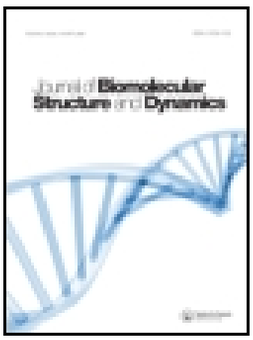

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Antioxidant, anti-quorum sensing, biofilm inhibitory activities and chemical composition of Patchouli essential oil: *in vitro* and *in silico* approach

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ABSTRACT

The interest in naturally occurring essential oils from medicinal plants has increased extremely over the last decade markedly because they possess antimicrobial and antioxidant protective properties against different chronic diseases. Extensive survival of drug-resistant infectious bacteria depends on quorum sensing (QS) signaling network which raises the need for alternative antibacterial compounds. The aim of this study was to examine the phytochemical compounds of patchouli essential oil (PEO) and to assess its antioxidant activity. Antioxidant studies estimated by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method showed that the PEO has effective antioxidant activity (IC_{50} 19.53 µg/mL). QS inhibitory activity of PEO was examined by employing the biosensor strain, *Chromobacterium violaceum* CV12472. At sub-lethal concentrations, PEO potentially reduced the QS regulated violacein synthesis in CV12472 without inhibiting its cell proliferation. Moreover, it also effectively reduced the production of some QS regulated virulence factors and biofilm development in *P. aeruginosa* PAO1 without hindering its growth. Phytochemical analysis of PEO was done by GC/MS technique. Molecular docking of PEO major compounds with QS (LasR and FabI) and biofilm regulator proteins (MvfR and Sialidase) of PAO1 was evaluated. These phytocompounds showed potential hydrogen binding interactions with these proteins. The overall results, *in vitro* and *in silico*, suggest that PEO could be applied as biocontrol agent against antibiotic resistance pathogens.

Abbreviations: ACP: acyl carrier protein; BHT: butylated hydroxytoluene; CFS: cell-free supernatant; CLSI: Clinical and Laboratory Standards Institute; DMSO: dimethyl sulfoxide; DPPH: 2, 2-diphenyl-1-picrylhydrazyl; ECR: E: lastin-Congo red; EO: essential oils; EPS: exopolysaccharide; GC/MS: gas chromatography mass spectrometry; H_2O_2: hydrogen peroxide; HCl: hydrochloric acid; IFD: Induced Fit Docking; LB: Luria Bertani; MIC: minimum inhibitory concentration; MOPS: 3-(N-morpholino) propanesulfonic acid; NIST: National Institute of Standards and Technology; OPLS: optimized potentials for liquid simulations; PBS: phosphate-buffered saline; PDB: Protein Data Bank; PEO: Patchouli essential oil; PQS: Pseudomonas quinolone signal; QGP: 4′-*O*-β-D glucopyranoside; QS: quorum sensing; ROS: reactive oxygen species; RT: room temperature; SP: Standard Precision; TCA: trichloroacetic acid; XP: Extra Precision

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Pogostemon cablin; Patchouli essential oil; antioxidant; quorum sensing inhibition; anti-biofilm; GC-MS analysis; molecular docking

1. Introduction

Essential oils (EOs) and their bioactive compounds have been widely used in several cosmetics, pharmaceutical and food industries due to their numerous biological activities (Burt, 2004; Salehi et al., 2018; Sharifi-Rad et al., 2018; Stojanović-Radić et al., 2016). EOs could be potential natural non-toxic antimicrobial compounds with better safety profiles. Due to increase in emergence of resistance to currently available antibiotics, medicinal phyto-chemicals are being recognized as a suitable alternative for the impediment of pathogenic bacterial growth (Salehi, Albaykar, et al., 2018; Sharifi-Rad, Tayeboon, et al., 2018; Szabó et al., 2010; Yap et al., 2014). Moreover, reports from the last decade show that plant based phytochemicals have promising antioxidant activity which can be explored to prevention of neurological and inflammatory diseases besides chronic bacterial infections (Sharifi-Rad, Sharifi-Rad, et al., 2018). Antioxidant compounds can reduce oxidative stress and prevent the development of diseases caused by excessive accumulation of reactive oxygen species and other free radicals that lead to the oxidation of important biological macro molecules thereby affecting the overall pathophysiology and metabolic pathways (Oliveira et al., 2016; Saeed et al., 2012).

Patchouli [*Pogostemoncablin* (Blanco) Benth.] plant, native of Asian tropical regions, is commonly found in Thailand, Philippines, China, Malaysia, India and Philippines. The dry

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leaves of patchouli on steam distillation yield aromatic oil called Patchouli Essential Oil (PEO). This oil is an important ingredient in several foods and fragrance products like perfumes, cosmetic products and soaps. In addition, the dried aerial part of this plant has been applied to treat gastrointestinal diseases since a long time in the aforementioned Asian regions. Numerous studies report that Patchouli extracts have antioxidant, antimicrobial, antibiofilm, anti-inflammatory, gastro-protective, and antiemetic properties (Farisa Banu et al., 2018; Nithyanand et al., 2015; Swamy & Sinniah, 2015).

The pathogenicity of chronic bacterial infections is mainly command by quorum sensing (QS), a complex and cell-density dependent signaling network which is a crucial etiological agent role in the colonization of the pathogen and invasion in the deeper tissues (Bridges & Bassler, 2019; De Kievit & Iglewski, 2000). Inhibition or disruption of the QS network is a novel strategy for warfare against multidrug-resistant pathogens (D'Angelo et al., 2018; Defoirdt et al., 2012; Tan et al., 2013). Till date, several medicinal plant extracts and EOs have been reported as QSIs (QS Inhibitors), since the resemblance in their structures with QS autoinducer signaling molecules (such as AHL, N-acyl homoserine lactone) and/or their potential to denatured QS signal receptors (such as LuxR/LasR) (Kalia et al., 2019; Poli et al., 2018; Ravichandran et al., 2018).

Pseudomonas aeruginosa, an opportunistic nosocomial pathogen, is frequently trigger fatal illness in immunocompromised patients (Gellatly & Hancock, 2013; Massai et al., 2019). Moreover, this bacterium is also involved in severe acute and chronic infections at different sites within the host body *viz*. skin (burn wounds), urinary tract and the respiratory system etc. with high mortality rate. In *P. aeruginosa* three different inter-linked QS signaling networks *viz*. Las, Rhl, and Pseudomonas quinolone signal (PQS) regulates the establishment of several life threatening infections (Lee & Zhang, 2015). In previous studies it is reported that *P. aeruginosa* defective in these signaling pathways are less virulent and produce antibiotic sensitive biofilm (D'Angelo et al., 2018; Paczkowski et al., 2017; Rajkumari et al., 2019; van Gennip et al., 2009).

Though numerous studies have reported QS inhibitory and anti-biofilm compounds from medicinal plants EOs and their extracts, many dietary sources still remain to be explored. Based on this information, the purpose of this study was to assess the aforesaid biological activities of PEO which broadens its pharmacological prospects.

2. Materials and methods

2.1. Patchouli essential oil (PEO)

PEO was acquired commercially from a local company (Kelkar Foods and Fragrances, Pune). It was stored in refrigerator at 4 °C in absence of light. All the biological experiments mentioned in this manuscript were conducted in a Luria Bertani (LB, Himedia laboratories, India) medium which was supplemented with different concentrations of PEO with 1% Tween 80 in order to increase oil solubility.

2.2. Antioxidant assay

The free radical scavenging activity of PEO was assessed by DPPH assay (Brand-Williams et al., 1995). Two-fold serial dilutions of the PEO samples (0.65–80 μg/mL) were assayed with DPPH methanol solution (100 μM). After 0.5 h incubation, the absorbance at 517 nm was recorded spectrophotometrically using methanol as a blank. The assay was performed in triplicate. Butylated hydroxytoluene (BHT), ascorbic acid and quercetin were used as positive control, whereas DPPH solution as negative control. DPPH IC_{50} was calculated and defined as the concentration of sample required to reduce DPPH activity by 50%. This parameter generally used to measure the antioxidant ability of sample. The antioxidant activity was calculated as a % inhibition using the following equation:

$$(\%) \;=\; [(\mathrm{Ac} - \mathrm{As}) * 100]/\mathrm{Ac},$$

where Ac and As is the absorbance of the control and sample, respectively.

2.3. Bacterial strains and culture media

P. aeruginosa PAO1 and *Chromobacterium violaceum* 12472 were purchased from Chandigarh and Pune, India, respectively. Both the cultures were regularly grown in LB broth with 120 rpm at 37 °C, except *C. violaceum* which was grown at 30 °C. Stock solution of both the cultures was kept at –80 °C in 50% (v/v) glycerol. Experiment tests were carried out in triplicates.

2.4. Determination of antibacterial activity of PEO

The MIC of PEO against mentioned strains was analysed using broth micro dilutions method (Clinical and Laboratory Standards Institute (CLSI), 2018). Two fold serial dilutions of PEO (from 10% to 0.04% v/v) were prepared in a 96-well plate (100 μL per well). Diluted bacterial suspensions were added to the wells, resulting in 1×10^7 CFU mL^{-1} concentration. Wells without PEO and bacteria were used as a positive and negative growth control, respectively. The 96-well plates were further incubated for 20 h and the bacterial growth was checked visually. The MIC is the lowest concentration of tested oil without visible bacterial growth. Further experiments were done at sub-MICs of PEO.

2.5. Growth assay

The effect of sub-MIC of PEO on bacterial cell proliferation of PAO1 and CV12472 was continuously evaluated over a period of 22 h by using growth assay method (Shah et al., 2019). Overnight grown cultures (OD600 = 0.05) were inoculated into LB broth medium supplemented with and without PEO (1.25% v/v). These assay flasks were incubated at aforementioned temperature in a lab shaker at 120 rpm. Then, the growth rate for both the cultures, treated and untreated, was recorded at OD_{600}.

2.6. QS-inhibitory activity in C. violaceum 12472

QS-inhibitory activity of PEO was determined by evaluating violacein inhibition in *C. violaceum* 12472 (Choo et al., 2006). Briefly, culture was grown with and without sub-MICs of PEO at 30 °C for 24 h. Next day, the culture was centrifuged (for 20 min at 7000 *g*) to precipitate the insoluble violacein. Then, DMSO (dimethyl sulfoxide) was added to this pellet to solubilize the violacein. The reaction sample was centrifuged (7000 g for 15 min) and the absorbance was recorded at 585 nm. The percentage inhibition in violacein production was quantified by the following formula:

$$\text{Violacein inhibition } (\%) = (OD_{585}\text{of the control} - OD_{585}\text{ of the treated sample})/(OD_{585}\text{of the control}) \times 100$$

2.7. Evaluation of anti-QS activity of PEO against PAO1

2.7.1. Pyocyanin assay

Production of pyocyanin, one of the many toxins produced by *P. aeruginosa*,was measuredas described by Imperi et al. (2013) method. *P. aeruginosa* PAO1 was grown with and without PEO and then incubated at for 20 h at 37 °C. After incubation, cultures were centrifuged at 8,000 *g* for 15 min at 4 °C. Obtained cell-free supernatant (CFS) were used for pyocyanin measurement. This CSF was mixed with 3 parts of chloroform and then mixed with hydrochloric acid (HCl, 0.2 M). Absorbance of the obtained solution was recorded at 520 nm. The percentage inhibition, if any, in pyocyanin production was calculated.

2.7.2. Elastase assay

Activity of elastase, a major virulence factor secreted by *P. aeruginosa*, was estimated using Elastin-Congo red (ECR) method earlier reported (Shah et al., 2019). Briefly, ECR dissolved in Tris-HCl buffer (pH 7.5) at a concentration of 10 mg/mL was mixed with same volume of CSF of PAO1 grown with and without PEO. Further, this reaction solution was incubated at 37 °C for 4 h and then centrifuge for 20 min at 7,000 *g* at 4 °C. The absorbance at 495 nm of the obtained supernatant is measure. The percent reduction in elastase activity, if any, with respect to control was then calculated.

2.7.3. Protease assay

The total protease activity of PAO1 CFS (both treated and untreated with PEO) was determined according to Kalia et al. (2015). In brief, 500 µL of CFS were mixed with 500 µL of azocasein (0.5% azocasein in 0.1 M Tris buffer) and the assay tubes were incubated for another 1 h at 37 °C. One millilitre of cold TCA (trichloroacetic acid) was mixed to terminate the reaction. The tubes were centrifuged for 15 min at 8000 *g*. Then, 1 M of NaOH was mixed to the obtained supernatant. Absorbance of the solution was measured at 420 nm for protease activity.

2.7.4. Swarming motility assay

This assay was carried out as reported by Shah et al. (2019). An overnight grown PAO1 culture was spot inoculated onto swarming agar plates supplemented with and without sub-MICs PEO. All the assay plates were incubated at above mentioned temperature. Next day, the reduction in swarming motility, if any, were recorded. Assay plates without PEO were reported as positive control.

2.7.5. Exopolysaccharide (EPS) assay

Bacterial biofilm is made up of self-produced EPS substances which play a key role in biofilm formation. PAO1 biofilm EPS, in the absence and presence of sub-MICs PEO, was quantified by the method of Banu et al. (2017). PAO1 culture was grown (with and without) sub-MICs of PEO and incubated for 20 h at static condition. Next day, the bacterial culture was centrifuged and the cell pellet was washed with 500 µl of NaCl (0.85%). To this suspension an equal amount of phenol (5%) was added and later five volume of concentrated H_2SO_4 was also added. The suspension tubes were incubated in the dark for 0.5 h. Tubes were then centrifuged at 8,000 *g* for 20 min. Absorbance of the obtained suspension was recorded at 490 nm.

2.8. Biofilm inhibitory activity of PEO

2.8.1. Crystal violet (CV) assay

PAO1 biofilm assay was carried out in 96-well polystyrene round bottom microwell plates (Shah et al., 2019). Overnight culture of PAO1 ($OD_{600} = 0.1$) was inoculated with and without sub-MICs PEO in sterile LB broth (total volume 200 µL) for 20 h at 37 °C without shaking. To enumerate PAO1 biofilm formation, bacterial cells were washed with PBS (phosphate-buffered saline) buffer to wash out all non-adherent cells. Adhare cells means biofilm was then stained with 0.1% aqueous CV solution for 20 min at RT. After this, the plates were rinsed two to three times with PBS buffer. Surface associated CV dye was then solubilized with ethanol. The percent inhibition of biofilm formation, if any, was evaluatedd using the following formula.

$$\text{Inhibition of biofilm formation } (\%) = [(OD_{570}\text{of U} - OD_{570}\text{of T})/OD_{570}\text{of U}] \times 100,$$

Where U is untreated and T is treated sample.

2.8.2. Microscopy assay

2.8.2.1. Light microscopy. Biofilms of PAO1 were allowed to form, with and without PEO, on 1 cm x 1 cm sterile cover slips which were placed into the 24-well titer plates. Plates were then incubated at 37 °C for 20 h at static condition. Next day, the cover slips with biofilm was stained using 0.1% CV dye solution for 5–10 min. After this, the cover slips were mildly washed with sterile miliQ water and allowed to dry for 10–15 min at RT. These stained cover slips were then monitored under light microscope (Nikon Eclipse Ti 100, Japan) (Banu et al., 2017).

2.8.2.2. Fluorescence microscopy. Biofilms of PAO1 were allowed to formed as mentioned in above part (Shah et al.,

2019). After incubation, the cover slips were gently washed with sterile MOPS (3-(N-morpholino) propanesulfonic acid) buffer to remove non-adherent bacteria from the cover slips. Cover slips were then dried at RT and stained with acridine orange. These stained cover slips were then monitored under fluorescence microscope (Olympus CX41, Hamburg, Germany).

2.9. Chemical analysis of PEO using GC-MS

GC-MS analysis was carried out by using Agilent 5977B GC/MSD system (Agilent Technologies, USA) equipped with Agilent 19091S-433 column (30 μm x 250 μm x 0.25 μm) to identify the components of patchouli essential oil (Adhavan et al., 2017). Temperature of the oven was encoded from 50 to 280 °C at 5 °C/min. Injector temperature was maintained at 250 °C and helium gas was used as carrier throughout analysis. The ionization source used was electron ionization at fixed electron energy of 70 eV. Identification of the components was ascertained based on their retention indices and their mass spectra by comparison with fragmentation pattern stowed in MS database of National Institute of Standards and Technology (NIST).

2.10. In silico docking analysis

2.10.1. Ligand structure retrieval and its preparation

As patchouli alcohol (patchoulol) (39.51%), δ-guaiene (15.7%) and α-guaiene (12.54%) are found to be a major component of PEO, we envisage that it might interfere with the QS and biofilm mechanism in *P. aeruginosa*. The 2 dimensional (2 D) chemical structures of patchouli alcohol (PubChem CID: 10955174), δ-guaiene (CID: 94275) and α-guaiene (CID: 5317844) were retrieved from PubChem database (Kim et al., 2019). These 2 D structures were subjected to appropriate bond order assignment and energy minimization with Optimized Potentials for Liquid Simulations (OPLS-2005) force field using the LigPrep (LigPrep: Schrödinger Release 2019-2) module of Schrodinger to get energetically stable confirmation.

2.10.2. Protein crystal structure preparation

Three-dimensional (3 D) crystal structures of *Pseudomonas aeruginosa* proteins such as LasR (PDB ID: 3IX3), MvfR (PDB ID: 4JVD), Sialidase (PDB ID: 2W38) and FabI (4NR0) was retrieved from RCSB Protein Data Bank (PDB) http://www.rcsb.org (Berman et al., 2002) with resolutions of 1.4 Å, 2.95 Å, 1.9 Åand 1.7 Å, respectively. Before performing the docking calculations, the crystal structures of LasR, MvfR, Sialidase, and FabI were prepared at pH 7.3, 6.0, 7.4 and 5.0, respectively by PROPKA program using protein preparation wizard of Schrodinger (Protein Preparation Wizard: Schrödinger Release 2019-2).The OPLS-2005 force field was used to optimize the crystal structure and the side chains atoms.

2.10.3. Glide SP, XP and induced fit docking

The binding cavities of targeted receptors were defined considering all residues which comes within the cut off distance of 5 Å from the crystal ligands which are present in retrieved crystal structures of LasR, MvfR, Sialidase and FabI receptors and also, the binding cavity information for these receptors was taken from PDBsum (Laskowski, 2009). The prepared ligand (patchouli alcohol, δ-guaiene and α-guaiene) were subjected to docking calculations within the defined binding cavity using the Standard Precision (SP) and Extra Precision (XP) mode of Glide program (Glide: Schrödinger Release 2019-2) with the OPLS-2005 force field. In the case of the LasR receptor, due to, a small binding cavity of the LasR receptor and complex structure of the patchouli alcohol, it fails to dock into the binding cavity using SP and XP mode of Glide. After that, patchouli alcohol subjected to Induced Fit Docking (IFD) using Glide and Prime (Prime: Schrödinger Release 2019-2) module of Schrodinger. The IFD program keeps binding pocket amino acid residues flexible within cut off distance of 5 Å from the crystal ligand to generate receptor conformations similar to the size and conformation of ligand. IFD program is able to do robust and minor conformational modification in the side chains of amino acid residues and backbone respectively in the binding pocket.

2.11. Statistical analysis

All the experiments were performed in triplicate. The significance differences in the average values between control and treated samples were evaluated using Student's test. ANOVA was performed for the analysis of multiple means (http://www.physics.csbsju.edu/stats/anova.html). Values of $p < 0.05$ and $p < 0.01$ were considered statistically significant.

3. Results and discussion

3.1. Antioxidant activity with DPPH assay

Over the last few years, the research of free radicals and reactive oxygen species (ROS) in biology has been accelerated remarkably because of their crucial role in disease mechanisms. It is strange that oxygen, a chemical element vital for life, under certain conditions has detrimental side effects on the human health (Lobo et al., 2010). Antioxidants are compounds which fight against these free radicals and protect us from various diseases such as inflammatory and neurological diseases, certain cancers, acute respiratory diseases syndrome, ischemic diseases, chronic obstructive pulmonary disease, as well as the process of aging. Antioxidant agents exert their action either by scavenging the ROS to reduce oxidative stress and to hinder the progression of diseases (Burt, 2004; Popović-Djordjević et al., 2019). Synthetic antioxidants have been reported to be hazardous for human health. EOs have been noted as natural, nontoxic and safe antioxidants due to their potential to reduce/scavenge these free radicals and their formation. In order to achieve this, the antioxidant potential of PEO was measured using DPPH test. DPPH (deep violet colour stable free radical) get reduced in the presence of antioxidant molecule and become colourless. The level of discoloration suggested the free radical scavenging

Table 1. Antioxidant activity of PEO by DPPH assay.

IC_{50} (μg/mL)				
PEO	Ascorbic acid[a]	Gallic acid[a]	Quercetin[a]	BHT[a]
19.53 ± 0.15	5.42 ± 0.21	7.335 ± 0.19	8.083 ± 0.3	12.655 ± 0.32

PEO, Patchouli essential oil; BHT, butylated hydroxytoluene.
Each value is an average of triplicate determination; ± standard deviation.
[a]Standard antioxidants.

potential of the test oil. Antioxidant potential of the PEO was evaluated as IC_{50} values. The present work showed that PEO exhibited DPPH scavenging activity at a concentration dependent manner with IC_{50} of 19.53 ± 0.15 μg/mL (Table 1). These results are consistent with the earlier reports (Dechayont et al., 2017; Luo et al., 2019; Manoj et al., 2011). A number of previously published reports showed that oxygenated monoterpenes and sesquiterpenes such as carvacol, thymol, limonene, and/or linalool etc. are responsible for the antioxidant activity of plant EOs (Barbieri et al., 2016; Miguel, 2010; Wei & Shibamoto, 2007). *P. cablin* EO is rich in sesquiterpenes, mainly the patchouli alcohol (patchoulol), a tricyclic sesquiterpene plus mixture of monoterpenoids, triterpenoids, phytosterols and flavonoids (Swamy & Sinniah, 2015). In addition to these, *P. cablin* potentially protected human neuroglioma cell line A172 against both necrotic and apoptotic cell death induced by H_2O_2 (hydrogen peroxide), recommend its application for treating neurodegenerative disorders (Kim et al., 2010). These findings suggest that aromatic plants are excellent sources of antioxidant compounds to scavenge the potential damage. In this sense, PEO can be a potential natural antioxidant supply for nutraceutical and/or cosmeceutical.

3.2. Antibacterial activity of PEO

Multidrug resistant strains are a substantial public health concern, thus, there is a worldwide demand to introduce non-toxic, natural and safe biological compounds for combating bacterial infections. Therefore, we examined the antibacterial potential of PEO against a nosocomial pathogen *P. aeruginosa* and QS biosensor strain *C. violaceum* 12742. The lowest tested concentration of the PEO which inhibit the visible growth of bacterial strains, was termed as MIC. The MIC of PEO against tested strains was 2.5% and sub-MIC was fixed at 0.31, 0.62 and 1.25%. Previous studies reported the presence of antimicrobial phytocompounds in PEO, therefore, it can be applicable in the treatment of antibiotic resistance microbial infections (Farisa Banu et al., 2018; Swamy & Sinniah, 2015; Yang et al., 2013). The obtained results were agreeing with the study of (Kalia et al., 2015), who reported antimicrobial activity of cinnamon oil against PAO1 and *C. violaceum*. EOs contain wide range of phytochemicals and it is believed that these compounds possess a strong binding affinity to different molecular structure of bacterial cell components, leading to the leakage of cell contents (Yap et al., 2014).

3.3. Growth assay

This assay was done in order to check that the obtained QS-inhibitory and biofilm reduction activities of PEO was not because of their antibacterial activity. For this, PAO1 and CV12472 growth curve was done in presence and absence of PEO (1.25% v/v).The obtained results (Figure 1) showed that the bacterial growth did not differ between control and treated cultures with PEO.

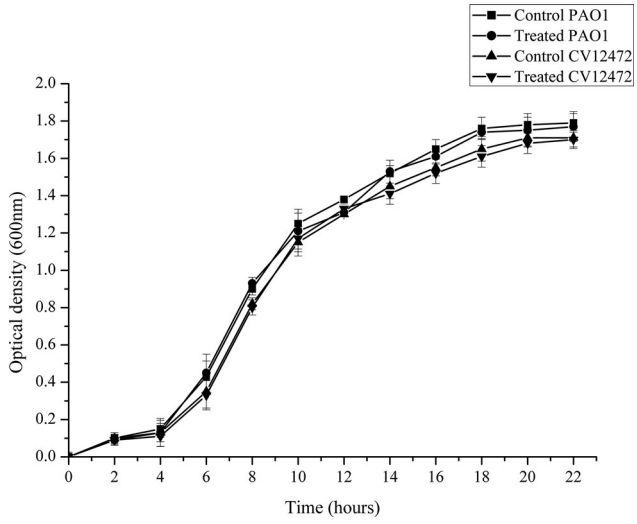

Figure 1. Growth curve of *P. aeruginosa* and *C. violaceum* with and without sub-MICs of PEO.

3.4. QS-inhibitory activity of PEO in C. violaceum 12472

Several biosensor stains have been implied for the screening of potential anti-QS activities (Rampioni et al., 2014). One of the biosensor strains is *C. violaceum* 12472. It is a Gram-negative bacterium produces violacein, a purple pigment that is responding to its C_6-AHL autoinducer molecule which is synthesized by its synthase CviI. This molecule binds and from a complex to CviR, its specific receptor protein, which activates the expression of violacein production (Stauff & Bassler, 2011). The reduction in of this pigment in this strain is generally used for anti-QS analysis (Ravichandran et al., 2018). Quantitative analysis of violacein reduction in this bacterium at tested sub-MICs of PEO is shown in Table 2. Inhibition of violacein is in a concentration-dependent manner. Maximum inhibition (94.37 ± 2.0%) was seen at 1.25% (v/v) of PEO. This validated the inhibitory effect of sub-inhibitory concentrations of PEO on QS-controlled violacein synthesis in *C. violaceum* 12472. Our results corroborate with previous report where eucalypt EOs (*Eucalyptus globulus* and *E. radiata*) significantly reducedproduction of violacein by 98.81% at a concentration of 5 μl/mL (v/v) (Luís et al., 2016). Similarly other natural products such as dietary phytochemicals (apigenin, curcumin, gallic acid, quercetin, luteolin and pyrogallol) (Bali et al., 2019), cinnamic acid (Rajkumari et al., 2018) and oleoropein glucoside, caffeic acid, and ferulic acid (Borges et al., 2014) are reported to inhibit QS .

3.5. Effect on QS-controlled virulence factors in PAO1

QS signaling network is one of a major regulator which effectively control the expression of several virulence genes in nosocomial pathogen *P. aeruginosa*. The secretion of the

Table 2. Effect of sub-MICs concentrations of PEO on violacein in CV12472 and different virulence factors in *P. aeruginosa* PAO1.

PEO (% v/v)	Violacein	Pyocyanin	Protease	Elastase	EPS	Swarming motility	Biofilm
0.00	–	–	–	–	–	–	–
0.31	48.91 ± 2.33	37.46 ± 5.87*	41.63 ± 3.22*	39.00 ± 5.13*	30.45 ± 4.44*	40.48 ± 3.15**	57.89 ± 4.12*
0.62	69.23 ± 4.18*	55.92 ± 6.99*	63.40 ± 3.61*	59.86 ± 5.97*	44.19 ± 4.38*	80.92 ± 5.10**	79.34 ± 3.58
1.25	94.37 ± 2.00**	85.18 ± 5.74*	76.49 ± 2.30*	70.13 ± 2.40*	63.46 ± 3.54*	90.00 ± 4.21**	88.13 ± 5.70*

The data represents mean values of three independent experiments. * indicates $p \leq 0.05$ and ** indicates $p \leq 0.01$ with respect to untreated sample.

majority of *P. aeruginosa* virulence factors is regulated by three inter-linked QS signaling pathwas, such as *las* and *rhl* pathways that use AHL and quinolone-based *pqs* pathway that uses 2-alkyl-4-quinolones (AQs) as QS signaling molecules. These QS signaling pathways regulate the transcription of genes coding for exotoxins, lectins, exoenzymes, other virulence factors (e.g. hydrogen cyanide, pyocyanin, pyoverdine, pyochelin, rhamnolipids) and antibiotic resistance biofilm development (Lee & Zhang, 2015; Williams & Cámara, 2009). *P. aeruginosa* strains defective in QS signaling pathways are potentially attenuated in animal infection models (van Gennip et al., 2009).

3.5.1. Pyocyanin inhibition

Pyocyanin, QS-regulated blue-green colored phenazine cytotoxic pigment, in PAO1 plays a key role during pathogenesis mainly in cystic fibrosis. Moreover, this pigment generates ROS by oxidizing reduced glutathione in host cells which is in correlation with severity of infection (Dietrich et al., 2006). Therefore, the inhibition of pyocyanin production is significant from the perspective of disease progression. The effects of sub-MICs of PEO on the production of pyocyanin in PAO1 were investigated. The obtained results indicate that the extent of pyocyanin production reduced with increasing concentrations of PEO, showing a maximum reduction of about 85.18 ± 5.74% at 1.25% (v/v) of PEO compared with the control, as shown in Table 2. The inhibition of QS controlled pyocyanin production in PAO1 suggested the efficacy of PEO in preventing the action of bacterial virulence factors during host infection. *Murraya koenigii* essential oil is reported to inhibit the production of PAO1 pyocyanin (Ganesh & Rai, 2016). Al-Yousef et al. (2017) demonstrated that quercetin 4′-*O*-β-D glucopyranoside (QGP) isolated from onion peel ethyl acetate fraction significantly reduced the production of pyocyanin in *P. aeruginosa* PAO1 and PAF79. Furthermore, EOs of *Cinnamomum tamala* were also reported to reduce the production of pyocyanin in *P. aeruginosa* PAO1 and in two clinical isolates of *P. aeruginosa* (AU07 and AU09) (Banu et al., 2017).

3.5.2. Protease inhibition

P. aeruginosa is known to produce a battery of QS regulated extracellular protease enzymes, *viz* alkaline protease and protease IV which are associated with the pathogenesis of this bacterium. These enzymes assist *Pseudomonas* for the colonization of the host tissues which in turn promotes the infection (Winson et al., 1995). Total protease activity was evaluated using azocasein method. In this method, the protease activity of PAO1 cultures grown with and without increasing concentrations of sub-MICs PEO was estimated. A reduction in protease activity of approximately 76.49 ± 2.30% was observed after treatment with 1.25% (v/v) PEO compared with the untreated culture (Table 2). These results are consistent with the observations of Parai et al. (2018) who reported significant decrease of protease production in sub-lethal concentrations of reserpine, a plant derived indole-alkaloid.

3.5.3. Elastase inhibition

Elastase is one of a major virulence factor in *P. aeruginosa,* regulated by the QS signaling pathway, causes extensive host tissue damage and promote bacterial invasion (Dulon et al., 2005). The efficacy of PEO to reduce the elastase activity was measured. A dose dependent reduction in elastase activity was examined on treatment with PEO compared to control (Table 2). PEO reduced elastase activity by 70.13 ± 2.40% at highest tested sub-MIC (1.25%) of PEO. Eugenol, an effective compound found in EOs, has been reported to curb QS regulated virulence in pathogenic bacteria. Rathinam et al. (2017) has shown that sub-MIC level of eugenol reduced significant elastase production in *P. aeruginosa* PAO1 and two clinical isolates of *P. aeruginosa* (RRLP1 and RRLP2). Recently, Al-Yousef et al. (2018) has reported 80% reduction of elastase activity at 3.2% concentration of avocado oil.

3.5.4. EPS reduction

EPS is one of the key virulence features which mostly facilitate the bacterial biofilm development which in turn facilitate the bacterial infection in tissue. EPS protects the bacterial cells by preventing the penetration of medicinal drugs and/or antibiotics in the biofilm. In the present report, PEO potentially inhibited the production of EPS in PAO1 to a significant level (Table 2). EPS content decreased by 63.46 ± 3.54% at a concentration of 1.25% v/v with compared to that of untreated culture. This reduced level of EPS may ultimately allow the penetration of drugs and/or antibiotics into the bacterial cells. Consistent with the present results, Husain et al. (2015) also reported significantly reduction in EPS production in PAO1 by 76% when treated with sub-MIC of *Mentha piperita* essential oil.

3.6. Anti-biofilm activity of PEO

3.6.1. Swarming motility assay

Although regulation of biofilm formation in *P. aeruginosa* largely depends on different environmental signals and EPS production, QS regulation of swarming motility also contributes to its development and formation (Shrout et al., 2006). Swarming motility controls the early adherence of

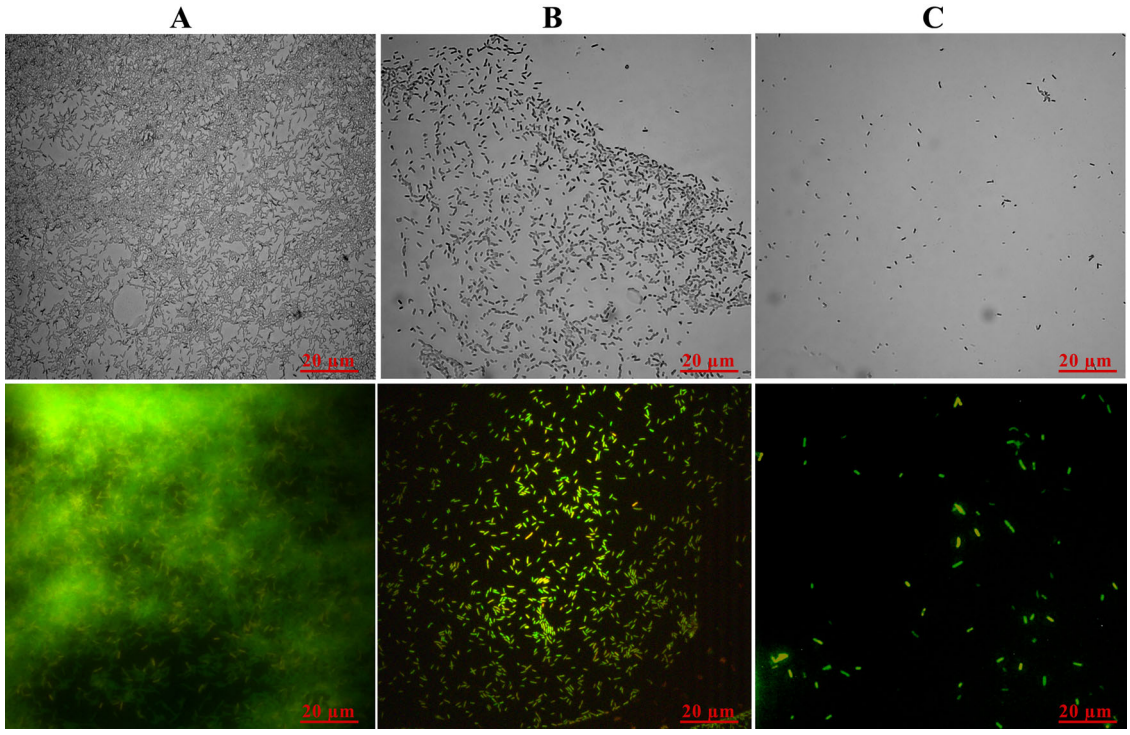

Figure 2. Light (upper panels) and fluorescence (bottom panels) imaging of *P. aeruginosa* biofilms. (a) Untreated, (b) 0.62% of PEO and (c) 1.25% of PEO.

Pseudomonas cells to either biotic or abiotic substratum and also promote infection via biofilm formation (Shah et al., 2019). In this study, the swarming motility of PAO1 was significantly reduced in the presence of sub-MIC concentration of PEO (Table 2). There was 40.48% reduction in swarm area at 0.13% PEO and subsequently reduced by around 90 ± 4.21% at 1.25% concentration. These observations are consistent with earlier results (Kalia et al., 2015), where swarming motility was inhibition at 0.2 µl/mL of cinnamon oil. Similarly, Al-Yousef et al. (2018) and Bali et al. (2019) reported the potential inhibition of swarming motility by avocado oil and curcumin, respectively.

3.6.2. Crystal violet (CV) assay

Conventional CV dye binding method was adopted to test the potential of PEO on PAO1 biofilm formation. PEO was shown to reduce biofilm formation at increasing concentrations without hampering bacterial cell growth, clearly demonstrating its anti-biofilm activity. As shown in Table 2, treatment with 0.31, 0.62 and 1.25% v/v PEO resulted in a biofilm reduction by 57.89 ± 4.12%, 79.34 ± 3.58% and 88.13 ± 5.70%, respectively. Similar observations have been reported by Zhou et al. (2018) where hordenine (phenolic phytochemical from sprouting barley) treatment significantly reduces the biofilm formation of PAO1 compared to control sample.

3.6.3. Microscopic assay

Sub-MIC concentrations of PEO potentially reduced the formation of biofilm which was further confirmed by light and fluorescence microscopic examination. The untreated culture formed a thick dense biofilm or mat on coverslips as observed through light microscope (Figure 2 upper panel). While as in treated culture efficient reduction in biofilm was observed with the deplete of cluster like architecture. Fluorescence microscopy also validate the effect of tested PEO concentrations on biofilm formation ability. It revealed the potency of PEO as an effective biofilm inhibitor. The observed images as shown in Figure 2 (lower panels) of treated cultures exhibited scattered bacterial cells compared to control clusters. Closely packed PAO1 bacterial cell appearing as a thick cluster mat were recorded in untreated sample, clearly suggesting the development of a well-clustered biofilm. The integrity of the observed biofilm in terms of EPS synthesis was also very less in the treated cultures. The observations of Shah et al. (2019) verified that sub-MIC concentrations of green synthesized silver nanoparticles potentially ($p < 0.05$) decreased the biofilm formation of PAO1 compared to the control sample.

3.7. GC-MS analysis of patchouli essential oil

In GC-MS analysis nineteen compounds (signifying 99.99% of the oil) were recognized by the matching process, on the basis of quality of the matching in terms of percentage, following the database of National Institute of Standards and Technology (Table 3). From GC MS analysis report, it can be witnessed that patchouli essential oil consists of a mixture of various chemical compounds. The non-oxygenated compounds form 59.91% and oxygenated compounds form 40.08% of the mixture with patchouli alcohol (patchoulol) 39.51%, δ-guaiene 15.7%, α-guaiene 12.54%, seychellene 7.61%, and methanoazulene 6.16%. These compounds signify the quality of patchouli oil, which provide its essential oil characteristics. Compounds found in the analysis are similar

Table 3. Chemical constituents of patchouli essential oil analysed by GC-MS.

No	Constituents	RT[a]	% Area
1	Caryophyllene	11.125	0.13
2	β-Patchoulene	12.376	2.92
3	β-Elemene	12.658	1.15
4	Cycloseychellene	13.152	0.87
5	Bicyclo[5.2.0]nonane, 2-methylene-4,8,8-trimethyl-4-vinyl	13.427	3.23
6	α-Guaiene	13.94	12.54
7	Seychellene	14.053	7.61
8	α-Patchoulene	14.409	6.16
9	α-Gurjunene	14.503	0.12
10	(-)-isocaryophyllene	14.741	0.44
11	α-Guaiene	14.966	0.78
12	β-Bulnesene	15.379	0.82
13	(-)-α-Selinene	15.567	3.69
14	δ-Guaiene	15.779	15.7
15	(-)-7-epi-α-selinene	16.067	0.34
16	(2R,8R,8aS)-8,8a-Dimethyl-2-(prop-1- en-2-yl)-1,2,3,7,8,8 a-Hexahydronaphthalene	16.461	0.11
17	Caryophyllene oxide	17.875	0.48
18	Patchouli alcohol	20.02	39.51
19	Pogostol	20.978	0.09

[a] Retention time.

Table 4. Molecular docking analysis of the patchouli alcohol, δ-guaiene and α-guaiene as per Glide calculations.

		Docking energy (kcal/mol)		
Compound name	Target name	SP	XP	IFD
Patchouli alcohol	LasR (PDB ID: 3IX3)	Not Docked	Not Docked	−7.385
α-Guaiene		−7.110	−6.755	NA
δ-Guaiene		−7.222	−6.881	NA
Patchouli alcohol	MvfR (PDB ID: 4JVD)	−4.220	−2.486	NA
α-Guaiene		−5.037	−4.249	NA
δ-Guaiene		−5.037	−4.519	NA
Patchouli alcohol	Sialidase (PDB ID: 2W38)	−2.882	−1.272	NA
α-Guaiene		−2.703	−0.632	NA
δ-Guaiene		−3.850	−0.269	NA
Patchouli alcohol	and FabI (PDB ID: 4NR0)	−6.843	−6.289	NA
α-Guaiene		−6.094	−5.121	NA
δ-Guaiene		−6.395	−5.462	NA

SP, Standard Precision; XP, Extra Precision and IFD, Induced Fit Docking.

to those reported by others (Review: van Beek & Joulain, 2018). Patchoulol was identified as the major constituent in the present study and also reported earlier (Adhavan et al., 2017; Verma et al., 2019). The high content of patchouli alcohol in the oil is notable as this oxygenated sesquiterpene alcohol is reported to possess anti-inflammatory, ulcerogenic, tumorigenic, influenza A (H2N2) virus, bacterial and fungal activities (Hu et al., 2017; Jeong et al., 2013; Kiyohara et al., 2012; Wei et al., 2018). Recently, Lian et al. (2019) has shown that patchoulol potentially suppressed intracellular *Helicobcter pylori* urease function and maturation, which increased macrophage digestion ability. Presently, it is widely utilized in different cosmetic goods such as soaps, skin care lotions and creams and also in perfumery products (Swamy & Sinniah, 2015).

3.8. Molecular docking analysis

Recently, most of the reports claiming several synthetic and/or natural compounds to be effective anti-QS drugs but very few reports have focused onto to the computational analysis of these compounds with QS regulatory proteins (Ali et al., 2017; Rajamanikandan et al., 2017; Wang et al., 2018). Keeping in view about the significance of computational docking and no report about PEO major compounds and QS controlled virulence genes we have executed the molecular docking calculations in order to reveal molecular interactions and to estimate the binding affinity of the patchouli alcohol, δ-guaiene and α-guaiene towards LasR, MvfR, Sialidase and FabI binding sites. These sesquiterpene alcohol i.e. patchouli alcohol, carbobicyclic compound, and sesquiterpene i.e. δ-guaiene and α-guaiene were successfully docked into the binding cavity of LasR, MvfR, Sialidase and FabI receptors successfully using SP and XP mode of Glide. The patchouli alcohol was docked into the MvfR, Sialidase, and FabI cavity with SP and XP, but in case of LasR receptor IFD program was used to predict the binding mode of patchouli alcohol with LasR.

The detailed molecular docking analysis of the patchouli alcohol, δ-guaiene and α-guaiene as per Glide calculations reported in Table 4. The results of docking simulations proved that, patchouli alcohol showed greater binding with LasR, Sialidase and FabI receptor with −7.358 kcal/mol, −1.272 kcal/mol and −6.289 kcal/mol (XP score) binding

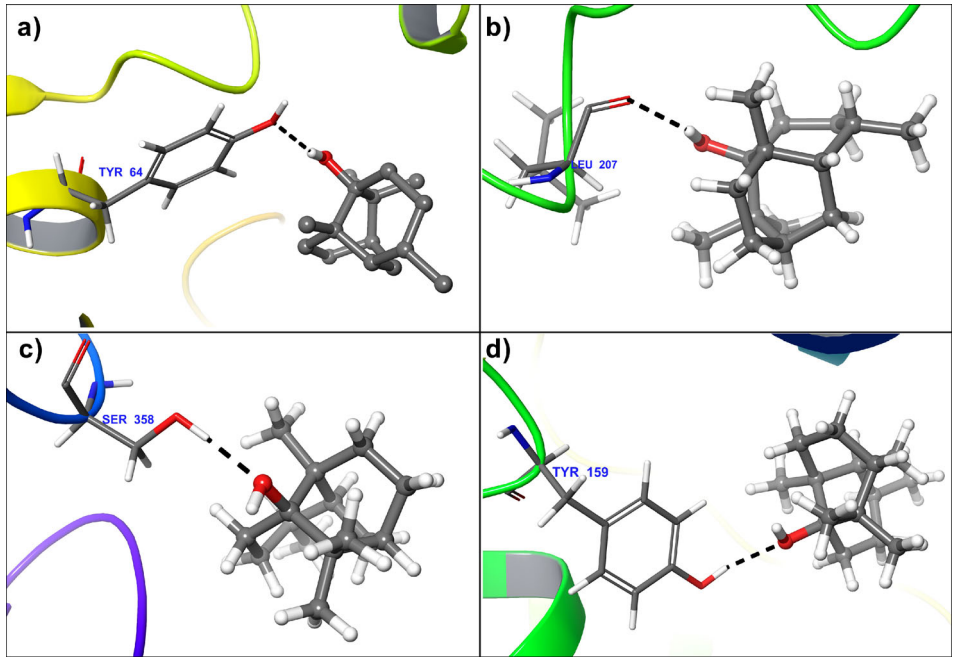

Figure 3. Docking analysis of potential biological targets of patchouli alcohol in *P. aeruginosa* PAO1. The hydrogen bonding from functional group of patchouli alcohol with amino acid residues. (a) Tyr64 of LasR, (b) Leu207 of MvfR, (c) Ser358 of Sialidase and (d) Tyr159 of FabI receptors.

affinities, respectively. On the other hand, δ-guaiene shows higher binding affinity –4.519 towards MvfR as compared to others. The hydroxyl functional group of patchouli alcohol shown to be making direct contact with LasR, MvfR, Sialidase and FabI receptors by forming hydrogen bonds. The hydrogen atom from the hydroxyl functional group of patchouli alcohol forms hydrogen bonding with amino acid residues Tyr64 of LasR (Figure 3(a)) and Leu207 of MvfR (Figure 3(b)) at bond distances of 2.03 Å and 2.04 Å, respectively. The oxygen atom from hydroxyl functional group of patchouli alcohol involved in hydrogen bonding with amino acid residues Ser358 of Sialidase (Figure 3(c)) and Tyr159 of FabI receptors (Figure 3(d)) at1.96 and 2.09 bond distances, respectively. Because of the carbobicyclic structures of δ-guaiene and α-guaiene and not containing any hydrogen bond donor, hydrogen bond acceptor, positively or negatively charged atoms in their structure, these compounds unable to form any type of interactions with LasR, MvfR, Sialidase, and FabI receptors.

From the molecular docking studies, stable binding interactions were observed for PEO compounds with *P. aeruginosa* virulence proteins. LasR in *P. aeruginosa* is a master QS signaling regulator which controls the expression of several virulence factors, such as elastases and proteases (Lee & Zhang, 2015). Moreover, this bacterium possess a type II or dissociated fatty acid synthase system. The final elongation step in fatty acid synthesis is catalysed by FabI enoyl-acyl carrier protein (ACP) reductase enzyme. The levels of QS signaling molecules such as C_4-AHL and N-(3-oxo)-dodecanoyl-AHL were significanty decreased in FabI mutant *P. aeruginosa* (Hoang & Schweizer, 1999). MvfR and sialidase contribute in biofilm formation by *P. aeruginosa* (Déziel et al., 2005; Xu et al., 2009). Since the interaction of PEO compounds with these proteins could be responsible for reduction in virulence factors and biofilm formation in *P. aeruginosa*. Overall, this study predicts that PEO compounds can interfere with the virulence of *P. aeruginosa* and assist to decrease the infection associated circumstance. Nevertheless, the actual activity of these compounds needs to be confirmed by *in vitro* and/or *in vivo* studies. Furthermore, these studies will help in further drug explorations against *P. aeruginosa* and other multidrug resistant bacteria that employ similar cell-cell signaling network.

4. Conclusions

The emergence of multidrug resistance in *P. aeruginosa* attributes a significant threat to human health. Quorum sensing is a signaling communication network in *P. aeruginosa*, which regulates an array of factors such as biofilm formation, and virulence phenotypes, which eventually contribute to drug resistance. Therefore, several targets of QS processes have been exploited to curb this network. In brief, the present study demonstrates that patchouli oil shows a decrease in the expression of QS-regulated phenotypes such as violacein, pyocyanin, elastase and protease production, swarming motility and biofilm formation, in a dose dependent fashion. Moreover, computational docking revealed that key components of this oil can function as a competitive inhibitor for QS signaling molecules towards LasR, MvfR, Sialidase and FabI receptor pathways. The present study appends additional knowledge of its potential antimicrobial and anti QS properties, contributing a novel approach to tackle antibiotic resistant pathogenic bugs in the health sector.

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Disclosure statement

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