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MOLECULAR AND *IN SILICO* STUDIES ON DHFR OF UROPATHOGENIC *KLEBSIELLA PNEUMONIAE*

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ABSTRACT

The most common uropathogenic Gram negative bacteria are *Escherichia coli* and *Klebsiella pneumoniae*. MDR *Klebsiella* infection poses a significant challenge for treatment. *K. pneumoniae* strains have become increasingly resistant to antibiotics, rendering infection by these strains very challenging to treat. Dihydrofolate reductase (DHFR) has been a target for treatment against a variety of pathogenic microorganisms, since the folate-dependent enzymes are essential for DNA synthesis and methylation. This study was designed to study promising antibacterial molecules viz; Chlorogenic acid, Hippuric acid, Ellagic acid, Gallic acid and Quercetin as alternatives to antibiotics. Antibiotic and molecular studies on *Klebsiella pneumoniae* were performed. Further, molecular docking using AutoDock, an automated suite of protein-ligand docking tools was used to identify potent antibacterial compounds to treat *Klebsiella pneumonia* mediated UTI.

KEYWORDS: Antibacterial activity, Chlorogenic acid, Hippuric acid, Ellagic acid, Gallic acid, Quercetin, DHFR, Molecular Docking, MDR, UTI.

INTRODUCTION

Urinary tract infection (UTI) is caused by Gram-negative bacteria such as *Escherichia coli*, *Klebsiella* species, *Enterobacter* species, *Proteus* species and Gram-positive bacteria like *Enterococcus* species, and *Staphylococcus saprophyticus*.[1]

MDR *Klebsiella pneumonia* is highly resistant to multiple broad-spectrum antibiotics such as ampicillin and cephalosporins, which were previously helpful in treatment.[2] *Klebsiella pneumoniae* has recently gained notoriety as an infectious agent due to a rise in the number of severe infections and the increasing scarcity of effective treatments.[3]

Current data indicates a rising incidence of multidrug-resistant (MDR) strains of urinary pathogens worldwide. Infections caused by MDR pathogens have become a therapeutic challenge for clinicians.[4] Trimethoprim- and sulfonamide-resistant bacteria confer resistance in bypassing inhibition of dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) enzymes (involved in tetrahydrofolate biosynthesis). They are inhibited by a trimethoprim and sulfonamides, respectively.[5]

The antibacterial efficacy of Chlorogenic acid (CGA), Hippuric acid (HA), Gallic acid (GA), Ellagic acid (EA) and Quercetin (Qu.) against *Klebsiella pneumoniae* has already been investigated.[6,7,8 & 9]

In this study, the molecular docking on DHFR of *Klebsiella pneumoniae* strains has been investigated. Further, computer-aided docking using Autodock was performed.[10] Modeling the structure of a protein-ligand complex is important for understanding the binding interactions between a potential medicinal compound (the ligand) and its therapeutic target (the protein). Computer-aided docking is a technique that explores the motion space of the protein ligand complex to compute energetically stable conformation(s) that model(s) the structure of the complex.[11] Chlorogenic acid (CGA), Hippuric acid (HA). Gallic acid (GA), Ellagic acid (EA) and Quercetin (Qu.) on the basis of antibacterial efficacy were selected as ligands for docking studies on Dihydrofolate Reductase (DHFR) of *Klebsiella pneumonia.*

MATERIALS AND METHODS

Microbial Studies was undertaken

80 urine samples of urinary tract infected patients were collected in sterile container from pathology laboratories in Nagpur, Maharashtra, India. 18 *Klebsiella pneumoniae* colonies were isolated by streaking diluted urine sample on a differential medium UTI agar plates (Himedia, SM1353) and then further on MacConkey Agar Plates. Characterization and identification of bacteria was done by Gram staining and Biochemical tests. The antibiotic sensitivity was checked for Trimethoprim (125µg) by Kirby-Bauer‟s Method (12). Further antibacterial efficacy of selected compounds was checked.

18 Clinical isolates of *Klebsiella pneumoniae* were tested by well diffusion method (12) at different concentrations (2mg, 5mg, 7mg, 10mg, 12mg, 14mg and 16mg) of Chlorogenic acid, Hippuric Acid, Ellagic acid, Gallic acid and Quercetin.

Enzymatic Studies: DHFR assay was performed

1ml of bacterial culture of 18 MDR *Klebsiella pneumoniae* clinical isolates (7 samples were sensitive to TMP and 11were resistant to TMP) were inoculated in 100 ml of sterile nutrient broth and incubated at 37°C overnight. The 100 ml bacterial culture was then centrifuged at 3000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet was resuspended in 5 ml of ice cold PBS (0.1M). Cell extracts were prepared by sonication of pellet for 2 min (PCiTM Analytics Ultrasonicator). These cell extracts were utilized for activity assay as described by Osborn and Huennekens, 1958. The specific activity of DHFR was determined by spectrophotometry (Eppendorf Bio Spectrometer) at 340 nm. The reaction mixture (1ml) consisted of: 100µMCell extract, 50µM TES (pH 6.8), 75µM Beta-mercaptoethanol, 100µM Dihydrofolate, 100µM NADPH with and without inhibitors i.e. Chlorogenic acid, Hippuric acid, Ellagic Acid**,** Gallic Acid and Quercetin at a concentration of 2mg, 5mg, 10mg and 12mg. Incubated at 37ºC for 3 minutes.[8,13]

Statistical Analysis

Statistical analysis for activity assay was performed using Student's "t" test for one tailed probability assuming unequal variance for significant differentiation. The criterion for significant difference was 0.05.

Molecular Studies

The genomic DNA was isolated by the method explained by *Wen-ping Chen* and *Tsong-the Kuo* (1993)[15] The bacterial culture was grown overnight for DNA isolation, 1.5 ml of culture was used to pellet down cells by centrifugation for 10 min at 12000 rpm. Pellet was suspended into 200µl of lysis buffer, 30mM Tris-acetate pH 7.8, 20mM sodium-acetate, 1mM EDTA, 1% SDS. 66µl of 5M NaCl was added, mixed well and centrifuged at 12000 rpm for 10 min at 30°C to pellet down proteins and cell debris. Supernatant was taken into a new vial and equal volume of chloroform was added and mixed by inverting. This was centrifuged again at 12000 rpm for 3 min. Supernatant was discarded and DNA was precipitated with salt precipitation overnight. DNA was washed twice by 70 % ethanol. Dried in air, to evaporate ethanol completely and re-dissolved in 100µl 1X TE (Tris-EDTA) buffer as described by Moon et al.[8]

Primer Designing

The primers for *folA Klebsiella pneumoniae* were designed using NCBI primer blast tool. These primers were procured from IDT (Integrated DNA technology, India) and PCR reagents were purchased from Bioline, USA. The primer pairs used for amplification of *folA Klebsiella pneumoniae* are shown in the Table-1.[9]

Table 1: Primers designed for amplification of *folA* of *Klebsiella pneumonia.*

Target gene	Primer	Tm	Amplicon Size
folA (Klebsiella pneumoniae)	F-5'GATATTGCCGATGAGCCGGA3' R-5'ACAAGCCGAAGGATCGTCTG3'	60.04ºC 60.11ºC	480 bp

PCR amplification

The reaction mixture contained 1µl genomic DNA extract of *K.pneumoniae*, 10þmoles (1.25µl) of each primer, 2mM (0.25µl) dNTPs, 1 U (0.25µl) Taq polymerase, 10X (2.5µl) buffer, 25mM (0.25µl), $MgCl_2$ solution and volume was make up to 25µl by double distilled water. A thermal cycler (PEQ LAB) was used and the reaction were run under following conditions: Hot start at 110° C, 5 min denaturation at 94°C, followed by 30 cycles of 10 min at 94°C, 1 min of annealing at 60.14°C and 1 min elongation at 72°C and final extension at 72° C. The resulting PCR products were run on 1.2% agarose gel and visualized.

Gel electrophoresis

1.2% agarose in TAE buffer solution was heated until it dissolved. Gel was poured into glass mould and comb was placed and rest mould as it is for solidification and well production. After solidification comb was removed delicately. Gel electrophoresis assembly was setup, flooded with TAE buffer until mould got submerged. 20 µl DNA samples with 5 µl 6X loading dye were poured in wells with the help of micropipette. Current was applied at 76 Volts from (-) to (+) direction. DNA bands were observed.

Molecular Docking

Preparation of Protein and Ligand

In silico studies were performed using Autodock 4 suite (version 1.5 6rC2). The ligands viz, Chlorogenic acid, Ellagic acid, Gallic Acid, Hippuric acid, Quercetin and standard antibiotics viz, Trimethoprim were docked with DHFR enzyme of *Klebsiella pneumonia* as described by Moon et al.[15,16] The structure of DHFR of *Klebsiella pneumoniae* was downloaded from PDB (protein data base) with PDB Id 4OR7 (*X ray diffraction* structure, 1.76 A^0). Refer Fig (1). The PubSum database, utilizes 4 characters, as inputs, to get the ligands with their

Ligplots. Ligplots indicate the interacting sites of the protein of interest i.e DHFR. Next a Ramachandran Plot to verify the protein structure was plotted. The structure of the ligands were downloaded from Pubchem (chemical structure data base) online portal and drawn in Marvin Sketch version 5.8.1. The docking results were analyzed on the basis of their binding energy and their interactions.[17]

The structures of DHFR *Klebsiella pneumonia* were imported in Biovia Discovery Studio 2016 version 16.1.0.15350. The structure of protein was cleared (i.e. the extra groups which includes water molecules, ligand groups were removed) by deleting the heteroatoms present in the protein.[17] Only the protein and active site for docking is required, hence was saved in the PDB format. The structure of ligand (Chlorogenic acid, Ellagic acid, Gallic Acid, Hippuric acid and Quercetin) in Marvin Sketch view version 5.8.1 were cleaned in 2D and 3D. This cleared the 2 Dimensional and 3 Dimensional structure of the ligand. For docking, the protein structure was obtained in PDB format and ligands in tripos-Mol format or PDB format.[15,16] Molecular docking of DHFR *Klebsiella pneumoniae* protein and ligands selected was performed using AutoDock4, whereas the inhibitor-enzyme interactions were estimated by the Lamarckian genetic algorithm. Grid points generate the coordinates or interaction points where the ligand is docked. The grid box was generated at 60x60x60 A^0 to cover all the active site residues, and allowed the flexible rotation of ligands. The GA (genetic algorithm) and number of generation were set to10 and 27000 for DHFR. The Lamarckian genetic algorithm was followed for ligand confirmation. All the above parameters decide the different confirmation of ligand in which the ligand will be docked. Other parameters for example, free energy (after docking is complete we get the value of free energy), rotatable bonds (number of rotatable bonds varies according to the ligand structure), number of torsions[15,16] were used as default.[15,16]

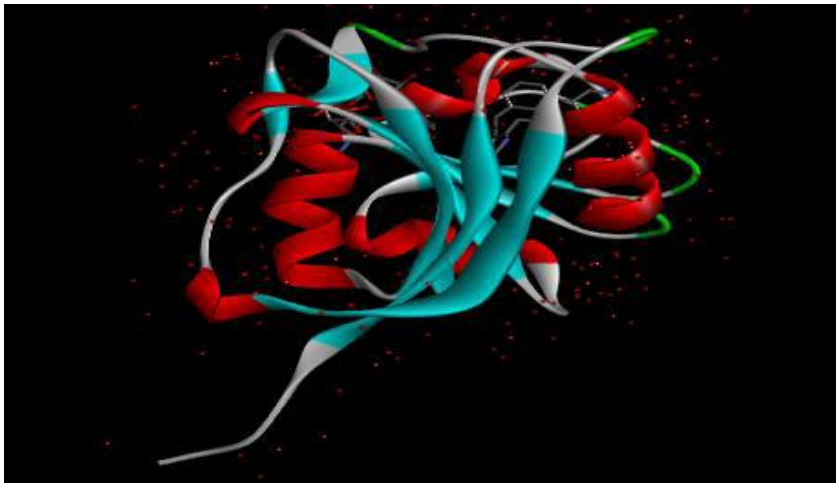

Figure No.1: *Klebsiella pneumonia:* PDBID: 4OR7; Resolution No. 1.76A^0.

RESULT AND DISSCUSSION

Bacterial identification and drug resistance profile of *K. pneumoniae*

The results of the drug resistance profile clearly indicate that 19% of the patients were infected with *K. pneumoniae.* The other predominant bacteria isolated from urine samples of patients were *E. coli* (37%), *Enterococcus faecalis* (32%), *Pseudomonas aeruginosa* (12%) and *S.aureus* (5%) Fig (2). *K. pneumoniae* isolates were selected for the present study. 18 samples were found to show mucous, opaque and blue colour colonies on UTI agar plates and pink coloured colonies on MacConkey Agar plates which indicate presence of *K. pneumonia* Fig (3). *K. pneumoniae* were further confirmed by Gram staining Fig (3). Out of 18 samples, 11 samples were found to be resistant and 7 sensitive to Trimethoprim (125µg).

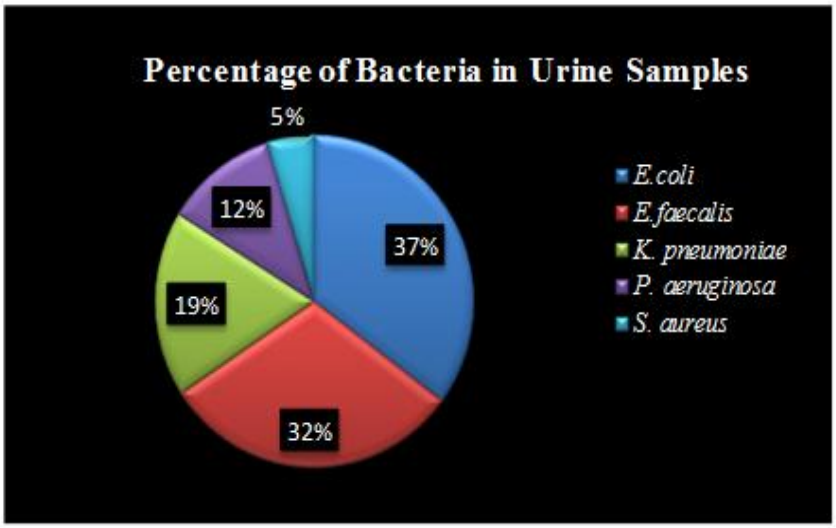

Figure 2: Causative bacteria for UTI in %.

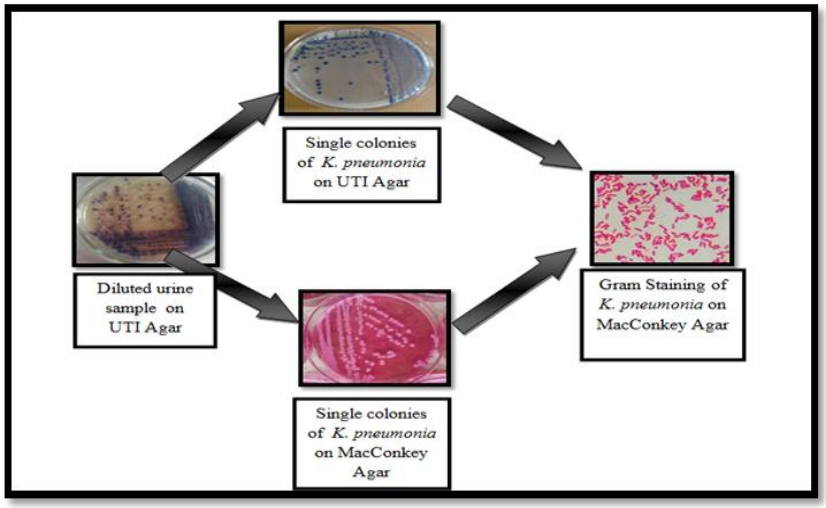

Figure 3: Isolation and Identification of *K. pneumonia.*

Antibacterial Activity of Inhibitors

The antibacterial activity of Chlorogenic Acid, Hippuric Acid, Ellagic Acid, Gallic Acid and Quercetin was tested by the disk diffusion method against *K. pneumonia* uropathogenic isolates. They showed commendable antibacterial activity as compared to Trimethoprim. All show sensitivity towards *K. pneumonia*. For further study, we selected trimethoprim resistant strains of *K. pneumoniae*.

The antibacterial activity of Chlorogenic Acid, Hippuric Acid, Ellagic Acid, Gallic Acid and Quercetin was found to be between 10-16 mg/ml, 2-16mg/ml, 1-16mg/ml, 2-16mg/ml, 1-16mg/ml respectively against *K. pneumonia* uropathogenic isolates.

DHFR Activity Assay

Specific activity of DHFR from *K. pneumoniae* isolates was estimated by measuring the ability of DHFR to carry out NADPH-dependant reduction of dihydrofolate to tetrahydrofolate in presence and absence of Gallic Acid, Hippuric Acid and Trimethoprim.

Activity of DHFR was found to be more in TMP resistant samples as compared to sensitive samples. This indicates that resistant samples show less response towards trimethoprim and are able to carry out folate synthesis easily. The Standard graph of activity assay is shown in Fig 4 and Specific activity of DHFR in isolates of *K. pneumoniae* is shown in presence of GA, HA and TMP (Fig 5,6 and 7) .

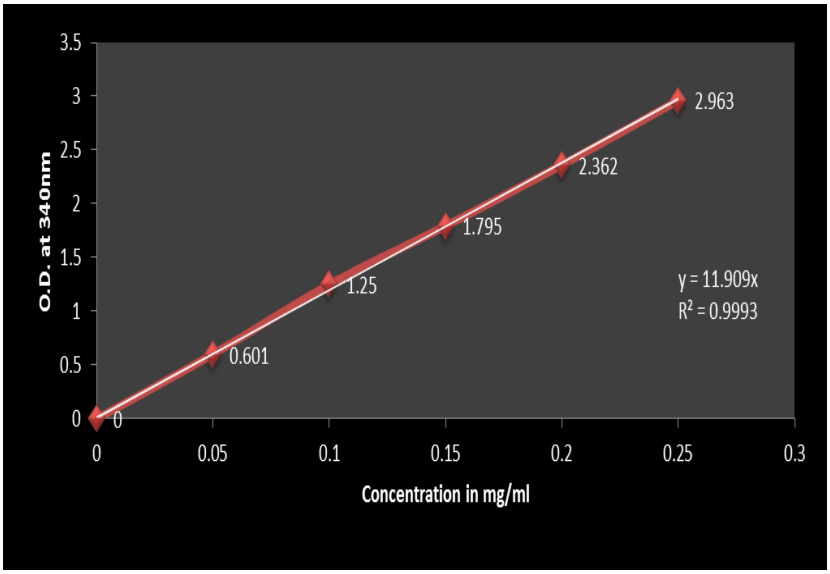

Figure No. 4: Standard Graph of DHFR assay.

Specific activity of DHFR in isolates of *K. pneumoniae*

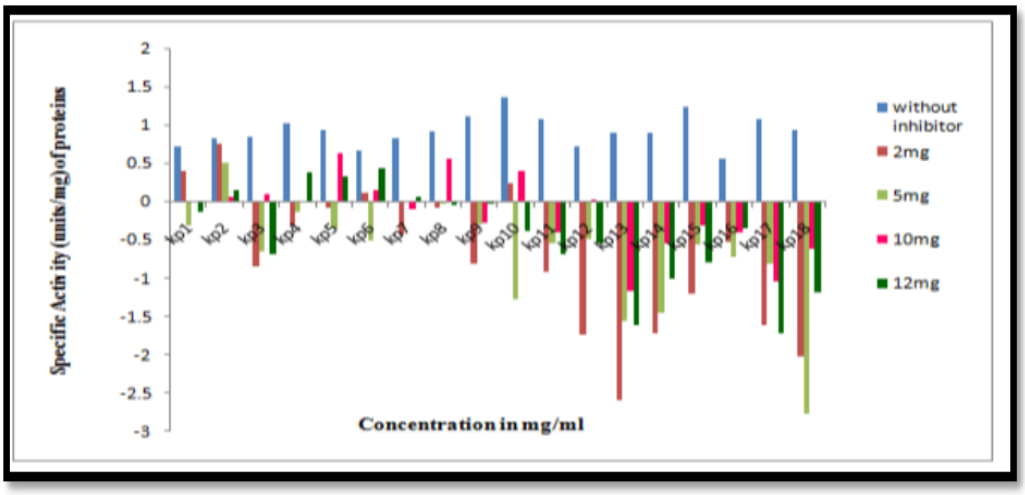

Figure No. 5: DHFR assay in presence of Gallic acid.

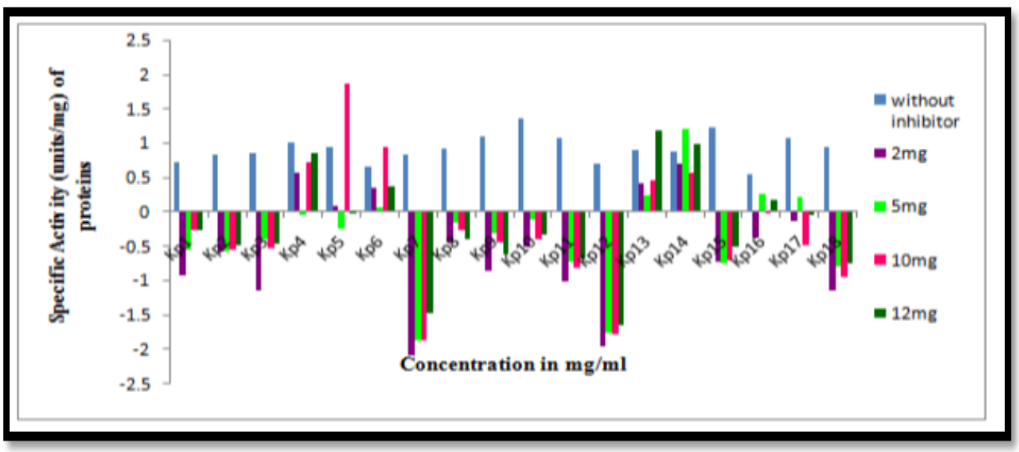

Figure No. 6: DHFR assay in presence of Hippuric acid.

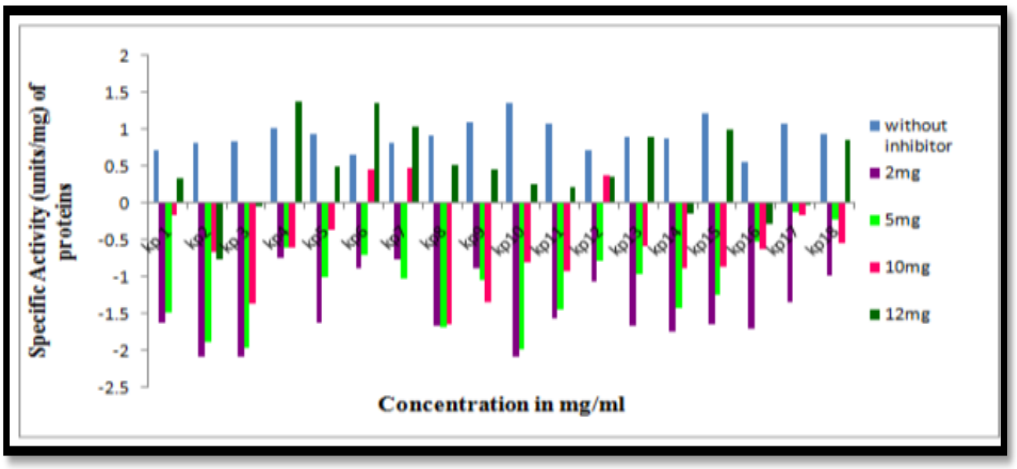

Figure No.7: DHFR assay in presence of TMP.

Genomic DNA isolation

The genomic DNA was isolated by Wen-ping Chen and Tsong-the Kuo (1993) Method. Total 18 samples of uropathogenic MDR *K. pneumoniae* were utilized. Sample no.2, 3, 5, 6, 8, 9, 15 and 16 were Trimethoprim sensitive and sample no. 1, 4, 7, 10, 11, 12, 13, 14, 17 and 18 were Trimethoprim resistant *K. pneumoniae*. The genomic DNA was measured in UV spectrometrically at 260 and 280 nm respectively and the 260/280 ratios were calculated found to be approximately 1.8 in all the samples of *K. pneumoniae*. After isolation of genomic DNA, it was run on a 1.2% agarose gel and observed under UV transilluminator. The bands of genomic DNA observed are shown in Fig 8.

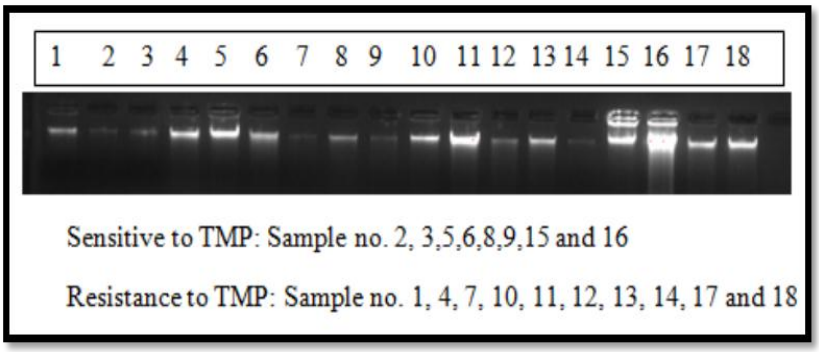

Fig No. 8: Genomic DNA of *K. pneumoniae* on 1.2% Agarose gel.

Primer designing

The primers were designed for *K. pneumoniae* DHFR. The expected amplicon size is 480bp for *K. pneumoniae* DHFR Fig. 9

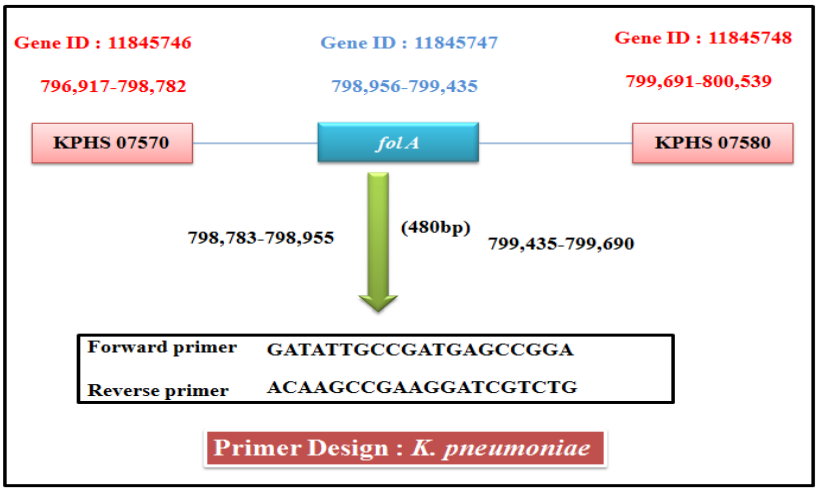

Fig No. 9: Primer for *K. pneumoniae Fol A*.

PCR Amplification of *folA* gene of *K. pneumoniae* isolates:

The PCR products were electrophoresed on 1.2% agarose gel and bands of the amplicons sized at 480bp corresponded to *folA* of *K. pneumonia* as shown in Fig (10).

Fig No.10: Agarose gel (1.2%) electrophoresis of PCR amplified products of *K. pneumonia* strains.

Molecular Docking

Docking Studies revealed the interaction of the protein with the ligand, binding energy, type of interaction and amino acids involved in the interactions. Binding energy should be negative. More negative the binding energy, better the binding affinity of ligand to the protein. To allow us to know whether phyto inhibitors Viz., Gallic Acid, Ellagic Acid, Hippuric Acid, Quercetin and Chlorogenic Acid bind to DHFR of *K. pneumoniae*, the docking study was undertaken. This result clearly indicates that among all phytochemicals studied, Quercetin shows highest negative binding energy. Molecular docking of Chlorogenic Acid, Gallic Acid, Ellagic Acid, Hippuric Acid, Quercetin showed good interaction with DHFR of *K. pneumoniae*.

Following figure no. 11 show the interactions. Following table (Table No. 2) indicates the binding energy of Ligands.

Sr. No.	Phytochemicals	Binding energy	Hydrogen bonds	Interacting sites(IS)
1.	Gallic Acid	-7.12	0 Hydrogen Atoms	12 Interacting sites
2.	Ellagic Acid	-6.89	3 Hydrogen Atoms	12 Interacting sites
3.	Hippuric Acid	-6.12	2 Hydrogen Atoms	12 Interacting sites
4.	Quercetin	-7.43	3 Hydrogen Atoms	12 Interacting sites
5.	Chlorogenic Acid	-6.21	3 Hydrogen Atoms	12 Interacting sites
6.	TMP	-7.70	2 Hydrogen Atoms	12 Interacting sites

Table No. 2: Binding Energy of Ligands with DHFR Protein of *K. pneumoniae.*

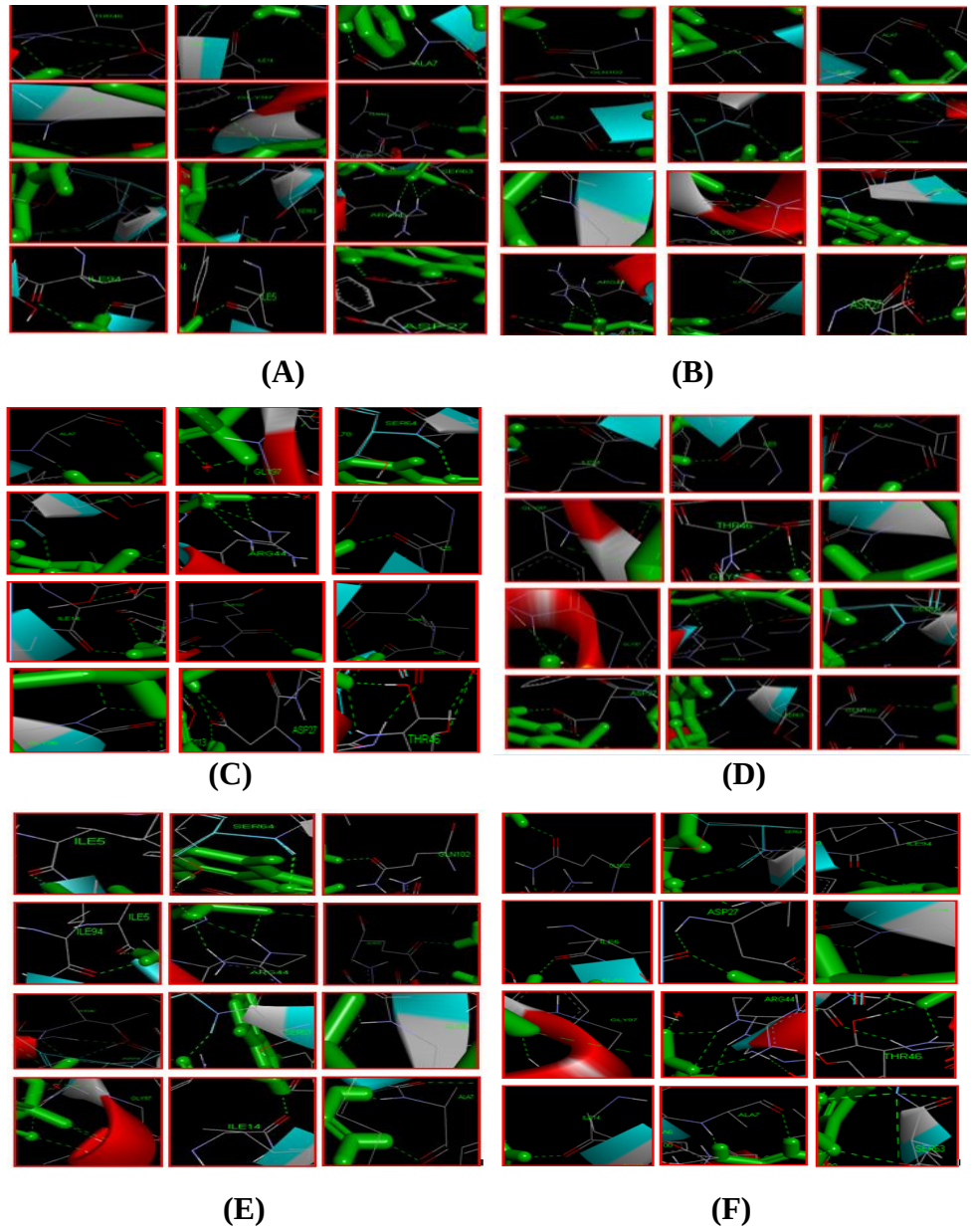

Figure No.11: Molecular interaction of (A) Gallic Acid, (B) Ellagic Acid, (C) Hippuric Acid, (D) Quercetin, (E) Chlorogenic Acid and (F) Trimethoprim with DHFR of *K. pneumoniae.*

CONCLUSION

Urinary tract infections (UTIs) are the major cause of bacterial infections, affecting millions of people throughout the world. Urinary tract infection (UTI) is the second most common infection that affects women majorly. Gram negative bacteria cause about 75 to 95% UTI, as gram negative bacteria are found more resistance towards UTI, hence we proceed our studies on *Klebsiella pneumonia*. The increasing –resistance of antibiotics, gram negative bacteria is a major global health care issue. It is true that the infections with multidrug-resistant (MDR) pathogens impose a significant and increasing burden on both patients and their healthcarer. Among MDR pathogens, *Klebsiella pneumoniae* (*K. pneumonia* or KP) is one of the world's most dangerous superbugs after *E.coli*. DHFR is a critical folate cycle enzyme targeted by antifolate medication used in the treatment of UTI. Dihydrofolate reductase is involved in conversion of 7, 8 dihydrofolate to 5, 6, 7, 8 tetrahydrofolate. Inhibition results in the reduction of dihydrofolic acid (DHF) to tetrahydrofolic acid (THF). THF is an essential precursor in thymidine synthesis pathway and interference of this pathway inhibits bacterial DNA synthesis. Inhibitors of DHFR is Trimethoprim, which showed resistance to all UTI bacteria. Recently, there has been a growing interest in the investigation and introduction of medicinal plants with various biological activities at the aspect of new drug development because of the advantages of ample materials source, ease of use, good efficacy, and small side effects. Hence we used a Phytoinhibitors against UTI *Klebsiella pneumonia*. We used Chlorogenic Acid, Hippuric Acid, Ellagic Acid, Gallic Acid and Quercetin as Phytoinhibitors which displayed effective antibacterial activities. We also compared these to the Standard antibiotic Trimethoprim. Molecular docking studies have helped to identify the molecules, which can be candidate drug molecules. Amongst the five compounds tested in this study, Quercetin showed best interaction energy. Hence, Quercetin can be used for further *invivo, invitro* and *insilico* studies for formulation of a suitable antibacterial drug to deal with recurrent UTI infections caused due to *Klebsiella pneumonia*.

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