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Screening of SdiA Inhibitors from *Melia dubia* Seeds Extracts Towards the Hold Back of Uropathogenic *E.coli* Quorum Sensing-Regulated Factors

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Abstract: Plants have always been a supreme source of drugs and India is endowed with a wide variety of them with high medicinal values. The Quorum Sensing (QS) quenching efficiency of various solvent extracts of *Melia dubia* seeds was investigated against uropathogenic *Escherichia coli* (UPEC) to screen the competitive inhibitor of SdiA, a transcriptional activator of quorum sensing in *E. coli*. In this study, potentiality of five different extracts of *Melia dubia* seeds for quorum sensing inhibitory activity was investigated against uropathogenic *Escherichia coli* (UPEC). Assays such as cell density, swarming motility, protein, protease, hemolysis, hemagglutination, hydrophobicity and biofilm inhibition were performed. Biofilm, hemolysis and swarming motility were found to be inhibited by 92.1%, 20.9 % and 48.52% respectively, when the medium was supplemented with 30 mg/ml of the ethanolic extract. GC-MS spectrum of the ethanolic extract showed an array of 27 structurally unlinked compounds with natural ligand C_8HSL. The docking against QS transcriptional regulator SdiA was predicted by *in silico* studies and the ligand C6 showed significant activity with -10.8 GScore. *In vitro* and *in silico* docking analysis showed fairly a good correlation, suggesting that the ethanolic extract showed potency to attenuate quorum sensing of uropathogenic *E. coli*. Further studies by *in vitro* and *in vivo* strategies are necessary to foresee the quorum quenching effect of the ligands.

Keywords: Quorum Sensing, quorum quenching, *Melia dubia*, uropathogenic *E.coli*, SdiA.

1. INTRODUCTION

Urinary tract infections (UTIs) are caused by several bacterial species that cause diseases like bacteriuria. *Escherichia coli* is a common inhabitant of human intestines whereas uropathogenic *Escherichia coli* (UPEC) is the causative for most of the UTI cases. UTI is known to affect over 60% of women population [1] and it is found to be a major problem in case of diabetic patients [2]. These UPEC strains are getting resistant towards almost every antibiotic that are currently in use and thus increasing the rate of morbidity and mortality [3].

Quorum Sensing (QS) is a cell-to-cell communication system through chemical signal molecules found in bacteria. Many bacteria regulate its biofilm and virulence related traits by means of QS. UPEC is a gram negative bacterium that coheres with all bacteria by means of LuxIR QS machinery. SdiA is a homologue of LuxR transcriptional regulator that modulates UPEC quorum sensing. It is a 238 aminoacid long protein which has an autoinducer binding domain (1-171) and a DNA binding motif (190-276) [4]. Over expression of SdiA increases expression of over 62 genes related to UV-resistance, adherence to a substratum and other major virulence factors [5]. SdiA amplification acted as a positive activator of ftsQAZ genes that are responsible for cell division and mitomycin C resistance [6]. The AcrAB genes which activate multidrug efflux pump, thereby increasing fluoroquinolones resistance property have also been up-regulated by SdiA [7]. SdiA also increases the curli synthesis that is responsible for hydrophobicity in *E.coli*. C terminus deletion of SdiA abolished the DNA binding activity, but retained the ligand binding domain. *E.coli* does not have its own autoinducer to activate SdiA with a folding switch [8]. Thus, *E.coli* was proved to detect the AHL of other bacteria [7]. Thereby, the virulence of UPEC can be corrupted by inhibiting its quorum sensing cascades. A few antagonist molecules are known to bind SdiA of *E.coli* that can break its quorum sensing called quorum quenching agents. Furanones act as effective antagonist in many other gram negative and gram positive bacteria [9]. There are many compounds which can act as antagonist to bacterial biofilms like carlacton, a natural molecule that inhibits biofilms [10]. Usage of natural herbal extracts against diseases is an age old traditional practice found in many places of Tamil Nadu. In this study, the extracts of the species *Melia dubia* is identified to control the UTI significantly. Previously, *Melia dubia* was used as an antidiabetic agent hypoglycemic drug [11], antifeedant [12] and antiviral drug [13]. Furthermore, it has recently been reported to have quorum sensing inhibitory potentiality [14, 15].

In this study, we aim to screen for SdiA inhibitors from the *Melia dubia* extracts which can act as an antagonist that blocks *E.coli* quorum sensing through various *in vitro* and *in silico* approaches.

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2. MATERIALS AND METHODS

2.1. Bacterial and Culture Conditions

UPEC strains were isolated from the outpatients affected by UTI in K.A.P. Vishwanatham Government Medical College, Trichy between September and December 2009. The strains were screened for their multidrug resistant properties and the strain that have showed utmost resistance (UPEC/QSPC/S4) against antibiotics like Ampicillin, Ciprofloxacin, Levofloxin, Nitrofurantoin and Trimethoprim was taken up for further studies. The strain was cultured in Luria Bertani (LB) medium for 24 hours at 37°C and used for further studies.

2.2. Plant Material and Extraction

The seeds of the plant *Melia dubia* were collected from nearby town Kumbakonam, Tamil Nadu, India between August and November 2009. Voucher herbarium specimen TUH 285 submitted to the Department of Environmental and Herbal Sciences, Thanjavur, Tamil Nadu, India and it was authorized by Dr. M. Jagadeesan, the Head of the Department. The seeds were dried under shade in a dust free condition then crushed into powder and taken up for further extraction by cold percolation method [16]. The powder was dissolved in five different solvents like water, ethanol (70%), petroleum ether (70%), methanol (70%) and hexane (70%) (1:10 W/V) at room temperature (25±1°C). The extracts were then agitated frequently and filtered after 72 hours using a muslin cloth to get the supernatants which are lyophilized and stored in amber colored bottles (-80°C) for further studies.

2.3. *In vitro* Assays

Five different concentrations (10, 20, 30, 40, 50 mg/ml) of the seed extracts of *M.dubia* were supplemented with LB media which act as tests. Addition of antibiotics like Ciprofloxacin (2mg/ml), Trimethoprim (2mg/ml) acts as references. Test strains were then inoculated in the media. Antibiotic addition differentiates the quorum quenching activity from that of antibiotic activity. Media without supplementation of antibiotics was taken as control. Cell density [17], swarming motility [18], protein [19], protease [20], hemolysis [21], hemagglutination [22], hydrophobicity [23] and biofilm inhibition [24] assays were performed at different time intervals such as 12, 24, 48 and 72 hours. Cell wet weight, cell dry weight and pH were also calculated. The entire test was triplicated for the purpose of statistical analysis.

2.4. GC-MS Analysis

To identify the active principle(s) present in the ethanolic extract of *M.dubia*, GC-MS analysis was carried out on PerkinElmer Clarus 500 with mass spectroscopy detector. The oven temperature was changed from 50°C (1min at 10°C/minute) to 150°C (1min at 8°C/minute), then to 250°C (1 min at 15°C/minute) and finally to 300°C for 3 minutes. Helium was used as the carrier gas at 280°C injector temperature. 1µl of the sample was dissolved in ethanol and it was injected into the system having a mass spectral range of 40-450 amu. The compounds were compared with the NIST (National Institute of Standard and Technology) mass spectral library and were further confirmed.

2.5. *In Silico* Studies

2.5.1. Homology Modeling of Uropathogenic E.coli SdiA

Amino acid sequence of UPEC SdiA (Swisprot accession number: Q8FGM5) and NMR solution structure coordinates of the *E.coli* SdiA (PDB Code: 2AVX) was loaded into the software, Modeller 9v8. Primary sequences of *E.coli* SdiA and UPEC SdiA were aligned carefully and further were checked to avoid deletions or insertions in the conserved regions. A series of UPEC SdiA models (100) were constructed independently.

2.5.2. Evaluation of Stereochemical Qualities of UPEC SdiA

Stereochemical qualities of UPEC SdiA were accessed using Ramachandran plot. To access the quality of the model further, Z-score was calculated using PROSA web server in order to check the overall model quality and to measure the deviation of total energy of structure with respect to an energy distribution derived from the random confirmations.

2.5.3. Ligand Preparation

27 compounds which were reported by the GC-MS were drawn using SymxDraw. Ligand files were prepared for docking using Schrödinger Ligprep software. In addition to the generation of energy minimized 3D structure, Schrodinger Ligprep was also used for adding hydrogen atoms. For computational studies, Ligprep was used to obtain low energy 3D structure for the set of ligands. OPLS_2005 force field was utilized to optimize the geometry and for minimization.

2.5.4. Docking Studies

All docking experiments were performed using the program GLIDE (Grid Based Ligand Docking with Energetics) module in Schrödinger. Coordinate of the modeled UPEC SdiA structure was prepared for Glide calculations by running protein preparation wizard. Energy Minimization was run until the average root mean square deviation (RMSD) of non hydrogen atom reached 0.290 Å. Glide uses two boxes that share a common centre to organize its calculations: a larger enclosing box and a smaller binding one. The grids are automatically calculated within the space defined by the enclosing box. The binding box defines the space through which the centre of the defined ligand will be allowed to move during the docking calculations. It provides to measure the effective size of search space. The only obligation on enclosing box is that, it should be large enough to contain all the ligand atoms, even when the ligand centre is placed at the edge or at the vertex of the binding box. Grid files were generated using C_8HSL (*N*-Octanoyl-L-homoserine lactone) at the centre of the two boxes. The size of the binding box was set at 20Å in order to explore a large region of the protein. Compounds were subjected to the flexible docking position using a precomputed grid files. For each compound 100 top score poses were saved and only the best scoring pose was analyzed.

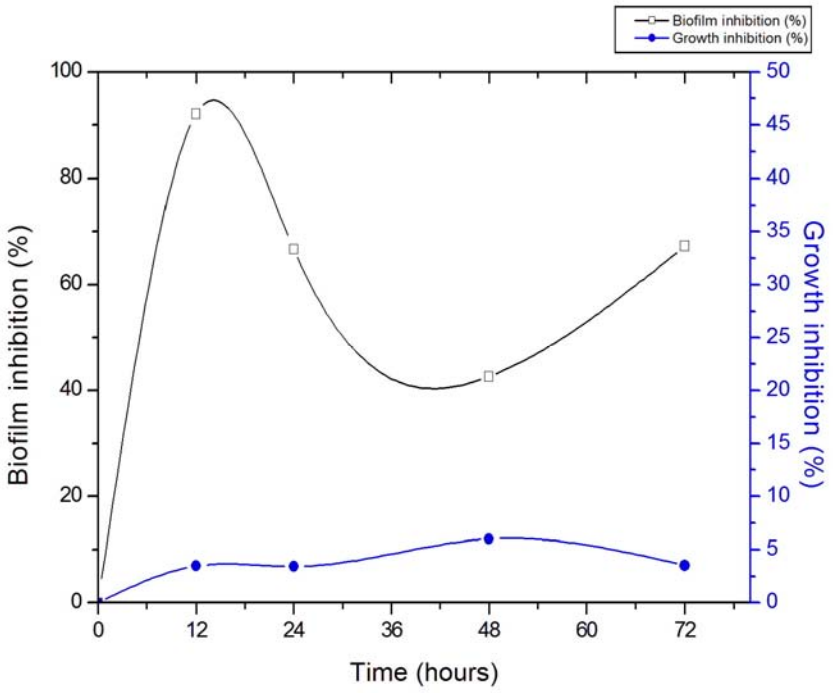


Fig. (1). Potential biofilm inhibiting efficacy of *M.dubia* seeds ethanolic extract (30mg/ml) at different time intervals. Maximum inhibition activity (92.1%) was recorded at 12th hour.

2.6. Statistical Analysis

The mean±SE was calculated and for the probability of differences below 5% was considered significant [25].

3. RESULT AND DISCUSSION

Cell density, swarming motility, protein, protease, hemolysis, hemagglutination, hydrophobicity and biofilm inhibition were the various parameters which were studied. The ethanolic extract of seeds of *M.dubia* showed the maximum quorum quenching activity and hence it was taken for further discussion.

3.1. *In vitro* Assays

Biofilm is the self synthesized polymeric matrix which thereby facilitates the attachment and resistance to the antimicrobial agents and to the host immune defenses. Ethanolic extract from the seeds of *M.dubia* was found to reduce the biofilm in *E.coli.* Maximum inhibition was recorded at 12th hour as 92.10% for 30mg/mL concentration of the extract (Fig. **1**). Reports were available on the biofilm inhibitory activity against *E.coli*. Inhibiting the biofilm of bacteria positively influences the virulence and thereby curbs the pathogenesis [9].

Hemolysin is one of the major virulence factors of *E.coli*. It is actually a pore forming toxin that belongs to the family of 'repeat toxin' (RTX). This toxin can not only destroy the erythrocytes but also leukocytes, endothelial and renal epithelial cells. The ethanol extract (30mg/ml) of the seeds inhibited the hemolysin production upto 17.50% at 72nd hour. (Fig. **2**). Supplementation of the herbicide 2, 4-dichlorophenoxyacetic acid to *E.coli* showed similar results as reported by Claudia *et al.,* 2002 [26].

Bacteria usually adhere to the water insoluble substrates as they are hydrophobic in nature. Bacterial adhesion to the surface decreases proportionally with the decrease in the hydrophobicity, thereby reducing biofilm efficiently. 30mg/mL of the ethanolic extract showed best results by reducing the hydrophobic nature of cells upto 36.05% at 72nd hour (Fig. **3**). Ciprofloxacin was reported to reduce hydrophobicity at 24th hour that coincides with our result [27]. Thus, the presence of active principle(s) in the seeds was found to disrupt bacterial quorum sensing.

Protease secretion in bacteria is also a known virulence factor which could degrade host proteins. Protease secretion was high at 24th hour which reduced gradually and reached the minimum level at 72nd hour (36.05%) (Fig. **4**). The garlic and onion extracts were also found to reduce protease activity which was reported by Teodar *et al.,* 2009 [28] that agree with our data.

Swarming motility allows the bacteria to navigate the intestine and to reach various parts, thereby damaging its functions. Ethanolic extract (30mg/ml) of the seeds was also found to control swarming motility of the *E.coli* upto 48.52% (72nd hour) (Fig. **5**). Cells wet weight, cells dry weight and pH were not altered significantly, whereas these characteristics were found to decrease with antibiotics supplementation. Thus it is confirmed that the ethanolic extract of *M.dubia* exerts quorum quenching activity rather than antibiotic effect.

3.2. GCMS Analysis

GC-MS analysis proved the richness of the secondary metabolites in the ethanolic extract of the plant seeds (Table **1**). 27 compounds were identified to have a retention time from 3.46 to 32.97.

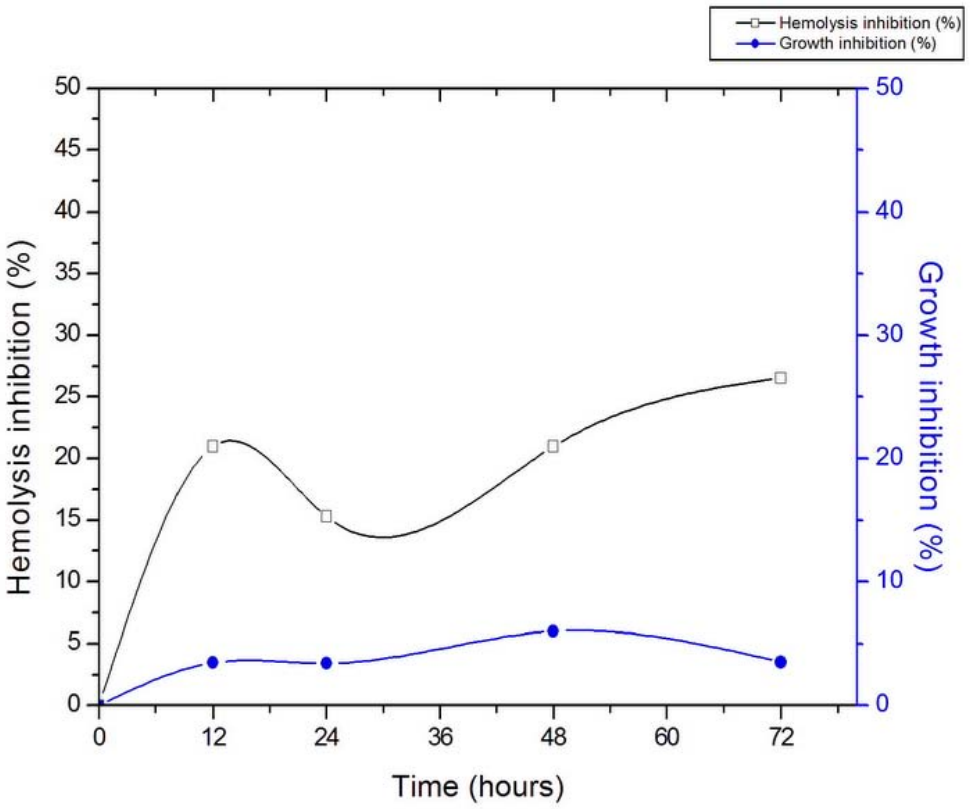


Fig. (2). Potential hemolysis inhibiting efficacy of *M.dubia* seeds ethanolic extract (50mg/ml) at different time intervals. Maximum inhibition activity (20.99%) was recorded at 12th hour.

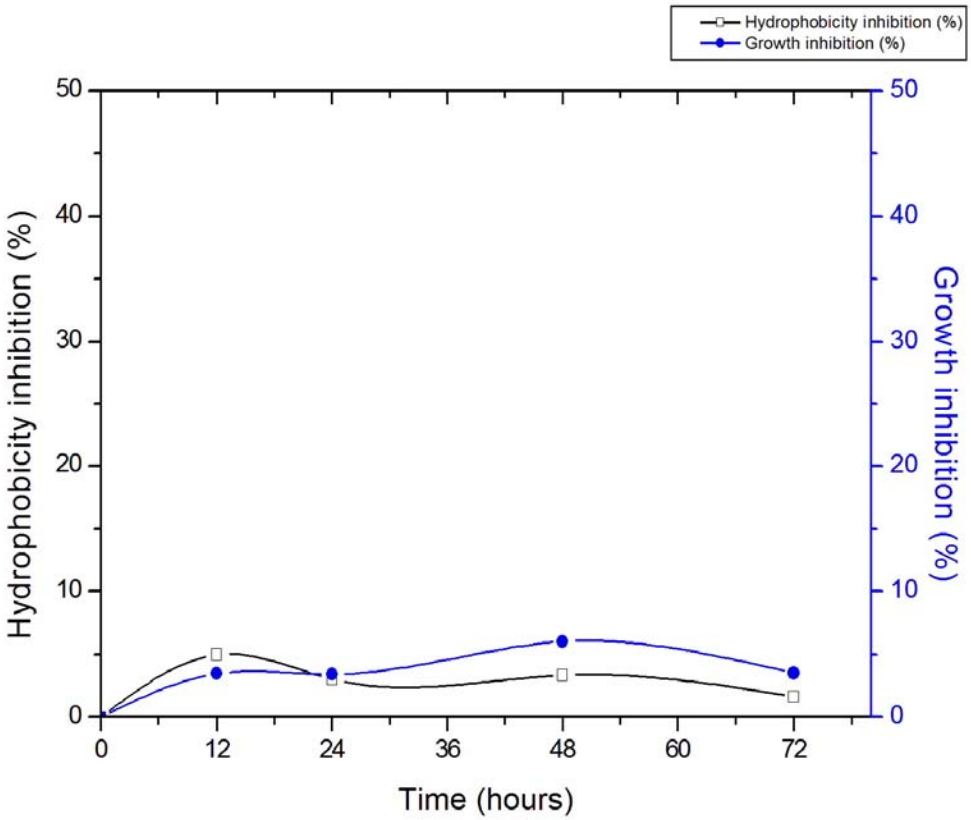


Fig. (3). Potential hydrophobicity inhibiting efficacy of *M.dubia* seeds ethanolic extract (30mg/ml) at different time intervals. Maximum inhibition activity (4.99%) was recorded at 12th hour.

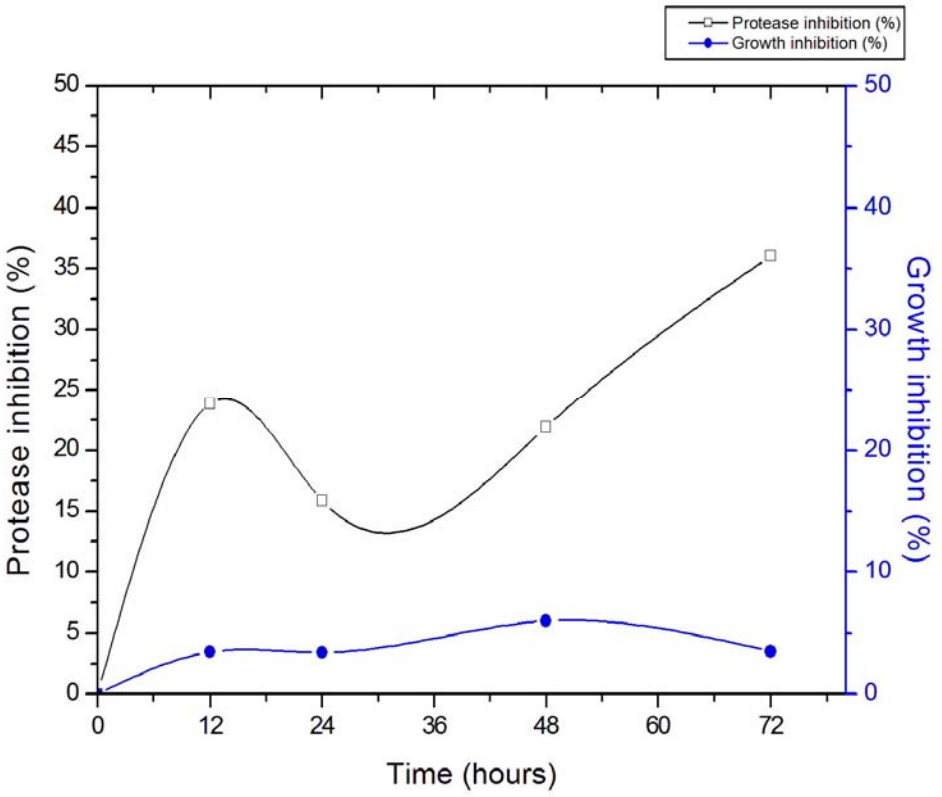


Fig. (4). Potential protease inhibiting efficacy of *M.dubia* seeds ethanolic extract (30mg/ml) at different time intervals. Maximum inhibition activity (23.88%) was recorded at 72 hour.

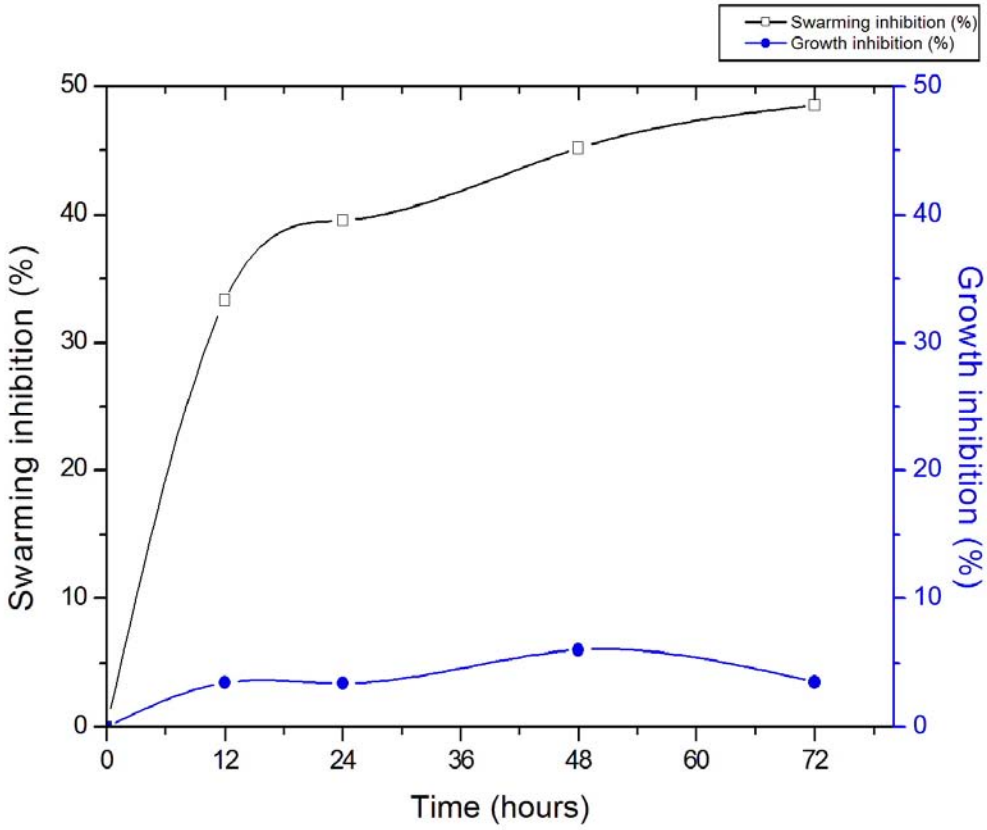
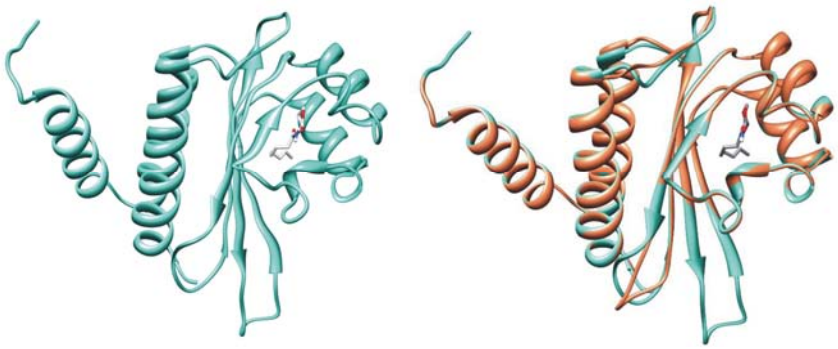


Fig. (5). Potential swarming inhibiting efficacy of *M.dubia* seeds ethanolic extract (30mg/ml) at different time intervals. Maximum inhibition activity (48.52%) was recorded at 72 hour.



a. **b.**

Fig. (6). (**a**) A ribbon diagram showing the overall structure of the UPEC sdiA. The bound C_8HSL molecular at active site is shown as stick. (**b**). Ribbon diagram of superposed *E.coli* sdiA (Coral) and UPEC sdiA (Turquoise).

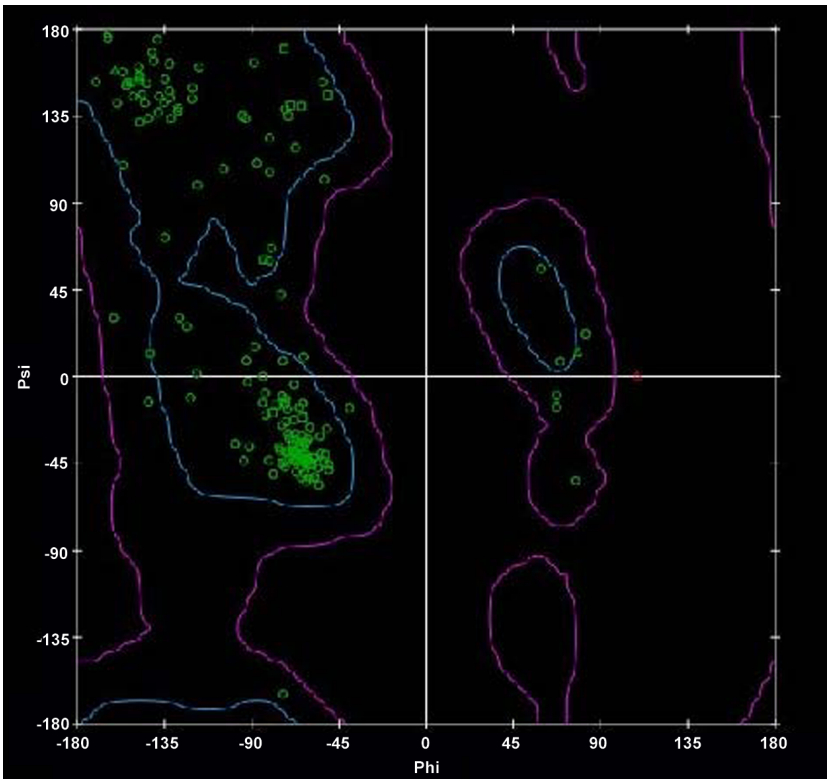


Fig. (7). The Ramachandran plot of the final model obtained by PROCKECK.

3.3. *In silico* Studies

3.3.1. Homology Modeling

The model which has best PDF total energy, PDF physical energy and DOPE function was selected and chosen for further stereochemical quality checks and docking studies (Fig. **6a** and **b**). Analysis of the Ramachandran plot revealed that 90.7 % of the residues were in the favored region, 7.3 % was in allowed region and only 1.9% was in disfavored region (Fig. **7**). Residues in the disallowed regions were located far away from the residue, which is in the ligand binding site (LBS). These results indicate that Phi and Psi backbone dihedral angles in the UPEC model were reasonably accurate. The Z-score for modeled UPEC SdiA is -6.12 and it is in the range of native conformation (Fig. **8**). Hence the model was chosen for further studies.

3.3.2. Identification and Analysis of Potential Compounds

As a control study, C_8HSL was docked to the protein and this exercise resulted in reproducing the NMR solution structure pose of the compound with -9.4, Gscore with 0.029 Å RMSD. GScore is the total GLIDE score: Sum of XP terms (Lipophilic EvdW, PhobEn, PhobEnHB, PhobEL, PairHB, HBond, Eleactro, SiteMap, Phi Stack, Cat, CLBR, LowM, Penalties, HBPenal, PhobicPenal, and RoatPNAL). Higher the contribution of XP term more will be the total GLIDE score. Score computed for this reference compound was used as reference value for identifying the possible leads. All the compounds that exhibited weaker binding in comparison with the reference compound were shortlisted for further analysis. From these docking studies, it was observed that the compound 6 is having better GScore of -10.6 than the

Table 1. List of Ligands Identified from *M. dubia* Using GC-MS Analysis

S.No.	Compound Name	Retention Time
1	Glycerin	7.63
2	Octanal, 7-methoxy-3,7-dimethyl-	9.04
3	Octanal	9.23
4	Octanoic Acid	10.52
5	4,5-Octanediol, 2,7-dimethyl-	11.60
6	3,4-Furandiol, tetrahydro-, trans-	12.36
7	Butanedioic acid, hydroxy-, (S)-	12.59
8	Benzaldehyde, 3-(chloroacetoxy)-4-methoxy-	15.23
9	Arabino-Hex-1-enitol, 1,5-anhydro-2-deoxy-	15.92
10	2H-1-Benzopyran-2-one	16.12
11	Xylitol	17.20
12	Undecanoic acid	17.90
13	3-Hydroxy-4-methoxybenzoic acid	18.11
14	Pentadecanoic acid, 2,6,10,14-tetramethyl-, methyl ester	18.35
15	Ethyl à-d-glucopyranoside	18.93,19.06
16	á-d-Mannofuranoside, methyl	19.81
17	Ethanone, 1-(1-hydroxy-2,6,6-trimethyl-2,4-cyclohexadien-1-yl)-	20.90
18	Nonanoic acid, 5-methyl-, ethyl ester	21.36
19	Hexanoic acid, 3-tetradecyl ester	21.53
20	Cyclopropane, 1,1-dimethyl-2-(1-methylethoxy)-3-(3-methyl-1-pentynyl)-	22.06
21	Dodeca-11,6-dien-12-ol,6,10-dimethyl-	22.48
22	1-Eicosanol	22.62
23	Tridecanoic acid, methyl ester	23.15
24	n-Hexadecanoic acid	23.74
25	Hexadecanoic acid, ethyl ester	24.01
26	9,12-Octadecadienoic acid,	25.28
27	Piperine	28.43

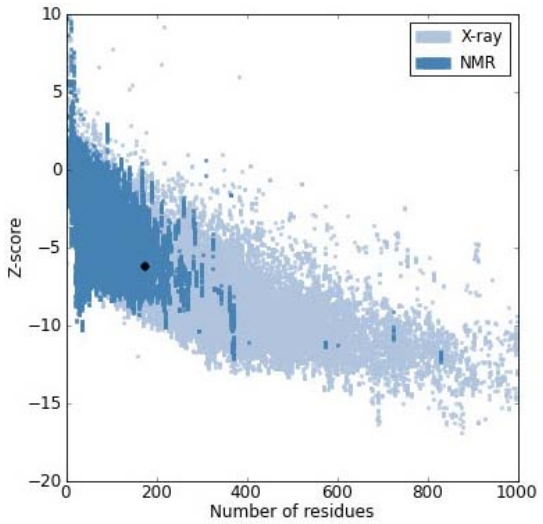


Fig. (8). Z-Plot of final model generated by ProSA-Web server.

native ligand C_8HSL (-9.4). Compound 6 (Fig. **9a**) was able to form three strong hydrogen bonds with amino acid residues SER43 and ASP84 (Fig. **9b**). From the previous experiment, it was proved that TRP67 and TYR 71 were highly conserved and the key residue for LuxR type proteins and SER 43 is a homologus residue of SdiA family. Since compound 6 (C6) was able to make strong hydrogen bond with these key residues, these compounds could be a possible reason for the quorum quenching activity. Hence, this compound can be further evaluated for its individual activities.

4. CONCLUSION

All the data obtained were verified and this study shows the fact that ethanolic extract of seeds of *M.dubia* contains rich active principles. The active principle may act as an antagonist of quorum sensing protein SdiA, thereby reducing its virulence as well as biofilm formation. Docking studies further confirmed that the ligand present in the *M.dubia* seed extract may be used as a lead compound to develop a new

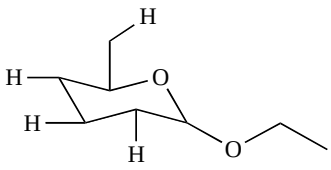


Fig. (9a). Compound **C6**.

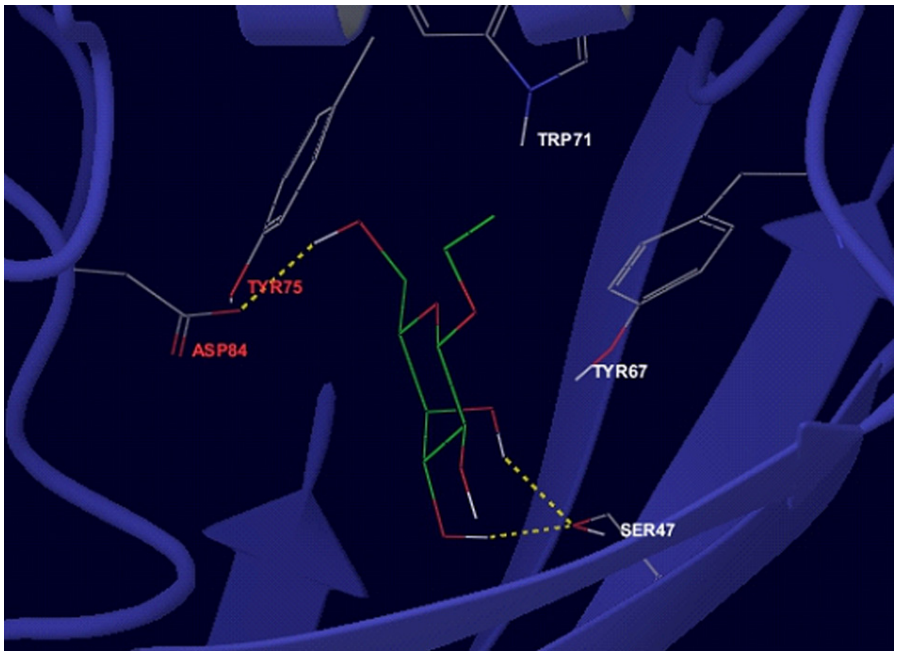


Fig. (9b). Docking model of UPEC sdiA-C 6. The hydrogen bond interactions with the key residues are show as dotted lines.

drug against UTI caused by UPEC. Future investigations will focus on the *in vivo* studies to prove the efficacy of the lead in an experimentally induced UTI animal model.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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