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Structure-based virtual screening for plant-derived SdiA-selective ligands as potential antivirulent agents against uropathogenic *Escherichia coli*

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ABSTRACT

The uropathogenic *Escherichia coli* pathogenecity is affected by quorum sensing transcriptional regulator SdiA. In this study, *in vitro* characterization of the active principles that could potentially antagonize with SdiA from the *Melia dubia* bark extracts has been described. After *in vitro* assays carried out to evaluate the inhibitory activities against the uropathogenic *E. coli*, the ethanolic extract (30 mg/ml) which showed the strongest suppression of haemolysis, swarming motility, hydrophobicity and biofilm formation, was subjected to GC–MS analysis and an array of 40 unrelated compounds was identified. Docking studies was conducted to screen for plant-based SdiA inhibitors. Five hits were assessed for their binding profiles and 7-(1-bromoethyl)-3, 3-dimethyl-bicyclo [4.1.0]heptan-2-one showed 66.95% binding ability with respect to C_8HSL.

1. Introduction

Urinary tract is the second most common site of bacterial infections in human, thus representing a major source of human discomfort. The incidence of urinary tract infection (UTI) in infants ranges from 10% in low birth weight infants to approximately 0.1–1.0% in new born infants [1]. After age one, both bacteriuria and UTI are more common in girls. Even when the patients with recurrent UTI and chronic pyelonephritis had no bacteriuria, they continued to show elevated antibody titres against standard strain of *Escherichia coli* (*E. coli*) [2]. The initiation of UTI is commonly due to the presence of *E. coli* in the gastrointestinal tract as commensals. Certain serotypes of *E. coli* (UPEC) are traditionally described to be associated with uropathogenicity and designated as uropathogenic *E. coli* (UPEC) [3]. In general, bacteria possess quorum sensing (QS) mechanism by which they gauge their own population density and adjust their behaviour accordingly. Researches on QS in diverse bacteria have shed light on the mechanisms by which cohorts of bacteria orchestrate their efforts during symbiosis with host organisms, respond to nutrient deprivation, and control multicellular behaviour and pathogenesis [4]. In QS, when external concentration increases as a function of increase in cell density, bacteria produce and release chemical signal molecules (i.e., autoinducers) [5]. Most common form of QS is mediated by the production and subsequent perception of acyl homoserine lactones (AHL) in gram negative bacteria [4]. The two quorum sensing systems of *E. coli*, AI-2/LsrR and AHL/SdiA were influence the gene regulation for their own QS network. Uropathogenic *E. coli*, within the bladder epithelium, forms bacterial community with many biofilm-like properties [6]. Different adhesins, haemolysin, and siderophore production forms various virulence factors of *E. coli* [7].

In the late 1970s, it was first ever recognized that despite the presence of mannose, *E. coli* strains that cause UTI typically agglutinate human erythrocyte mediated by fimbriae [7]. All strains of pathogenic and non-pathogenic *E. coli* as well as *Salmonella enterica* serovar *Typhimurium* require the LuxS gene for the synthesis of Autoinducer-2 (AI-2). The LuxI gene induces the synthesis of autoinducer AHL [8] and further detected by the LuxR encoded receptor/transcriptional activator which is responsible for bioluminescence [9]. *E. coli* has no gene for the LuxI-type autoinducer (Acyl Homoserine Lactones), but a gene homologue for the LuxR receptor, known as *SdiA*, is expressed in some strains of *E. coli* [10].

SdiA of *E. coli*, a 240 amino acid protein, named for its ability to suppress cell division, belongs to the LuxR family of transcriptional regulators that induces ftsQAZ locus in cell division [11]. P1 and P2 promoters regulate the ftsQAZ gene cluster. It was observed that SdiA over expression affects only the P2 promoter [12]. SdiA protein of *E. coli* shows a marked sequence homology similar to the well known

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QS proteins such as TraR of the crown gall bacterium *Agrobacterium tumefaciens* and the LuxR of luminescent bacterium *Vibrio fischeri* [13]. *E. coli* uses SdiA to monitor indole-producing strains as well as to monitor strains like *Pseudomonas fluorescens* (produces N-octanoyl-L-homoserine lactone), *Pseudomonas syringae* (produces N-hexanoyl-L-homoserine lactone), *Pseudomonas aeruginosa* (produces N-butyryl-L-homoserine lactone) [14]. SdiA then activates two *Salmonella* specific loci, *srgE* (SdiA-regulated gene) and the *rck* (resistance to complement killing) operon present in the *Salmonella* virulence plasmid [15]. Variation in the SdiA induction and its mediated transcription upon indole addition, lack of response in the biofilm formation by the SdiA mutant upon the addition of homoserine lactones and the fact that biofilm of the SdiA mutant was not affected by indole suggest that indole may bind to SdiA [14]. *E. coli* has been used as host organism for several AHL biosensor strains, despite the presence of SdiA due to its inability to synthesize AHL [14].

Reports worldwide highlight the problem of resistant uropathogens and their influence on empiric therapy, such as high prevalence of trimethoprim-sulfamethoxazole resistance in the community [16]. In *E. coli* O157:H7, expression of virulence factors by interacting with unknown stationary phase signals has been repressed by SdiA and it enhances multidrug resistance by stimulating efflux pumps in *E. coli* [14]. In *E. coli*, SdiA induces multidrug efflux pump AcrAB [14]. The need for the development of a new strategy altogether is due to emergence of antibiotics resistant in wide range of strains. Hence for the effective treatment of UTI, we are in urge to develop a novel strategy targeting quorum sensing of UPEC to just curb the pathogenesis rather killing the bacteria.

The Indian subcontinent is known for its rich and diverse plant genetic sources. Even though the bioactivity of various plant products is unknown, these products are widely used because of the faith in folk medicine with proven efficacy in traditional practices. *Melia dubia* is a plant from *Meliaceae* family and its various parts have been used in folk medicine for the treatment of UTI in the southern part of Tamil Nadu. Since the *M. dubia* is being used as a treatment for UTI, we have hypothesized that there might be few compounds which can curtail the biofilm formation and virulence factor by controlling the quorum sensing. Ethanol extract of the fruit of *M. dubia* showed anti-viral activity and anti-neoplastic activity [17]. Ethanol extract of total fruits showed hypoglycemic and anti-diabetic effects [18]. Toosendanin, a limonoid with a C-19/28 oxygen bridge from *M. dubia*, was known as an antifeedant and growth inhibitor [19]. In 2002, it was reported that the *Meliaceae* family in general and the genus *Melia* in particular had shown a great potential among botanical pesticides for pest management in terms of allelochemicals in its various species [18]. In addition to this, it was also found that the ovicidal action of 10% methanolic extract of *M. azedarach* on hatching (32.77%) was higher [20].

The current paper aimed to examine the antivirulent potentiality of *M. dubia* and then to evaluate the potential of the principles present for antagonizing the SdiA of uropathogenic *E. coli* by virtual screening through docking analysis of interactions between the ligands and SdiA.

2. Material and methods

2.1. Bacteria and culture conditions

Hospitalized patients from K.A.P. Vishwanathan Government Medical College, Trichy who acquired urinary tract infections were examined from September to December, 2009. Samples of uropathogenic *E. coli* causing the infection were isolated. Isolated strains of uropathogenic *E. coli* were subjected to screening for multidrug resistance (MDR) against the antibiotics, ampicillin, aiprofloxacin, levofloxin, nirofurantoin and trimethoprim. Results showed that the strain UPEC/QSPL/S4 showed maximum resistance, and this isolated strain was cultured in LB (Luria Bertani) broth at 37 °C for 24 h, and was examined throughout the study.

2.2. Extraction of plant material

M. dubia, a species from the nearby town Kumbakonum from Thanjavur, Tamil Nadu was gathered from August to November, 2009. Dr. M. Jegadeesan identified and authenticated the plant materials. The voucher herbarium (TUH 285) specimens of the plant were deposited in the Department of Environmental and Herbal Science, Tamil University, Thanjavur, Tamil Nadu, India. The cleaned patch of bark was cut into pieces and dried in a dust free environment and powdered. Cold percolation method [21] was employed for extraction.

Five different solvents, water, ethanol (70%), methanol (70%), petroleum ether (70%), and hexane (70%) (1:10 W/V), were used for the extraction at room temperature (25 ± 1 °C). The obtained extracts were agitated frequently; the supernatant was filtered through a muslin cloth. The filtrates thus obtained were dried and stored in an amber coloured bottle, for further analysis after lyophilization at −80 °C in a freezer.

2.3. In vitro assays

To test the activity of various extracts of *M. dubia* bark, the LB broth was supplemented with five different extracts of varying concentrations (10, 20, 30, 40 and 50 mg/ml). To differentiate quorum quenching activity from antibiotic activity, the antibiotics ciprofloxacin (2 mg/ml) and trimethoprim (2 mg/ml) were considered as positive control. For blank control, LB media without supplementation was employed. The activity of the extract was evaluated at different time intervals of 12, 24, 48, 72 h and the extracts were dissolved in PBS. Various assays such as cell density [22], swarming motility [23], protein [24], protease [25], haemolysis [26], haemagglutination [27], hydrophobicity [28] and biofilm inhibition [29] were performed. Cell dry weight, cell wet weight, and pH were also evaluated. All the tests were carried out in triplicates.

2.4. GC–MS analysis

The ethanolic extract of *M. dubia* bark was subjected to GC–MS analysis for its chemical constituents using a PerkinElmer Clarus 500 GC–MS system. The oven program was kept at the temperature of 50 °C for 1 min and ramped at 10 °C/min to 150 °C (hold for 1 min), at 8° C/minute to 250 °C (hold for 1 min), at 15° C/minute to 300 °C (hold for 3 min). Helium (1 ml/min) was used as carrier gas. The injector temperature was adjusted to 280 °C and the mass range was set at 40–450 amu. One microlitre of sample dissolved in ethanol was injected into the system. The compounds were identified by the comparison of their spectra with those in the NIST (National Institute of Standard and Technology) mass spectral library.

2.5. Computational studies

2.5.1. Homology modelling of UPEC SdiA

The amino acid sequence of UPEC SdiA (Uniprot Accession No: Q8FGM5) and then the NMR solution structure coordinates of *E. coli* sdiA (PDB code: 2AVX) were loaded into modeller 9v8. The primary SdiA sequences of *E. coli* and UPEC were aligned and carefully checked to avoid deletions or insertions in conserved regions. A series of UPEC SdiA models (100 models) were independently constructed with Modeller. The model with the best molpdf (molecular probability density function) scores and DOPE (Discrete

Optimized Protein Energy) scores were selected for further docking studies.

2.5.2. Ligand and protein preparation

The 40 compounds reported by GC–MS of *M. dubia* bark extract were drawn using ACD Chemsketch. The energy minimized 3D ligand file was prepared for docking using Schrödinger LigPrep software. The modelled UPEC SdiA was prepared for docking by the addition of polar hydrogen and Kollman charges. The macromolecule was treated to be completely rigid for all docking studies to reduce the extensive computation cost. A grid box encompassing the sites ($60 \times 60 \times 60$; 0.375 Å spacing) was constructed and used for all the docking runs. Our definitions of the site as input for the docking program encompasses amino acids TYR 63, TRP 67, TYR 71, ASP 80, and TRP 95 which were previously identified as the binding site residues of SdiA.

2.5.3. Docking studies

The docking studies were conducted by using AutoDock 4.0 software (http://autodock.scripps.edu/). The docking log files (.dlg) were parsed using pearl script to scan the clustering histogram, and the ligands that have docked pose with binding energy lower than that of the natural ligand C_8HSL were identified and deemed the potential leads.

2.5.4. Statistical analysis

The experimental outputs were interpreted as mean ± SE. *P* values less than 5% ($P < 0.05$) were considered statistically significant [30].

3. Results and discussion

The extract of *M. dubia* bark is known to inhibit the major virulence factors in a significant manner. Biofilm formation, haemolysin production, hydrophobicity, protease activity, and swarming motility are the factors that actively contribute to the pathogenesis of UPEC. As the ethanolic extract of the bark showed many interesting results and hence it is discussed in detail.

3.1. In vitro assays

Biofilms are the attachment 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. The bark ethanolic extract showed outstanding biofilm formation inhibition results at 30 mg/ml, with the highest inhibition (64.81%) being recorded at 48th hour (Fig. 1).

Hemolysins are the exotoxins that are produced by bacteria which lead to the lysis of red blood cells. HlyA, a haemolytic and leukotoxic exotoxin of the RTX (repeat toxin) exotoxin family elaborated by gram negative bacteria, is a primary virulence factor produced by pathogenic *E. coli* strains. Haemolysis, also known as cytotoxic necrotising factor, is strongly proinflammatory leading to the secretion of IL-6 and chemotoxins which sets the pace for pathogenesis of renal diseases [31]. In the present investigation, inhibition of haemolysin production was found to be 14.32% at 48th hour which gradually increased up to a maximum level of 22.81% at 72nd hour (Fig. 2). These data indicate that the ethanolic bark extract possibly reduced the exotoxic enzyme production, thereby decreases the pathogenesis of *E. coli*. Balague and team interpreted similar results with herbicides [32].

In many gram negative bacteria, lectin-mediated interactions were found to be involved in adherence with the animal cells. 30 mg/ml of ethanolic bark extract showed better activity at 12th hour (Fig. 3), a maximum suppression (10.81%) of hydrophobicity, which indirectly affects the biofilm formation in bacteria as ciprofloxacin [33]. Protease activity suppression was also recorded for the ethanolic extract of *M. dubia.* Maximum inhibition of protease synthesis was found to be at the 12th hour, with a peak suppression of 31.65% (Fig. 4).

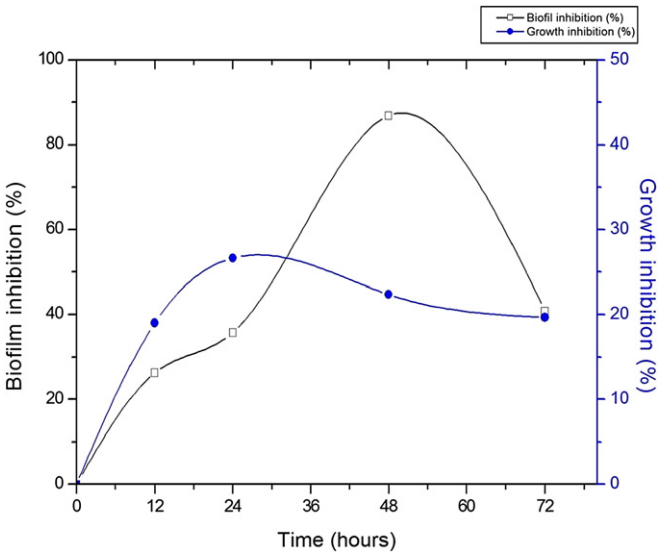

Fig. 1. Potential biofilm inhibiting efficacy of *M. dubia* bark ethanolic extract (30 mg/ml) at different time intervals. Maximum inhibition activity (86.81%) was recorded at 48th h.

When the population is too large to inhabit a single given niche, quorum sensing regulation of swarming presumably allows optimal dissemination of bacterial cells, which indirectly contributes to the biofilm formation. The ethanolic bark extract showed maximum swarming motility inhibition of 75.61% at the 24th hour (Fig. 5), which suggested that the ethanolic bark extract has a potency to shutdown intercellular communication through an unknown mechanism. Ruth Daniels and his team found that the furanone influences the growth rate and inhibits swarming motility, which supported our data [34]. Similar results were also stated by Thomas and his team which confirmed that the furanones

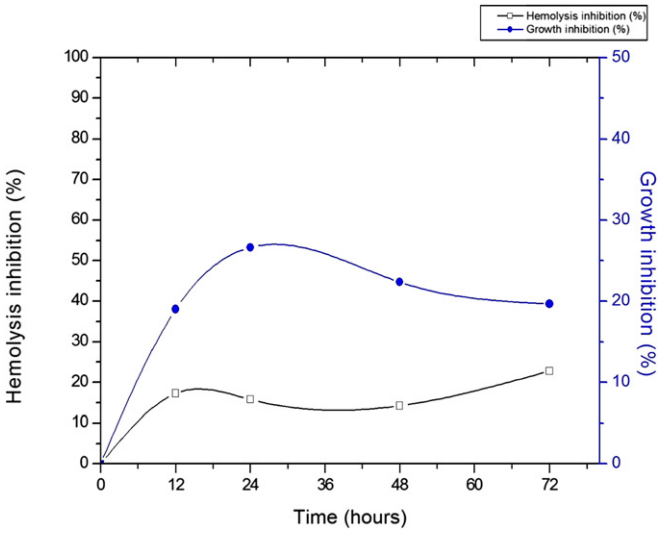

Fig. 2. Potential haemolysis inhibiting efficacy of *M. dubia* bark ethanolic extract (30 mg/ml) at different time intervals. Maximum inhibition activity (17.28%) was recorded at 12th h.

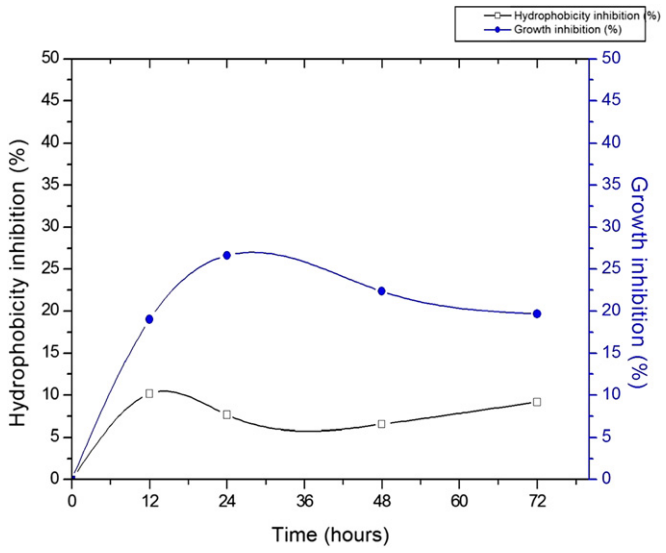

Fig. 3. Potential hydrophobicity inhibiting efficacy of *M. dubia* bark ethanolic extract (30 mg/ml) at different time intervals. Maximum inhibition activity (10.18%) was recorded at 12th h.

have ability to shutdown intercellular communication [35]. All these data suggested the presence of one or more quorum quenching compounds against *E. coli* quorum sensing network in the ethanolic extract of *M. dubia* bark.

Contrary to the above discoveries, selected parameters like cell weight, cell density, and cell growth of *E. coli* against the ethanolic bark extract remained unaltered. However, all these factors greatly decreased when subjected to antibiotics. These findings confirm that the ethanolic bark extract of *M. dubia* does not act as an antibiotic but as an antiquorum sensing herbal preparation.

3.2. GC–MS analysis

The ethanolic extract of *M. dubia* was studied thoroughly by GC/MS and total of 40 secondary metabolites present were identified by doing NIST library search of the acquired mass spectral data (Table 1).

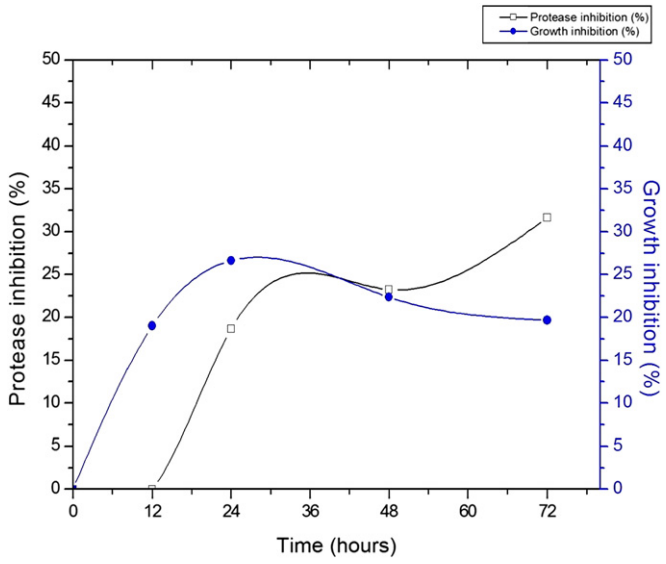

Fig. 4. Potential protease inhibiting efficacy of *M. dubia* bark ethanolic extract (30 mg/ml) at different time intervals. Maximum inhibition activity (31.65%) was recorded at 72 h.

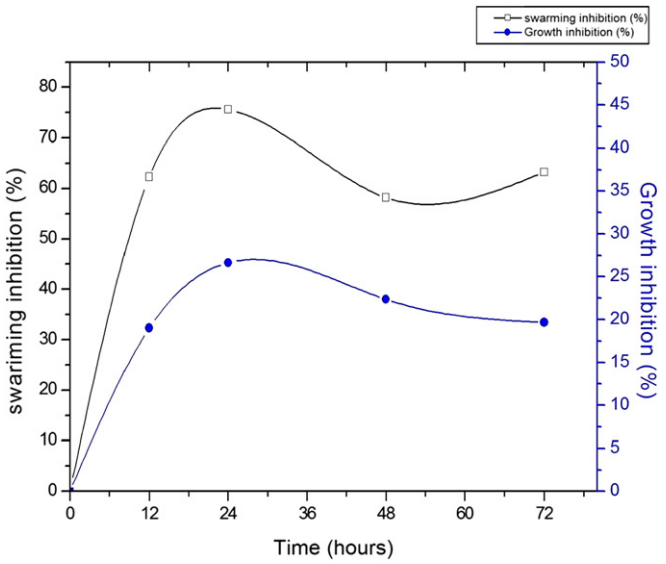

Fig. 5. Potential swarming inhibiting efficacy of *M. dubia* bark ethanolic extract (30 mg/ml) at different time intervals. Maximum inhibition activity (62.31%) was recorded at 24th h.

Table 1
List of ligands identified from *M. dubia* using GC–MS analysis.

S. No	Compound name	Retention time
1	Ethylbenzene	06.57
2	1,3-Cyclopentadiene, 5-(1-methylethylidene)	06.76
3	7-(1-Bromoethyl)-3-3-dimethyl-bicyclo [4.1.0]heptan-2-one	09.31
4	1-Hexene, 3-methyl	13.45
5	Undecane	13.54
6	1-Heptene, 5-methyl	13.63
7	1-Hexanol, 3-methyl	13.82
8	3-Tetradecene	20.90
9	3-Tetradecene	20.97
10	Methyl a-D-ribofuranoside	18.45
11	1-Undecene, 9-methyl	21.08
12	4-Tetradecanol	21.28
13	[1,1′-Biphenyl]-3-amine	21.57
14	Hexadecanal	22.86
15	3-Pentanol, 2,4-dimethyl	23.52
16	3-Tetradecene	23.92
17	Oxalic acid, allyl hexadecyl ester	24.07
18	13-Heptadecyn-1-ol	25.09
19	Tetradecanoic acid, ethyl ester	26.56
20	Benzene, 1-3-bis(1,1-dimethylethyl)-2-methoxy-5-methyl-	26.70
21	Hexadecanoic acid, ethyl ester	27.78
22	Heptacosane	27.84
23	9,12-Octadecadienoic acid, methyl ester	28.64
24	(E)-9-Octadecanoic acid ethyl ester	28.70
25	Ethyl 9-hexadecenoate	28.78
26	Octadecanoic acid, ethyl ester	29.02
27	Hexadecane	30.15
28	Nonadecane	30.32
29	Octadecanoic acid, ethyl ester	31.39
30	Nonadecanoic acid, ethyl ester	32.68
31	1,16-Hexadecandiol	32.92
32	1,2-Benzenedicarboxylic acid, diisooctyl ester	33.00
33	9,12-Octadecadienoyl chloride	33.18
34	Octadecanoic acid, ethyl ester	33.60
35	Pentadecanal	34.16
36	Nonadecanoic acid, ethyl ester	34.95
37	Octadecanoic acid, ethyl ester	36.54
38	Hexadeca-2,6,10,14-tetraen-1-ol, 3,711,16-tetramethyl	36.96
39	1-Heptatriacontanol	39.15
40	Eicosanoic acid, ethyl ester	40.85

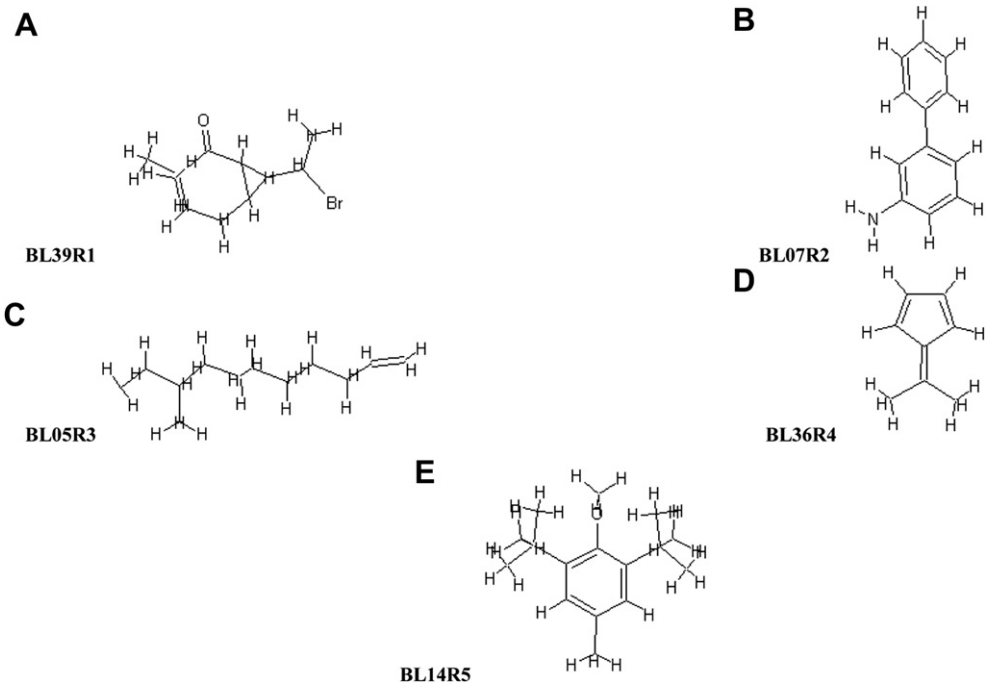

Fig. 6. The ligands identified from ethanolic extract of *M. dubia* bark that showed best binding ability to sdiA. A. (BL39R1) 7-(1-Bromoethyl)-3-3-dimethyl-bicyclo [4.1.0]heptan-2-one (BL07R2) 4-Tetradecanol C. (BL05R3) 1-Undecene, 9-methyl D. (BL36R4) 1,3-Cyclopentadiene, 5-(1-methylethylidene) E. (BL14R5) Benzene, 1-3-bis(1,1-dimethylethyl)-2-methoxy-5-methyl.

3.3. Docking studies

Plants play an essential role in healthcare, it has been estimated by the World Health Organization (WHO) that around 80% of the world's inhabitants rely mainly on traditional medicines for their primary healthcare. An array of secondary metabolites produced by the plant renders its defense mechanism against various threats. The chemical diversity renders natural products which are most promising source of drug leads.

Docking analysis was carried out to screen the potential active principle(s) that could interact with the quorum regulator, SdiA. The ligands showing appreciable binding affinity and binding energies of these ligands are presented in Figs. 6 and 7, respectively. Among the chemical principles studied, BL39R1 showed highest free energy (−5.41 kcal/mol), which is comparable to that of the native ligand C_8HSL (−8.08 kcal/mol). Result suggests that this ligand may be as a promising lead and further structural modifications may lead to the discovery of an effective SdiA inhibitor. The binding free energies are −5.35, −4.28, −4.08, and −3.75 kcal/mol for BL07R2, BL05R3, BL36R4, and BL14R5, respectively. BL39R1 was unable to form a hydrogen bond but the amino acids PHE 63, TYR 75, TYR 67 and VAL 86, which were identified as the nearby amino acids (Fig. 8), may help in the interaction. In the case of BL07R2, it can form a single hydrogen bond with the amino acids GLU 153 and ASN 131 in two different conformations.

We noticed that PHE 63, TYR 67, TRP 71, VAL 86 are playing vital roles in bond formation and they are the crucial amino acids for efficient binding. The mode of interaction for each ligand is different but the amino acids involved in interaction were relatively similar for all the identified ligands from *M. dubia* and even for cognate ligand too. One exception is BL14R5 since its interaction pattern is entirely different from others. The ligand that showed best binding pattern is 7-(1-Bromoethyl)-3,3-dimethyl-bicyclo [4.1.0]heptan-2-one since the ligand BL39R1 which can effectively bind to the SdiA; thus, it can be used as a competitive inhibitor. The

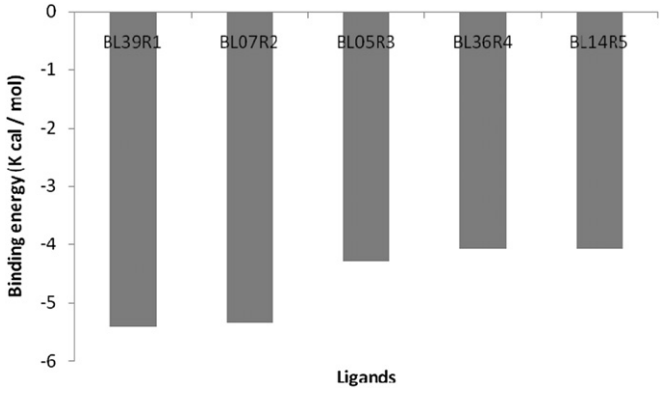

Fig. 7. The predicted binding energy of ligands identified from ethanolic extract of *M. dubia* bark.

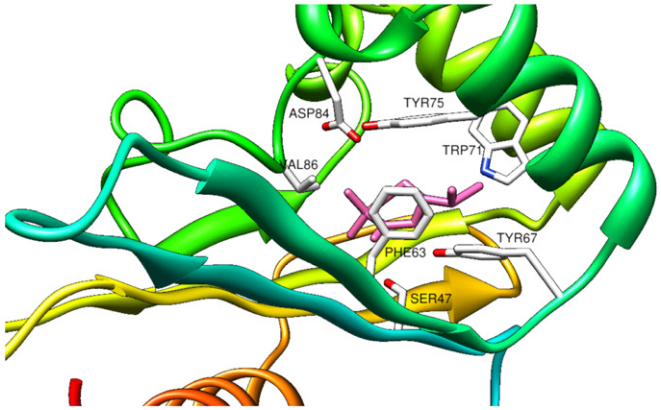

Fig. 8. Docking structure between ligand BL39R1 and sdiA.

ketone functional group has the ability to increase metabolic efficiency, while decreasing production of the free radicals and damaging byproducts of normal metabolism. Hence, the compound can serve as a potential lead compound for the development of new drug which can be used against uropathogenic *E. coli*. It can competitively bind to SdiA instead of C_8HSL, which plays a vital role in quorum sensing of *E. coli*; consequently it can inhibit cell-to-cell communication, quorum sensing.

4. Conclusion

Through *in vitro* and *in silico* experiments, the quorum quenching activity of *M. dubia* has been proved by effectively inhibiting the biofilm formation and virulence factors. Through *in silico* screening, a potential quorum quenching compound in the ethanolic extract of the bark of *M. dubia* against uropathogenic *E. coli* has been identified. This compound can be a potential drug lead against *E. coli* that causes urinary tract infections.

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