Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 7, Supple 1, 2015

Original Article

INHIBITORS FROM *MELIA DUBIA* AGAINST SDIA MEDIATED QUORUM SENSING OF UROPATHOGENIC *E. COLI*

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Received: 24 Jun 2015 Revised and Accepted: 21 Sep 2015

ABSTRACT

Objective: To investigate the potentiality of *Meliadubia*stem extracts for quorum sensing (SdiA-selective) inhibitory activity against uropathogenic *Escherichia coli* (UPEC).

Methods: The antimicrobial (cell-density) and anti-virulent (swarming motility, protein, protease, hemolysis, hemagglutination, hydrophobicity and biofilm inhibition) properties of the *Meliadubia* stem extracts were performed.

Results: The biofilm, hemolysis, swarming motility were inhibited by 45.71%, 12.97 % and 33.33% respectively when the media were supplemented with 30 mg/ml of ethanolic extract. The GC-MS spectrum of ethanolic extract showed an array of 49 structurally unrelated compounds with the natural ligand, AHL. Their interaction with the quorum regulator, SdiA, was predicted by Glide module of Schrödinger suite and the ligands C 7, C 20, C 28 showed appreciable activity with the following G-Score 11.4, 10.7, 9.9 respectively. *In vitro* and *in silico* molecular docking analysis data showed fairly good correlation, suggesting that the ethanolic extract has the potential to attenuate the quorum sensing of UPEC. Further investigation is desired to study the antagonistic effect of the above ligand by *in vitro* and *in vivo* strategies.

Conclusion: The quorum quenching activity of *Meliadubia* stem was proven from the overall analysis and its effect towards the inhibition of biofilm and virulence factors were analyzed.

Keywords: Quorum sensing, Quorum quenching, *Meliadubia*, Uropathogenic *E. coli*, SdiA.

INTRODUCTION

E. coli O157:H7, O25:H4 are the major strains that cause the Urinary Tract Infections(UTI) [1]. The rapid increase in antibiotic resistant strains of the above causative strains raises the need to search for a new path to control the UTI. It is also evident that their pathogenicity overlie on the signal molecules that communicate [2] and regulate the pathogenicity island of the uropathogenic *E. coli* [3].

The SdiA, a transcriptional regulator protein present in *E. coli* is a lux R homologue and is associated with multiple quorum sensing signals like indole, AHL's and AI-2 [4] and also regulates interspecies communication. It is reported that SdiA has 240 amino acids starting from methionine and it is likely to have two domains, amino acids 1-171 has the AI binding domain [5] and 197-216 has the helix-turn-helix DNA binding domain [6]. SdiA represses the inhibitors of cell division and induces ftsQAZ locus which is responsible for the formation of the septum during cell division through AI-2 [7]. The C-terminus deletion abolishes the DNA binding domain of SdiA but retaining the ligand binding domain, enhancing control of SdiA over biofilm formation [7].

It would be rather effective in screening a potent antagonist against the transcriptional quorum regulator to repress the pathogenesis of UPEC [8, 9]. So, we intend to excerpt the antagonist from traditional Indian medicinal plant *Meliadubia*, belonging to *Melicaceae* family as an effective antivirulent against UPEC. Earlier reports show that the plant is found to have antidiabetic effects [10,11] and antifeedant properties [12].

MATERIALS AND METHODS

Bacteria and culture conditions

Uropathogenic *Escherichia coli* (UPEC) strains were isolated from patients of K. A. P Vishwanathan Government Medical College Hospital, Trichy from September to December, 2009. The multidrug resistance profile of *E. coli* against the commonly used antibiotics ampicillin, ciprofloxacin, levofloxacin, nitrofurantoin and trimethoprim was screened and the most resistant strain (UPEC/QSPL/S4) was selected. This strain was cultured in Luria Bertani (LB) broth at 37 ° C for 24 h and used for further experiments.

Plant material and extraction

Raw stem was taken from *Meliadubia* from August to November 2009, which is a common inhabitant of nearby town Kumbakonam, TamilNadu, India. Identification and authentication ofthe plant materials were done by Dr. M. Jegadeesan. The herbarium specimen (TUH 285) of the plant was given to Department of Environmental and Herbal Science, Tamil University, Thanjavur, Tamil Nadu, India. The cleaned stem was cut into pieces and dried under the shade in a dust free environment and later it was powdered. Cold percolation method of extraction [13] was used for the extraction of plant material from the stem powder.

Water, ethanol (70%), methanol (70%), petroleum ether (70%) and hexane (70%) (1:10 w/v) are the five different solvents used for extraction at room temperature (25±1 °C). The extracted samples were agitated frequently and the supernatants were collected through the filtration using a muslin cloth after 72 h. The filtrate was stored in amber-colored bottles after lyophilization at-80 ° C using deep freezer and was analyzed further.

In vitro assays

LB medium was prepared and supplemented with five different concentrations (10, 20, 30, 40, 50 mg/ml) of the stem extracts of *Meliadubia*as test along with 100 µl of UPEC/QSPL/S4. The antibiotics ciprofloxacin (2 mg/ml) and trimethoprim (2 mg/ml) were also added which makes the quorum quenching activity significant by differentiating with antibiotic activity. The unsupplemented LB medium was used as control. Phosphate buffer is added to the extract and the efficacy of the extract is evaluated at different time intervals (12, 24, 48, 72 h). Cell density [14], swarming motility [15], protein [16], protease [17], hemolysis [18], hemagglutination [19], hydrophobicity [20], biofilm inhibition [21] assays were performed. Cell wet weight, cell dry weight and pH was

also estimated. To favor the statistical analysis of the data, all the assays were carried out in triplicates.

GC-MS analysis

The ethanolic extract of the stem of *Meliadubia* was taken for GC-MS analysis in PerkinElmer Clarius 500 with mass spectroscopy detector to find the various active principle(s) present in the extract. The program for oven temperature was 50 °C for 1 minute at 10 °C/minute to 150 °C for 1 minute at 8 °C/minute to 250 °C for 1 minute at 15 °C/minute to 300 °C for three minutes. The extract was injected at 250 °C with helium as the carrier gas. 40-450 amu of the spectral range is used in mass spectroscopy. One microliter of the dissolved sample was injected into the system. The compounds present in the extract were found by comparison with NIST (National Institute of Standard and Technology) mass spectral library.

***In silico* studies**

Homology modeling of uropathogenic *E. coli* SdiA

The amino acid sequence of UPEC SdiA (Swisprot accession number: Q8FGM5) and the NMR solution structure coordinates of *E. coli* SdiA (PDB Code: 2AVX) were loaded into the Modeller 9v8. The primary sequence of *E. coli* SdiA and UPEC SdiA was a ligned carefully and was checked to avoid deletions or insertions in the conserved regions [22]. A series of the UPEC SdiA model (100 models) was constructed independently. There were no differences in the number and organization of the secondary structural elements and no significant main chain deviations among 100 models. The model with the best PDF total energy, PDF physical energy and DOPE function was selected and chosen for the further stereochemical quality checks and docking studies (fig. 1A and B).

Evaluation of the streochemical qualities of UPEC SdiA

The streochemical qualities of the UPEC SdiA were accessed by Ramachandran plot. Analysis of Ramachandran plot revealed that 90.7 % of the residues were in the favoured region, 7.3 % in the allowed region and only 1.9 % were in the dis favored region (Fig.2). The residues in the disallowed regions are located far away from the residue in the ligand binding site (LBS). These results indicate that the Phi and Psi backbone dihedral angles in the UPEC model are reasonably accurate.

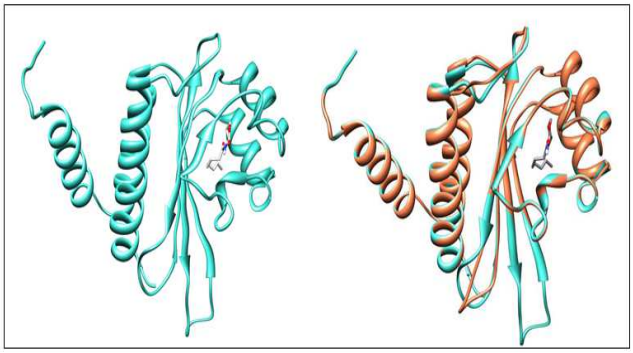

Fig. 1A: A ribbon structure of the Modelled Protein UPEC SdiA. The bound C8HSL molecule at its active site is shown as a stick. B: Ribbon diagram of superimposed *E. coli* sdiA (Coral) and UPEC SdiA (Turquoise)

To access the quality of the model further, the Z-score was calculated using PROSA web server in order to check the overall model quality and to measure the deviation of the total energy of the structure with respect to an energy distribution derived from random confirmations. Fig. 3 shows the location of the Z-score for UPEC SdiA. The value-6.12 is in the range of native conformation. Hence the model was chosen for the further docking studies.

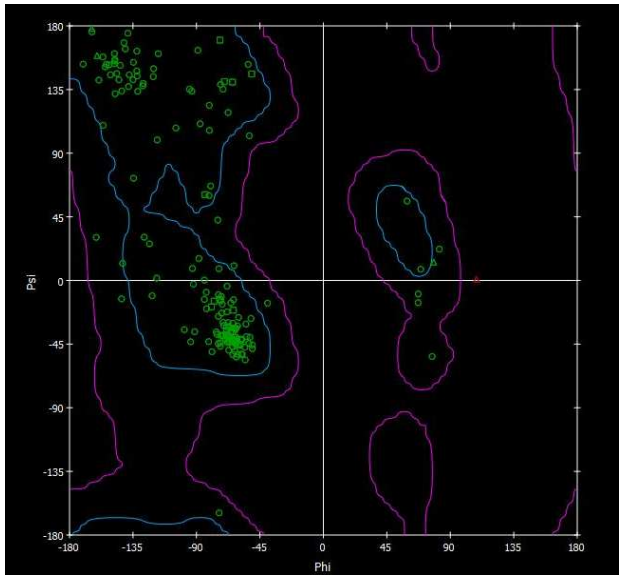

Fig. 2: The Ramachandran plot of the final model obtained by prockeck

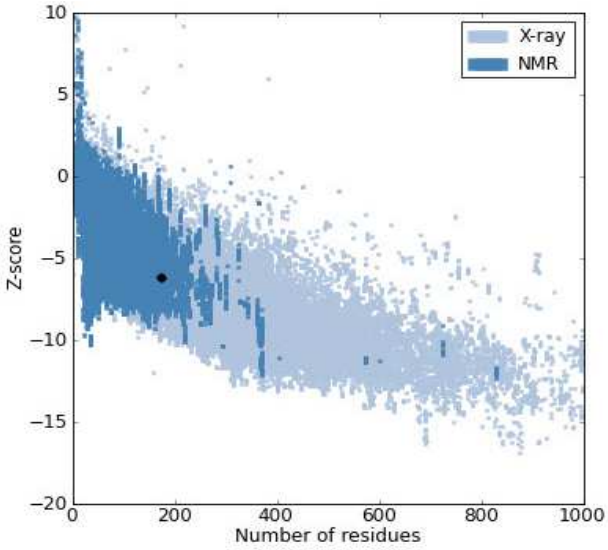

Fig. 3: Z-Plot of final model generated by ProSA-Web server

Ligand preparation

49 compounds reported by the GC-MS were drawn using the SymxDraw. The Ligands files were prepared for docking using Schrodinger Ligprep software. In addition to the generation of energy minimized 3D structure, Schrodinger Ligprep was also used for adding hydrogens. For further 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 minimize the energy.

Docking studies

Docking studies, as listed in table 1 were performed using the modeled UPEC SdiA structure. All the docking experiments were performed using the program GLIDE (Grid Based Ligand Docking with Energetics) module in Schrodinger. Coordinate of the modeled UPEC SdiA structure was prepared for glide calculations by running the protein preparation wizard. Energy Minimization was run until the average root mean square deviation (RMSD) of the non-hydrogen atom reached 0.290 A. Glide uses two boxes that share a common center to organize its calculations: alarger enclosing box and a smaller binding box. The grids themselves are 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 docking calculations. It provides a measure of the effective size of the search space. The only obligation on the enclosing box is that it should be large enough to contain all ligand atoms, even when the ligand is placed at the edge or vertex of the binding box. Grid files were generated using the C_8HSL to the center of the two boxes. The size of the binding box was set at 20 Å in order to explore a large region of the protein. The compounds were subjected to flexible docking using the pre computed grid files. For each compound, the 100 top score poses were saved and only the best scoring pose was analyzed.

Table 1: List of ligands identified from *M. dubia* stem using GC-MS analysis

S. No.	Compound name	Retention time
1	Formamide, N-methoxy-	3.44
2	Diethoxymethyl acetate	3.68
3	Ethylbenzene	3.81
4	Oxalic acid, isobutyl pentyl ester	4.98
5	Furfural	4.11
6	1-Butanol, 3-methyl-acetate	4.56
7	4,6,10,10-Tetramethyl-5-oxatricyclo[4.4.0.0.(1,40)]dec-2	6.20
8	Propanamide, 2-hydroxy	7.10
9	3-Hexen-2-one, 3,4-dimethyl	8.15
10	1-Pentanol, 2-ethyl-4-methyl	8.47
11	Levoglucosenone	8.77
12	3,6-Dimethyl-5-hepten-1-ol-acetate	8.90
13	3,4-Furandiol, tetrahydro-, cis-	9.30
14	Decane	9.92
15	2,3-Dimethyl-1-hexene	10.00
16	Benzaldehyde,3,5-dimethyl	10.70
17	4-Hydroxy-3-methylacetophenone	12.11
18	1-Octene, 6-methyl	13.24
19	Hexadecane	13.37
20	Sucrose	14.23
21	Phenol, 2-methoxy-4-(1-propenyl)-,(z)-	14.48
22	1-Heptanol, 6-methyl	14.71
23	Ethanone, 1-(2,4,6-trimethylphenyl)-	15.07
24	1,6-Anhydro-a-D-glucopyranose	15.27
25	Undecanoic acid	16.09
26	3-Undecene, 3-methyl	16.34
27	Ethyl-a-d-riboside	17.20
28	d-Glycero-d-tallo-heptose	17.58
29	3-Tetreadecene	17.89
30	1-Undecene, 9-methyl	18.20
31	Oxalic acid, allylhexadecyl ester	19.46, 20.71
32	Oxalic acid, allylpentadecyl ester	19.61
33	3-Caren-10-al	20.79
34	Didodecyl phthalate	21.24
35	Octadecanoic acid, ethyl ester	22.08
36	Propanamaide,3-[3,5-di(tert-butyl)-4-hydroxyphenyl]	22.22
37	9-Ecosene	22.80
38	7-Hexadecanal	23.65
39	1,E-11,Z-13-Octadecatrine	24.14
40	10-Octadecenal	25.26
41	1-Doddecanol, 3,7,11-trimethyl	25.96
42	Ethanol, 2-(9-octadecenyloxy)	26.48
43	Eicosane	27.06
44	Undecane, 5-ethyl	29.36
45	Oxirane, octyl	23.35

MBEC determination and adherence assay

The Minimum Biofilm Eradication Concentration (MBEC) of the compound, sucrose was determined as described by Subhankari Prasad Chakraborty *et al.*, 2012 [23]. For the adherence assay the test strains (*ΔsdiA*, *sdiA*$^+$, UPEC, MTCC 729) individually cultured in the LB medium were supplemented with sucrose in three different doses (Low 5 µg/ml, Medium 10µg/ml, high 15µg/ml) in relation with the calculated MBEC concentration [24]. The dose response effect of sucrose was assessed in triplicates as compared with the negative (C_8HSL) and positive control (indole, furanone).

Cytotoxicity assay

The kidney carcinoma cell line (A498) was obtained from the National Centre for Cell Science (NCCS), Pune and grown in Eagles Minimum Essential Medium (EMEM) containing 10% fetal bovine serum (FBS). All cells were maintained at 37ºC, 5% CO_2, 95% air and 100% relative humidity. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week.

The potential influence of QSI-MD on cell viability was tested by using the MTT assay [24]. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] is a yellow water soluble tetrazolium salt Succinate-dehydrogenase, a mitochondrial enzyme in living cells, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Once the cell density reached $1x10^7$ cells/ml, 100 µl per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37ºC, 5% CO_2 and 100% relative humidity. After 24 h, the cells were treated with different concentrations (5, 10, 15, 100 µg/ml respectively designated as Low dose, medium dose, high dose and very high dose) of sucrose. The plates were incubated for an additional 24 h at 37º C, 5% CO_2, 95% air and 100% relative humidity. 15 µl of MTT (5 mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37ºC for 3 h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100 µl of DMSO and then measured the absorbance at 570 nm using microtitre plate reader. The medium containing without sucrose served as control and the cell viability was estimated against control. All assays were performed in triplicate and mean±SD values were used to estimate cell viability.

Statistical analysis

A mean±SE was calculated for the experimental results. The values less than 5% probability ($P<0.05$) [25] were significant statistically for the differences obtained.

RESULTS

The major virulence factors of UPEC were found as biofilm formation, hemolysin production, hydrophobicity and protease synthesis and swarming motility. The extract from the stem of *Meliadubia* is known to control all these major factors in a significant manner. Since the stem ethanolic extract showed maximum activity among other extracts, it is discussed in detail.

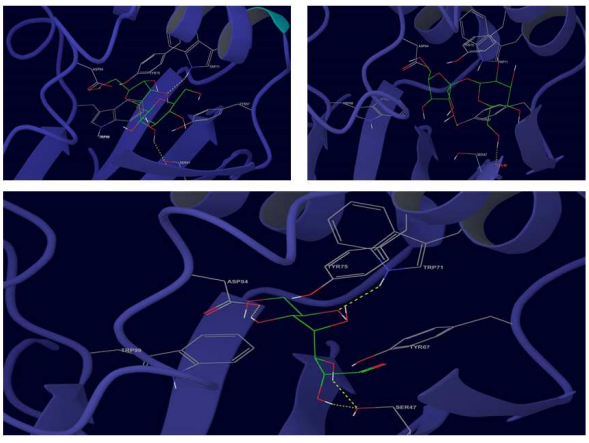

Fig. 4A: Docking model of UPEC SdiA-C 7. The hydrogen bond interactions with the key residues are shown as dotted lines, fig. 4B: Docking model of UPEC SdiA-C 20. The hydrogen bond interactions with the key residues are shown as dotted lines, fig. 4C: Docking model of UPEC SdiA–C28. The hydrogen bond interactions with the key residues are shown as dotted lines

***In vitro* assays**

Bacterial communities in ecological niches spread over a natural or artificial surface as single or multiple species are called as biofilm. The data showed that the biofilm formation was inhibited in a significant range (data not shown). The inhibition was found to be the highest at the 12th h (46.17%). The extract showed best results at 30 mg/ml. 30 mg/ml of the ethanolic extract of the stem is showing maximum inhibition to the hemolysin production. At 12th h, the hemolysin production is recorded to be the least with an enhanced activity of 12.97% (data not shown). The increase in the hemolysin level was noted along the 24th h which shows an increased level of production of the enzyme. The maximum reduction of hydrophobicity was noted at the 12th h with an activity of 9.40% (data not shown). The inhibition of the protease enzyme by the ethanolicextract was also studied in the present investigation. The protease synthesis reached a minimum value at the 12th h of the growth towards 30 mg/ml concentrations (26.64%) (data not shown). Another major factor for biofilm formation is the swarming motility of the bacterial species. 30 mg/ml of the ethanolic extract is found to control the swarming motility upto 33.33% (data not shown) and it also shows maximum activity at the 12th h.

MBEC determination and *in vitro* Biofilm adherence assay

The MBEC of sucrose was found to be 10µg/ml and that taken into further studies. Biofilms are attached of microorganisms to a surface of polysaccharides, proteins, and nucleic acids to form a community. The intracellular biofilms are responsible for a dormant reservoir of pathogens inside the bladder cells, which outlast the strong host immune response. So, time dependent response of the lead compound C39, sucrose was studied to elicit its mode of SdiA selective biofilm inhibition on polystyrene plates at 12, 18 and 24 h (data not shown). The data showed a consistent effect on SdiA null strain with no response over the behavioral change in adhering the plastics irrespective of treating with or without C39 as compared with the wild type strain. Inconsistent effect on the wild type strain was recorded at the 24th h, when administered a high dose of lead (41.89%) which relatively higher than the known biofilm inhibitor (33.18%) (fig. 5).

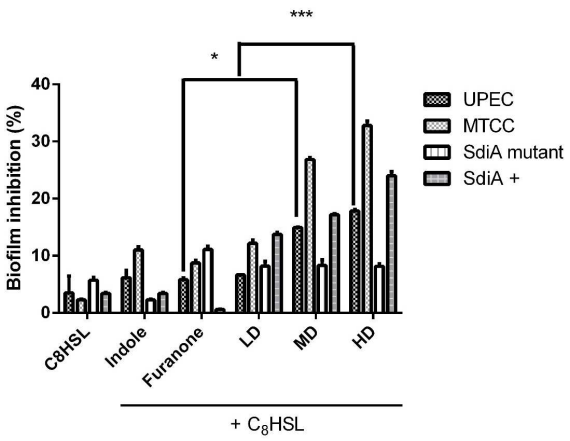

Fig. 5: Biofilm inhibitory efficiency of sucrose against various strains of *E. coli* at 24th h. Data revealed that the sucrose inhibited the biofilm forming ability of all the strains significantly especially the UPEC. The SdiA mutant is the only strain where no alteration found that supports the mode of action of the drug i.e., through sdiA

GC-MS analysis

The richness of the presence of significant quantity of secondary metabolites in *M. dubia*ethanolic extract was clearly understood. The comparison was made between the mass spectrums of each compound with the NIST library. Finally, 49 compounds were identified from the ethanolic extract. The presence of active principle(s) in *M. dubia* stem was clearly observed in GC-MS results.

***In silico* studies**

Identification and Analysis of potential compounds

As a control study, the C_8HSL was docked to the protein, and this exercise which resulted in reproducing the NMR solution structure poses of the compound that yields-9.4 as the with G score with 0.029A ° RMSD. G score 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). A higher contribution of XP term will increase the total GLIDE score. The score computed for this reference compound was used as reference value for identifying the possible leads. All those compounds that exhibited weaker binding in comparison with the reference compound were shortlisted for further analysis. From the docking studies, it is observed that compounds 7, 20 and 28, are having better G score of 11.4, 10.7 and 9.9 respectively than the native Ligand C_8HSL (2). Compound 7 (C 7) forms three strong hydrogen bond interactions with the amino acid residues SER47, TRP71 and ASP84 (fig. 4 A) Compound 20 (C 20) forms two hydrogen bond interactions with TRP 67 and LEU48 (fig. 4 B). Compound 28 (C 28) forms three hydrogen bond with SER47, TRP71 and ASP84 (fig. 4 C). From the previous experiment, it is proved that TRP67 and TYR 71 are highly conserved and the key residue for LuxR type proteins and SER 43 is a homologus residue of SdiA family. Since compounds 7 and 20 and 28 were able to make strong hydrogen bond interactions with these key residues, these compounds could be a possible reason for the quorum quenching activity. Hence this compound can be further evaluated for their individual activities.

Cytotoxicity

Sucrose was tested for its toxic effects on human kidney carcinoma cell line (A498). It is found that it has not inhibited the cell growth at all the tested concentrations. Even in very high dose of sucrose (100µg/ml), there is no significant change in the cell viability (fig. 6). This confirms that the compound sucrose is efficient drug against UPEC quorum sensing with no toxic effects.

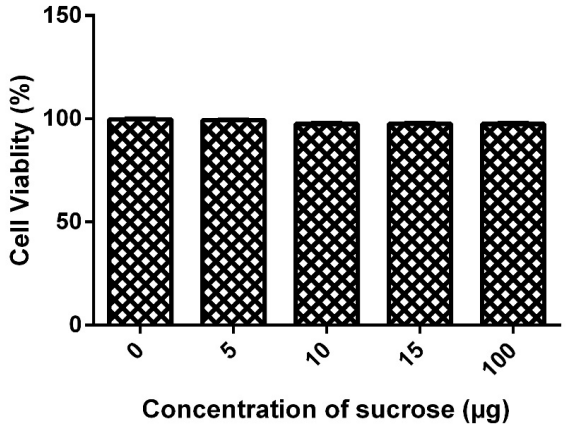

Fig. 6: The MTT assay showed no significant decrease in the cell viability thereby proved that the sucrose is nontoxic to the cell line

DISCUSSION

The treatment for UTI through inhibition of biofilm in bacteria gains more importance in the medical field. Halogenated furanone was proven to be an inhibitor for bacterial biofilm and [26]. Thus furanone was shown to be an inhibitory compound for bacterial biofilm. *Vibrio* biofilm was inhibitory to marine actinomycete strains [27]. Carolacton, a secondary metabolite from the *Sorangiumcellulosm* is recently screened for biofilm inhibitory properties which are also thought to show quorum quenching activity rather than antibiotic activity [28]. *E. coli* is known to control its virulence factors and biofilm through the activity of quorum sensing. Thus, by inhibiting biofilm and reducing the virulence, the pathogenesis of the bacteria is decreased. In our study, we could observe

that the ethanolic extract of the *Meliadubia*to show anti-virulence/biofilm potential against the uro-pathogenic *E. coli*. Hemolysin is the enzyme that can lyse the erythrocytes of higher organisms. One of the most important virulence factors in *E. coli* is the production of hemolysins. It belongs to the family of 'repeat toxin' (RTX) and the damage extend to leucocytes and renal epithelial cells. Similar results were obtained with herbicides by Balague *et al.* [29].

The formation of biofilm mainly depends on the hydrophobic character of the bacterial surface. The decrease in the hydrophobicity shows a proportional decrease in the biofilm formation. The synthesis of curlifibres in *E. coli* influences the hydrophobicity thereby increasing the attachment to the substrate [30]. Similar effect of hydrophobicity with ciprofloxacin antibiotic was studied by Evans *et al.,* [31]. Thus, the hydrophobicity of bacteria relates to the biofilm structure which in turn influences the pathogenicity.

According to Bakri and Douglass [32] the garlic extract showed an inhibitory action towards protease enzyme which coheres with our findings. Usage of (5z)-4-bromo-5-(bromomethylene)-3-Butyl-2(5H)-furanone to inhibit swarming and biofilm formation was done by Ren and coworkers [32]. Similar efforts were taken to control swarming with resveratrol (3, 5, 4-trihydroxy-trans-stilbene) was tried by Won-Bo *et al.,*[33]. Branched chain fatty acid also tends to decrease the swarming motility [34]. All these results cohere with our data and confirm the presence of active principle(s) in the ethanolic extract of the plant in a significant quantity. The cell growth, cell density, cell wet weight, dry weights were not much altered due to supplementation of the stem extract of *Meliadubia*. But these parameters tend to decrease greatly with the addition of antibiotics (data not shown). This proves the quorum quenching activity of the extract rather than the antibacterial activity.

The adhesion pattern of the strains was reported as discussed above are in accordance with the earlier report by Stepanovic*et al.,* 2004 [35]. Based on the optical density (OD) measured against bacterial films, strains were classified into the following categories: no biofilm producers, weak, moderate or strong biofilm producers, as previously described [35]. Briefly, the cut-off OD (OD_c) was defined as three standard deviations above the mean OD of the negative control. Strains were classified as follows: O. D ≤ OD_c = no biofilm producer, OD_c<OD ≤ (2 x OD_c) = weak biofilm producer, (2 x OD_c)<OD= (4 x OD_c) = moderate biofilm producer and (4 x OD_c)<OD = strong biofilm producer. It is found that all the tested strains except SdiA null mutant were under no biofilm producing category (data not shown) when administered with sucrose [36].

The overall result shows that the lead does not affect the ΔsdiA strains as it is acting through as sdiA. It is been reported by Ren *et al.,* 2001 that the quorum-sensing disrupter (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone (furanone) of the alga *Deliseapulchra* inhibited the biofim and swarming of *Escherichia coli*. It has been recently reported that natural products-inspired organosulfur compounds inhibits biofilm by inhibiting quorum sensing [23]. The drug lead sucrose showed a better efficacy profile than the known inhibitors like indole and furanone as well [37].

CONCLUSION

The quorum quenching activity of *Meliadubia*stem was proven from the overall analysis and its effect towards the inhibition of biofilm and virulence factors was analyzed. The analysis also proved that the ligands present in *Meliadubia* can be raised as an efficient drug against the *E. coli* that causes urinary tract infection. Mutant strains revealed the SdiA mediated quorum sensing inhibition by sucrose in a dose dependent manner. The *in vitro* and *in vivo* analysis of sucrose and its derivatives will be focused in future research.

ACKNOWLEDGEMENT

The authors are grateful to the management of SASTRA University for the support and infrastructure facility. We thank Prof. Thomas K. Wood for providing the SdiA mutant strains.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest

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