Indian Journal of Biochemistry & Biophysics
Vol. 56, August 2019, pp. 276-286

In silico and *in vitro* analysis of quorum quenching active phytochemicals from the ethanolic extract of medicinal plants against quorum sensing mediated virulence factors of *Acinetobacter baumannii*

S Karthick Raja Namasivayam[1]*, K Gowri Shankar[2], JM Vivek[1], Mohideen Nizar[1] & Sudarsan AV[1]

[1]Centre for Bioresource & Development (C-BIRD), Department of Biotechnology, Sathyabama Institute of Science and Technology, Chennai- 600 119, Tamil Nadu, India

[2]Stem Cell Research Centre, Institute of Surgical Gastroenterology, Government Stanley Medical Hospital, Chennai- 600 001, Tamil Nadu, India

Received 10 October 2018; *revised 10 February 2019*

Inhibition of quorum sensing called quorum quenching (QQ) is now extensively utilized in the prevention of bacterial infections. In the present study, *in silico* and *in vitro* analysis of quorum quenching (QQ) or anti-Quorum sensing (QS) activity of ethanolic extract of medicinal plants against QS mediated virulence factors of human pathogenic bacteria *Acinetobacter baumannii* has been investigated. The effect of plant extracts on QS by acyl homoserine lactone (AHL) has been carried out by quantification of secreted AHL by high-pressure liquid chromatography (HPLC). Measurement of QQ activity was determined by maximum inhibition of virulence factors and AHL production which was recorded in *E. globules* and *A. indica* extracts. *In silico* analysis was studied with possible bioactive compounds in the ethanolic extract of respective plant material that were characterized by gas chromatography equipped with mass spectroscopy (GCMS) against the enzyme responsible for the production of signaling molecule which mediates QS AHL synthase. Distinct reduction of all the QS-mediated virulence factors was recorded in the *E. globules* and *A. indica*. Among the different bioactive compounds, the ethanolic leaf extract of *E. globules* of GCMS analyzed compound, Hexadeconoic acid, 1-(hydroxymethyl), 1, 2-ethannediyl ester interacted with 1KZF protein (AHL synthase) and showed binding energy of −11.2 kcal/mol to MET 42 and TYR 54. Phytochemicals mediated inhibition of AHL synthase activity which was responsible for AHL production would suggest the possible utilization of plant extracts as an antibacterial agent to fight against disease-causing pathogenic bacteria.

Keywords: *Acinetobacter baumannii*, Acyl homeserine lactone (Ahl) Synthase, *In silico*, Quorum quenching, Virulence factors

Effective antimicrobial chemotherapy for the life-threatening fatal infectious diseases has primarily based on inhibition of growth of pathogenic microorganisms. But, the continuous or misuse of chemotherapeutics leads to emergence of multiple antibiotics resistant (MDR) strains Recent studies reveals that many pathogenic bacteria carry out cell-cell communication systems that mediated by production of signaling molecules as density-dependent manner to express certain phenotypic traits in a density-dependent manner which signaling principle is called as quorum-sensing (QS). A novel strategy is to inhibit bacterial infections without preventing the growth is to an effective suppression of bacterial cell communication-quorum sensing using certain chemicals modulators[1]. Certain Gram-negative pathogenic bacteria produces an important signaling molecules which mediate QS. Among the molecules, *N*-acyl-homoserine lactones (AHLs) are the most important signaling molecules and synthesis and detection of AHLs which promotes many gram-negative bacteria to engage in QS, an intercellular signaling mechanism that activates various virulence factors production like biofilm lifestyles. Synthesis of AHL *via* the catalytic activity of AHL synthase plays an important role in QS. Acylation of S-adenosyl-L-methionine by acyl-acyl carrier protein and lactonization of the methionine moiety through the catalytic activity of AHL synthases leads to the production of AHLs[2]. AHL-based signaling systems typically mediated by two proteins, an AHL synthase, (usually a member of the LuxI family of proteins) and an AHL receptor (protein belonging to the LuxR family of transcriptional regulators). The basal level of AHL by AHL synthase activity is produced at less bacterial population densities and the level of AHL increased in the growth medium at high bacterial population

*Correspondence:
E-mail: biologiask@gmail.com

densities. When the level of AHLs is reaching a threshold concentration, the AHL molecule binds to its cognate receptor followed by activation or inhibition of target genes which mediates pathogenesis of bacterial pathogens[3].

In this study, inhibition of AHL synthase activity of major human bacterial pathogen *Acinetobacter baumannii* by the ethanolic extracts of medicinal plants under *in silico* and *in vitro* analysis. Among the diverse Gram-negative bacteria, *Acinetobacter* sp. *via* an emerging pathogen which can be isolated fromvarious environmental, clinical sources and the various strains of this genus have emerged as multi-drug resistant pathogen since the 1970s. However, recent reports reveal that strains of *Acinetobacter* sp. play a major role in the removal of toxic inorganic,organic pollutants,.bioengineering of enzymes and diagnostic materials[4-5]. In recent years, pathogenic strains of *Acinetobacter* sp. known to cause life-threatening infections like pneumonia/pulmonary infections, septicemia, endocarditics, meningitis, burns[6].

Antimicrobial resistance among *Acinetobacter* sp. has been raised in the past decade and the ability of *Acinetobacter* sp. to exhibit high resistance is due to the presence of impermeable outer membrane and its exposure to a large reservoir of resistance genes. A wide variety of genotypes and phenotypes are formed among *Acinetobacter* sp. is the result of resistance mechanisms. Bacterial strains that shows extreme resistance to all antimicrobial agents, including polymyxins, have been reported in the recent studies making more complication in treatment against the life-threatening infections caused by the bacterial strains[7]. Pathogenesis of many bacterial diseases mediated by quorum sensing mechanism has been reported in recent studies indicating the importance of QS in the production of extensive virulence factors Most of the human pathogens use QS systems to regulate processes associated with virulence factors this increases the viability or survival in the host body when the bacterial population reaches a high population density, increasing the likelihood that the hosts defenses will be successfully overwhelmed. In the case of *Pseudomonas aeruginosa*, well studied QS model organism reveals that QS is the major regulatory process which mediates the formation of biofilms which act as a major barrier against antibiotics or other chemotherapeutics and the host immune system[8-10].

Inhibition of quorum sensing by disrupting the bacterial communication through degradation of signaling molecules called as Quorum quenching (QQ) is an effective strategy of prevention of pathogenesis. Bioactive metabolites derived from plants and microorganisms acts as the QQ compounds[11]. Recent reports on AHL degradation kinetics using rapid resolution liquid chromatography shows that various chemicals and enzymes from diversified sources effectively brought about QQ by degradation of AHL. Several chemicals and enzymes have utilized in recent years that target the key components of bacterial QS systems. These QQ chemicals explain the mechanism of preventing bacterial infections by blocking bacterial cell-cell communications and will undoubtedly stimulate further research and biotechnological innovation in formulating practical ways to control bacterial diseases[12]. Plants or plant based l products from diverse parts of the world are effective sources of OS inhibitors[13]. Among the biological sources, plants and their derived chemicals act as a large and attractive reserve for the discovery of QQ compounds. Potential phytochemicals from the plants that have been used for treatment of disease for very long time, their less side effects or lower toxicity, biocompatibility are the major reason for selecting QS inhibitors[14].

In our present study we have evaluated the interaction of GCMS analyzed compounds of ethanolic extract of *Curcuma longa*, leaves of *Ocium tenuiflorum*, *Aegle marmelos*, *Eucalyptus globules*, *Azadirachta indica*, and *Cynodon dactylon* with the target protein AHL synthase (PDB ID: 1KZF) and amino acids involved in interactions with the most favorable pose within the binding site, in energy terms. The developments in molecular characterization and bioinformatics have made possible to “dock” small molecules (*i.e.* ligands) to proteins and “score” their potential binding efficiency[15]. Protein-ligand docking is a well known molecular modeling technique which is a rapidly emerging area that globally covers the development of techniques using software to capture, analyze and integrate biological and medical data from various diverse sources that ultimately provide discoveries or advances in medicine and therapeutics[16]. Drug development strategy is now heavily depends on the protein-ligand docking which is used to predict the position and orientation of ligands to select as likely drug candidates when it is bound to a protein receptor or enzyme[17]. The present study, *in silico* and *in vitro* screening of metabolites from the medicinal plant

extracts against acyl homoserine lactone synthase –an enzyme mediates QS of *Acinetobacter baumannii* has been carried out and this study will helpful to formulate bioactive metabolites from the traditional plant extracts as an anti infectious agents against life threatening disease causing organisms.

Materials and Methods

Bacterial strain and growth condition

Acinetobacter baumannii used in the present study was isolated from urinary tract infection patient (46 years non-HIV post-operative Male) by standard methods. The bacterium was identified based on cultural, biochemical and molecular characteristics. Isolated pure culture was maintained on trypticase soy agar slant.

Crude extraction of metabolites

Curcuma longa, leaves of *Ocium tenuiflorum*, *Aegle marmelos*, *Eucalyptus globules*, *Azadirachta indica* and *Cynodon dactylon*, were collected from Agriculture college and research institute, Madurai. Extraction was carried out by the modified method of Hussaini and Mahasneh[13]. A Known quantity of dried powder of respective plant material (100 g) was extracted twice at room temperature with ethanol 95 % (100 mL/10 g of plant material). The final ethanol extract of each plant part was filtered using (Whatman No.1) filter paper and evaporated under vacuum at 40°C using a rotary vacuum evaporator; the concentrated extract thus obtained was collected in a screw-cap vial and used for further studies.

Screening of anti-QS activities

Effect of plant extracts on QS mediated virulence factors such as biofilm formation, iron acquisition on biofilm formation, twitching motility, cell adhesion, and total proteolytic activity was studied in the present work.

Inoculum preparation

An inoculum of bacterial strain was prepared by inoculating a loopful of bacteria from nutrient agar slant to nutrient broth, kept under shaking condition (REMI, India) for 18 to 24 h at 30°C and 150 rpm and the grown bacterial culture was adjusted to 0.5 McFarland standard (10^8 CFU/mL).

Biofilm inhibition

Biofilm inhibitory effect of plant extracts was studied by spectrophotometric microtitre plate assay18. 1 mL of bacterial inoculum thus prepared was inoculated into wells followed by the addition of respective plant extracts under aseptic condition followed by incubation at 37°C for 72 h. After the incubation period, the content was removed and 0.1 mL of 0.1 % *v/v* crystal violet was added, incubated at room temperature for 30 min. After the incubation period, the stained wells were washed with distilled water followed by ethanol addition (95 %). Ethanol solubilized mixture thus obtained was read spectrophotometrically at 579 nm. Biofilm inhibitory effect was determined by measurement of differences in optical density of ethanol solubilized mixture of control and treatment groups.

SEM analysis of biofilm

Scanning electron microscopy analysis was done to confirm plant extracts mediated antibiofilm effect. Microtitre plate with adherent biofilm was isolated adopting ultrasonication method and the disturbed biofilm aggregates were fixed using glutaraldehyde (2.5%) and examined under scanning electron microscopy using Carl Zeiss Subra (Germany) scanning electron microscope.

Iron effect on biofilm formation

M9 minimal media supplemented with 40 µM $Fecl_3$ inoculated with 100 µL of mid-log phase bacterial strain, 200 µL of respective plant extract and nano particle suspension. The inoculated tubes were incubated without shaking at 25°C after the incubation period. The tubes were stained with 0.1% crystal violet and incubated for 20 min. The stained biofilm was solubilized by adding 1ml of ethanol. Ethanol solubilized solution was read at 570 nm and the biofilm formation was calculated by the formula as described earlier.

Twitching motility

Solid agar medium was prepared with Luria Bertani agar. After sterilization, bacterial culture was stab inoculated into the medium. The plates were incubated at 37°C for 24 h. The bacterial growth was identified in the interface of the medium and the plate surface. These can be visualized by removing the agar from the plates and staining the plates with 1% crystal violet, these were kept undisturbed for 10 min and the radius was measured.

Cell adhesion

Plant extracts mediated inhibition of cell adhesion was carried out by a modified method of NCCL[19] using 96 well flat bottom microwell plates. Prior cell adhesion, wells were coated with 150 µL of freshly

prepared 1.0% BSA, incubated at 30°C for 30 min followed by washing thrice with sterile phosphate buffered saline (PBS). Bacterial inoculum (50 µL) thus, prepared in nutrient broth was transferred to the well followed by the addition of 50 µL of the respective plant extracts. Seeded microtitre plate was incubated at 37°C for 24 h. Cells were allowed to adhere and the non-adhered cells were washed 5 times with PBS at room temperature. Adhered cells were detected by adding 50 µL of 0.1% crystal violet per well, incubated at room temperature for 30 min. Wells were washed with sterile distilled water to remove excess stain. 10 µL of ethanol was added to fix the adhered cells. 50 µL of 0.2% Triton X was added to the wells for the lysing of cells and the absorbance was read at 570 nm.

Proteolytic activity

Evaluation of plant extracts on the proteolytic activity of *A. baumannii* was carried out by a colorimetric determination by the standard method[20]. 100 mL of protease production medium was prepared in 250 mL of conical flask, sterilized by autoclaving followed by inoculation of bacterial inoculum (0.1 mL). Seeded flasks were incubated at 30°C under shaking condition for 36 h. After the incubation period, the broth was centrifuged at 10000 rpm for 10 min, collected supernatant was used as the source of protease. Determination of protease enzyme activity was done using casein as the substrate. The reaction mixture consisted of 0.25 mL of 50 mM sodium phosphate buffer (pH 7.0) containing 2.0% (w/v) of azocasein and 0.15 ml of enzyme solution. After incubating at 25°C for 15 min, the reaction was stopped by adding 1.2 mL of 10.0% (*w/v*) TCA, incubating at room temperature for an additional 15 min, and then the precipitate was removed by centrifugation at 8000 g for 5 min. 1.4 mL of 1.0 M NaOH was added to 1.2 mL of the supernatant, and its absorbance was measured at 440 nm.

HPLC analysis of effective plant extracts on signaling molecule AHL

100 µL of *A. baumannii* culture was inoculated in 100 mL of LB broth medium followed by the addition of 0.1 mL of respective plant material. Inoculated flasks were incubated at 30°C under shaking condition for 24 h. After the incubation period, the culture was centrifuged and the supernatant was extracted twice with equal volumes of acetonitrile and the extract was concentrated with a rotary evaporator. Percent composition of AHL was determined by HPLC (Shimadzu LC 10AT VP). The concentrated extract was suspended in 1 mL of the acetonitrile water mixture, and filtered through 0.45 micron membrane filter. The filtrate (20 µL) was injected through C18 ODS (Octa Decyl Silane) column worked at the reverse phase mode.

Molecular docking studies

The 3D crystal structure of the targeted AHL synthase (Protein Data Base PDB ID: 1KZF) was retrieved from the www.rcsb.org/pdb. The PubChem and Chem Spider database was used for retrieving the structure of ligand molecules[16]. The selected chemical structures were generated from SMILES notation (Simplified Molecular Input Line Entry Specification) by using the Chem 3D pro 12.0.2.1076. The molecular docking was performed using AutoDock Vina is an open source program for doing molecular docking. All the ligands and target protein were geometrically optimized and docked using docking program AutoDock Vina using the method of Gowri Shankar *et al.*[17], Maicol Ahumedo *et al.*[21]. Structural and active site studies of the protein were done by using PyMOL molecular visualization software.

Results and Discussion

Pharmacologically active products from natural sources have been used by humanity to treat various diseases for a long time and the various chemotherapeutic agents derived from these sources having potential pharmacological activities[22]. Molecular docking is an important analysis method to predict the drug interactions at the molecular levels with the target proteins[23]. In this study, evaluation of QQ activities or inhibition of quorum sensing of ethanol extract of medicinal plants like biofilm formation, iron acquisition on biofilm formation, twitching motility, cell adhesion and total proteolytic activity of *A. baumannii* has been studied using protein docking studies using acyl-homoserine lactone synthase as a target.

Ethanol extracts of respective plant extracts were characterized by GCMS analysis which reveals the presence of various bioactive metabolites as reported by our earlier works[24]. Effect of the extracts on the QS mediated factors was studied and the results were presented in (Fig. 1). Among the extracts, maximum effect on the biofilm was observed in *Eucalyptus globules* extract followed by *Azadirachta indica*. Both the extracts brought about significant inhibition

(P> 0.05). Scanning electron microscopic images of the biofilm treated with plant extracts show degeneration of biofilm with weakened cell masses while the control exposed compact tightly packed cell aggregates (Fig. 2). Similar inhibition of other QS based virulence factors was also observed in *Eucalyptus globules* and *Azadirachta indica*, Namasivayam and Roy[25] reported that leaf extracts of neem brought about maximum inhibition of biofilm which might be due to phytochemicals in the extract inhibited quorum sensing which mediates biofilm formation. *Aegle marmelos* and *Curcuma longa* exhibited a moderate inhibitory effect on all the tested QS mediated virulence factors. Significant inhibition on the tested QS mediated virulence factors was not recorded in *Ocium tenuiflorum* and *Cynodon dactylon* extracts.

Effect of plant extracts on the Acyl-homoserine lactone was studied by measurement of AHL in control and plant extracts treated culture. AHL extracted from the respective plant extracts treated bacterial culture grown in nutrient broth and the cell-free supernatant was extracted with a suitable solvent system. HPLC study shows AHL of the control was eluted at 4.622 min retention time (Rt) at the above mentioned condition. The percentage of AHL was measured by the same parameters and calculated by using the Rt which reveals 98%. AHL from the bacterial culture treated with *Eucalyptus globules* and *Azadirachta indica* were eluted at 4.545 and 4.485 min Rt with 5.24 % and 11.67%, respectively, (Fig. 3).

The possible presence of bioactive compounds in the extracts was characterized by gas chromatography-mass spectroscopy (GCMS) has reported in our previous work Namasivayam *et al.*[24]. The crystal structures of AHL synthase (1KZF) were downloaded from PDB database was shown in (Fig. 4). Molecular docking analysis of 1kzf protein

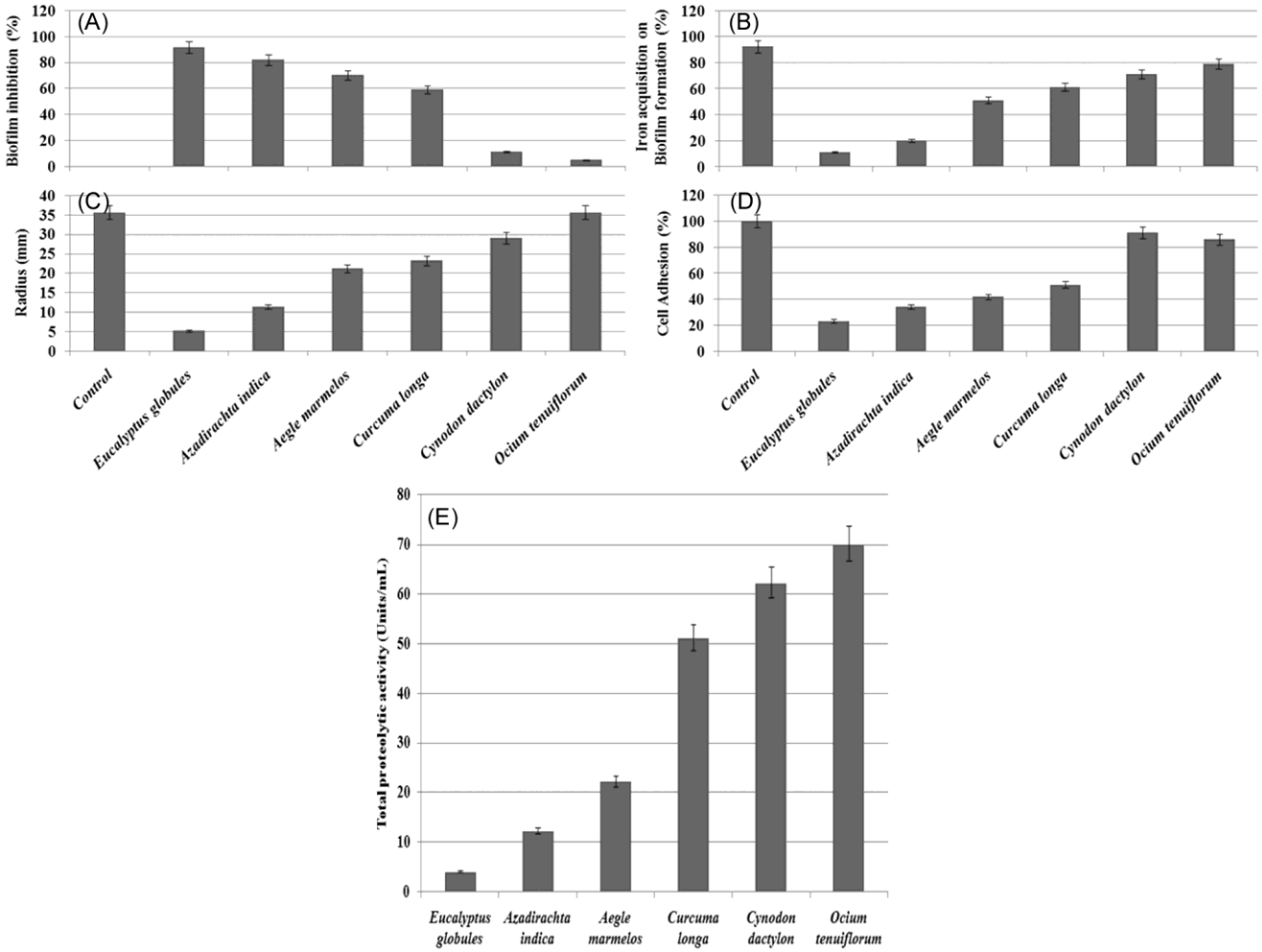

Fig. 1 — Effect of plant extracts on the (A) Biofilm inhibition; (B) Iron acquisition (%) of biofilm formation; (C) The twisting motility (radius); (D) Cell adhesion; and (E) Proteolytic activity

Fig. 2 — Scanning electron microscopic image of biofilm treated with plant extract (A) Control; (B) *Azadirachta indica*; and (C) *Eucalyptus globules*

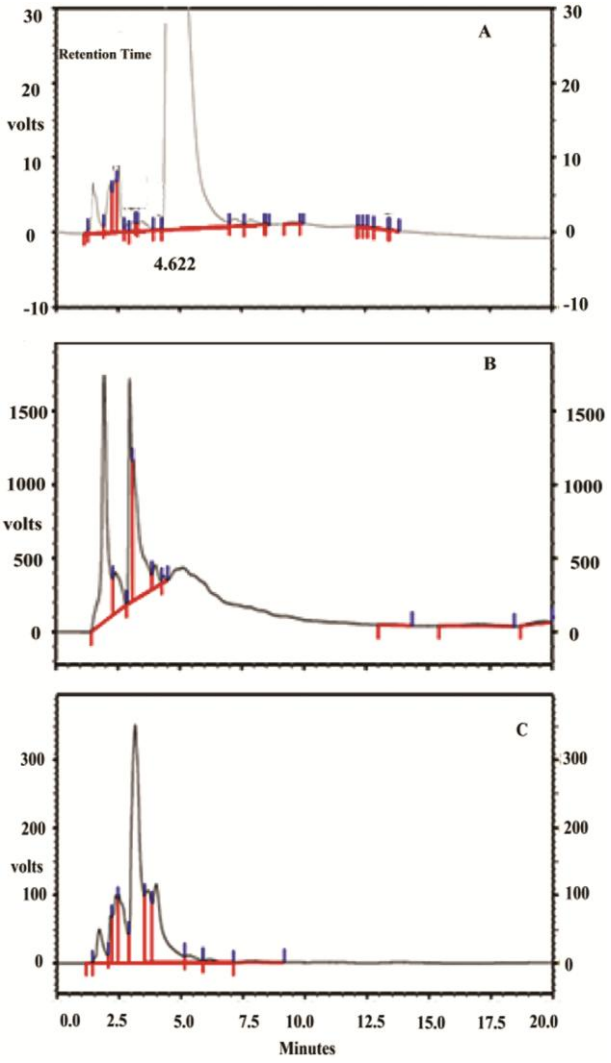

Fig. 3 — HPLC chromatogram of acyl homoserine lactone extracted from *A. buymani* (A) Control; (B) *Azadirachta indica*; and (C) *Eucalyptus globules*

using GCMS analyzed compounds of ethanolic extract of the respective plants was presented in (Table 1). Ethanolic leaf extract of *Eucalyptus globules* of GCMS analyzed compound, Hexadeconoic acid,1-(hydroxymethyl), 1, 2-ethannediyl ester interacted strongly with 1KZF protein and

Fig. 4 — Crystal structure of the Acyl-homoserine lactone synthase (PDB ID: 1 Kzf)

showed the least binding energy of −11.2 kcal/mol to the amino acids MET 42 and TYR 54, when compared all other ligands shown (Fig. 5A).

Ethanolic leaf extract of *Azadirachta indica* & *Aegle marmelos* GCMS analysed compounds of Ethanol, 2-(9-octadececenyloxy)-(z) & l-[+] Ascorbic acid 2, 6-dihexadecanoate interacted strongly with 1KZF, which showed least binding energy of −8.2 & −8.1 kcal/mol to the aminoacids MET 42 and SER 44 & ARG 100, PHE 101 and ILE 141, when compared all other ligands, respectively, shown in (Table 1 and Fig. 5B & C). The ethanolic whole plant extract of *Cynodon dactylon* & *Ocium tenuiflorum* GCMS analysed compounds of Eichosanoic acid-ethyl ester & Ethyl 9, 9-diformylnona-2, 4, 6, 8-tetraenoate interacted strongly with 1KZF, which showed least binding energy of −7.8 & −6.1 kcal/mol to the amino acids MET 42 & GLN 13, SER 66, ARG100, ARG 68, and VAL 67, when compared all other ligands,

Table 1 — Molecular docking analysis of 1kzf protein using GCMS analyzed compounds of ethanolic extract of the whole plant

Name of the compound	Parameters		
	Binding energy (Kcal/mol)	No. of bonds	Amino acid residues
Cynodon dactylon			
2-Penta,6,10,14-trimethyl	-	-	-
1-Dodecanol,3,7,1 1-trimethyl	-	-	-
Hexadecanoic acid- ethyl ester	−7.1	2	SER 44, TYR 54
3,7,11,15-Tetramethyl-2-hexadecen-1-ol	−6.4	4	MET1, ILE 58, GLU 3
Ethyl Oleate	−5.9	1	GLN13
Heptadecanoic acid 15-methyl-ethyl ester	-	-	-
Eichosanoic acid- ethyl ester	−7.8	1	MET42
Eucalyptus globules			
Patchoulene	-	-	-
Globulol	-	-	-
a-phellandrene	-	-	-
Pentadecanoic acid,14-methyl-methylEster	-	-	-
1,2-benzenedicarboxilic acid, butyl octy ester	−9.1	2	SER 44, TYR 54
8,11-Octadecadienoic acid, methyl ester	−6.1	3	MET 42, SER 66
Ethanol,2-(9-octadecenyloxy)-,(z)-	−8.2	3	MET 42, SER 44
Oleic acid	−7.9	1	CYS 38
2,3-Dihydroxypropyl elidate	−7.9	5	TYR 9, GLN 13, SER 44
Hexadeconoic acid,1-(hydroxymethyl) 1,2-ethannediyl ester	−11.2	2	MET 42, TYR 54
9-Octadecenoic acid(z)-,2-hydroxy-1-(hydroxymethyl)ethyl ester	−6.7	4	ARG 152 & 193
Azadirachta indica			
Pentadecanoic acid,14-methyl,methyl ester	-	-	-
Hexadecanoic acid, ethyl ester	−7.1	2	SER 44, TYR 54
8,11-Octadecadienoic acid, methyl ester	−6.1	3	MET 42, SER 66
10- Octadecadienoic acid, methyl ester	−6.4	2	ARG100,PHE 101
Ethanol,2.(9.octadececenyloxy).(z)	−8.2	3	MET 42, SER 44
Oleic acid	−7.9	1	CYS 38
Hexadecanoic acid,1-(hydroxymethyl)1-2 ethanediyl ester	−7.4	2	SER 44, TYR 54
9-Octadecenoic acid(z)2-hydroxy 1-(hydroxymethyl)ethyl ester	−7.9	1	CYS 38
Ocium tenuiflorum			
9-Octadecene,1,1-(1,2-ethanediylbis(oxy)) bis-,(ZZ).	-	-	-
Ethyl 9,9-diformylnona-2,4,6,8-tetraenoate	−6.1	5	GLN 13, SER 66, ARG100, ARG 68, VAL 67

(*contd.*)

Table 1 — Molecular docking analysis of 1kzf protein using GCMS analyzed compounds of ethanolic extract of the whole plant (*contd.*)

Curcuma long		Parameters	
Name of the compound	Binding energy (Kcal/mol)	No. of bonds	Amino acid residues
Benzene,1-methyl-4-(1,2,2-trimethylcyclopentyl)-,(R)	-	-	-
6-(p-Toly)-2-methyl-2-heptenol	−7.7	3	ASP 48, ARG 100
7-Oxabicyclo(4.1.0)heptane,1-(1,3-dimethyl-1,3-butadienyl)-2,2,6-trimethyl-(E)	−6.4	2	MET 42, SER 44
Acetic acid,3-hydroxy-6-isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydronaphthalen-2-yl ester	−7.2	5	GLN 13, SER 44, ASP 48, MET 42
7-(1,3-Dimethylbuta-1,3-dienyl)-1,6,6-trimethyl-3,8-dioxatricyclo[5.1.0.0(2,4)]loctane	-	-	-
10-Octadecenoic acid, methyl ester	−6.4	2	ARG100,PHE 101
Heptadecanoic acid, 16-methyl-,methyl ester	−5.8	2	MET 42, SER 66
Aegle marmelos			
Pentadecanoic acid, 14-methyl-, methyl ester	-	-	-
Hexadecanoic acid, ethyl ester	−7.1	2	SER 44, TYR 54
l-[+]Ascorbic acid 2,6-dihexadecanoate	−8.1	4	ARG 100, PHE 101, ILE 141
Oleic acid	−7.9	1	CYS 38
Hexadecanoic acid,2,3-dihydroxypropyl ester,(n)-	−7.4	2	SER 44, TYR 54
9-Octadecenoic acid(Z)-,2-hydroxy-1(hydroxymethyl)ethyl ester	−7.9	1	CYS 38

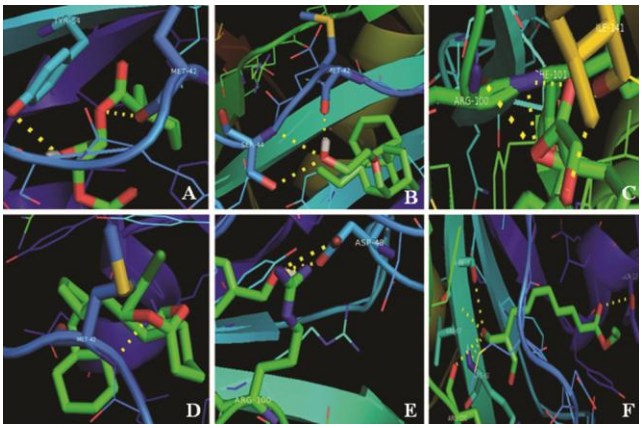

Fig. 5 — Binding Pose (PyMol Viewer) of selected ligand molecules docked with 1KZF. (A) Hexadeconoic acid,1-(hydroxymethyl), 1, 2-ethannediyl ester; (B) 2-(9-octadececenyloxy)-(z); (C) l-[+] Ascorbic acid 2, 6-dihexadecanoate; (D) Eichosanoic acid-ethyl ester; (E) 6-(p-Toly)-2-methyl-2-heptenol; and (F) Ethyl 9, 9-diformylnona-2, 4, 6, 8-tetraenoate. Green stick model indicates ligands, Yellow dots indicates the bonding

respectively, shown in (Table 1 and Fig. 5D & F). The ethanolic extract of C*urcuma long* GCMS analysed compound, 6-(p-Toly)-2-methyl-2-heptenol interacted strongly with 1KZF, which showed least binding energy of −7.7 kcal/mol to the amino acids ASP 48 and ARG 100 when compared all other ligands, respectively, shown in (Table 1 and Fig. 5E).

Recently, several inhibitors of QS have been discovered from natural sources like microorganisms and plants which interfere with QS. Natural products especially plants used in traditional medicines are a promising source for deriving molecules that can potentially inhibit quorum sensing. These plants can offer a large and attractive repertoire for the discovery of quorum sensing inhibitors[12]. They are of particular importance as these have been used for thousands of years for the treatment and management of diseases and may have few side-effects and toxicity issues as with many antibiotic regimens and currently known QS inhibitors. Herbs, Spices and Medicinal Plants used in Hispanic cultures have been used for several centuries to treat common ailments, are well known for their antimicrobial effects on a variety of human pathogens. However, few reports have been studied regarding anti-QS activities of medicinal plant extracts against human pathogens In the present investigation, we evaluated *in silico* and *in vitro*

analysis of ethanolic extract of medicinal plants against QS mediated virulence factors like biofilm inhibition, iron acquisition on biofilm formation, twitching motility, cell adhesion, total proteolytic activity and measurement of signaling molecule mediates QS acyl homoserine lactone (AHL). The anti-QS activity of herbal plant extracts against human pathogenic bacteria revealed the presence of various phytochemicals in the extracts to interfere with the activity of acyl homoserine lactone (AHL) a signaling molecule controls quorum sensing activities. Inhibition of QS-mediated virulence factors of *P. aeruginosa* by bud extract of Clove (*Syzygium aromaticum*) has been reported[2].

Results indicate that all the QS-mediated virulence factors were highly inhibited by *Eucalyptus globules* extract followed by *Azadirachta indica*. Among the different QS mediated virulence factors, the present study focused on five major factors like biofilm inhibition, iron acquisition on biofilm formation, twitching motility, cell adhesion, total proteolytic activity. Microbial biofilm develops when microorganisms irreversibly adhere to a submerged surface and produce extracellular polymers that facilitate adhesion and provide a structural matrix by the QS-mediated signaling which prevents the entry of the drug and resistant towards the mechanical stress. Biofilm formation is also the critical problem in medical devices which leads to life-threatening infection. Inhibition of biofilm is an effective strategy of microbial infection treatment. In this study, ethanolic extract of *Eucalyptus globules* and *Azadirachta indica* showed effective inhibition and the scanning electron microscopic image reveals the plant extracts treatment weakened the biofilm by damaging the dense aggregates of cell masses and the number of free cells were observed instead of the dense biofilm matrix. Iron acquisition is an important factor of biofilm development by *Acinetobacter* sp., which play a crucial role in biofilm formation. *Eucalyptus globules* and *Azadirachta indica* extracts used in this study were highly reduced the iron acquisition on the biofilm formation. Cell adhesion is another important QS mediated virulence factor studied by the method of NCCLS using 96 well flat bottom microwell plate which resembles the adhesion of bacteria to the suitable cell receptor or tissues which initiates the pathogenesis process. Like the other factors, *Eucalyptus globules* and *Azadirachta indica* treatment highly reduced cell adhesion. A similar effect was also observed in total proteolytic activity. Protease is an important hydrolytic enzyme produced by the pathogenic bacteria strain which degrades the protein of the target tissues which may help in spreading of the organism to the host body system. Proteolytic activity was found to be highly reduced in the plant extracts treatment. Influence of plant extracts on AHL a signaling molecule responsible for quorum sensing was also studied by quantification of AHL in the culture free supernatant extracted with the suitable solvent system. Results clearly indicate that *Eucalyptus globules* and *Azadirachta indica* treated plant extracts highly reduced AHL production which was determined by the least production of AHL. Phytochemicals are modulating the bacterial synthesis of AHL and in turn inhibit QS. Many natural extracts are believed to inhibit QS by either interfering with AHL activity by degradation of the LuxR/LasR receptors for the AHL molecules[26]. The availability of the 3D molecular structure of an AHL synthase, particularly the region responsible for its chemical interactions, makes it possible to identify a compound capable of binding to the active site of the enzyme using computational molecular modeling techniques[17]. The 3D structures of transcriptional regulators involved in QS from *P. aeruginosa C. violaceum* and *A. tumefaciens* have been analyzed[27,28]. Aliyu *et al.*[29] explained the molecular docking was conducted to explore the binding conformations of sesquiterpene lactones into the binding sites of QS regulator proteins, CviR and CviR0; and the binding energy data suggested that the blumeoidolides have a tendency to inhibit both CviR and CviR0 with varying binding affinities[30-32]. In order to substantiate the experimental observations and probe the binding modes of ethanolic extracts of GCMS analyzed ligands into the binding site of AHL synthase (PDB ID: 1KZF), molecular docking simulations were performed. All compounds docked successfully into the binding sites of 1KZF with good binding affinity as evidenced by the lower computed binding energies as shown in (Table 1).

Conclusion

Ethanolic extracts of *Eucalyptus globules* and *Azadirachta indica* exhibited QQ activities against all the QS-mediated virulence factors of clinical isolate *A. baumannii* under *in vitro* condition and the finding was supported by *in silico* analysis by ethanolic leaf extract of *Eucalyptus globules* of GCMS analyzed

compounds showed the least binding energy towards AHL synthase (PDB ID: 1KZF) which was responsible for AHL production. This work reveals the effective inhibition of bacterial cell signaling molecular mechanism which subsequently leads to the production of various virulence factors by the phytochemicals in the medicinal plant extracts which provided potential new information to develop non-antibiotics dependent effective safe drugs which help in the treatment of various bacterial diseases.

References

1 Tiwary BK, Ghosh R, Moktan S, Ranjan VK, Dey P, Choudhury D, Dutta S, Deb D, Das AP & Chakraborty R, Prospective bacterial quorum sensing inhibitors from Indian medicinal plant extracts. *Lett Appl Microbiol*, 65 (2017) 2.

2 Harbottle H, Thakur S, Zhao S & White DG, Genetics of antimicrobial resistance. *Anim Biotechnol*, 17 (2008) 111.

3 Kalyoncu F, Cetin B & Saglam H, Antimicrobial activity of Common mader (*Rubia tinctorum* L.). *Phytother Res*, 20 (2006) 490.

4 Rao RS, Karthika RU, Singh SP, Shashikala P, Kanungo R, Jayachandra S & Prashanth K, Correlation between biofilm production and multiple drug resistance in imipenem resistant clinical isolates of *acinetobacter baumannii*. *Indian J Med Microbiol*, 26 (2008) 333.

5 Di Popolo A, Giannouli M, Triassi M, Brisse S & Zarrilli R, Molecular epidemiological investigation of multi drug resistant *Acinetobacter baumannii* strains in four Mediterranean countries with a multilocus sequence typing scheme. *Clin Microbiol Infect*, 17 (2011) 197.

6 Perez F, Terashima R, Adams MD & Bonomo RA, Are we closing in on an 'elusive enemy. The current status of our battle with *Acinetobacter baumannii*. *Virulence*, 2 (2011) 86.

7 Chuang YC, Sheng WH & Li SY, Influence of genospecies of *Acinetobacter baumannii* complex on clinical outcomes of patients with *Acinetobacter* bacteremia. *Clin Infect Dis*, 52 (2011) 352.

8 Mendes RE, Bell JM, Turnidge JD, Castanheira M & Jones RN, Emergence and widespread dissemination of OXA-23,-24/40 and -58 carbapenemases among *Acinetobacter* spp. in Asia-Pacific nations: report from the SENTRY Surveillance Program. *J Antimicrob Chemother*, 63 (2009) 55.

9 Peleg AY, Seifert H & Paterson DL, *Acinetobacter baumannii*. Emergence of a successful pathogen. *Clin Microbiol Rev*, 21 (2008) 538.

10 Mendes RE, Farrell DJ, Sader HS & Jones RN, Comprehensive assessment of tigecycline activity tested against a worldwide collection of *Acinetobacter* spp. *Diagn Microbiol Infect Dis*, 68 (2010) 307.

11 Zhang L, An R, Wang J, Sun N, Zhang S, Hu J & Kuai J, Isolation of novel bioactive compounds from marine microbes. *Curr Opin Microbiol*, 8 (2005) 525.

12 Mihalik K, Chung SH, Crixell RJ & McLean DA, Quorum sensing modulators of Pseudomonas aeruginosa characterized in *Camellia sinensis*. *Asian J Trad Med*, 3 (2008) 12.

13 Hussaini A & Mahasneh M, Microbial Growth and Quorum Sensing Antagonist Activities of Herbal Plants Extracts. *Molecules*, 14 (2009) 3425.

14 Ren D, Sims JJ & Wood TK, Inhibition of biofilm formation and swarming of *Escherichia coli* by (5Z)4-bromo-5-(bromomethylene)-3-butyl-2(5H)- furanone. *Environ Microbiol*, 3 (2001) 731.

15 Pripp AH, Docking and virtual screening of ACE inhibitory dipeptides. *Eur Food Res Technol*, 225 (2007) 589.

16 Ekins S, Mestres J & Testa B, *In silico* pharmacology for drug discovery: Methods for virtual ligand screening and profiling. *Brit J Pharmacol*, 152 (2007) 9.

17 Trott O & Olson AJ, AutodockVina: improving the speed and accuracy of docking with a new scoring function, efficient optimization and multithreading. *J Comput Chem*, 31 (2010) 455.

18 Gowri Shankar K, Albin Fleming T, Vidhya & Namrata Pradhan R, Synergistic efficacy of three plant extracts, *Bergeniaciliata*, *Acoruscalamus* and *Dioscorea bulbifera* L. for antimicrobial activity. *Int J Pharm Bio Sci*, 7 (2016) 619.

19 National Committee for Clinical Laboratory Standards. *Methods for Disk Susceptibility Tests for Bacteria that Grow Aerobically*. (NCCLS M2-A7. NCCLS: Wayne; PA, USA), 45 (2003) 7.

20 Lowry OH, Rosenbrough NJ, Farr AL & Randall RJ, Protein measurement with the folin phenol reagent. *J Biol Chem*, 193 (1951) 265.

21 Ahumedo M, Drosos JC & Vivas-Reyes R, Application of molecular docking and ONIOM methods for the description of interactions between anti-quorum sensing active (AHL) analogues and the *Pseudomonas aeruginosa* LasR binding site. *Mol Biosyst*, 10 (2014) 1162.

22 Aditya G & Ajay S, *In silico* interaction of rutin with some immunomodulatory targets;a docking analysis. *Indian J Biochem Biophys*, 55 (2018) 88.

23 Prabhakar MM, Manoharan S, Ignacimuthu S & Stalin A, *In silico* docking analysis to explore the proapoptotic and anti cell proliferative potential of ferulic acid. *Indian J Biochem Biophys*, 53 (2016) 17.

24 Namasivayam SKR & Vivek J, Screening of quorum sensing (qs) modulatory effect of medicinal plant extracts against quorum sensing mediated virulence factors of human pathogenic gram negative bacteria. *Int J Pharm Phytochem Res*, 8 (2016) 263.

25 Namasivayam SKR & Roy EA, Biofilm effect of medicinal plant extracts against clinical isolate of biofilm of *Escherichia coli*. *Int J Pharm Pharm Sci*, 5 (2013) 486.

26 Hazan Z, Zumeris J, Jacob H, Raskin H, Kratysh G & Vishnia M, Effective prevention of microbial biofilm formation on medical devices by low-energy surface acoustic waves. *Antimicrob Agents Chemother*, 50 (2006) 4144.

27 Thiba K, Wai F.Y & Kok G.C, Inhibition of Quorum Sensing-Controlled Virulence Factor Production in Pseudomonas aeruginosa PAO1 by Ayurveda Spice Clove (*Syzygium Aromaticum*) *Bud Extract Sensors* (*Basel*), 12 (2012) 4016.

28 Barbosa da Silva AC, Romário da Silva D, De Macêdo A, Ferreira S, Agripino GG, Albuquerque T & Gaudencio R, *In silico* approach for the identification of potential targets and specific antimicrobials for *Streptococcus mutans*. *Adv Biosci Biotechnol*, 5 (2014) 373.

29 Aliyu A.B, Koorbanally N.A, Moodley B, Singh P & Chenia HY, Quorum sensing inhibitory potential and molecular docking studies of sesquiterpene lactones from *Vernonia blumeoides*. *Phytochemistry*, 126 (2016) 23.

30 Bottomley MJ, Muraglia E, Bazzo R, & Carfì A, Molecular insights into quorum sensing in the human pathogen Pseudomonas aeruginosa from the structure of the virulence regulator LasR bound to its autoinducer. *J Biol Chem*, 28 (2007) 13592.

31 Chen G, Swem LR, Swem DL, O'Loughlin CT, Jeffrey PD, Bassler BL & Hughson FM, A strategy for antagonizing quorum sensing. *Mol Cell*, 42 (2011) 199.

32 Vannini A, Volpari C, Gargioli E, Muraglia R, Cortese R, De Francesco P, Neddermann & Marco SD, The crystal structure of the quorum sensing protein TraR bound to its autoinducer and target DNA. *EMBO J*, 21 (2002) 4393.