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Evaluation of Anti-quorum Sensing Potential of *Saraca asoca* (Family Caesalpiniaceae) against *Chromobacterium violaceum* and *Pseudomonas aeruginosa* PA01

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Authors' contributions

This work was carried out in collaboration among all authors. All authors contributed equally to carry out research work. Author BSP design the work and performed all the experiments. Authors BNS and JM coordinated experiments, analyzed results and wrote the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The present study was performed to evaluate the anti-quorum sensing (QS) potential of traditional medicinal herb *Saracaasoca* (family Caesalpiniaceae) stem bark extract against *Chromobacterium violaceum* and *Pseudomonas aeruginosa* PA01.
Study Design: First, the test sample (bark extract) was screened for anti-QS activity. Then systematic *in-vitro* and biochemical tests were performed to evaluate the effect of the test sample on the QS mediated virulence factors.
Place and Duration of Study: All the experimental works were performed in Lab 311, pharmacology division, CSIR-NBRI Lucknow from June 2019 to October 2019.

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Methodology: The samples of *Saraca asoca* stem bark were washed, dried and extracted using 70% methanol. The minimum inhibitory concentration (MIC) of the prepared *Sarca asoca* bark extract was determined using the Alamar blue assay, and the anti-QS activity was screened using standard agar overlay method against CV 12472 at subinhibitory concentrations 100, 200 and 300 µg (< MIC value). SAE effect on biofilms formation was assessed by growing biofilms on glass slides in a static culture of PA01. Anti-virulence effect of SAE on the production of QS-regulated virulence factors such as Pyocyanin, proteases, elastases, rhamnolipid and alginate in *Pseudomonas aeruginosa* was determined using the supernatant of a 24 hours old broth culture of PA01 supplemented with SAE. Using the agar plate technique, the swimming and swarming motility assays were conducted on 0.3% and 0.5% agar plates respectively. One-way ANOVA was used to analyze the data, presented as mean ± SD (standard deviation) of three independent experiments.

Results: Preliminary screening results showed significant QS inhibition against CV 12472 in an agar overlay disk diffusion assay in a concentration-dependent manner. Data from the biofilm assay showed loose, distorted, irregular PA01 biofilm formation at 200 µg (48%) and 300 µg (65%). SAE caused a significant drop in virulence factor production, with maximum reduction in pyocyanin (58%), proteases (67%), elastases (52%), rhamnolipid (53%), and alginate (44%) observed at 300 µg concentration. At SAE sub-lethal concentrations (200 and 300 µg), both the swimming and swarming motility of PA01 were significantly inhibited.

Conclusions: The present study demonstrates the broad-spectrum anti-QS potential of SAE, reported for the first time, suggesting that SAE could be considered as an alternative herbal source to develop antimicrobial agents which can be either solitary or synergistically with conventional antimicrobial drugs.

Keywords: Anti-quorum sensing; virulence factors; biofilms; P. aeruginosa.

1. INTRODUCTION

In nature, constant interactions occur between lower and higher organisms. To survival, organisms make adaptions, producing unique phenotypic changes by regulating their gene expression according to their interaction with the extrinsic environment [1]. Microorganism such as bacteria develops quorum sensing (QS) as an adaptive response to colonize and coordinate themselves during interaction with higher organisms like plants and animals. When the bacterial population reaches a certain threshold density, this intracellular communication network triggers a change in phenotypic characteristics via the expression of virulence genes. QS mechanism determines the intensity of infection among hosts by producing various virulence factors such as antibiotic-resistant biofilm formation, production of exoenzymes (proteases, elastases) and motility for spreading infection [2-3]. So, inhibition of QS Natural plant products (metabolites) has been well studied in pathogens has been generally considered as a strategic approach to address the global multidrug resistance problem for anti-QS properties. Herbal medicines target QS in various ways due to the complexity of their biochemical composition. Plants produce these metabolites as an evolutionary adaptive response against bacterial infection. Plants modify their DNA/chromatin status to develop phenotypic change via modulating gene expression and transcriptional factors [4]. Therefore the demand for new biologically active plant metabolites/compounds that possess anti-QS activity is constantly increasing.

Saraca asoca (*Roxb*.), an evergreen plant widely found all over the Indian subcontinent, belongs to the family Caesalpiniaceae. Traditional therapeutic practices of the plant have been texted in Charak Samhita (100. AD) [5]. The different parts of *S. asoca* showed diversified biological properties like antibacterial, antipyretic, antihelminthic, anti-inflammatory and analgesic activity [6-7]. Methanolic extract of its bark contains alkaloids, tannin, glycosides, flavonoids and terpenoids [8]. Its bark extract reported potent antibacterial [9], antimutagenic [10] and antigenotoxic properties [11].

Though the plant has been extensively used for indigenous pharmacological purposes, the anti-QS property of bark extract has not been explored yet. Therefore current study evaluates anti-QS property of *S. asoca* bark extract of against biomonitor strain *Chromobacterium violaceum* 12472. Further broad spectrum anti-virulence and antibiofilm activity were explored

against opportunistic human pathogen *P. aeruginosa*.

2. MATERIALS AND METHODS

2.1 Collection of Plant Samples

Samples of *S. asoca* barks (stem) were collected from the garden of CSIR-National botanical research institute, Lucknow, Uttar Pradesh, India.

2.2 Preparation of *S. asoca* Bark Extracts (SAE)

Stem Bark samples of *S. asoca* were washed properly with tap water to remove any mud/dust particles and left to dry in shade. Dried barks were cut into small pieces and grind to powder form in the mixer grinder (Cello GNM Elite Mixer Grinder, India).10 grams of sample powder was mixed with 100 mL of 70% methanolic solvent and kept on a rotator shaker (Scientech SE-140 D.C. Drive, India) at 150 rpm for 24 hours, for extraction of metabolites. Then the mixture was filtered through Whatman filter paper no 1and process was repeated to obtain a clear filtrate. This 70% methanolic filtrate was subjected to evaporation under reduced pressure in a rotatory evaporator sat 50^{o}C. Further remaining moisture was removed by using a lyophilizer under a vacuum evaporator (labconco, FreeZone 2.5 Liter Benchtop Freeze Dryers, Kansas city, USA) at -84^{o}C.

2.3 Bacterial Strains and Culture Maintenance

C. violaceum ATCC 12472 and *P. aeruginosa* (PA01) cultures stock were maintained in nutrient agar slants, stored at 4^{o}C. Experiments were performed using standard cell suspension (SCS, 1x 10^5 cell/mL) of CV12472 and PA01. To prepare SCS, single colony of bacteria picked from overnight grown agar culture plates and dissolved in 0.85% saline and OD_{600} adjusted to 0.5 nm.

2.4 Determination of Minimum Inhibitory Concentration

MIC test was performed against *C. violaceum* and *P. aeruginosa* using standard alamar blue assay. Alamar Blue dye contains an oxidation-reduction (Redox) indicator. Cellular proliferation induces chemical reduction of the media which results in a change in redox color from blue to red [12]. Briefly, 100 µL of autoclaved Luria broth was added to the 96 well microtitre cell culture plates (nunc, Thermo scientific). SCS of bacterial strains CV12472 and PA01 were prepared by overnight grown fresh cultures. 10% v/v of SCS was used to inoculate wells broth media. Each well was supplemented with various concentrations of SAE (100, 150, 200, 250 and 300 µg) and the control well was left untreated. Culture plates (96 well) were left incubated at 37^{o}C for 24 hours. To the well (cultured bacterial cell) 10 µL of alamar blue (invitrogen, DAL1025) was added according to the manufacturer's instructions and kept in dark for 3 hours for redox reaction carried out by live cells in media. Cell viability was determined by taking absorbance 570 nm (reduced form) and 600 nm (oxidized form).[https://www.thermofisher.com/in/en/home/references/protocols/cell-and-tissue-analysis/cell-profilteration-assay-protocols/cell-viability-with-alamarblue.htmL].

2.5 Screening for Anti-QS Activity

Chromobacterium violaceum 12472 was used to screen anti-QS activity of SAE under the overlay agar disk diffusion method described earlier [13]. First, SCS of overnight grown CV 12472 culture was prepared and used to inoculate 0.3% autoclaved Luria agar. When it is in a molten state (35-38^{o}C),10 mL, of this culture mixed media was immediately poured over pre-cooled 1.5% Luria agar plates to form a uniform upper layer. Then plates were allowed to cool and after solidification 6 mm sterile paper disc was placed on agar. The disks were loaded (15 µL) with various concentrations of SAE samples (100, 200, 300 µg) prepared in 10% DMSO (Dimethyl sulfoxide). The culture plates were incubated for 24 hours at 37^{o}C. Anti-QS activity was analyzed by observing a clear halo zone formed around the sample loaded disk due to inhibition of violacein production.

2.6 Quantification of Violacein Production

C. violaceum 12472 naturally produces violet color pigment violacein, regulated by QS mechanism. Anti-QS agents disrupt QS-mediated signaling and subsequently violacein production in CV 12472 culture. Effect of SAE on violacein production CV 12472 was quantified in B broth culture. Briefly, 50 mL of freshly prepared sterile Luria broth was inoculated with SCS (10% v/v) of CV 12472 culture and supplemented with different concentrations of SAE (100, 200 and 300 µg) followed by incubation at 37^{o}C for 24

hours. To this culture 10% SDS was added to lyses cell and mixed vigorously and left for 10 minutes. Violacein was extracted by mixing water-saturated n-butanol and quantified by measuring absorbance at wavelength 585 nm [13].

2.7 Determination of Anti-virulence Activity of SAE in *P. aeruginosa*

In *P. aerugnosa* QS-regulated gene expression, many virulence factors such as Proteases, elastases, pyocyanin, rhamnolipid, alginate, motility, and biofilms that help bacterial to colonized infection areas in a host, are produced.To determine virulence factors production,SCS of overnight grown *P. aerugonosa* broth culture (LB media) was used to inoculate 250 mL fresh sterile Luria broth, supplemented with different concentration of SAE (100, 200 and 300 µg)and incubate at 37 ^{o}C on a rotator shaker (Scientech SE-140 D.C. Drive, India) at 150 rpm for 24 hours. Then treated cultures were centrifuged at 5000 rpm to sediment bacterial cells and supernatant was filtered through whatmann filter paper (0.45 µm). Pyocyanin was extracted by adding chloroform to one-third volume of the culture supernatant followed by vigorous mixing: the bottom organic layer was further extracted with 0.2 N HCl. The light pink color was developed on the upper aqueous layer and absorbance of it was taken at 520 nm [14]. Elastase activity was determined by the method described earlier with some modification [15]. 100 µL culture supernatant mixed with 900 µL Elastin congo-red buffer (100mM Tris, 1mM $CaCl_2$, pH 7.5and 10 mg Elastin congo-red). The mixture was left for incubation at 37 ^{o}C for 3 hours. After centrifugation at 5000g absorbance of the supernatant was measured at 495 nm. Azocasein substrate was used to estimate protease activity. Briefly, to 100 µL of culture supernatant 900 µL of 0.5%azocasien substrate solution was added (50 mM Tris buffer and 2 mM $CaCl_2$), after 2 hours of incubation at 30^{o}C, reaction was stopped by adding 10 % TCA (100µL). Insoluble azocasien was removed by centrifugation at 8000 rpm for 15 minutes and absorbance of the supernatant was taken at 440 nm [16]. For rhamnolipid estimation, culture supernatants of treated samples are acidified by sulphuric acid to maintain pH 2. An equal volume of methanol:chloroform (1:2) was mixed vortex and the organic layer was separated and dried. The further dried extract was dissolved in methanol and 1 mL of orcinol reagent was added and incubates at 80^{o}C for 30 minutes.After adding methanol absorbance was taken at 421 nm [17-18]. Alginate production was measured by iso-propanol precipitation of culture supernatant. The precipitate was isolated by centrifugation and the pellet was dry at 37^{o}C. The dried pellet was resuspended into 0.2% carbazole reagent prepared in 10 mM boric sulfuric acid and the mixture was left for 15 minutes. Alginate production was quantified by measuring the absorbance of the solution at 500 nm [19].

2.8 Effect of SAE in Biofilms Formation

Biofilm inhibition test was performed in 6 well cell culture plates. Sterile square-shaped glass coverslips were placed in each well and 2 mL autoclaved Luria broth was added. To this media, SAE samples were supplemented with 100, 200 and 300 µg concentrations. Each well was inoculated with SCS of *P. aeruginosa* culture leaving the control well untreated. The plates were incubated statically at 37^{o}C. After 24 hours, broth media was discarded from wells carefully without disrupting formed biofilms on glass slides. The slides were washed with phosphate buffer saline (PBS, 7.4 pH) and the biofilms were fixed with 50% isopropanol followed by air drying. Fixed biofilms were stained with 10% crystal violet and washed again with PBS. Biofilms inhibition was observed by taking images of glass slides into bright field microscopy (Leica DM2000 & DM2000 LED). Further, biofilms production was quantified by resuspending crystal violet stained biofilms in 0.5 mL ethanol (95%) for five minutes and OD of dissolved crystal violet was measured at 650 nm [14].

2.9 Swimming and Swarming Motility

P. aeruginosa shows a synchronized way of motility on specialized agar media. Motility facilitates the spreading of bacterial infection into a host. *P. aeruginosa* possesses single polar flagellum (helps in swimming motility) and several short pili all-around the cell (provide swarming motility). SAE effect on swimming motility was observed by inoculating point culture on Luria agar plates (0.5% agar, 0.5% glucose) treated with SAE (100, 200 and 300 µg) and then plates were incubated at 37 ^{o}C for 3 days [18]. For swimming motility, *P. aeruginosa* culture was spot inoculated on agar plates (0.05% yeast, 0.5% NaCl, 0.1% tryptone and 0.3% agar) supplemented with different concentrations of SAE [14]. Inhibition to swimming and swarming

motility was monitored by comparing culture growth from the center compared to untreated control plates.

2.10 Statistical Analysis

The results were expressed as mean ± standard deviation (SD), taken from at least three independent experiments. The student's t-test was used to calculate the difference between two mean values. Whereas, one-way analysis of variance (ANOVA) was performed for comparative analysis of multiple means values, using online link http:// www.physics.csbsju.edu/stats/anova.htmL.

3. RESULTS AND DISCUSSION

3.1 Minimum Inhibitory Concentration

MIC of SAE against CV 12472 and PA01 strains was determined by Alamar blue assay Fig. 1(I). The dye contains an oxidation-reduction indicator, resazurin which undergoes color change due to cellular metabolic reduction by live cells. The pink color, a reduced form resorufin is highly fluorescent and is directly proportional to the number of living cells respiring. The intensity of fluorescent is used to measure live cells quantitatively. The result of MIC assay showed that SAE did not exert any cidal effect against both CV 12472 and PA01 at all concentrations (100-300 µg). Therefore MIC of SAE was expected to be much higher than 300 µg. Previous studies also reported a higher MIC of 500 µg (methanolic extracts) and 2 mg (aqueous extracts) of *S. asoca* stem bark [20-21].

3.2 Anti-QS Activity of SAE

Anti-QS activity of SAE was evaluated by using the standard agar overlay method. The results show significant dose-dependent activity in SAE-treated culture plates with clear halo zone formed around the within the tested concentration range (100-300 µg) Fig. 1(II). Since, CV 12472 violacein pigment is regulated by QS system, therefore strain is considered as a sophisticated model microbe for anti-QS activity screening. Quantification results of violacein production in SAE-treated broth cultures show 50% (200 µg) and 65% (300 µg) inhibition Fig. 1(III & IV). Previous studies suggested anti-QS agents' action takes place via one or more of the following: (i) inhibition of autoinducer synthesis (AHL) (ii) competitive inhibition via molecular symmetry with autoinducers and (iii) suppressing downstream AHL receptors [22-27]. For plants metabolites, Terpenoids and flavonoids have been shown to be effective QS inhibitors [28-30]. The stem bark of *S. acoca* contains quercetin, quercetin-3-O-α-lrhamnoside, kaempferol, beta-sitosterol [31]. Studies have shown that quercetin and its derivates posses significant anti-QS properties against *C. violaceum* and *P. aeruginosa* [32-33]. Possibly, the anti-QS activity of SAE may be attributed to its bioactive flavonoid contents.

3.3 Effect of SAE on Virulence Factors Production in *P. aeruginosa*

P. aeruginosa produces several virulence factors that are cumulative responses of downstream expression of QS-regulated virulence genes. This virulence secretion plays important role in host invasion, adhesion and disruption of host immune signaling. A blue-green color pigment, Pyocyanin, secrete by *P. aeruginosa* is a virulence factor that causes inflammation, impaired host mitochondrial and catalase functions, provides defense for the bacterial cells against host phagocytic cells and exerts ciliary dysfunction in the respiratory tract [34-35]. SAE treatments cause a significant decrease in the production of pyocyanin. Our results show 38%, 49%, and 58% less production of pyocyanin at 100, 200 and 300 µg respectively. A previous study reported that flavonoid fraction of *P. guajava* extract inhibited pyocyanin in *P. aeruginosa* by 50% at 25 µg/mL and complete inhibition at 200 µg/mL. *P. aeruginosa* type II and III secretion system is responsible for secreting exotoxin A that causes death in case of severe lung infection. Furthermore, type II secretion system comprises a group of proteases secreted in the extracellular environment. They are hydrolyzing enzymes and facilitate bacterial membrane disruption, internalization, adhesion and pathogenicity [36-37]. These multiprotein include alkaline protease, lipolytic enzymes, LasA and LasB elastase. Elastase destroys host first-line defense by degrading collagen proteins and host tissue [38]. Studies demonstrated the role of exotoxins and proteases in tissue damage and invasion during infection in an animal model [39-40]. Our test results indicate a significant drop in protease (66%) and elastase (52%) secretion in SAE-treated culture at 300 µg concentration. Nonetheless, SAE show some inhibition on protease and elastase production at all tested concentrations. Rhamnolipid and alginate are extracellular polysaccharides produce by *P. aeruginosa* that showed assistive function in initial attachment, biofilms formation

and antibiotic resistance. Alginate protects bacteria from host unfavorable environment during cystic fibrosis lung infection and host immune defense macrophage cells. Both polysaccharides form a network-like matrix in which bacterial cells get embedded and protected against antibiotics, neutrophils and reactive oxygen species [41-43]. The effect of SAE treatment on exopolysaccharide secretion indicates a significant decrease in their production with a percentage inhibition of 53% (rhamnolipid) and 44% (alginate) at 300 µg concentration Fig. 1(V).

3.4 Effect of SAE on Biofilms Formation and Motility *in P. aeruginosa*

P. aeruginosa is an excellent biofilms producer. Its biofilms are composed of exopolysaccharides and act as protective shield to those bacterial cells entrapped inside biofilms matrix compared to single planktonic cells. Biofilms develop an interface between biotic and abiotic surroundings and form a barrier to most antibiotics treatments. Therefore, *P. aeruginosa* pathogenicity much relies on biofilms production at the infection site [44]. QS Plays important role in biofilms formation via LasR-LasI system at an initial stage and RhlR-RhlI system at the developing stage [45-46]. We examined anti-biofilm potential of SAE against PA01.Results of biofilms inhibition test were examined under bright field microscopy, Fig. 2(II,A). SAE-exposed P. aeruginosa static cultures demonstrate a significantlylow formation of biofilms with loose adherence ability. The biofilms in treated cultures show distorted and disrupted architecture compare to the untreated control that shows thick and continuous, uniform biofilms formed on glass slides. Further quantification of biofilms production results revealed 48% and 65% inhibition in biofilms production at 200 µg and 300µg, Fig. 2(I). It has been reported that anti-QS activity of plant extract is attributed to some of the plant phytochemicals, which have structural similarities with QS signaling molecules and their efficacy to inhibit signals receptors (LauxR/LasR) [47-48]. Other studies also reported a significant effect of plant extracts on biofilms production through inhibition of AHL production and flagella genes [49-52]. Whereas, flavonoids includes quercetin, apigenin, sinensetin has reported for antibiofilm activity in V. harveyi BB120 and E. coli O157:H7 [53-54]. In *P. aeruginosa*, flavonoids such as catechin and flavanes-3-ol showed reduction in biofilms production [47]. In another report bioactive fraction of Saraca asoca flower extract showed inhibition of Type II protein secretion system and chemical stimuli in *P. aeruginosa* at 312 µg. they also found that extract treatment impaired cell membrane and inhibited QS with low production of virulence rhmanolipid, pyocyanin Pseudomonas quinolone signal and HSLs [55]. Therefore, anti-biofilms activity of SAE could be due to one of the above possible mechanisms. Swimming and swarming activity in SAE-treated plates also indicate that both types of motility were significantly affected, evidently shown in how the bacteria become less motile on agar culture plates, Fig. 2(II, B&C), while in non-treated control plates showed high swimming and swarming (zig zag) motility compared to SAE treated plates. These results further validate the anti-biofilm properties of SAE by inhibiting PA01 motility, which has been shown to play an important role in biofilms formation, spreading and adhesion [56].

The current study qualitatively and quantitatively demonstrated the anti-QS potential of SAE. Saraca species are found to be rich in flavonoid and tannins [31,57]. *In Silico* studies suggested the metabolites (tannins, flavonoids, and alkaloids) present in plant extract showed anti-QS activity through antagonistic work against LuxS [58]. In the present work, we report broad-spectrum anti-QS and anti-virulence factors production potential of SAE at subinhibitory concentrations (< 300 µg). Previous studies reported anti-QS activity at much higher concentrations; 80% violacein inhibition against CV JCM1249 at 2000µg (in *Terminalia catappa* methanolic extract) [59], Bhargav N et al reported that Glycyrrhiza. glabra water extract showed 41% quorum quenching activity at 1000 µg and 70% activity in ethyle acetate fraction at 500 µg against A. tumefaciens A136 biosensor [60]. Similarly, water extract from stem bark of *ommiphora leptophloeos* and fruits of *Senna macranthera* allowed 32.7% and 43.3% biofilms formation only in *S. epidermidis at 400* µg [61]. Numerous natural products and biosynthesized nanomaterials have been reported for potential anti-quorum sensing and anti-biofilm activities against human pathogenic microorganisms [62-73]. However, proper fractionation needs to be done with a sophisticated instrument to identify the most active compounds. Also, detailed studies are required for evaluating the exact mechanism of the active compound in SAE, to define whether its activity is based on the inhibitory effects on AHL synthesis or AHL receptor antagonism.

Inhibiting bacterial QS is considered as a promising approach for developing next-generation antimicrobial drugs that will address multidrug resistance (MDR) pathogenic strains. Extensive researches have been going on to discover potential anti-QS agents. Plants metabolites are natural complex compounds and their synergistic application with conventional antibiotics could boost antimicrobial properties. SAE showed a strong effect on QS signaling in pathogenic *P. aeruginosa* therefore can be considered as a potential source to augment currently available drugs for therapeutic purposes.

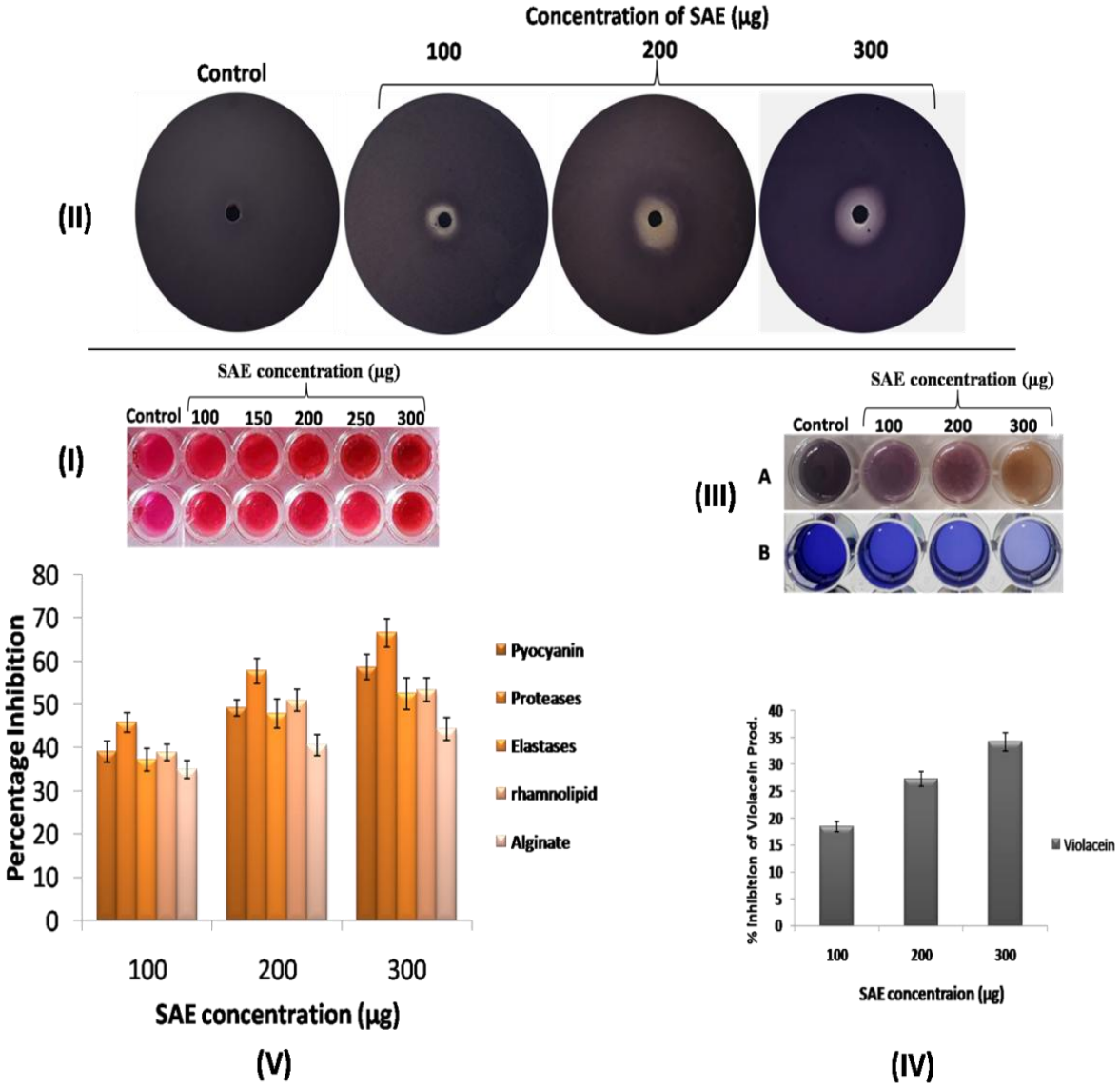

Fig. 1. (I) MIC using Alamar blue assay. Anti-QS and anti-virulence effect of SAE. (II) the anti-QS activity of SAE against CV12472. A halo zone around the disk represents inhibition of QS-regulated violacein productions. (III) Concentration-dependent inhibition of violacein production (A) CV12472 culture images supplemented with SAE. (B) Extracted violacein from SAE treated CV12472 culture. (IV) violacein quantification: showed percentage inhibition of violacein production. (V) Effect of SAE on QS-mediated virulence factors productions. Bar graph shows mean ± SD, where error bars represent SD (n=3), significant at *P=0.05*

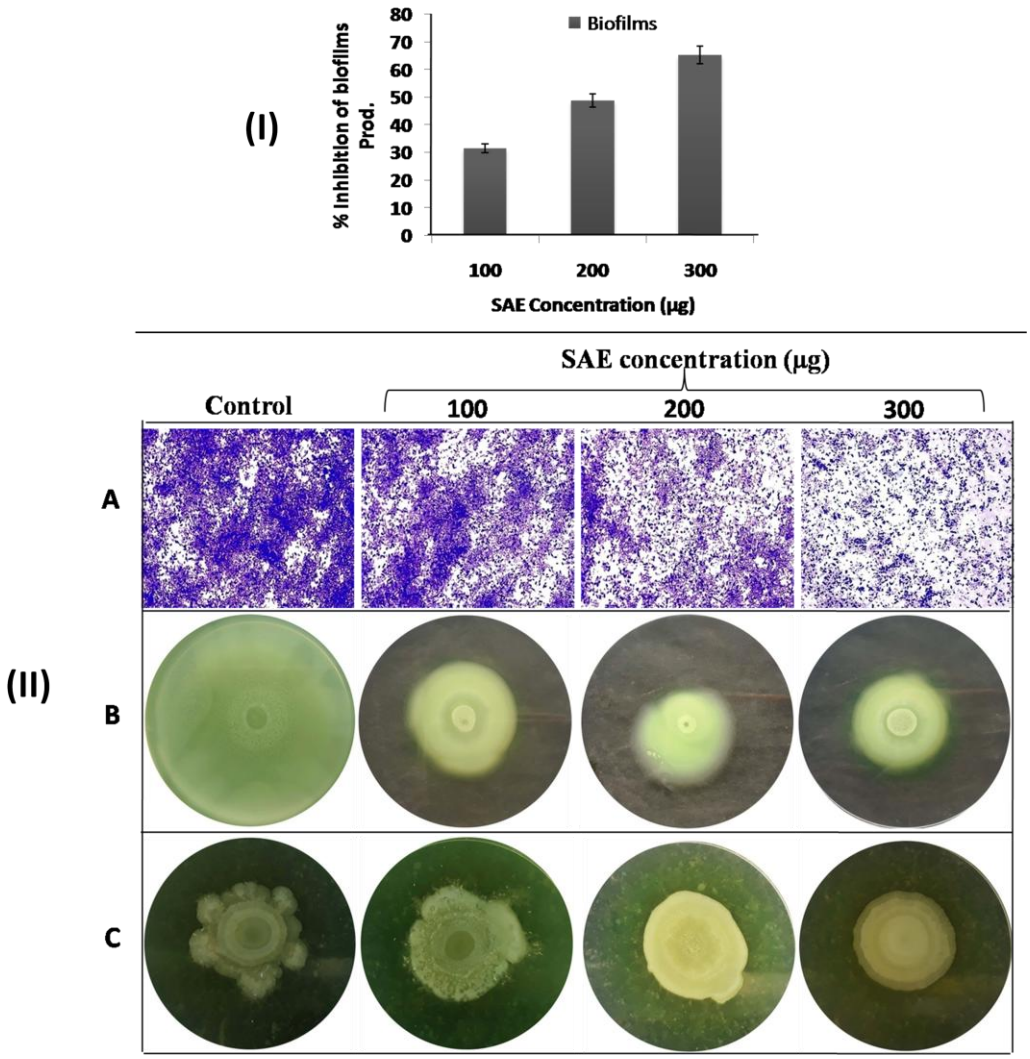

Fig. 2. Effect of SAE treatment on biofilms production and motility against PA01. (I) Percentage inhibition of biofilms production. Results are represented as mean ± SD. Indicated error bars represent standard deviation of three independent experiments. (II). (A) bright field microscopic image of SAE-treated Biofilms on glass coverslip. (B) Swimming motility on 0.3% agar (C) Swarming motility on 0.5% agar

4. CONCLUSIONS

Multidrug-resistant strains necessitate a review of currently available antimicrobial drugs. Targeting microbial pathogenesis via QS inhibition is a promising approach toward combating emerging mutant strains. Plants' products and their metabolites can serve as a potential source of new drugs that are ecofriendly with complex molecular topology. The present study first time demonstrated broad spectrum anti-QS properties of SAE against CV12472 and *P. aeruginosa.* Anti-biofilm and anti-virulence test results suggest that SAE could be a promising herbal source of anti-QS agents. Further studies on SAE's pharmacodynamic mechanism, bioavailability, and toxicity could provide valuable information for its application synergistically with other antimicrobial drugs.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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