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INHIBITION OF NDM-1 IN SUPERBUGS BY FLAVONOIDS- AN INSILICO APPROACH

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ABSTRACT

NDM-1 stands for New Delhi metallo beta lactamase-1 is a novel beta lactamase enzyme produced by certain strains of Gram negative bacteria especially members of Enteobacteriaceae family. This family includes common bacteria like *Escherichia coli (E.coli), Klebsiella* and *Pseudomonas*. The gene that encodes for NDM-1 is called bla_{NDM-1} and has been identified on bacterial chromosomes and plasmids. NDM-1 is a newly identified problem, only recognized since about December 2009 in the medical literature. To date, there have more than 1000 cases identified outside of the Indian subcontinent. However, the number of cases is growing and the concern is that these highly resistant bacteria could supplant more antibiotic-sensitive strains. At present, there are no effective antibiotics against NDM-1 positive pathogen. This study provides clues to investigate the molecular basis of extended antibiotics resistance of NDM-1 and then accelerate the search for new antibiotics against NDM-1 positive strain in clinical studies. For this study the tertiary structure of NDM-1 was retrieved from PDB database. Docking analysis of NDM-1 was performed with certain flavonoids using Molegro Virtual Docker (MVD), Argus lab and Autodock. The analysis of the results of all three docking softwares suggested that the flavonoid Quercetin and its analog penta-O-ethylquercetin are potential inhibitors of NDM-1. NDM-1 containing Zn^{2+} and other divalent cations as cofactors, which inactivate almost all classes of beta-lactam antibiotics including carbapenems by catalyzing the hydrolytic cleavage of the substrate amide bond. In order to remove the zinc ions some of the chelating agents were used, BAPTA showed best inhibition activity towards zinc ions. These studies can be further validated using wet lab for NDM-1inhibition.

Keywords: NDM-1, *Klebesiella pneumonia*, Flavonoids, Softwares, Quercetin analogues, Chelating agents.

INTRODUCTION

New Delhi metallo-beta-lactamase-1 (NDM-1) is an enzyme that makes bacteria resistant to a broad range of beta-lactam antibiotics. The gene for NDM-1 is one of the members of a large gene family that encodes beta-lactamase enzymes called carbapenemases [1]. Bacteria that produce these are often referred to as “superbugs” because infections caused by them are difficult to treat. NDM-1 was first identified in a Swedish national of Indian origin, who had recently travelled to New Delhi, and acquired a urinary infection caused by resistant carbapenem *Klebesiella pneumonia* [2]. Antibiotics such as Carbapenems are a class of beta-lactam which are capable of killing most of the bacteria by inhibiting the

synthesis of cell wall layers. These beta-lactamase enzymes mediate the antibiotic resistance. However, the bla_{NDM-1} gene that produces the enzyme hydrolyzes and inactivates a wide range of carbapenem antibiotics.

NDM-1 shares very little identity with other Metallo Beta Lactamases (MBLs), with the most similar MBLs being VIM-1/VIM-2, with which it has only 32.4% identity. As well as having unique residues near the active site, NDM-1 also has an additional insert between 162 and 166 those are not present in other MBLs. NDM-1 has a molecular mass of 28 kDa , it is of monomeric and can hydrolyze most of the beta lactams [3]. NDM-1 belongs to the Metallo-b-lactamase (MBL, class B) family containing Zn2+ and other divalent cations as cofactors. It inactivates almost all classes of beta-lactams antibiotics including carbapenems by catalyzing the hydrolytic cleavage of the substrate amide bond. Due to the presence of two zinc ions the NDM-1 catalytic activity will be increased.NDM-1 activity depends upon zinc and the ability of zinc chelating agents like EDTA for decreasing activity.

However, bacteria that produce NDM-1 are resistant to all commonly used beta-lactam antibiotics, including carbapenems. Some antibiotics like aminoglycosides and fluoroquinolones do not contain beta-lactam rings. Unfortunately, the bacteria that have acquired NDM-1 have also acquired other resistance factors and most are already resistant to aminoglycosides and fluoroquinolones. NDM-1 strains have been sensitive to tigecycline, but this agent should be used cautiously in serious infections because it does not achieve high levels in the bloodstream. A few strains have also been sensitive to aztreonam and some of polymyxin antibiotic such as colistin [4]. Resistance to colistin is currently rare but it has some side effects including nephrotoxicity and neurotoxicity. In future the NDM-1 may have a chance getting resistance power towards colistin. The growing increase in the rates of antibiotic resistance needs an alternative for NDM-1 treatment.

For the treatment of NDM-1 there is a need to investigate new alternatives. For our studies the flavonoids present in green tea were chosen for carrying out the docking studies. Flavonoids are a class of plant secondary metabolites having antioxidant activity [10].

The information about the flavonoids present in green tea was obtained from USDA Database for the Flavonoids Content of Selected Foods [5]. (Table 1)

Table 1: List of flavonoids & their classification found in green tea (obtained from USDA Database)

S. No	FLAVAN-3-OLS	FLAVONES	FLAVONOLS
1	Epicatechin	Apigenin	Kaempferol
2	Epicatechin-3-gallate	Luteolin	Myricetin
3	Epigallocatechin		Quercetin
4	Catechin		
5	Theaflavins		
6	Theaflavin-3,3'-digallate		
7	Theaflavin-3'-gallte		
8	Theaflacin-3-gallate		
9	Thearubigins		

Materials and Methods

The NDM-1 sequence was retrieved from uniprot database with accession number **C7C422 which is having a length of 270 amino acids.**

1. Prediction of Protein Secondary Structure:

In first step, secondary structure of the protein was predicted through "SOPMA" program (Self-Optimized Prediction Method), the program determined the role of individual amino acid for building the secondary structure with their positions (http://npsapbil.ibcp.fr/cgibin/npsaautomat.pl?page=/NPSA/npsa_sopma.html) and "TMpred" were used to predict membrane-spanning regions of the protein and their orientation. (http://www.ch.embnet.org/software/TMPRED - form.html).

The crystal structure of NDM – 1 (3SPU) was retrieved from Protein Databank (PDB). The

NDM – 1 having two zinc divalent ions these are acts as cofactors.

2.1 Preparation of Ligands:

Based on the Anti-microbial activity, Flavonoids, were considered as possible inhibitors of the NDM-1 protein. The CID files of the Ligands were downloaded from PUBCHEM.

2.2 Active Site Analysis*:*

Pocket Finder, an online tool which uses hydrophobic probes, was used to predict possible binding sites. Energetically favorable probes sites were clustered and then ranked according to the sum of interaction energies.

3. Docking Studies:

In the present study the nature of interactions, binding mode and selectivity of NDM-1 protein with the flavonoids docking was carried out with MVD, Autodock, and Argus lab.

3.1 Autodock 4.0

Auto Dock is a molecular modeling simulation software. It is especially effective for Protein-ligand docking [6]. AutoDock performs the docking of ligand to a target protein. The energy grid was built within a cubic box of dimensions 40X30X40 A° with a spacing of 1.0 A°.

3.2 Argus lab 4.0.1

Argus lab 4.0.1 is molecular and drug docking software [7]. It is a very useful, highly-featured and easy-to-use molecular modeling, graphic and drug design programmes was used to carry out docking studies of NDM-1.

3.3 Molegro Virtual Docker (MVD)

MVD is an integrated environment for studying and predicting how ligands interact with macromolecules. The identification of ligand binding modes is done by iteratively evaluating a number of candidate solutions (ligand conformations) and estimating the energy of their interactions with the macromolecule [8]. The highest scoring solutions are returned for further analysis. MVD requires a three-dimensional structure of both protein and ligand (usually derived from X-ray/NMR experiments or homology modeling) [9]. MVD performs flexible ligand docking, so the optimal geometry of the ligand will be determined during the docking.

Results and Discussion

Prediction of Protein Secondary Structure:

The result obtained from "SOPMA" is presented in the form of graphics (Figure 1). The tool described that about 34.81% of amino acids presented in helix, 38.15% of amino acids in random coil, 19.26% of amino acids in extended strand and remaining rest of all amino acids in turn that is 7.78%. "TMpred" characterized secondary structure orientation of the helices was inside to outside. There were five helices formed by the amino acids from 13-31, 64-84,126-146, and 153-175 and 231-250. (++) symbol indicates a strong preference of this orientation.

```
        10        20        30        40        50        60        70
         |         |         |         |         |         |         |
MELPNIMHPVAKLSTALAAALMLSGCMPGEIRPTIGQQMETGDQRFGDLVFRQLAPNVWQHTSYLDMPGF
hhcchhcchhhhhhhhhhhhhhttccccccccchhhhhhhhhhccceeeehcctteeeeeeeecccccc
GAVASNGLIVRDGGRVLVVDTAWTDDQTAQILNWIKQEINLPVALAVVTHAHQDKMGGMDALHAAGIATY
ccccttteeeeettteeeeecccchhhhhhhhhhhhhhccceeeeeeecccccccchhhhhhttcchh
ANALSNQLAPQEGMVAAQHSLTFAANGWVEPATAPNFGPLKVFYPGPGHTSDNITVGIDGTDIAFGGCLI
hhhhhhhhhhtttccccccceecccccccccccccccccceeeeecccccccceeeeettcceeetteee
KDSKAKSLGNLGDADTEHYAASARAFGAAFPKASMIVMSHSAPDSRAAITHTARMADKLR
ccccccccccchhhhhhhhhhhhhhhhhcttceeeeetcccccchhhhhhhhhhhhhh

Sequence length :   270

SOPMA :
   Alpha helix     (Hh) :    94 is  34.81%
   3(10) helix     (Gg) :     0 is   0.00%
   Pi helix        (Ii) :     0 is   0.00%
   Beta bridge     (Bb) :     0 is   0.00%
   Extended strand (Ee) :    52 is  19.26%
   Beta turn       (Tt) :    21 is   7.78%
   Bend region     (Ss) :     0 is   0.00%
   Random coil     (Cc) :   103 is  38.15%
```

Fig1: NDM-1 secondary structure prediction by using SOPMA

The Mol Inspiration data of the compounds was then analyzed using Lipinski's Rule of Five. Those compounds that had more than one violation (i.e.thearubigins, theaflavins and its derivatives,

Epicatechin-3-gallate, Epigallocatechin, and Epigallocatechin-3-gallate) were eliminated.

Pocket finder predicted 10 different sites. The first site was considered the most probable binding site. The residues that formed the binding site in NDM-1 were identified as HIS 122 ,ASP 124 , HIS 189 ,GLY 207 ,CYS 208,ILE 210 ,LYS 211,ASP 212 ,ALA 215, LEU 218, GLY 219, ASN 220 ,LEU 221 ,ASP 223 ,ALA 224 ,TYR 229 ,MET 248 , SER 249 ,HIS 250 ,SER 251 . The NDM-1 protein will have two zinc molecules. Zn-I bound to three histidine residues at 120,122,189 positions and the Zn-II bound to Asp-124, Cys-208 and His-250 residues (Fig:2)

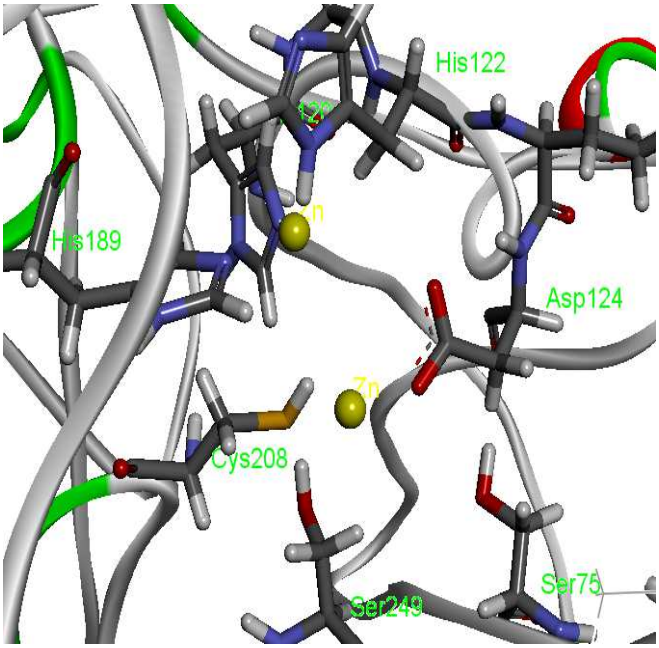

Fig2: Structure of NDM-1 with two Zn ions

The Proteins and ligands are docked using each of the three docking software's. The energy values from the softwares are indicated in Table 1. From the results it was shown that the flavonoid Quercetin is the best inhibitor. The interaction of quercetin with NDM-1 is indicated in Figure 3

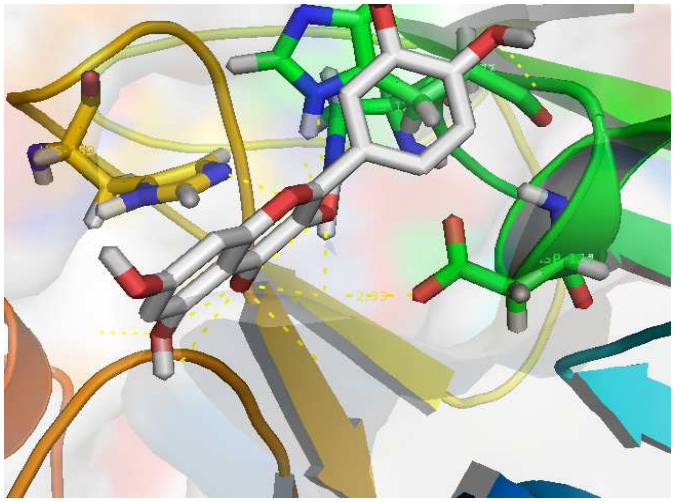

Fig3: Docking between quercetin and NDM-1

Table1: Docking studies between flavonoids and NDM-1 with their binding affinities

S.No	Affinity (kcal/mol)			
	Ligand Name	Autodock	Argus Lab	MVD
1	Apigenin	-6.6	-8.19	-94.52
2	Caffeine	-4.8	-5.79	-77.96
3	Catechins	-6.6	-8.27	-94.09
4	Kaempferol	-6.9	-8.08	-94.12
5	Luteolin	-7	-8.43	-95.83
6	Quercetin	-7.1	-8.61	-100.95

The docking studies were further carried out with the quercetin analogues. Out of these the analogue penta-O-ethyl quercetin had shown best inhibition activity. Docking was carried out for quercetin with different side groups. The possible best results tabulated below.

Table2: Docking studies between Quercetin analogs and NDM-1 with their binding affinities

S.No	Affinity (kcal/mol)			
	Quercetin Analog	Autodock	Argus Lab	MVD
1	penta-O-ethylquercetin	-8.4	-9.8	-132.99
2	Quercetin acetate	-7.1	-9.11	-103.07
3	quercetin 3 -O- sulfate	-7.4	-9.17	-109.96
4	Q–OCH3	-6.8	-7.71	-93.46

Next docking was carried out by using zinc chelating agents in those BAPTA had shown highest inhibition activity towards NDM-1.**BAPTA** (1,2-bis-o-aminophenoxy) ethane-N,N,N',N'-tetra acetic acid) having higher affinity towards zinc.

Table3: Docking studies between chelating agents and NDM-1 with their binding affinities

S.No	Affinity (kcal/mol)			
	Chelating agent	Autodock	Argus lab	MVD
1	2,3-dimercaptosuccinic acid	-4.4	-7.12	-62.33
2	BAPTA	-6.1	-9.86	-136.43
3	Disulfiram	-3.5	-6.07	-89.97
4	EDDS	-5.1	-8.38	-107.28
5	EDTA	-4.1	-7.92	-100.47
6	EDTMP	-4.9	-5.58	-95.83
7	Gluconic_acid	-4.8	-7.76	-105.66
8	Nitrilotriacetic_acid	-4.6	-7.31	-63.83
9	Penetic acid-DTPA	-5.1	-5.46	-94.68
10	Penicillamine	-3.4	-6.78	-46.94

The aim of this study is to find out the best and most potential inhibitor of the flavonoid and its analogues. Docking results indicated that quercetin and penta-O-ethyl quercetin are best potent inhibitors of NDM-1 and BAPTA the chelating agent has shown best inhibition activity. The chelating agents may have the chance to remove our body metal ions. Further analysis can be carried out in the wet lab.

Conclusion and Future prospects

From many years we have a bleak window and using antibiotics very wisely, but also grapple with the reality that we have nothing to treat these infections with. It is the first time it has got to this stage with these types of bacteria. So now there are no antibiotics in the pipeline to treat this. From this study we know that the Flavonoids are powerful agents against oxidative stress, inflammation, allergies, microbes and some of the dreadful diseases. The medicinal effects of many flavonoid compounds and chelating agents and probably lots of others in nature waiting to be analysed for their potential therapeutic or preventive use.

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