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Research Article

MOLECULAR STUDIES ON*FOLA*AND *FOLP*GENES OFUROPATHOGENICMULTI DRUG RESISTANT *E. COLI*

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Abstract

The urinary tract infection (UTI) is a bacterial infection of the urinary tract affecting both men and women. *E. coli* is the cause of 80–85% of urinary tract infections.Urinary tract infectionsare easily treated with a short course of antibiotics, although resistance to antibiotics is increasing.One of the components of Co-trimoxazole, Sulfamethoxazole (SMX) induces its therapeutic effect by competing with p-aminobenzoic acid (PABA) in the biosynthesis of dihydrofolate by inhibiting theenzyme **DihydropteroateSynthase**(DHPS). While the other component Trimethoprim (TMP) serves as a competitive inhibitor of **DihydrofolateReductase**(DHFR), thereby inhibiting the *de novo* synthesis of tetrahydrofolate, the biologically active form of folate.In this study, folate pathway enzymes have been targeted to inhibit bacterial growth.We have isolated *E. coli* from urine samples of UTI patients. Antibacterial activity of chlorogenic acid (CGA) and hippuric acid (HA) was checked against *E. coli*uropathogenic isolates. Genomic DNA was isolated from the*E. coli*. The genomic DNA was then used to PCR amplify 1500bp and 990bp DNA fragments of *E. colifolA* and *folP* respectively. The DHFR activity assay was performed with CGA and HA as inhibitors. *Insilico* docking of Chlorogenic Acid on*E. Coli*DHFR and DHPS proteins has been performed using Autodock.

Keywords: Urinary tract infection (UTI),Antibacterial activity, DihydrofolateReductase(DHFR), Dihydropteroate Synthase(DHPS), molecular docking, MDR, chlorogenic acid, hippuric acid.

Introduction

Urinary tract infection (UTI) is the second most common infectiouspresentation in community medical practice. Worldwide, about 150 million people are diagnosed with UTI each year, and UTI are classified as uncomplicated or complicated(1).The NationalKidney & Urologic Diseases Information Clearing house (NKUDIC) reports that UTIs

account for over 8 million doctor visits annually (2). Dihydrofolatereductase, DHFR [E.C. No. 1.5.1.3] is an enzyme from thereductase family, that reduces 5,6 dihydrofolic acid to 5,6,7,8 tetrahydrofolic acid. DHFR is found ubiquitously in all dividing cells of prokaryotes and eukaryotes. DHFR from *Escherichia coli* is a monomeric protein of 18kDa. (3). Dihydropteroate synthase (EC 2.5.1.15) (DHPS) catalyses the condensation of 6 hydroxymethyl-7,8-dihydropteridine pyrophosphate to para-aminobenzoicacid to form 7, 8-dihydropteroate. This is the second step in the three-step pathway leading from 6- hydroxymethyl-7, 8-dihydropterin to 7,8- dihydrofolate. DHPS is the target of sulphonamides, which are substrate analogues that compete with para-aminobenzoic acid. Bacterial DHPS (gene sul or *folP*) is a protein of about 275 to 315 amino acid residues that is either chromosomally encoded or found on various antibiotic resistance plasmids.

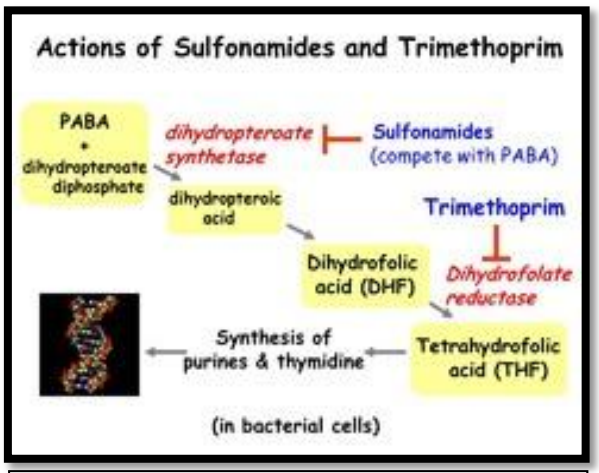

Figure 1: Folate Pathway.

The folate synthesis pathway is a crucial pathway for synthesizing amino acids. Obviously, without amino acids, bacteria cannot function and hencehas been envisaged as an efficient method to inhibit bacterial growth.

Molecular docking is one of the most frequently used methods in Structure based drug designing(SBDD) because of its ability to predict, with a substantial degree of accuracy, the conformation of small molecule ligands within theappropriate target binding site (4).Following the development of the first algorithms in the 1980s, molecular docking became an essential tool in drug discovery (5). Furthermore, molecular docking algorithms execute quantitative predictions of binding energetics, providing rankings of docked compounds based on the binding affinity of ligand-receptor complexes (5, 6). Chlorogenic acid (CGA) has been reported to possess commendable antibacterial activity (7).The antibacterial activity of Hippuric acid has been investigated in our lab. Thiswas also selected as a ligand for docking studies using molecular docking tool Autodock. The DihydrofolateReductase(DHFR) and Dihydropteroate synthase (DHPS) of *E. coli*have been selected as targets for molecular docking simulation.

Coffee is a complex mixture of chemicals that provides significant amounts of chlorogenic acid and Caffeine (14).Chlorogenic acids are a family of esters formed between quinic and *trans*-cinnamic acids, which are an important group of dietary phenols. The most common individual chlorogenic acid is 5-*O*-caffeoylquinic acid, which is still often called chlorogenic acid. Coffee represents the richest dietary source of chlorogenic acids and cinnamic acids (caffeic acid) (14).The quinic acid moiety in chlorogenic acid is the major precursor of hippuric acid (15). Hippuric acid itself, deriving from both caffeic and quinic acids moieties accounted for 36.5% of the chlorogenic acid intake (15).

Materials and Methods

- **Isolation, identification and antibiotic susceptibility testing of bacterial isolates:**

The bacterial colonies were isolated by streaking dilutedurine sample on a differential medium UTI agar plates (Himedia, SM1353) and then further on EMB agar Plates (Himedia, M317). The antibacterial activity of chlorogenicacid and Hippuric Acid was checked against isolated MDR *E. coli.*The antibiotic sensitivity was checked for Amplicillin (10µg), Cefazolin (30µg), Nalidixic Acid (30µg), Norfloxacin (10µg), Ciprofloxacin (5µg), Co-Trimoxazole (25µg), Levofloxacin (5µg), Nitrofurantoin (300µg), Augmentin (30µg), Cefuroxime (30µg), Gentamicin (10µg), Cefixime (5µg), Amikacin (30µg), Colistin (10µg), Netillin (30µg), Ceftriaxone (10µg), Cephotaxime (30µg), Furazolidone (50µg), Amoxycillin (10µg) and Vancomycin (30µg) by Kirby-Bauer's Method (9).

- **Antibacterial Activity of Chlorogenic Acid and Hippuric Acid**

Antibacterial activity of chlorogenic acid and hippuric acid against isolated bacteria was tested by well diffusion method (Kirby–Bauer, 1950) (9).Chlorogenic acid of different concentrations(viz; 1mg, 2mg, 5mg, 10mg, 12mg, 14mg, 16mg, 18mg, 20mg and 40mg) and Hippuric Acid (viz; 2mg, 5mg, 10mg, 12mg, 14mg, 16mg, 18mg, 20mg and 40mg) was utilized.

- **DHFR Activity Assay**

1ml of bacterial culture of 15 MDR *E. coli*clinical isolates (nine samples were sensitive to cotrimoxazole and six were resistant to cotrimoxazole)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 (PCi^{TM} Analytics ultrasoniccleaner) of pellet for 2 min. These cell extracts were further used for the activity assay as

described by Osborn and Huennekens, 1958.The specific activity of DHFR was determined by spectrophotometry (eppendorfBioSpectrometer)at 340 nm. The reaction mixture (1ml) consisted of: 100µMCell extract, 50µMTES (pH 6.8), 75µMBeta-mercaptoethanol, 100µMDihydrofolate, 100µMNADPH and inhibitors i.e. CGA (conc. 2mg, 5mg, 10mg and 12mg) and Hippuric acid (conc. 5mg, 10mg, 12mg and 15mg).Incubated at 37ºC for 3 minutes(10).

- **Statistical Analysis**

Statistical analysis for activity assay was done using Students't' test. Students't' test for one tailedprobability assuming unequal variance was used for significant differentiation. The criterion for significantdifference was 0.05.

- **Genomic DNA Isolation**

The genomic DNA was isolated by the method explained by *Wen-ping Chen* and*Tsong-the Kuo* (1993)(11).The bacterial culture was grownovernight 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, 20mMsodium-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, till smell of ethanol vanished and re-dissolved in 100µl 1X TE (Tris-EDTA) buffer.

- **PCR amplification**

The primers for*folA* and*folPE.coli*were designed using NCBI primer blast tool. These primers were procured from IDT (Integrate DNA technology, India) and PCR reagents were purchased from Bioline, USA.The primer pairs used for amplification of*folA* and*folPE.coli*are shown in the table-1 and 2

Table 1: Primers designed for amplification of *folA* of*E. coli.*

	Sequence (5'->3')	**Tm**	**Amplicon size**
Forward primer	TAGGTCTGGGGCCGTATGAA	57°C	1500bp
Reverse primer	CTTCCGGCGATTCTTTGCTG	56°C	

Table 2: Primers designed for amplification of *folP*of *E. coli.*

	Sequence (5'->3')	**Tm**	**Amplicon size**
Forward primer	ACACGACCACGAATCCCATC	57.12	990bp
Reverse primer	AGGCGACAAGTAAGTTCCCG	56.09	

folA and *folP* gene amplification of all fifteen MDR *E. coli* was done by RT-PCR.A single reaction mixture contained 500ng of genomic DNA extract for *E. coli*, 10 µl SYBR Green master mix, 0.5 µl Forward primer, 0.5 µl reverse primer, and reaction volume of 25µL by double distilled water. Amplification was done under following conditions: Hot start at 110° C, 5 min denaturation at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min of annealing at 60.07° Cand 2 min final extension at 72°C. The resulting PCR products were run on a 1.2% agarose gel.

- **Molecular docking**

The structures of DHFR and DHPS *E. coli* were opened 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 (13). Only the protein and active site for docking is required, hence was saved in the PDB format. The structure of ligand (Chlorogenic acid and Hippuric acid) were downloaded from Pubchem and drawn in Marvin Sketch view version 5.8.1 and 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 (12, 8).

Automated molecular docking betweenDHFR and DHPS *E. coli*protein and Chlorogenic acid was performed by using the advanced docking program 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 A0 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 60 and 27000 for DHPS respectively. 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 (12, 8) etc. were used as default (12, 8).

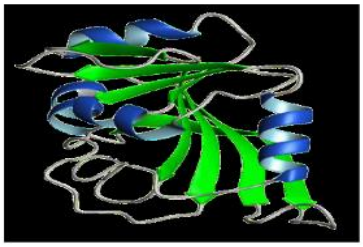

Figure 2: PDB Structure of *E. coli*DihydrofolateReductase (PDB 1RX2)

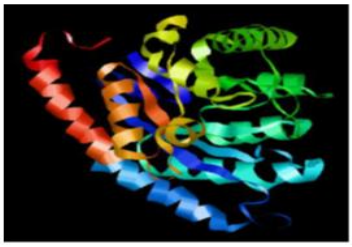

Figure 3: PDB Structure of *E. coli*Dihydropteroate Synthase (PDB 1AJ2)

Results and Discussion

- **Bacterial identification and drug resistance profile of *E. coli***

The results after performing antibacterial assay indicate that 37% of the patients were infected withMDR *E. coli.* The other predominant bacteria isolated from urine samples of patients were *Pseudomonas aeruginosa*(12%), *Klebsiella pneumonia* (19%) and *Enterococcusfaecalis* (32%).

E .coli isolates were selected for the present study. These *E. coli* were found completely resistant toAmplicillin (10µg), Cefazolin (30µg), Nalidixic Acid (30µg), Norfloxacin (10µg), Ciprofloxacin (5µg), Co-Trimoxazole (25µg), Levofloxacin (5µg), Nitrofurantoin (300µg), Augmentin (30µg), Cefuroxime (30µg), Gentamicin (10µg), Cefixime (5µg), Amikacin (30µg), Colistin (10µg), Netillin (30µg), Ceftriaxone (10µg), Cephotaxime (30µg), Furazolidone (50µg), Amoxycillin (10µg) and Vancomycin (30µg).

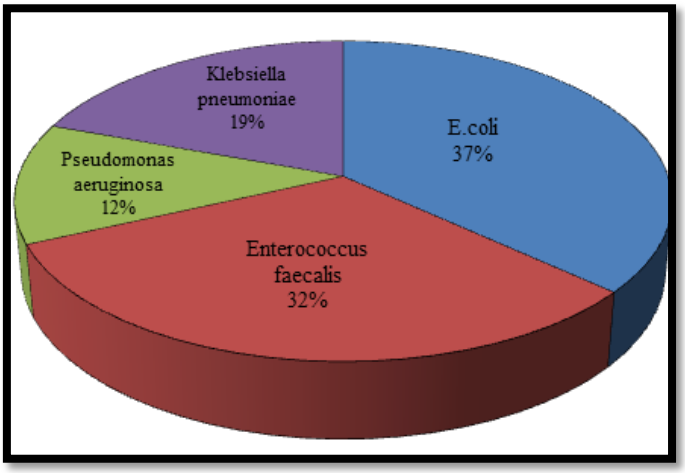

Figure 4: Percentage of bacteria found in urine samples

- **Antibacterial Activity of Chlorogenic Acid and Hippuric Acid**

The antibacterial activity of Chlorogenic Acid and Hippuric acid was found out to be between 10-40 mg/ml and 5-40mg/ml of chlorogenicacid and hippuric acid respectively against *E. coli* uropathogenic isolates.

- **The DHFR Activity Assay**

The DHFR activity in *E. coli* was checked in presence of inhibitors, chlorogenic acid and hippuric acid and in absence of inhibitors.

It was found that *E. coli*isolates have significant p values (p value < 0.05) in samples containing 5mg (p value 0.04), and 12mg (p value 0.05) of hippuric acid and in 2mg (p value 0.006), 5mg (p value 0.01), 10mg (p value 0.01), and 12mg (p value 0.008) of chlorogenic acid. Fig. no. 5, 6 and 7.

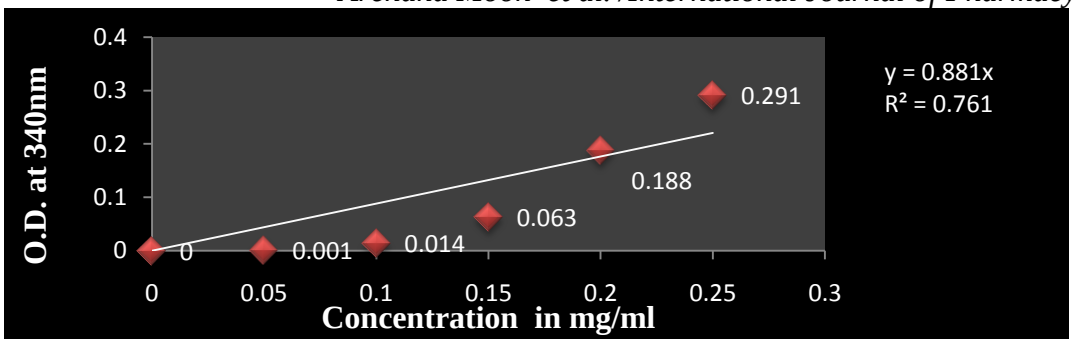

Figure 5: Standard Graph of DHFR assay

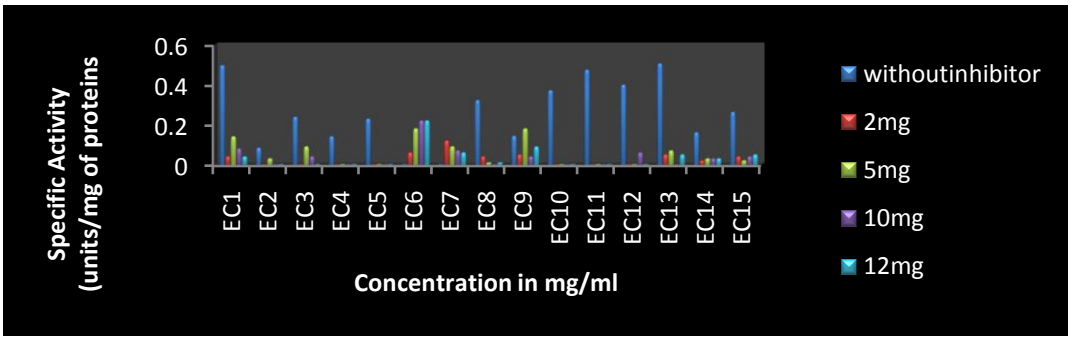

Figure 6: DHFR assay of Chlorogenic acid

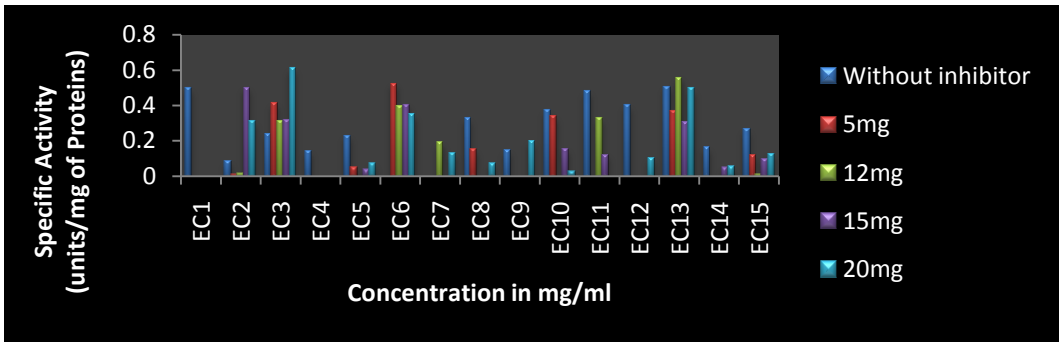

Figure 7: DHFR assay of Hippuric Acid

Genomic DNA isolation

The genomic DNA was isolated by Wen-ping Chen and Tsong-the Kuo (1993) Method.Total 15 samples of uropathogenic MDR *E. coli*were utilized sample no. 1, 2, and 4 were Cotrimoxazolesensitive and sample no. 12 and 15 were Cotrimoxazole resistant samples of *E. coli*. The genomic DNA was measured in UV spectrophotometer at 260 and 280 nm respectively and the values of 260/280 ratios was found to be approximately 1.8 in all the samples of *E. coli*. Genomic DNA was loaded on 1.2% Agarose gel stained with Ethidium bromide. (Figure 7)

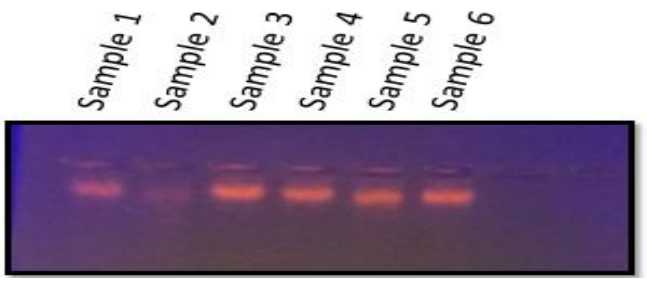

Figure 8: Genomic DNA of *E. coli*

- **PCR Amplification of*folA* and *folP* gene of *E. coli* isolates**

The PCR products were electrophoresed on 1.2% agarose gel and bands of the amplicons sized at ~990bp corresponded to *folP* of *E. coli*. No amplification in *E. colifolA* gene was obtained.

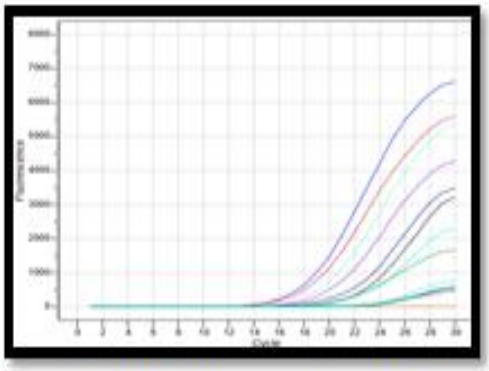

Figure 9: PCR Amplification Plot of folA *E. coli*

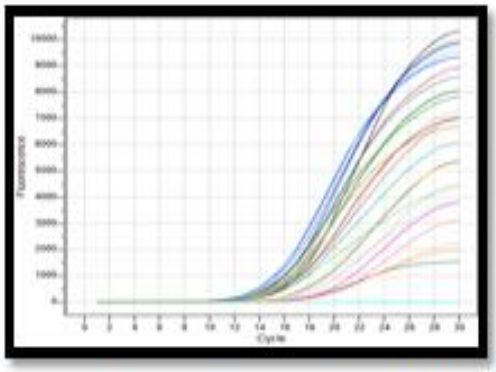

Figure 10: PCR Amplification Plot of folP *E. coli*

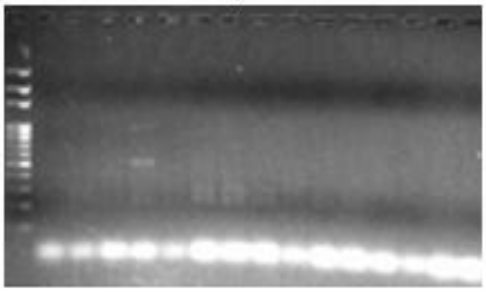

Figure 11: PCR Amplification of folA *E. coli*

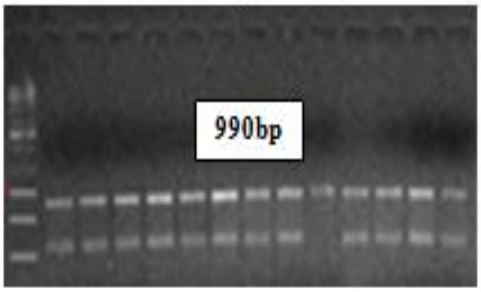

Figure 12: PCR Amplification Plot of folP *E. coli*

- **Molecular Docking**

Docking Studies revealed the interaction of the protein with the ligand, binding energy, type of interaction and amino acids involved in interactions. Binding energy should be negative. More negative the binding energy, better the binding affinity of ligand and protein (12). In our study,Chlorogenic Acid (CGA)and Hippuric acid (HA)showed direct interaction with active site Ala7 (CGA) and Thr113 (HA)residue of DHFRand their docking energy is -8.70 (CGA) and -6.45 (HA), which indicates that the chlorogenic acid and hippuric acid were docked with DHFR*E. coli*. But,chlorogenic acid and hippuric acid shows weak interactions with DHPS*E. coli* and their docking energy is +4.98 (CGA) and -5.91 (HA). Also, the standard antibiotics i.e. Trimethoprim and Sulfamethoxazole shows direct interactions with Ala7 (CGA) and Thr46 (HA) residues of DHFR which indicates that chlorogenic acid and hippuric acid were docked with DHFR *E. coli* and their docking energies is -7.91 (CGA) and -7.50 (HA). But, Trimethoprim and Sulfamethoxazole show weak interaction with DHPS and their docking energies is -5.58 and -3.99 respectively.

Figure 13: Interacting Sites of DHFR with Chlorogenic acid

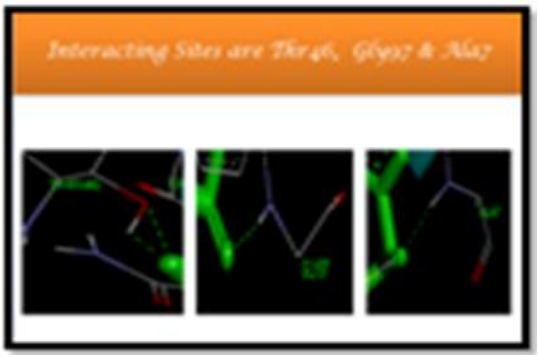

Figure 14: Interacting Sites of DHFR with Hippuric acid

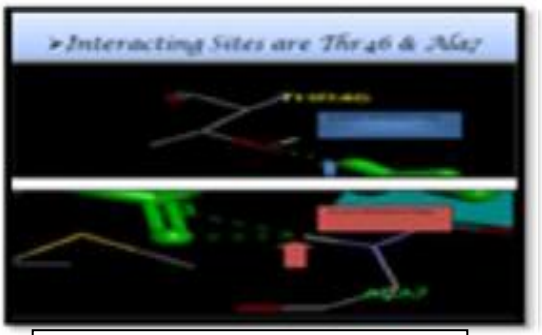

Figure 15: Interacting Sites of DHFR with Sulfamethoxazole

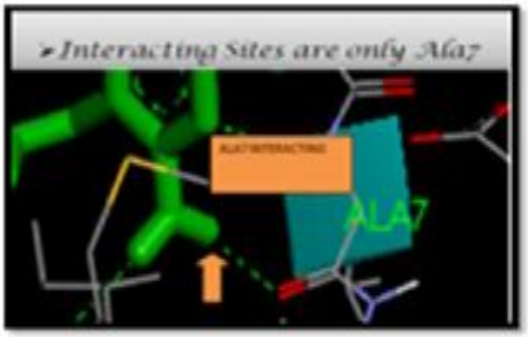

Figure 16: Interacting Sites of DHFR with Trimethoprim

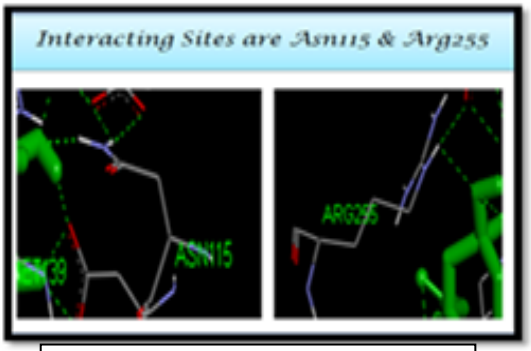

Figure 17: Interacting Sites of DHPS with Chlorogenic acid

No Interaction ofDHPSwith Hippuric acid, Sulfamethoxazole and Trimethoprim

Table 3: Interaction of ligands with DHFR and DHPS protein i.e. Hydrogen bond length, name and amino acid involved in interaction.

Ligand	DHFR *E. coli*			DHPS *E. coli*		
	Hydrogen Bond Length In A°	Hydrogen Bond Name	Interacting Sites	Hydrogen Bond Length In A°	Hydrogen Bond Name	Interacting Sites

Chlorogenic Acid	2.202 2.083 1.846 1.888 2.205	Thr46 Thr46 Gly97 - Ala7	Ala7	1.969 1.824	Asn115 -	Asn115 Arg255
Hippuric acid	2.148	Ala7	Thr113 Ala7	1.978	-	-
Sulfamethoxazole	2.127 1.938	- Ala7	Thr46 Ala7	1.919	Met223	-
Trimethoprim	1.878 1.835	- -	Ala7	1.827	-	-

Conclusion:

UTI is a serious bacterial infection of the urinary tract, which damages the kidney, if left untreated. UTI bacteria show MDR towards antibiotics. Hence, it is important to study the antibiotic resistance patterns and possible mechanisms that bacteria use to resist antibiotic action. In the current study, we have isolated uropathogenic MDR*E. coli*. The antibacterial activity of Chlorogenic Acid and Hippuric acid was found to be between 10-40mg/ml and 5-40mg/mlofchlorogenic acid and hippuric acid respectivelyagainst *E. coli*uropathogenic isolates. It was found that chlorogenic acid lead to an inhibitory action of MDR bacteria.

The DHFR activity has been investigated and it was found thatin presence of inhibitors (CGA and HA) *E. coli* isolates have significantly less DHFR activity (p value < 0.05), which indicates that chlorogenic acid and Hippuricacid inhibit the folate pathway of *E. coli*bacteria.

Genomic DNA was isolated from *E. coli* isolates and PCR amplification was performed for *folA* and *folP* gene. A ~990bp corresponding to *folP* of *E. coli*was successfully amplified. No amplification in *E. colifolA*was seen.

The second part of this study dealt with molecular docking of CGA and HA and standard antibiotics such as Trimethoprim and Sulfamethoxazole to DHFR and DHPS. This willaid to establish new ligands/inhibitors for the selected target receptor proteins from the different available databases, based on their efficiency to bind the active sites on the receptor (14). Our studies show that Chlorogenic acid and Hippuric acid compared to standard antibiotics

i.e. Trimethoprim and Sulfamethoxazolegive best interaction with DHFR *E. coli*, whileChlorogenic acid gives best interaction with DHPS *E. coli*as compared to Hippuric acid, Trimethoprim and Sulfamethoxazole(8).The interactions between ligand and proteins have been observed for different poses.Binding energy is reported to be negative for DHFR protein which is indicative of better binding affinity of CGA and HA to DHFR and is reported positive for DHPS(8). These *in silico* studies supported with *invivo, invitro*and ADMET testing will certainly help towards developing new treatment of MDR-UTI in the future.

Acknowledgements:

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