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Studies on epidemiology and screening of a quorum quencher from *Melia dubia* against urinary tract infections during pregnancy

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ABSTRACT

Urinary Tract Infection (UTI) is accountable for maternal and neonatal morbidity and about a third of the pregnant women have UTI at some point of gestation. *Staphylococcus aureus* is one of the microorganism commonly found causing UTI and is of high concern due to high occurances of multi-drug resistance. The epidemiology of the disease among pregnant women in the region of Thanjavur was studied to obtain an understanding of the prevelance of UTI and the organisms causing it. *S. aureus* uses Quorum sensing as a means of communication, which is regulated by the pleiotropic pathways of Agr/ Sar. To tackle the microbe, the efficacy of the extracts of *Melia dubia*, a plant known for its antimicrobial properties, has been employed. The crude library of compounds from *Melia dubia* extracts was discovered to have anti-biofilm rather than bactericidal properties. Docking studies were executed with the characterized compounds binding with Sar and Agr receptors to predict the pathway of quorum quenching activity.

KEYWORDS

Quorum sensing;
Biofilms;
Auto inducing peptides;
Agr;
Sar pathway.

INTRODUCTION

Quorum sensing is an association of microorganisms as a result of their response to signals molecules. A bacterium employs the quorum sensing mechanism to coordinate various vital processes through controlled gene expression. The mechanism is highly sophisticated and plays a significant role in the process of bacterial communication. This can be achieved through various factors which differ among the Gram positive and negative bacteria. Though the mechanism is quite simple in Gram negative bacteria, involving active or passive transport of lipids through diffusion, the phenomenon is complex in Gram positive bacteria[1]. Among Gram positive bacteria the secreted signals requires suitable carriers for its transportation across the membrane to trigger the mechanism of quorum sensing. The mechanism allows the assemblage of bacteria to interact and organize a variety of processes needed for its existence[2,3]. This highly sophisticated mechanism comes to play at higher cell densities. The Gram negative bacterium uses small neutral lipid molecules as signals to trigger quorum sensing whereas larger peptide act as the essential signals to effect the mechanism in Gram positive bacte-

ria. This cell to cell interaction is instrumental in the production of biofilm. Biofilms consist of mixed groups of microorganisms adhered to a solid surfaces, usually enclosed in a matrix of lipopolysaccharides which serve as a potential barrier for many antimicrobial agents[4]. Research studies in the past have confirmed the presence of dental biofilms comprising of >500 different bacterial species[5]. The extent of antibiotic resistance exhibited by biofilm producing strains can be 10 to 1000 times higher in contrast to the planktonic cells[6]. Biofilms are responsible for causing 65% of nosocomially acquired infections and involves a cost of $ 1 billion for treatment annually[7,8]. This in turn has created an interest in studies on biofilm based anti microbial agents. Research pioneers has made use of infrared spectroscopy to illustrate the extent of diffusion of antibiotic ciprofloxacin on a colonized surface[9]. The study has revealed reduced levels of antibiotic diffusion on a colonized surface when compared to a sterile surface. Similar results were obtained in case of antibiotic piperacillin when used against the biofilms of *Pseudomonas aeruginosa*[10]. Antimicrobial agents like rifampicin and vancomycin were effective against *Staphylococcus epidermidis* biofilms[11]. The inhibition of antibiotic diffusion also relies on the nature of biofilm based on cell densities. Based on their cell compactness, they are classified as thin and thick biofilms and *KatA* is an important factor conferring resistance against hydrogen peroxide due to the production of enzyme catalase under the influence of *KatA* gene[12].

Studies in the past have demonstrated the role of β-lactamase enzyme in offering resistance against antibiotics like ampicillin, as was observed in the biofilm of *Klebsiella pneumoniae*[12]. Hence in addition to lipopolysaccharide matrix enclosing the cells, there are certain factors at the genotypic level which are capable of producing the products conferring the anti biocide property. Slow growth and stress response also enhances the resistance against the antimicrobial agents[13]. The exact reason is unknown but researchers believe that nutrient deprival results in physiological changes which in turn imparts the property of biocide resistance. Studies in the past have been able to illustrate the significance of slow growth rate in relation to biofilm resistance against a variety of antimicrobial agents. Researchers have studied the similar effect in the biofilms of *P. aeruginosa*, *E. coli* and *S. epidermidis* and have revealed that the cells sensitive to antimicrobial agents under normal conditions were resistant under adverse conditions due to slow growth as a consequence of nutrition limitation[14]. The resistance of biofilm producing and non biofilm formers during the various stages of the exponential phase has been evaluated and the resistance of microbial cell cultures to antimicrobial agents was found to be enhanced as the cells enter the stationary phase[15].

The emergence of multidrug resistance in S. aureus poses a significant threat to the infections in which it gets involved, that the host often gets immuno compromised and treatment becomes increasingly difficult. The phenomenon of quorum sensing and biofilms in *S. aureus* are regulated by the pathways of *agr* (accessory gene regulator) and *sar* (*Staphylococcus* accessory regulator). The signaling molecules in the control of these genes are known to induce quorum sensing, which in turn triggers a cascade of reactions resulting in regulated gene expression[16]. The general paradigm involves the recognition of small lipids to larger peptides by Gram negative and positive bacteria resulting in quorum sensing[17]. The composition and configuration of the peptide signals among Gram positive bacteria relies on the type of bacteria and the nature of function performed. The competence in *Streptococcus pneumoniae* is triggered by a linear peptide 17 residues long[18]. In addition to linear peptides, cyclic lactones and thiolactones also serve as signaling molecules resulting in bacterial communication. The *agr* in *S. aureus* comprises of two transcripts called RNA II and RNA III, under the control of P2 and P3 promoters respectively. The RNA transcript comprises of a set of four genes constituting an operon essential to activate a cascade of reactions. The genes *agrBDCA* plays a vital role in the production of a mature AIP molecule (auto inducing peptide) responsible for virulence. The activation of *agr* pathway increases the production of factors responsible for virulence and production of surface proteins[19]. In addition to *agr* pathway, research studies have revealed an alternative pathway called as the *sar* pathway which is considered as a global regulatory loci. The *sar* pathway is also known for regulating various genes involved in the production of a variety of determinants like the fibronectin binding protein, α-hemolysin through *agr*

independent mechanism.

The major regulatory proteins encoded within the *sar* pathway are capable of binding to a highly conserved sequence similar to the SarA binding site on the agr promoter and triggers the expression of several genes coding for α-hemolysin, surface protein A (*spa*), fibronectin binding protein (*fnb*) and enterotoxin C gene (*sec*)[20]. Research studies have revealed the fact that the removal of SarA recognition motif of *agr* allows the transcription of these genes that were untraceable in the *agr* mutants. Similarly the activity of surface protein A (*spa*) which is usually repressed by *sar* under normal condition was found to be inhibited even after the removal of the SarA binding site from the spa promoter region. This reveals the influence of the SarA binding site on spa promoters. The *sar* locus is highly significant for *agr* dependent quorum sensing and transcription of RNA II and III relies on the *sar* locus that flanks overlapping *sarA*, *sarB* and *sarC* transcripts[21]. Despite the fact that *agr* and *sar* controls a collection of virulence factors, researchers have demonstrated the role of SarA as a regulatory protein that binds to its consensus sequences leading to the induction or inhibition of the transcription of the target genes. The current study was carried out with an attempt to explore the specific pathway responsible for the formation of biofilm and the efficacy of *Melia dubia* plant extract in inhibiting the biofilm formation by obstructing the pathway. The choice of *M. dubia* as a cure stems from the fact that the bioactive substances present in it, has already been employed for centuries as a folklore medicine against UTI during pregnancy, in South India.

MATERIALS AND METHODS

Sample collection

Urine samples (N=100) were collected at random from pregnant women during their pre-natal visit at Mother and Child care maternity hospital, Thanjavur between May 2012 to July 2012 and the status of infection and pyuria was analysed. Pathogenic strains causing confirmed cases of urinary tract infections were isolated. The strains were then screened for multidrug resistance (MDR) against antibiotics like ampicillin, amoxicillin, cefotaxime, ciprofloxacin, clindamycin, gentamicin, penicillin, trimethoprim, and vancomycin. These isolated strains were cultured in enriched nutrient broth and used for the subsequent study.

Extraction of plant material

The *Melia dubia* root specimen collected from Thanjavur, Tamil Nadu was subjected to the process of extraction at room temperature (30 ± 1^0C), through cold percolation methods using different solvents (water, hexane, petroleum ether, ethanol, and methanol). The extract was thoroughly mixed and the supernatant was filtered through a fine cloth. The filtrate lyophilized and was stored at -80^0C[22].

In vitro assay

The efficacy of *M. dubia* extract was examined at different concentrations to reveal the minimum inhibitory concentration required to inhibit the biofilm formation. Tryptic soya broth (TSB) was supplemented with various concentrations of the extract and its impact on biofilm formation was examined at various time intervals ranging from 0^{th} to 24^{th} hour. The extract was dissolved in phosphate buffer saline (PBS). Tryptic soya broth devoid of the extract served as blank. Anti-biofilm activity was pitted against dithiothreitol (5mM), which is an established anti-biofilm compound, taken as a positive control[23]. In addition biochemical analysis was performed to reveal the identity of the clinical isolates from infected samples.

Gas chromatography and mass spectrometry (GC-MS)

GC-MS analysis was carried out for the root ethanolic extract of *M. dubia* in order to reveal the various chemical constituents present in the extract using a PerkinElmer Clarus 500 GC-MS system. The program was set at a temperature of 50^0C for a duration of 1 min and raised at 10^0C/min to 150^0C (1 min hold), at 8^0C/minute to 250^0C (1 min hold), at 15^0C/minute to 300^0C (3 min hold). Helium (1 ml/min) was used as carrier gas. The injector temperature was maintained at 280^0C and the mass range was 40-450 amu. 1µl of sample dissolved in ethanol was injected into the system. The identification of the compounds was made by comparing their spectra with the National Institute of Standard and Technology (NIST) spectral library.

Computational analysis

Derivation of interaction sites

The 3D structure of SarA was retrieved from PDB database [Accession ID: 2FRH]. The primary target constraints were receptor based, in which interactions of the SarA with ligand form the basis for design and scoring. The de-novo receptor and binding site in SarA were defined from highly conserved winged helix regions responsible for DNA binding (R90) within a 7.7A^0 radius sphere.

Ligand preparation

Compounds reported by the GC-MS analysis were drawn using Symyx Draw™. Ligand files were prepared for docking using Schrodinger Ligprep™ software. In addition to the generation of energy minimized 3D structure, Schrodinger Ligprep™ software was also used for adding hydrogen atoms. Ligprep was used to obtain low energy 3D structure for the set of ligands for computational studies. OPLS-2005 force field was utilized to optimize the geometry and for minimization.

Docking studies

All docking experiments were performed using the GLIDE (Grid Based Ligand Docking with Energetics) program in Schrodinger Suite™. Grid files were generated targeting the interaction sites. The size of the binding box was set at 7.7A^0 in order to explore a large region of protein. Compounds were subjected to the flexible docking position using a precomputed grid file. For each compound 100 top score poses were saved and the best scoring pose was analyzed[24].

RESULTS AND DISCUSSION

The demonstrative study is aimed at revealing the specific pathway responsible for favoring the biofilm production in pathogens causing urinary tract infection during pregnancy. The study presents the epidemiology of prevalence of UTI among pregnant women in the region (TABLE 1). Thirteen of the hundred random samples tested positive for UTI and it was found that gram negative pathogens causes nearly 70% of the infections. This outcome agrees with the finding reported in the earlier studies[25] which has shown 66.7% of Gram negative isolates in contrast to 28% of Gram positive isolates. The most effective antibiotics were vancomycin among gram positives and gentamycin among gram negative, *S. aureus* exhibiting maximum antibiotic resistant. All the isolates were resistant to ampicillin and this agrees with the findings reported in previous experimental studies (TABLE 2)[26]. In addition, all the *E. coli* isolates were resistant to trimethoprim and penicillin. Even thought *S. aureus* causes less than 10% of the urinary infections, its multidrug resistance could hamper curing the infection. Various demographic parameters have been considered in the study to predict the occurrence and the specific group vulnerable to the infection (TABLE 1). The emerging trait of antibiotic resistance among various pathogens has posed a great threat and has provoked the discovery of various novel techniques to overcome the problem[27,28]. It is quite evident that women in their late twenties and early thirties are at higher risk of encountering urinary tract infection during pregnancy (TABLE 1). Studies in the past have confirmed the prevalence of the infection among women in their late twenties and early thirties during pregnancy and are higher during the first

TABLE 1 : Prevalence of UTI and demographic characteristics among the study population (N=100)

Age	Num tested	Negative	Positive	Chi sq	p-value
15-24	10	10	0		
25-34	69	56	13	6.74	0.034
35-44	21	21	0		
Address					
Rural	22	18	4	0.72	0.40
Urban	78	69	9		
Gravidity					
1	51	44	7		
1-3	42	36	6	1.12	0.60
4-6	7	7	0		
Parity					
Nullipara					
Single	51	44	7		
Multipara	42	36	6	1.12	0.60
Grand MP	7	7	0		
Trimester					
1st	35	30	5		
2nd	31	27	4	0.0932	0.95
3rd	34	30	4		
Education					
Primary (1-8)	14	12	2		
Secondary (8-12)	47	41	6	0.03	0.98
Higher (>12)	39	34	5		

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TABLE 2 : Antimicrobial resistance pattern (in %) of pathogens in samples infected with UTI

Bacterial isolate	AMOXICILLIN	AMPICILLIN	CEFOTAXIME	CIPROFLOXACIN	CLINDAMYCIN	GENTAMICIN	PENICILLIN	TRIMETHOPRIM	VANCOMYCIN
	Interpretation of zone diameter (mm)								
	Amc R≤18 S≥26	Amp R≤16 S≥24	Ctx R≤18 S≥26	Cip R≤20 S≥30	Cd R≤24 S≥30	Gen R≤16 S≥22	P R≤24 S≥30	W R≤18 S≥26	Va R≤17 S≥21
E. coli	70	100	43.3	0	13.3	0	100	100	100
P. aeruginosa	89.5	100	68.4	0	15.9	0	100	89.5	100
K. pneumoniae	75	100	58.3	0	0	0	58.3	33.3	100
S. aureus	61.9	100	80.9	0	9.5	0	71.4	100	0
E. faecalis	52.5	100	25	0	0	0	50	100	0

BI: Bacterial Isolate; Amoxicillin (Amc 30µg); Ampicillin (Amp 10µg); Cefotaxime (Ctx 30µg); Ciprofloxacin (Cip 5µg); Clindamycin (Cd 2µg); Gentamicin (Gen 10µg); Penicillin (P 10units/disc); Trimethoprim (W 5µg); Vancomycin (Va 30µg)

trimester[29,30].

In vitro studies

The study involved the testing of the efficacy of root ethanolic extract of *M. dubia* in suppressing the traits responsible for the establishment of microbial biofilm. Root ethanolic extract served to be the best among all the extracts as a biofilm inhibitor. Biofilm formation was

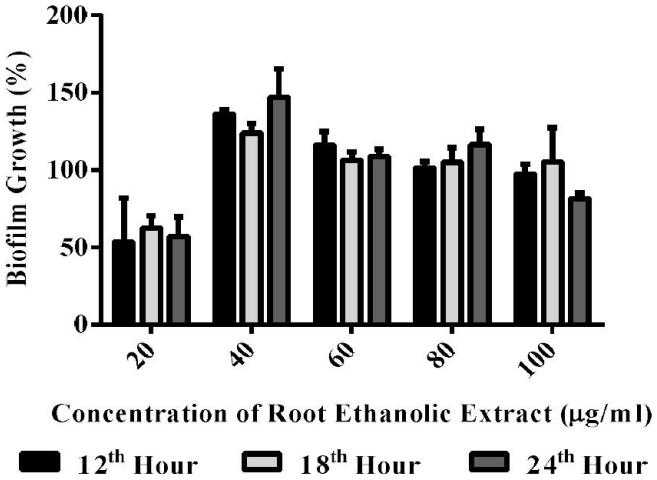

Figure 1 : Biofilm formation in response to the minimum inhibitory concentration of Root Ethanolic Extract in ATCC 25923 (reference strain)

retarded by nearly 50% in the presence of 20µg/ml concentration of the root ethanolic extract (Figure 1).

GC-MS Analysis

In silico studies have revealed the presence of organic compound derivatives which restrains the biofilm formation. The gas chromatography mass spectroscopy

TABLE 3 : List of ligands obtained from *Melia dubia* root extract from GC-MS analysis

S. No.	Peak name	Retention time
1	Glycerin	6.7
2	Propanoic acid, 2-oxo-, methyl ester	9.99
3	Octanediamide, N,N'-di-benzoyloxy-	10.52
4	Piperazine, 1-(aminoacetyl)-	11.25
5	Dianhydromannitol	11.59
6	2-Butanone, 4-phenyl-	12.03
7	L-Galactose, 6-deoxy-	12.31
8	2-Nonen-1-ol	13.43
9	Cyclohexanecarboxylic acid, 3-(acetyloxy)	15.03
10	5,6-Epoxy-6-methyl-2-heptanone	15.14
11	Vanillin lactoside	15.22
12	Sucrose	15.88
13	2-Propenoic acid, 3-(2-hydroxyphenyl)-	16.13
14	1,7-Octanediol, 3,7-dimethyl-	16.4
15	Hexanoic acid, 2-methyl-	17.19
16	Benzene, 1,2,3-trimethoxy-5-(2-propenyl)-	17.68
17	Undecanoic acid	17.88
18	Benzenepropanol, 4-hydroxy-à-methyl-, (R)-	18.19
19	Ethyl à-d-glucopyranoside	18.91

S. No.	Peak name	Retention time
20	1-Cyclohexanol, 1-[5-hydroxy-4-methyl-2-hexenyl]	19.34
21	d-Mannose	19.43
22	[1,1'-Bicyclopropyl]-2-octanoic acid, 2'-hexyl-, methyl ester	19.74
23	3,7,11-Trimethyl-dodeca-2,6,10-trienoic acid	19.87
24	1,2-15,16-Diepoxyhexadecane	20.01
25	4-Cyclononen-1-one	20.27
26	Decanoic acid, 3-methyl-	20.39
27	2-Butyl-5-methyl-3-(2-methylprop-2-enyl)cyclohexanone	21.13
28	Tetradecanoic acid, ethyl ester	21.35
29	2-Indanone, 4,5,6,7-tetrahydro-	23.26
30	n-Hexadecanoic acid	23.69
31	2-Cyclohexen-1-one, 2-(2-methyl-2-propenyl)-	24.46
32	(E)-9-Octadecenoic acid ethyl ester	26.12
33	3,7,7-Trimethyl-1-penta-1,3-dienyl-2-oxabicyclo[3.2.0]hept-3-ene	26.47
34	Menthol, 1'-(butyn-3-one-1-yl)-, (1S,2S,5R)-	26.78
35	2-(3,4-Methylenedioxyphenyl)cyclohexanone	28.45
36	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	30.19
37	Hexadecanoic acid, ethyl ester	31.16
38	Docosanoic acid, ethyl ester	31.74
39	Stigmasterol	32.97
40	Cholesta-22,24-dien-5-ol, 4,4-dimethyl-	33.79

(GC-MS) analysis revealed the variety of ingredients embedded in the extract (TABLE 3).

MOLECULAR DOCKING STUDIES

The antimicrobial property processed by the plants is an undeniable fact and the present demonstrative study has made use of plant extract to disclose its anti biofilm nature. As the preceding analysis involving the GC-MS study revealed the presence of an organic compound (MDR^{C12}) as a potential anti biofilm agent. Further analysis was carried out at the molecular level by employing computational techniques. The active site on SarA was disclosed through these computational approaches to explore the mode of ligand binding on to the active site. The ligand designing was based on the fragmental approach in order to provide interphase plots for copious fragments. The *in silico* methods for revealing the potential SarA inhibitors employed Schrödinger suite software to dock small fragments at the binding site. A methodical investigation to probe the structural fragments relied on non bonded contact linking the SarA functional group and the ligand. This in turn illustrates the indispensible association between the ligand and the active SarA site. The outcome of molecular docking has demonstrated the binding mode of SarA inhibitor and possible docking sites within SarA active site resulting in the formation of a hydrogen bond (Figure 2, 3). The three dimensional (3D) structures of SarA were retrieved from protein data bank (PDB) database from its unique identity based on its accession ID (2FRH) and processed. The chief restraints were receptor related where the binding of SarA with its ligand is the source for design and scoring.

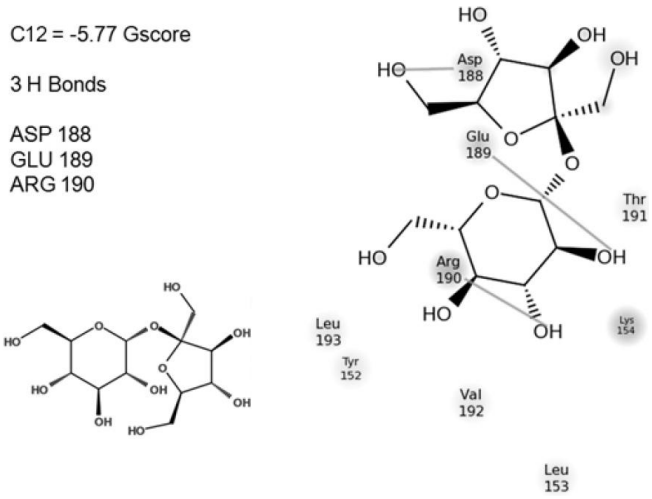

Figure 2 : Docking pattern between sucrose and Sar protein

The active binding site of the SarA is well determined and it not only describes the shape constraint but theoretical binding sites supported by hydrogen bonds, electrostatic and additional non covalent communications.

The very much conserved helix region accountable for DNA binding (R90) as well as induction (D88 and E89) characterized the *de novo* receptor and the binding site. The X-ray structures of SarA served as the protein coordinates for molecular docking. The bound divalent cations were impounded and the hydrogen atoms were constituted.

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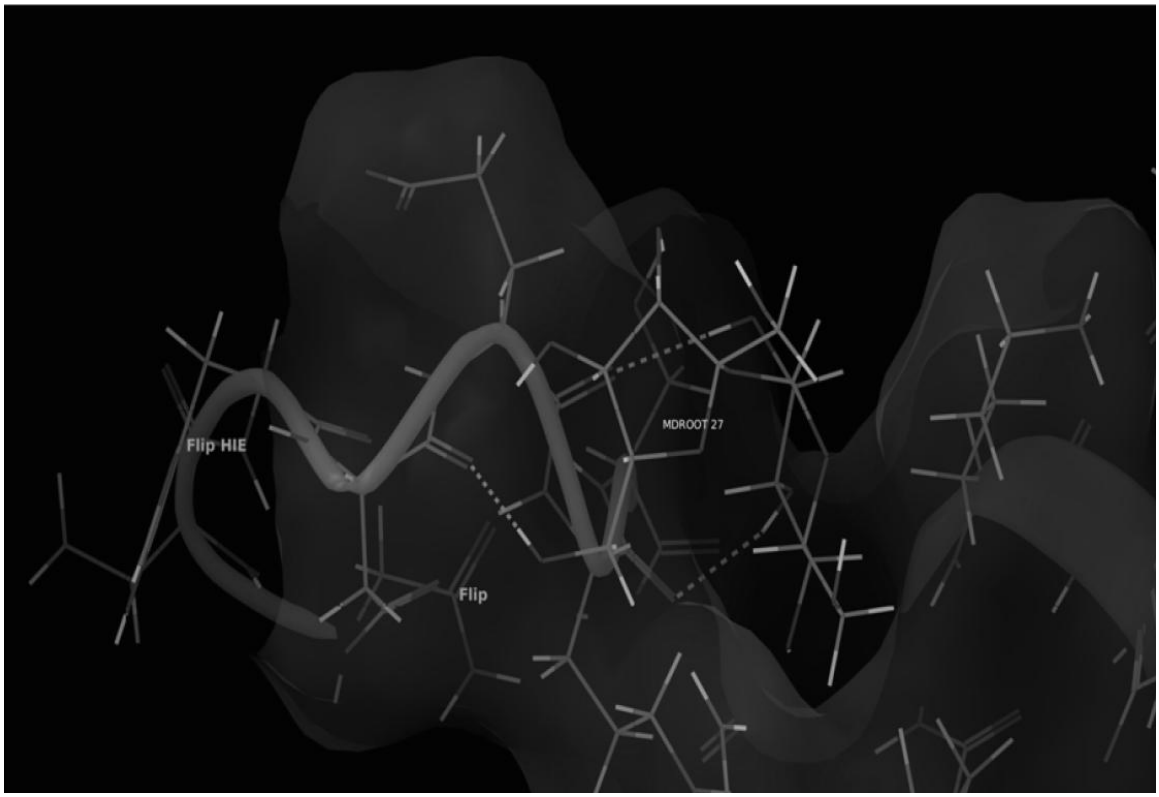

Figure 3 : Graphical image representing the interaction between sucrose and Sar protein

CONCLUSION

It is evident from the present study that UTI is a common infection in pregnant and non pregnant women and its prevalence is enhanced during pregnancy due to various physiological factors. The onset of the infection is in the 6th week of pregnancy through 24th week[30]. Despite the fact that the prevalence of bacteriuria during pregnancy is similar to that in non pregnant women, pregnancy enhances the possibility of infection among women[31,32].

The rate of occurrence varies from 2 to 13% during pregnancy and it starts during the 6th week of pregnancy and reaches peak during the 24th week. *E. coli* is the predominant pathogen responsible for the infection and *S. aureus* predominates among the Gram positive bacteria. Untreated asymptomatic UTI can lead to symptomatic UTI up to 40 to 70% among women during pregnancy. Sensitivity pattern of the pathogens isolated from the urine samples of the patients shows resistance to some commonly prescribed drugs during pregnancy. Appropriate use of antimicrobial agents can be administered only after culture and sensitivity pattern of the pathogens. The *agr* and *sar* pathway are extensively studied due to their role in favoring quorum sensing leading to biofilm formation and confers the pathogen with properties which can mask the effect of a variety of anti microbial agents. The inhibition of quorum sensing mechanism can lead to effectual novel approaches to pacify the bacterial pathogenesis[33]. The existence of two pleiotropic pathways (*agr* and *sar*) responsible for the expression of virulent genes[34].

The antibiotic sensitivity pattern demonstrates the existence of resistance to β-lactum antibiotics like ampicillin and amoxicillin. Molecular docking studies have disclosed the specific active sites on SarA responsible for triggering significant corroborations. Despite the fact that the present study has investigated the rate of prevalence of UTI among women during pregnancy and has revealed the antimicrobial pattern exhibited by the pathogen, the study involved a small number of samples. Further studies with large sample size are required in order to widen the facts of this study. Further studies will provide stronger evidences to confirm the role of these pleotrophic pathways in regulating the various genes associated with virulence.

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