to standard for their binding energies. These are given in Table no. 1 [7-11].

2.10 NDM-1 Assay

All reagents were equilibrated to 30^{0}C in a water bath before adding them to the reaction tubes [20 x 150 mm. Pyrex test tubes] in the following order: first 1 ml of gelatin Solution [1 per cent c. p. grade, E. Merck in 0.1*M* phosphate buffer, P^H 7.0], 50 µl of NDM-1 enzyme, 1 drop of Starch Solution [1 per cent soluble starch], 1 ml of Penicillin Solution [Crystalline Sodium Penicillin G obtained from Hindustan Antibiotics Ltd., 1660 µ /mg, dissolved in 3ml of phosphate buffer, pH 7.0, to contain not less than 5,000 µ/ml], 3 ml of sample solution TA1-TA12 and finally added 5 ml of iodine [0.01*N* iodine in 0.1*M* potassium iodide]. Then the time of decolorization of iodine was recorded with a stop-watch, after

addition of substrate, blank should always be determined using water in place of sample solution [see table 2] [12-14]
The activity of the enzyme sample tested was calculated from the following formula:

NDM-1 Activity (Activity µ/ml):

$$\frac{A - Sample}{B} \times \frac{40}{F} \times \frac{1}{T} \times \frac{1}{V}$$

In this equation,
A= Buffer + substrate solution + Enzyme + Iodine
B = Buffer + Iodine
Sample = TA1- TA12
F = Consumed iodine in ml.
T = Time in minutes.
V = Volume of enzyme solution (in ml) added to the assay.

Table 1: Binding affinities of Docked (TA1-TA12) compounds

Compound Code	Binding affinity (Kcal/mol)
TA1	-6.9
TA2	-6.8
TA3	-5.9
TA4	-5.4
TA5	-5.7
TA6	-5.6
TA7	-7.6
TA8	-7.2
TA9	-7.4
TA10	-6.9
TA11	-6.3
TA12	-6.9
Standard (Aztreonam)	**-7.2**

Table 2: NDM-1 inhibitory activity of compounds (TA1-TA12)

Sr. No.	Comp. Code	Time for decolorization of I_2 in Sec.	Activity µ/ml
1	Control	79.5	91.32
2	Standard (Aztreonam)	179.7	40.4
3	1	125.5	57.86
4	2	125.3	58.17
5	3	120	60.5
6	4	83.1	87.68
7	5	95.5	76.1
8	6	89.4	81.2
9	7	192.8	37.69
10	8	182.9	39.8
11	9	187.4	38.78
12	10	125.3	58.17
13	11	107.7	67.59
14	12	135.5	53.77

3. Result and Discussion

All the synthesized compounds [TA1-TA12] were subjected to Docking studies for finding the binding affinity to the binding site of NDM-1 protein and were screened for NDM-1 inhibitory activity. The results of NDM-1 inhibitory activity are presented in Table 2. From the results it is clear that compounds TA7 and TA9 show better activity while compound TA 8 shows equal activity than the standard antibiotic Azetreonam. Azetidine moiety is present in Azetreonam structure. Similarly our compounds TA7, TA8 and TA9 also have Azetidine moiety. Thus the better NDM-1 inhibitory activity in these compounds can be attributed to the presence of Azetidine ring sandwiched between N-hydroxy carboxamide and 3-(3, 4-dimethoxyphenyl) prop-2-enoyl moiety. During the docking studies, compounds TA7 and TA9 have shown better binding affinity to NDM-1 protein while compound TA8 shows equal binding affinity. Thus we can say that our docking results are in concurrence with the NDM-1 inhibitory activity.

4. Conclusion

From the results it is clear that compounds TA7 and TA9 show better activity while compound TA8 shows equal activity than the standard antibiotic Azetreonam. These compounds should be further explored for safety, toxicity and pharmacokinetics studies. If the compounds show positive results for these studies then they would be breakthrough drugs for treating NDM-1 resistant pathogens.

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