Molecular and in Silico Studies on DHFR of Uropathogenic Staphylococcus Aureus

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Abstract- Urinary tract infections (UTI) are the infection caused by microbes and women are especially prone to it than men due to anatomical reasons. Uropathogenic S.aureus have developed resistant to presently prescribed antibiotics viz., Trimethoprim and cotrimoxazole. S.aureus clinical isolates were utilized in this study.Antibacterial activity for selected phytochemicals was studied.Plasmid DNA isolation and PCR amplification of dfrAgene of DHFR S.aureus was done. In silico studies were performed to analyze the binding efficiencyof various phytochemicals and antibiotics on the DHFR protein of S.aureus.

Index Terms- Urinary Tract infection (UTI); Trimethoprim; S.aureus; Antibacterial activity; Dihydrofolate reductase(DHFR); Docking

I. INTRODUCTION

Urinary Tract Infection (UTI) is caused by the existence and growth of microorganisms in the urinary tract which occurs in all age groups and inboth gender[1]. UTI accounts for around 8.1 million visits to a doctor every year[2].Causative microbes include *Staphylococcus*, *Streptococcus*,*Corynebacterium*, *Lactobacillus*, *Enterococcus* and members of the familyof *Enterobacteriaceae*, and the fungus *Candida* (yeast).*Staphylococcus aureus* is a relatively rare cause of urinary tract infection in the general population[3]

Antimicrobial resistance among the pathogensthat cause UTI is growing and is a most important health problem in the management of UTI.Several antibiotics are used to treat UTI[4]. Oral antibiotics such as Trimethoprim/ Sulfamethoxazole (TMP/SMX) called as cotrimoxazole, nitrofurantoin, or fosfomycin are typically first line. Staphylococcus aureuscommonly shows resistance to the following drugs; Trimethoprim, Cotrimoxazole, Nitrofurantoin, Amoxycillin, Clindamycin, Oxacillin, Penicillin, Amoxycillin/clavulanic acid, erythromycin, ceftazidime[5]. *S. aureus* resistance to antibiotics is a serious medical problem worldwide[6].

One of the component of co-trimoxazole, sulfamethoxazole (SMX) induces its therapeutic effect by competing with p-aminobenzoic acid (PABA) in the biosynthesis of dihydrofolate by inhibiting the enzyme Dihydropteroatesynthetase (DHPS). While the other component which is trimethoprim (TMP) serves as a competitive inhibitor of Dihydrofolatereductase (DHFR), thereby inhibiting the de novo synthesis of tetrahydrofolate, the biologically active form of folate.

DHFR Dihydrofolatereductase, [E.C. No. 1.5.1.3] is an enzyme of reductase family that reduces 5, 6 dihydrofolic acid to 5,6,7,8 tetrahydrofolic acid.[7]

Antibiotic resistance is a seriously growing problem in medicine. Hence, there is anneed to search for potent therapeutic antibacterial agents. Thecurrent study includes isolation and identification of *S.aureus* and investigation of antibacterial activity of different compounds *on S.aureus* and DHFR gene activity assay.DNA isolation and PCR amplification of *dfr*Agene.

Molecular docking is a method which predicts the preferred relative orientation of one molecule (key) when bound in an active site of another molecule (lock) to form a stable complex such that free energy of the overall system is minimized. It exploits the concept of molecular shape and physicochemical complementarity. The structures interact like a hand in a glove, where both shape and physicochemical properties contribute to the fit.

This study proposes phytochemicals possessing inhibitory activity on DHFR and thus block the folate pathway. This strategy may bring forth novel biomolecules which could curb antibiotic drug resistant *S.aureus* causative UTI infections.

II. MATERIALS AND METHOD

COLLECTION AND PROCESSING OF URINE SAMPLES

55 urine samples of patients were collected in a sterile container from pathology laboratories from Nagpur, Maharashtra, India. The collected samples were diluted 1:100 using autoclaved distilled water and were stored at 4º C till further use.

BACTERIAL ISOLATION

18 bacterial colonies were isolated by streaking diluted urine sample on differential medium UTI agar plates (Himedia M118) followed by Mannitol salt differential and confirmatory agar.[7]

BACTERIAL INOCULATION

A single bacterial colony was inoculated into 5ml of sterile nutrient broth and incubated at 37^0C for 16-18 h to match turbidity with 0.5 McFarland's Nephelometer Standard. This culture was then used for antibiotic susceptibility testing.[8]

IDENTIFICATION OF BACTERIA

Characterization and identification of bacteria was done by Gram staining and differentbiochemical testing methods.[9] (Table 1)

Table 1: Biochemical test for Identification and Characterization of *Staphylococcus aureus*

Sr.	Test	Principle
1.	Gram staining	Gains and makes a complex of crystal- violet and iodine in cell wall.
2.	Catalase	Presence of catalase in aerobic bacteria leading to breakdown of hydrogen peroxide to oxygen and water.
3.	Citrate	Bacteria produce citrate permease and are able to utilize citrate as carbon source.
4.	Coagulase	*S. aureus* produce catalase enzyme which converts fibrinogen to fibrin.

ANTIBIOTIC SENSITIVITY ASSAY

Antibiotic sensitivity test is a procedure of determining the sensitivity of bacterial culturetowards specific antibiotics. Trimethoprim was purchased from Himedia, Mumbai. The sensitivity testing was performed by agar disc diffusion method described by Kirby-Bauer.75ml of Muller-Hilton agar was inoculated with 50µl of bacterial culture and poured onto thesterile petri plate. After solidification of agar, antibiotic discs were kept on it and incubated for16-18 hours.The diameter of zone of inhibition around the disc was measured (Himedia antibiotic scale: PW096) to determine the sensitivity towards TMP at 5 mcg.

DETERMINATION OF ANTIBACTERIAL ACTIVITY OF PHYTOCHEMICALS

Antibacterial activity of Chlorogenic acid (CGA),Hippuric acid (HA),Gallic acid (GA),Ellagic acid (EA), and Quercetin (Qu) was tested against all the 18 isolates of *S. aureus* at 1,2,5,7,10,12,14,16 mg/ml. Compounds were used by dissolving them in 100% ethanol.Well diffusion method was employed for this test. Muller Hinton agar (30ml) was poured onto a sterile petri plate of 90 mm diameter. After solidification of agar, bacterial inoculum (40µl) was spread on agar plate by spread plate technique then wells were prepared and solution of phytochemicals at different concentration were added to the wells. The plates were incubated for 16-18 hours at 37˚C.The diameter of zone of inhibition around the wells was measured using antibiotic scale.

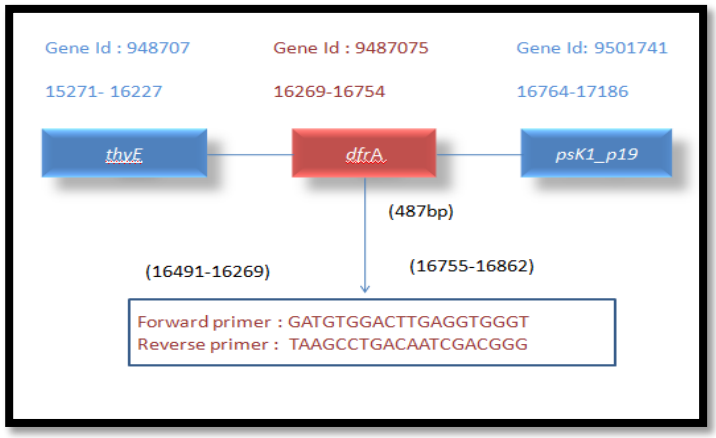
PLASMID DNA ISOLATION

The plasmid DNA was isolated by alkaline lysis method as explained Birnboim and Doly (1979). 1.5 ml bacterial culture was grown overnight for the DNA isolation.The resulting products were run on 1.2% agarose gel and visualized under UV transilluminator.

PRIMER DESIGNING

Primers were designed for DHFR gene (*dfrA)* using online 'NCBI Primer designing tool'. These primers were procured from IDT (Integrate DNA Technology) (Fig 1).The DHFR gene of *S. aureus* is flanked by *thyE* and *psK1_p19* genes.The forward and reverse primers designed would yield an expected amplicon size of 487bp.

Fig 1: Primers for *dfrA* gene of *S.aureus*



PCR AMPLIFICATION

The reaction mixture contained variable amount of plasmid DNA of *S. aureus*, 10þmoles (0.5µl) of each primer, 10mM (0.5µl) dNTPs, 1 U (0.125µl) Taq polymerase, 10X (2.5µl) buffer, solution. Volume was made up to 25µl by DEPC treated water. A thermalcycler (PEQ LAB) was reaction was run under following condition: 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 59.88° C and 1 min elongation at 72°C and final extension at 72° C for 10 min. The resulting PCR products were run on 1.2% agarose gel and visualize under UV transilluminator.

DHFR ACTIVITY ASSAY

1 ml of bacterial culture was inoculated in 100 ml of sterile nutrient broth and incubated at 37°C for overnight. The 100 ml bacterial culture was then centrifuged at 3000 rpm for 10 min at 4°C. The supernatant was then discarded and the pellet was re-suspended in 5 ml of ice cold PBS (0.1M). Cell extracts were prepared by sonication of pellet for 2 min. These cell extracts were further used for the DHFR activity assay.[10]

The specific activity of DHFR was determined by spectrophotometry. The reaction mixture (1ml) consisted of cell extract 100µM, TES(pH 6.8) 500mM, betamercaptoethanol

75mM, dihydrofolate 100µM, NADPH 100µM. Incubated at 37˚C for 3 min. Read adsorbance at 340 nm using UVvisible spectrometer (Bio Red Eppendof)

MOLECULAR DOCKING

Docking is a molecular modeling technique that is used to predict how a protein (enzyme) interacts with small molecules (ligands). The ability of a protein (enzyme) to interact with small molecules to form a supramolecular complex plays a major role in the dynamics of the protein, which may enhance or inhibit its biological function. The method aims to identify correct poses of ligands in the binding pocket of a protein and to predict the affinity between the ligand and the protein.Molecular docking process can be separated into two major steps i) searching and ii) scoring.

Search algorithm

The search algorithm implemented in any docking tool should enumerate an optimum number of ways two molecules can be put together. The size of the search space grows exponentially with the increase in the size of the molecules.

Scoring functions

The scoring function measures and ranks the binding of a ligand-receptor complex. The score should directly correspond to the binding affinity of the ligand for the protein so that the top scoring compounds are also the best binders.

This study was performed using AutoDock 1.5 version 6rc2.AutoDock is a molecular docking suite consisting of automated docking tools. AutoDock consists of two main programs: AutoDock and AutoGrid. AutoDock docks the two molecules according to the grid, which is precalculated and set by AutoGrid. AutoDock is considered one of the best programs when it comes to docking and virtual screening[11].Docking study is cost effective for speeding up the process of drug discovery and development.[12]

RESULTS AND DISCUSSION

BACTERIAL ISOLATION

The urine samples collected from pathology labs from Nagpur city and thus diluted were streaked on UTI agar. The bacterial mixed population was observed on HiCrome UTI agar (Figure 2). Out of 55 samples, only 18 samples were found to give white to cream coloured, opaque, small, and spherical colonies on UTI agar. Confirmatory test results show yellow coloured colonies surrounded by yellow zones on Mannitol agar plates which indicative of presence of *S.aureus*.

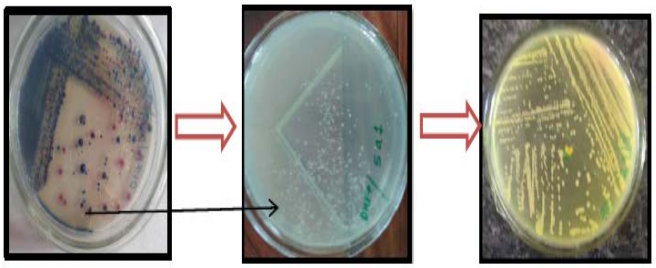


Fig 2a: Diluted urine sample on UTI agar | Fig 2b: *S. aureus* | Fig 2c: Mannitol salt agar confirming *S. aureus*

Fig 2: Isolation of bacteria

IDENTIFICATION OF *S.AUREUS*

The isolated samples of *S. aureus*were further confirmed by Gram staining and several biochemical tests.The combined results of all the biochemical tests confirmed *S. aureus.*

Table 2: Biochemical Tests for Identification and Characterization of *S. aureus*, Gram positive bacteria.

Sr.No.	Test	Result
1.	Gram staining	Violet, Spherical shape
2.	Catalase	Bubbles are generated on slide.
3.	Citrate	Colour changes to blue.
4.	Coagulase	Formation of clumps on the slide.

Fig 3b: Catalase test

Fig 3a: Gram staining of S.aureus | Fig 3b: Catalase test | Fig 3c: Coagulase test

Fig 3: Characterization and identification of bacteria

ANTIBIOTIC SENSITIVITY ASSAY

Trimethoprim, a commonly used antibiotic against UTI, was selected as an inhibitor of DHFR.Out of 18 clinical isolates 12 were resistant and 6 were sensitive to TMP at 5 mcg. Trimethoprim inhibit the bacterial growth by inhibiting the DHFR of the folate synthesis pathway.

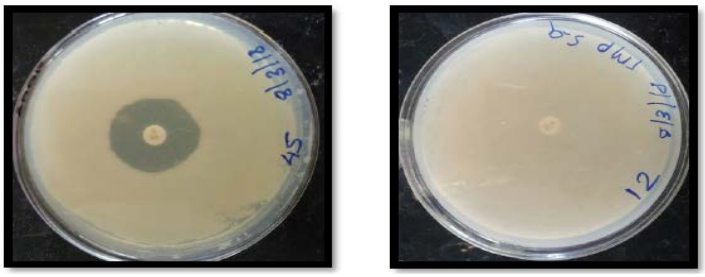


Fig4: Sensitivity and resistance towards TMP by agar diffusion method

ANTIBACTERIAL ACTIVITY OF PHYTOCHEMICALS

The most effective antibacterial activity of gallic acid is at 14 mg/ml, ellagic acid is 5 mg/ml, hippuric acid is 7mg/ml, quercetin is 10 mg/ml, chlorogenic acid is 14mg/ml.

DHFR ACTIVITY ASSAY

In order to study the response of DHFR in trimethoprim sensitive and resistant strains of *S.aureus*, specific activity of DHFR was determined with and without TMP and selected phytochemicals showing commendable antibacterial activity viz., ellagic acid and gallic acid. while other phytochemicals namely, quercitin, hippuric acid and chlorogenic acid were not used as inhibitors as they showed comparatively less antibacterial effect. Activity assay of DHFR is based on the ability of DHFR to reduce dihydrofolic acid to tetrahydrofolic acid. Activity of DHFR was found to be more in resistant strains as compared to sensitive strains.

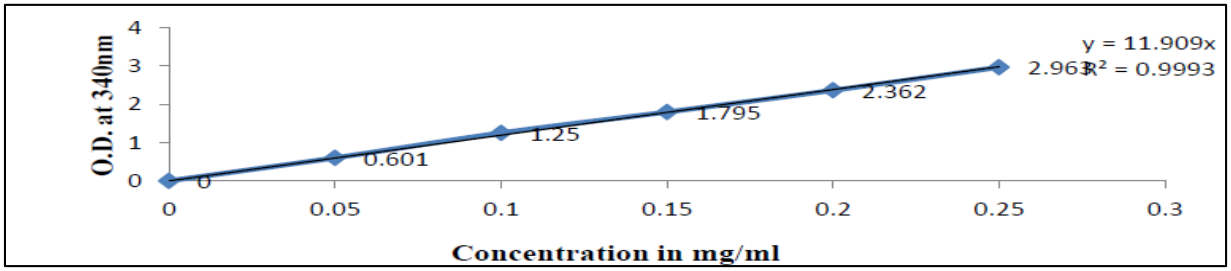


Fig 5: Standard graph of DHFR assay

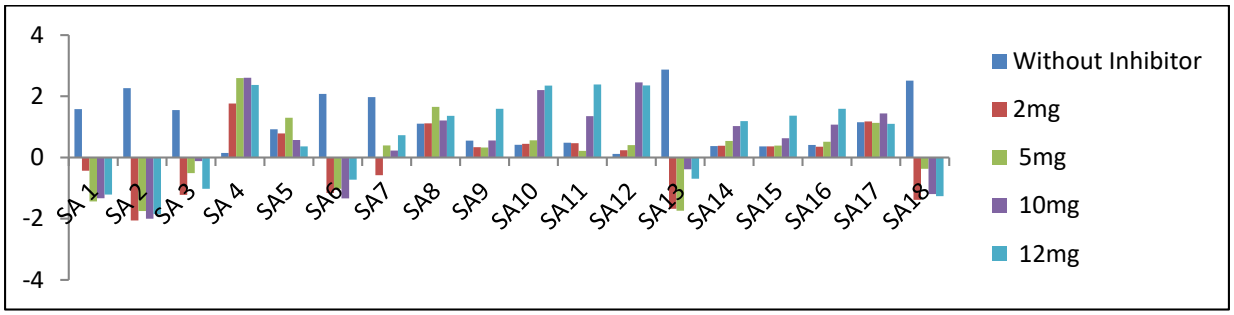


Fig 6: DHFR assay in presence of TMP

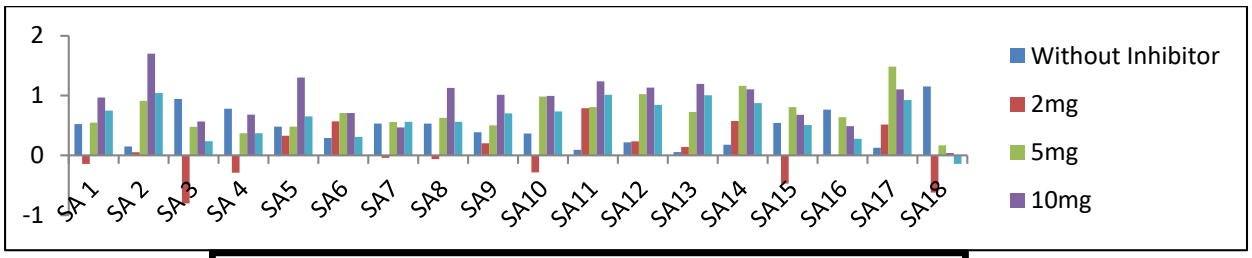


Fig 7: DHFR assay in presence of Ellagic acid

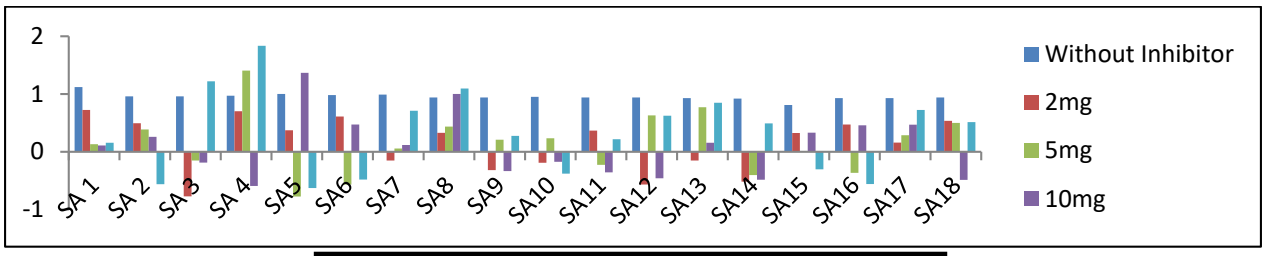


Fig 8: DHFR assay in presence of Gallic acid

Table 3: Specific activity of DHFR *S.aureus* isolates

Samples	Sensitivity towards TMP	Activity of DHFR (Units/mg protein)
Control	Sensitive	0.4146
SA 1	Resistant	0.52
SA 2	Sensitive	0.143
SA 3	Resistant	0.943
SA 4	Resistant	0.780
SA 5	Resistant	0.479
SA 6	Sensitive	0.291
SA 7	Resistant	0.532
SA 8	Resistant	0.538
SA 9	Sensitive	0.387
SA 10	Sensitive	0.366
SA 11	Sensitive	0.093
SA 12	Sensitive	0.217
SA 13	Sensitive	0.054
SA 14	Sensitive	0.177
SA 15	Resistant	0.543
SA 16	Resistant	0.763
SA 17	Sensitive	0.126
SA 18	Resistant	0.55

Since, the antibacterial assays and the DHFR activity assay showed promising results, it is appropriate to study the molecular aspects of DHFR from the TMP resistant clinical isolates of*S. aureus.* Hence, the plasmid DNA isolation was done for 18 *S.aureus* isolates.

PRIMER DESIGNING

The primers were designed for *S. aureus* DHFRwith Tm 59.93 and 59.83 for forward and reverse primers respectively. *dfrA* is flanked by *thyE* and *psK1_p19* genes, and with the primers designed the expected amplicon size is 487bp for *S.aureus* DHFR.

PLASMID DNA ISOLATION

After isolation of plasmid DNA, it was run on 1.2% agarose gel and observed under UV transilluminator. The bands of plasmid DNA are shown in the figure below.

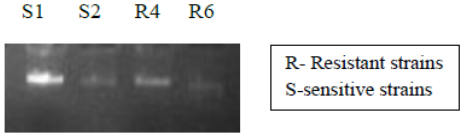
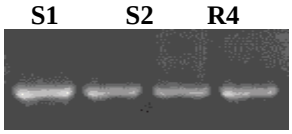


Fig 9: Plasmid DNA of S.aureus on 1.2% agarose

PCR AMPLIFICATION

PCR amplification was observed at an annealing temperatrue of 59.88ºC for all *S. aureus* isolates. The PCR products were electrophoresd on 1.5 % agarose gel and the bands of the amplicon size at 487bp corresponds to *dfrA*of *S.aureus* respectively were obtained. Plasmid DNA for 4 samples of *S.aureus* was amplified. Following gel image shows the amplification result.



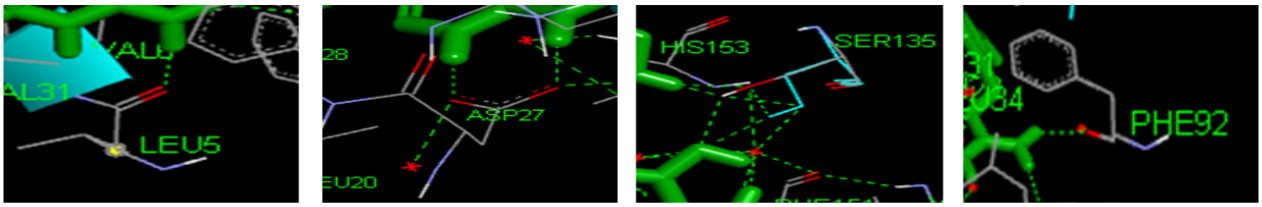
R- Resistant
S- Sensitive

Fig 10: Agarose gel (1.5%) electrophoresis of *dfrA* in *S. aureus* strains.

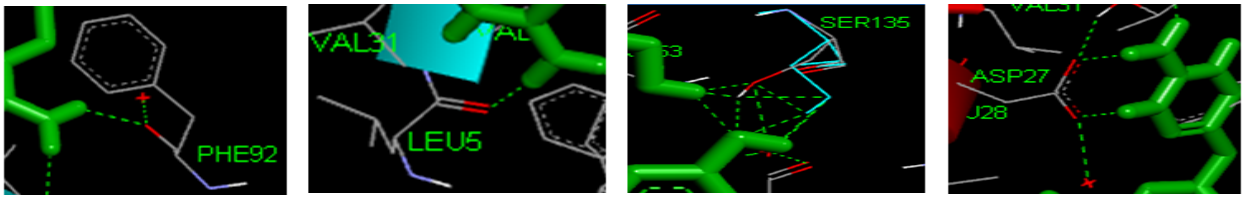
MOLECULAR DOCKING

Docking of various phytochemicals and antibiotics with DHFR of *S.aureus* was performedusing various softwares viz., Discovery Studio visualize, AutoDock 1.5.rC2,etc. Phytochemicals which were used for docking were ellagic acid, gallic acid, quercitin, hippuric acid and chlorogenic acid. The antibiotic used for docking was TMP. The structure of *S. aureus* DHFR was obtained from Uniprot and was found to have four amino acids which may interact with its ligands

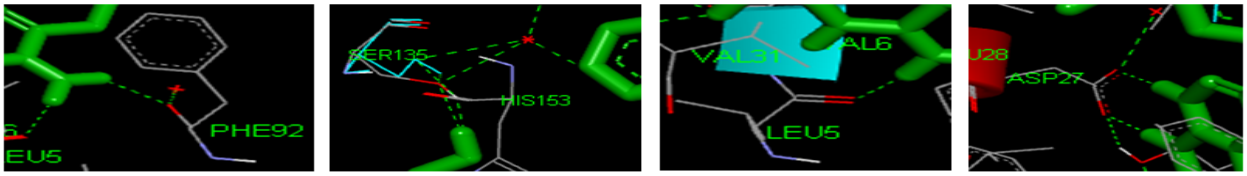
ELLAGIC ACID



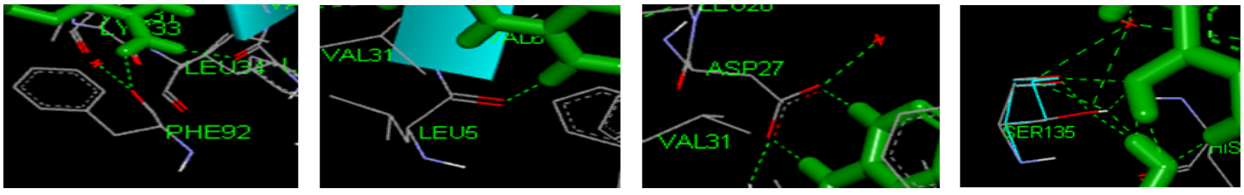
QUERCETIN



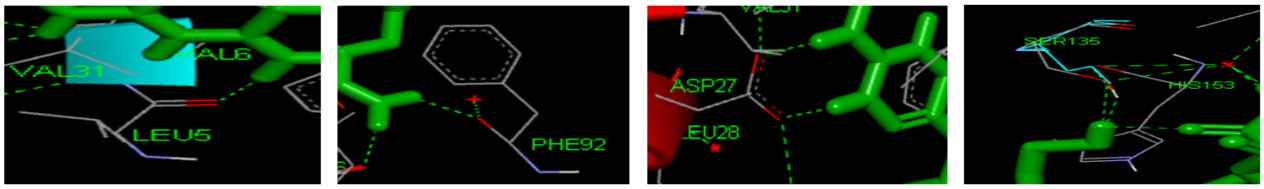
HIPPURIC ACID



GALLIC ACID



CHOROLOGENIC ACID



TRIMETHOPRIM

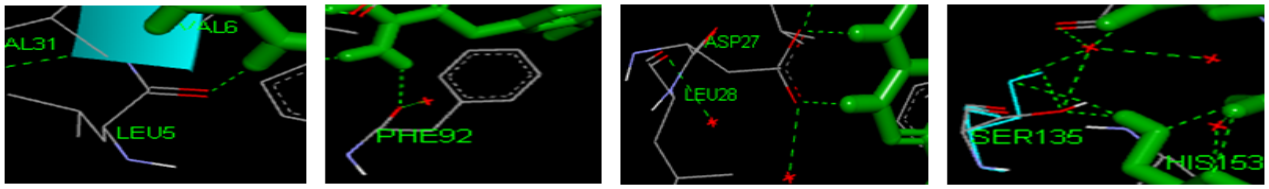


Fig11: Molecular docking images of DHFR with various inhibitors

Table 4: Binding energy of ligands with DHFR of *S.aureus*

Sr. no.	Ligand	Binding energy	Hydrogen Atom	Interacting Sites
1	Chlorogenic acid	-4.80	2	4
2	Hippuric acid	-4.78	1	4
3	Ellagic acid	-6.59	1	4
4	Quercetin	-6.50	2	4
5	Gallic acid	-4.64	2	4
6	Trimethoprim	-5.47	2	4

Most effective binding energy

Table 5: Interpreting values of ligplot of *S. aureus*

Interacting sites of Ligplot	Atoms	X-VALUE	Y-VALUE	Z-VALUE
Ligand-1:TOP				
Phe 92	O	12.163	1.254	14.122
Leu 5	O	9.180	0.498	11.939
Asp27	O	2.888	6.585	11.205
Ligand-2:EDO				
Ser 135	O	3.136	5.267	5.833
Average		6.841	3.401	10.774

The docking of phytochemicals and TMP clearly indicates that quercetin and ellagic acid have more –ve binding energy. The lower the dock score, higher is the affinity towards binding on the protein.

III. CONCULSION

Urinary tract infection (UTI) is the second most common infection experienced by humans after respiratory and gastro-intestinal infections.Since the urethra is shorter in women when compared to men they are more prone to infections associated with the urinary tract. Although *E.coli* responsible for about 80 to 90 percent of infections, incidence of UTI due to *S. aureus* is usually considered as a secondary infection next to blood borne infections. Most common antibiotic treatement used against UTI is trimethoprim and cotrimoxazole for infective organisms like *E.coli, S.aureus*, etc.

Trimethoprim were tested individually against various isolates of *Staphylococcus aureus.* The study revealed that out of 18 isolates of *S.aureus*, 6 isolates were found tobe resistant while 12 isoltes were sensitive. Being a key enzyme in folate metabolism, it has been used as a drug target for treatment of various diseases as an antifolate target. This enzyme is targeted in UTI by trimethoprim and co-trimoxazole. In order to view the activity of DHFR in trimethoprim sensitive and resistant strains an activity assay of DHFR was carried out.

Activity ofDHFR was found to be more in trimethoprim resistant samples as compared tosensitive samples. In order to know about the response of DHFR inhibitors towards the enzyme it requires the study of DHFR at molecular level.

Hence, Plasmid DNA of the clinical isolates of *Staphylococcus aureus* was processed for gene amplification with the help of primers designed for *dfrA*gene. PCR amplification results shows that bands of the amplicon size at 487bp corresponds to *dfrA*of *S.aureus* were obtained.

As there is a rise in MDR strains of *Staphylococcus aureus*, as is evident from the studies, it is necessary to look for new safe, cost-effective antifolate antibacterials from plants. Reviewing various literatures, the antibacterial efficacy of the photochemicals was performed against *S. aureus* resistant and sensitive strains. It was found that some of the phytochemicals lead to an inhibitory action of TMP resistant bacteria.

Hence, Molecular docking studies were performed using software to elaborate the biological data obtained by our antibacterial assay of phytochemicals. The future prospective of the study involves the sequencing of *dfrA*gene and study of mutations if any, leading to development of plant based DHFR inhibitors as drugs.

REFERENCES

[1] Oladeinde, B.H., R. Omoregie, M. Olley and J.A. Anunibe, 2011. Urinary Tract Infection in a Rural Community of Nigeria. North American Journal Medical Science, 3(2): 75-77.

[2] Urinary Tract Infections in Adults. (n.d.). “National Kidney and Urologic diseases Information Clearinghouse.”Retrieved May11, 2012.

[3] Demuth, P.J., G.N. Gerding and K. Crossley, 1979.Staphylococcus aureus Bacteriuria. Arch InternalMedicine, 139: 78-80.

[4] Grigoryan L, Trautner BW, Gupta K. Diagnosis and management of urinary tract infections in the outpatient setting: a review. jama. 2014 Oct 22;312(16):1677–84.

[5] T.E. Ayogu, J.O. Orji,et.al.Antibiotic Resistance Pattern of Escherichia coli and Staphylococcus aureusfrom Pregnant Women with Urinary Tract Infection (UTI) in Ezza South L.G.A. of Ebonyi State, Nigeria. Middle-East Journal of Scientific Research 25 (5): 1120-1135, 2017.

[6] Felten, A., B. Grandy, P. Lagrange and I. Casin, 2002.Evaluation of three Techniques for Detection of low-level Methicillin-Resistant Staphylococcus aureus.A disc Diffusion Method with Cefoxitin and Moxalactam, the Vitek 2 System and the MRSA.-screen Latex Agglutination Test. Journal of Clinical. Microbiology, 40: 2766-2771.

[7] Sawaya MR & Kraut J (1997) Loop and subdomain movements in the mechanism of Escherichia coli dihydrofolatereductase : crystallographic evidence. Biochemistry 36, 586–603.

[8] Bollela VR et al. McFarland nephelometer as a simple method to estimate the sensitivityof the polymerase chain reaction using Mycobacterium tuberculosis as a research tool. Braz J Med Biol Res, 1999 Sep;32(9):1073-6.

[9] 1 Sanjay Bhattacharya,2 Bärbel Christiansen,3 Jürgen Gebel,1Peter Goroncy-Bermes,4 Philippe Hartemann,5 Peter Heeg,6 Carola Ilschner,1Axel Kramer,7 Elaine Larson,8,9 Wolfgang Merkens,4 Martin Mielke,10Peter Oltmanns,4 Birgit Ross,11 Manfred Rotter,12 Ricarda Maria Schmithausen,1Hans-Günther Sonntag,13 and Matthias Trautma. GMS Hyg Infect Control. 2017; 12: Doc05. Published online 2017 Apr 10. doi: 10.3205/dgkh000290PMCID: PMC5388835PMID: 28451516Language: English | German

[10] Identification of Gram Negative BacteriaMs. Banan A. Atwah 1431 - 1432 H Fall 2015 – Jackie Reynolds, Richland College, BIOL 2421 McFarland, J., J.Amer.Med.Assoc. 14:1176, 1907.

[11] Park H, Lee J, Lee S (2006) Critical assessment of the automated AutoDock as a new docking tool for virtual screening. Proteins 65: 549–554.

[12] Sudha Ramachandra1, Vinay Chavan2 A Genetic Algorithm for Conformation Search Optimization in Molecular Docking (IJCSIT) International Journal of Computer Science and Information Technologies, Vol. 6 (6) , 2015, 5547-5551.

[13] Himedia biosciences catalogue 2018-2019, Antibiotic susceptibility testing zone size interpretive chart Pg no. 271-281.

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