Brazilian Journal of Microbiology (2020) 51:15–27
https://doi.org/10.1007/s42770-019-00169-0

BACTERIAL AND FUNGAL PATHOGENESIS - RESEARCH PAPER

In vitro anti-biofilm activity of 14-deoxy-11,12-didehydroandrographolide from *Andrographis paniculata* against *Pseudomonas aeruginosa*

Moumita Majumdar[1] · Tarun Kumar Misra[1] · Dijendra Nath Roy[2]

Received: 6 February 2019 / Accepted: 3 October 2019 / Published online: 6 November 2019

Abstract

14-Deoxy-11,12-didehydroandrographolide is a biologically active molecule present in the extract of *Andrographis paniculata* (Kalmegh), a classic ethnic herbal formula, which has been used for over thousand years as therapeutics to treat numerous infectious diseases like upper respiratory tract infection, urinary tract infection, and many more health issues. The present study is designed to ascertain an inhibitor against biofilm formation from the major metabolites of *Andrographis paniculata*, because the extract of this herb shows inhibition of bacterial quorum sensing (QS) communication and biofilm development against microorganisms. 14-Deoxy-11,12-didehydroandrographolide at 0.1 mM (sub-MIC dose) with azithromycin (6 μg/mL, sub-MIC) or gentamicin (4 μg/mL, sub-MIC) synergistically inhibits 92% biofilm production by a 48-h treatment against *Pseudomonas aeruginosa*. Further investigation carried out by atomic force microscopy shows promising reduction in roughness and height of biofilm in the presence of 14-deoxy-11,12-didehydroandrographolide compared with the control group. The content of extracellular polymeric substances, level of pyocyanin production, and synthesis of extracellular protease by *P. aeruginosa* have also been reduced significantly at around 90% in 14-deoxy-11,12-didehydroandrographolide-treated group. In conclusion, 14-deoxy-11,12-didehydroandrographolide could be used as a drug molecule against biofilm development by inhibiting QS pathway in *Pseudomonas aeruginosa*.

Keywords *Pseudomonas aeruginosa* · Quorum sensing (QS) pathway · 14-deoxy-11,12-didehydroandrographolide (DDAG) · Biofilm · QS inhibitor · Antibiotic resistance

Introduction

Andrographis paniculata (family Acanthaceae), commonly known as the "King of Bitters," is widely used in India, Japan, Korea, China, and many other Asian countries for 2000 years for treatment of human diseases including influenza, sore throat, ulcers in the mouth, ulcer in the tongue, acute or chronic cough, colitis, upper respiratory tract infection, urinary tract infection, rheumatoid arthritis, diarrhea, diabetes, inflammation, and hepatic disorders [1–9]. The two major bioactive diterpenoids of *A. paniculata* are andrographolide (AG) and 14-deoxy-11,12-didehydroandrographolide (DDAG). Andrographolide and its derivatives have been used to treat bacterial infections for many years but these drug molecules have been found to have minimal or no direct effect to inhibit bacterial growth, especially against biofilm formation by *Pseudomonas aeruginosa* [10, 11]. Andrographolide and 14-deoxy-11,12-didehydroandrographolide are extracted from the aerial parts of *A. paniculata*. Andrographolide is a diterpene containing a β-lactone ring connected to a decalin ring system via an unsaturated C2 moiety. 14-deoxy-11,12-didehydroandrographolide is different from andrographolide in respect to dehydrogenation at the 11th and 12th positions in the diterpene, and deoxygenation in the 14th position of the β-lactone ring (Fig. 1). According to an earlier report, DDAG

Responsible Editor: Marina Baquerizo Martinez.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s42770-019-00169-0) contains supplementary material, which is available to authorized users.

✉ Dijendra Nath Roy
dnr_20@hotmail.com

[1] Department of Chemistry, National Institute of Technology, Agartala, Tripura, India

[2] Department of Bio Engineering, National Institute of Technology, Agartala, Tripura, India

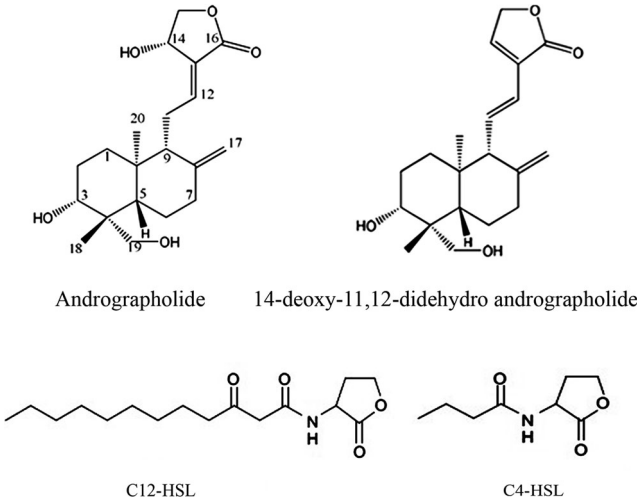

Fig. 1 Chemical structures of compounds. Andrographolide (AG), 14-deoxy-11,12-didehydroandrographolide (DDAG), C12-HSL, and C4-HSL

has less cytotoxicity than AG in HepG2 hepatoma cells [12]. So, the antibacterial activity of DDAG might be achieved through some alternative process, which is still unknown. According to reports, one of the major reasons behind failure of antimicrobial treatment is drug resistance of microbes due to biofilm formation [13]. Bacterial community attached to biofilm is not susceptible to a large extent like planktonic bacteria during antibiotic treatment, because it is almost impossible for them (antibiotics) to reach the bacterial cell by invading the multiple layers of biofilm [14, 15]. Biofilm is usually pathogenic in nature and is the major cause of nosocomial infections all over the world. However, among all, percentage of microbial and chronic infections associated with biofilm formation is 65% and 80%, respectively [16]. The biofilm primarily starts to develop when the cell density of the respective microbes reached at certain threshold. Under biofilm, the microbes are connected and communicate to each other via chemical signals through quorum sensing (QS) pathway. The whole bacterial community with biofilm attaches to a suitable surface to survive and proliferate. The adherent cells become encapsulated by self-producing extracellular polymeric substances composed of proteins, polysaccharides, and nucleic acids. *Pseudomonas aeruginosa* is one of the principal model microorganisms well-known to the clinicians as a cause of many human diseases including urinary tract infection, cystic fibrosis, and pneumonia, which have high mortality rate due to biofilm development at the site of infection. The process of biofilm formation consists of following steps like initiating with bacterial attachment to a living or non-living surface that leads to microcolony development, giving rise to matured three-dimensional structures, and finally dispersed after detachment [17, 18]. Like other bacteria, *P. aeruginosa* also use quorum sensing during host infections. For example, QS system is the dynamic influence for pathogenesis of *P. aeruginosa* in lung infection of cystic fibrosis patient [19]. Noticeably, *P. aeruginosa* encapsulated in biofilm predominantly executes an intracellular and extracellular QS communications for colonization, which is well characterized by many research groups [20–23]. Quorum sensing of *P. aeruginosa* controls biofilm formation and is followed by virulence expression during infection to animal, which makes it a strong pathogen in nature. *P. aeruginosa* possesses two canonical LuxI/R-type quorum sensing systems, LasI/R and RhlI/R, which produce and detect 3-oxo-C12-homoserine lactone (C12-HSL) and C4-homoserine lactone (C4-HSL), respectively [24]. In brief, LasI primarily catalyzes the synthesis of 3-oxo-C12-homoserine lactone, and RhlI directs synthesis of C4-homoserine lactone in *P. aeruginosa*. At low bacterial population, LasI produces a basal level of 3-oxo-C12-HSL, followed by increment of bacterial cell density, 3-oxo-C12-HSL builds to a critical concentration, at which point it interacts with LasR. The LasR-3-oxo-C12-HSL complex then activates transcription of a number of genes, including LasB, ToxA, RhlR, and LasI, followed by production of a variety of secondary metabolites such as hydrogen cyanide and pyocyanin. 14-deoxy-11,12-didehydroandrographolide and andrographolide both have functional lactone moiety group in their chemical structure. On the other hand, 3-oxo-C12-HSL and C4-HSL are important indigenous molecules of microbe helping in biofilm formation [24, 25]. We assume that these externally added DDAG and AG may create competition to microbial native C12-HSL and C4-HSL in binding phenomenon with the ligands (LasR and RhlR), leading to inhibition of biofilm formation.

In the present study, we evaluated the anti-biofilm efficiency of the compounds DDAG and AG against *P. aeruginosa* biofilm formation by carrying out detailed investigation of biochemical and structural studies in combination with azithromycin or gentamicin antibiotics. The synergistic effects of DDAG in the presence of antibiotics were estimated by quantification of virulence factors involved in pathogenesis. Finally, the ability of DDAG to inhibit the swarming motility of *P. aeruginosa* was also evaluated to understand the efficacy of the drug molecule to stop the translocation of the bacterial population across the surfaces during antimicrobial treatment.

Materials and methods

Maintenance of microbial cultivation

In the present study, *Pseudomonas aeruginosa* wild-type (MTCC 7814) was used, which is maintained in the Department of Bioengineering, NIT-Agartala. Bacteria were initially grown on tryptone soya agar (TSA) plate (HiMedia) from glycerol stock. A single colony of *P. aeruginosa* was isolated from TSA plate and it was inoculated in tryptone soya

broth (HiMedia) at pH 7.4 followed by incubation for 24 h at 37 °C. From this culture, 10^6CFU/mL cell suspension of mid-log phase was prepared by medium dilution for all subsequent experiments following standard protocols [26, 27].

Assessment of biofilm development under laboratory condition and its estimation

Sterile 96-well plates (polypropylene tissue culture plates with flat-bottom) were used for the development of biofilm by *P. aeruginosa* in our study. Initially, inoculum (10^6CFU/mL) from fresh 24-h culture was added in each well of 96-well plate containing 200 μL of TSB media, and it was incubated horizontally under aerobic condition at 37 °C for 48 h. After the incubation, the content of each well was discarded, rinsed thrice with sterilized distilled water, and kept for 30 min to be air-dried. Finally, biofilm of each well was stained with safranin (HiMedia) 0.5% (w/v) for 10 min. After that, excess stain was washed out with 0.9% saline and the well plate was kept overnight at 37 °C to become completely dry. The glacial acetic acid 30% (w/v) was added to dissolve the stain from adherent biofilm. The OD of each well was measured using a microplate reader (Company: Diatek; Model: LWR96) at 492 nm [28, 29].

Optimization of minimum inhibitory concentration

The minimum concentration of azithromycin (AZI) and gentamicin (GEN) to prevent the growth of a planktonic microbial population was determined using broth microdilution method as per CLSI [30]. Similarly, minimum inhibitory concentration (MIC) value of andrographolide (Sigma-Aldrich) and 14-deoxy-11,12-didehydroanrdographolide (Sigma-Aldrich) was also tested for interest of the present study.

Determination of minimum biofilm eradication concentration of drug molecules

The minimum biofilm eradication concentration (MBEC) was defined as the minimal concentration of compound required to eradicate the biofilm. Briefly, different doses of AG and DDAG ranging from 0.025 to 0.175 mM, individually and with combination of sub-MIC dose of AZI (6 μg/mL) and GEN (4 μg/mL), were used to the bacterial cell suspension (10^6CFU/mL), followed by incubation for 48 h at 37 °C. Wells were rinsed with sterile PBS and the content of each well was transferred in a fresh sterile 96-well plate containing AG and DDAG alone and in combination with AZI and GEN sub-MIC doses. The plate was incubated for 24 h at 37 °C. MBEC was then determined by measuring the turbidity at 650 nm [31].

Determination of anti-biofilm development activity of drug molecules

The assay was performed to determine the percentage of anti-biofilm activity of individual compound like AG and DDAG, and their combination with either sub-MIC dose of AZI (6 μg/mL) or GEN (4 μg/mL). These drug combinations were applied individually to the bacterial cell suspension (10^6CFU/mL) and incubated for 48 h at 37 °C. Biofilm estimation was evaluated at 492 nm following standard methods as mentioned earlier [26, 28]. Percentage of biofilm inhibition in all treated wells in respect to untreated control was determined.

Studies of biofilm topography by atomic force microscopy

Mica sheets containing treated and untreated bacterial (*P. aeruginosa*) suspension were air-dried before atomic force microscopy (AFM) analysis [29, 32]. The images were captured by atomic force microscopy with the help of silicon tip (Company: Bruker Multimode 8) and evaluated with compatible software (NanoScope Analysis v1.40). The parameters such as R_a, R_t, R_q, and R_{max} of biofilm were measured by using software (Gwyddion-2.50 and NanoScope Analysis version-1.40) following standard method.

Estimation of total exopolysaccharide of biofilm

Total polysaccharide concentration of biofilm was estimated by phenol-sulfuric acid test. Mixture of 1 mL of freshly prepared 5% phenol and 5 mL of 96% sulfuric acid was incubated with 200 μL of sonicated biofilm sample, as mentioned earlier at 25 to 30 °C for 15 min. The absorbance was measured at 490 nm [33].

Estimation of extracellular free deoxyribonucleic acid

Extracellular DNA (eDNA) was determined by UV spectrophotometric method by measuring absorbance at 260 nm after extraction carried out with bacterial genomic DNA purification kit (HiMedia) following standard method [33].

Estimation of total protein of biofilm

Estimation of total protein of biofilm of *P. aeruginosa* was determined by Bradford protein estimation assay. Briefly, 10^6CFU/mL cell suspension was incubated in 96-well plates at 37 °C for 48 h in the presence or absence of drugs. Untreated set with drug was considered as control. Planktonic cells were discarded, and adhered cells of biofilm were gently rinsed with sterile PBS. Finally, the content of each well was sonicated for 45 min with 50 Hz, followed by

protein estimation done with the help of Bradford reagent at 595 nm [33].

Quantification of pyocyanin

The quantification of pyocyanin was carried out by the following method described by Essar et al. *P. aeruginosa* cell suspension (10^6CFU/mL) was incubated for 48 h at 37 °C with or without drugs. Culture supernatant was collected after centrifugation at 10,000 rpm for 5 min. After that, 5 mL of supernatant was added with 3 mL of chloroform and the extraction was re-extracted using 0.2 N HCL, followed by the production of orange to pink color solution. The absorbance of the resultant solution was measured at 520 nm [34], and it was multiplied by a factor 17.07 to get the pyocyanin concentration in μg/mL [35].

Determination of LasB activity

Elastinolytic activity of *P. aeruginosa* was determined by elastin congo red (ECR) assay. A total of 50 μL of culture supernatant was added to ECR (10 mg/mL in 0.1 M Tris-HCL, pH 7.0) and incubated for 17 h at 37 °C under shaking condition. Finally, absorbance of centrifuged sample was measured at 495 nm [36].

Estimation of exoprotease activity

P. aeruginosa was cultured for 48 h at 37 °C in the presence and absence of drugs. The culture supernatant was collected after centrifugation at 10,000 rpm for 5 min. A total of 1 mL of azocasein (0.3% in 0.05 M Tris-HCl, pH 7.5) was mixed with 150 μL of supernatant and incubated for 15 min at 37 °C. A total of 0.5 mL of l0% trichloroacetic acid was added to the reaction mixture, followed by centrifugation at 10,000 rpm for 5 min. Optical density of supernatant was measured at 400 nm [37].

Measurement of swarming motility of *Pseudomonas aeruginosa*

Swarming motility of *P. aeruginosa* was performed in small 35 × 10 mm polystyrene plates containing nutrient agar (8 g/L) supplemented with glucose (5.0 g/L) (HiMedia). A total of 2 μL cell suspension from a 48-h *P. aeruginosa* culture (treated and untreated) was added at the center of the plate following point inoculation methods. The plates were incubated at 37 °C for 48 h [26, 29]. The rapid and coordinated translocation of a bacterial population across semi-solid surfaces was checked in triplicates.

Statistical analysis

Each experiment was performed independently in triplicate to obtain the statistical justification. Results of experimental outcomes were expressed as mean ± standard deviation. Statistical significance was calculated by using one-way ANOVA and Student's *t* test. Significance was mentioned as $^{**}P$ value < 0.01 and $^{***}P$ value < 0.001. Correlation coefficient (*r*) was calculated by Pearson's correlation method to demonstrate the correlation between different data sets. Statistical analysis was done by one-way ANOVA using Turkey's multiple comparison test using Graphpad Prism 7.00 software.

Results

Biofilm eradication activity of andrographolide and 14-deoxy-11,12-didehydroandrographolide in combination with azithromycin or gentamicin

For this study, 14-deoxy-11,12-didehydroandrographolide and andrographolide were used separately to find out the effectiveness in inhibition of biofilm formation mediated by *P. aeruginosa*. The range of concentration of each drug was chosen from 0.025 to 0.175 mM which is completely nontoxic to the animal body as per research reports [38]. During the experiment, we observed that there was a sharp boost in inhibition of biofilm formation from 0.025 to 0.1 mM of the DDAG and AG compounds (Fig. 2a). Interestingly, further biofilm inhibition was not observed following the increasing dose of individual drug and reached a steady state at higher doses, for example, 0.125 mM, 0.15 mM, and 0.175 mM. The result showed around 56% inhibition during DDAG treatment, whereas AG shows around 40% inhibition. This outcome created further interest to check the status of biofilm production in the presence of different antibiotics including AZI and GEN. In our study, selected sub-MIC doses of DDAG, AG, AZI, and GEN were individually tested on the planktonic cells of *P. aeruginosa* to evaluate their antibacterial activity. In the case of antibiotics, it was observed that the application of AZI (6 μg/mL) and GEN (4 μg/mL) at sub-MIC did not have no lethal effects on bacteria (Table S1) and also incapable of inhibiting biofilm formation (48 h at 37 °C), which is very less, around 10% (Fig. 2b). Moreover, we found that these AG and DDAG did not have antimicrobial activity at sub-MIC dose against planktonic cells of *P. aeruginosa* (Table S1). Not only that, we checked the zone of inhibition by using DDAG and AG but no promising growth arrest was observed at sub-MIC doses (data is not represented).

The synergistic effects of DDAG or AG along with antibiotics AZI or GEN on biofilm formation were evaluated (Fig. 2b). Percentage of biofilm inhibition was measured in the

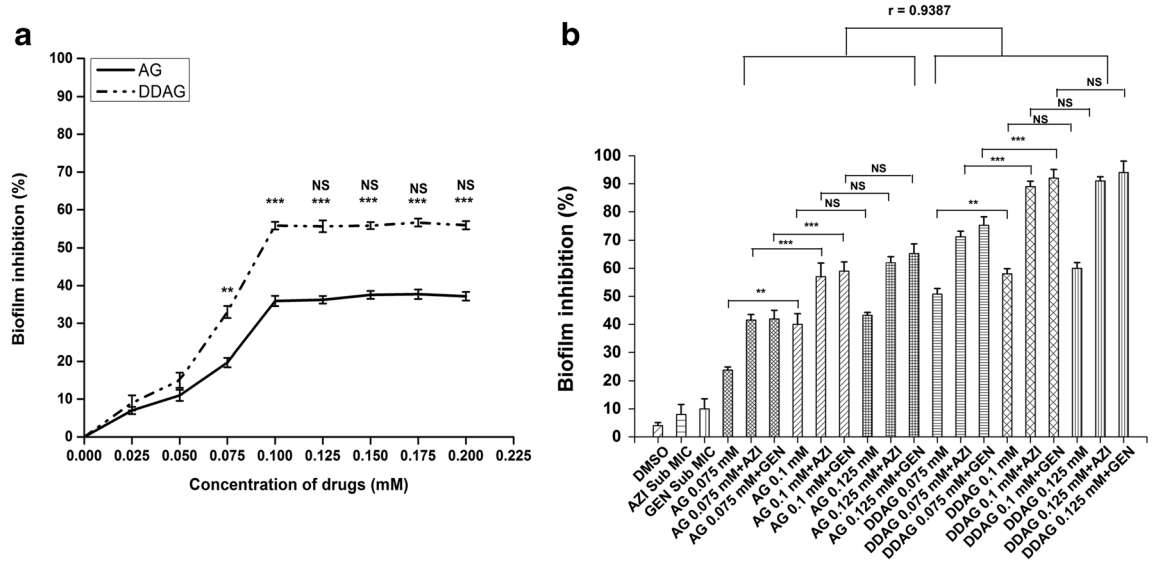

Fig. 2 Studies of synergistic effects of andrographolide (AG) or 14-deoxy-11,12-didehydroandrographolide (DDAG) along with either azithromycin (AZI) or gentamicin (GEN) in inhibiting biofilm development by *P. aeruginosa.* **a** Andrographolide (AG) and 14-deoxy 11, 12-didehydroandrographolide (DDAG) were used considering the following concentrations such as 0.025 mM, 0.05 mM, 0.075 mM, 0.1 mM, 0.125 mM, 0.15 mM, and 0.175 mM against biofilm formation. Percentage of inhibition of biofilm formation was calculated after 48 h of treatment compared with the untreated control. Each value represents as mean ± SD of seven independent experiments. Compared with similar concentration of AG, $***P < 0.001$; $**P < 0.01$; and NS, non-significant change. Further, percentage of inhibition by using different concentrations of DDAG was compared with 0.1 mM of DDAG; NS, non-significant change. **b** Sub-MIC doses of AZI (6 μg/mL) and GEN (4 μg/mL) were used separately and combined with AG or DDAG. Drug molecules AG and DDAG were used in different concentrations such as 0.075 mM, 0.1 mM, and 0.125 mM. Percentage of biofilm inhibition by AG at 0.1 mM is compared with other two (0.075 mM and 0.125 mM) concentrations of drugs. Each value represents as mean ± SD of seven independent experiments. $***P < 0.001$; $**P < 0.01$; and NS, non-significant change. Correlation coefficient of experimental data between experimental sets was calculated using Pearson's correlation method

presence of AG or DDAG, in a dose-dependent manner from 0.075 to 0.125 mM concentration of each compound alone or in combination with sub-MIC dose of AZI or GEN. The result was obtained in a fashion that DDAG at 0.1 mM along with either AZI or GEN synergistically inhibited biofilm formation at around 92%, but AG at 0.1 mM showed lesser percentage of biofilm inhibition in the presence of AZI or GEN, which was closed to 60%. Using statistical approach, it was found that DDAG significantly blocked the biofilm formation at higher percentage compared with the parent compound AG, which was justified by a strong correlation coefficient value (> 0.9) between the two experimental sets. This result has further established that the synergistic role of DDAG at 0.1 mM with sub-MIC dose of antibiotic was beneficial in inhibiting the biofilm formation, which was not achieved by killing the microbes but by inhibiting the process of biofilm development. These optimum concentrations were chosen for further experiments.

Microscopic studies on structural alterations of biofilm

The previous results on reduction of biofilm formation by *P. aeruginosa* insisted us to focus on the structural alteration of biofilm after 48 h of drug treatment (Fig. 3). For this purpose, we have chosen atomic force microscopic (AFM) to visualize the texture, height, and roughness parameters of biofilm [32]. The average roughness (R_a) is calculated considering the entire measured length/area, known as mean height [39], and is used to explain the roughness of surfaces. The maximum peak to valley height roughness (R_t) is the measurement of vertical distance between the highest and lowest points of the evaluated length/area of biofilm and is used to explain the overall roughness of the biofilm surface [40]. Furthermore, root mean square roughness (R_q) is known to calculate the square root of distribution of surface height, and this characteristic is considered for more sensitive determination of roughness for larger deviations from the mean surface [39]. Accordingly, we carried out AFM study thoroughly to get the idea of the four parameters R_a, R_t, R_q, and R_{max} of biofilm for all groups such as the control group (without treatment); AG, AG + AZI, AG + GEN, DDAG, DDAG + AZI, and DDAG + GEN (Table 1). In comparative demonstration of roughness parameters of seven different groups, it is distinguished that DDAG successfully reduced the R_a, R_t, R_q, and R_{max} of biofilm produced by *P. aeruginosa*. Further investigation detailed that DDAG reduced the biofilm roughness in the presence of antibiotic following synergistic behavior. Less roughness indicates the presence of lesser thickness in treated groups compared with the control group. This is also

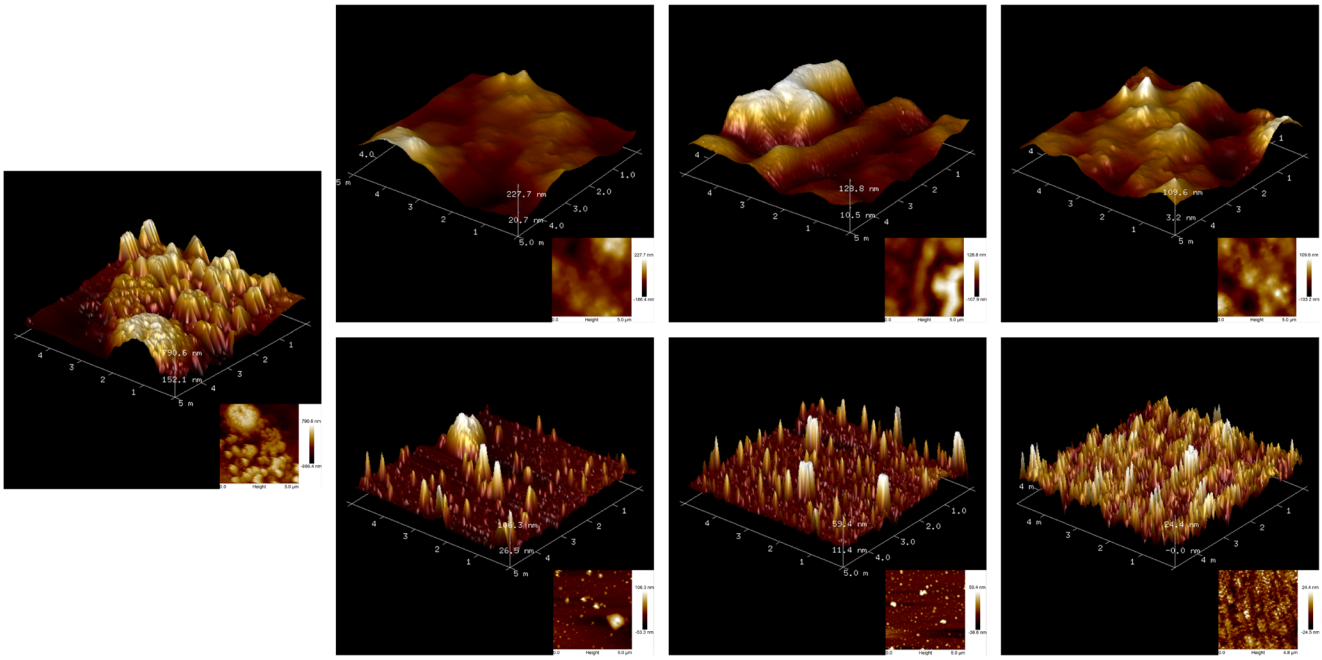

Fig. 3 AFM studies for structural analysis of biofilm in the presence of andrographolide and 14-deoxy-11,12-didehydroandrographolide. Biofilm was grown on mica sheets for 48 h at 37 °C as control sample. Similarly, other experimental sets were treated with AG (0.1 mM) or DDAG (0.1 mM) individually and combined with sub-MIC dose of AZI (6 μg/mL) or GEN (4 μg/mL). The representative biofilm structures are demonstrated as control (without treatment); andrographolide (AG); AG with AZI; AG with GEN; 14-deoxy-11,12-didehydroandrographolide (DDAG); DDAG with AZI; and DDAG with GEN. Scan speed was performed at 0.996 Hz. Nanoscope analysis version 1.40 was used to examine the topographic images of biofilm surface of respective groups. *X*-axis and *Y*-axis represent cross-sectional area for consideration, which is 40.4 μm^2, and *Z*-axis represents the height of the biofilm in nm scale. Representative scale bar for height is also displayed in each 2D image, respectively. The demonstrated illustration is the representation of three independent experiments

clearly noted that the average height of the biofilm is significantly decreased in the drug-treated group compared with that in the control group. The represented figures demonstrate that there was a clear reduction in height of biofilm formation in DDAG-treated group and DDAG with AZI or GEN in combination. This result has justified our hypothesis to address the role of DDAG in inhibition study of biofilm formation. This diminution of biofilm thickness deciphers the effectiveness of DDAG treatment to get the synergistic inhibitory effect in biofilm formation in the presence of antibiotics.

Table 1 Comparative demonstration of roughness parameters of biofilm developed by *P. aeruginosa* MTCC 7814

Different groups	Roughness parameters of biofilm (nm)			
	R_a	R_q	R_t	R_{max}
Untreated control	182.1 ± 61.08	214.2 ± 67.29	674.9 ± 129	1092
AG	39.42 ± 25.29	52.56 ± 30.27	153.2 ± 75.32	287
AG + AZI	33.69 ± 9.8	40.18 ± 10.53	143.8 ± 31.19	218
AG + GEN	23.42 ± 9.4	30.03 ± 9.5	104.8 ± 38.98	184
DDAG	11.78 ± 8.2	18.96 ± 11.7	73.29 ± 36.08	182
DDAG + AZI	5.935 ± 3.16	10.18 ± 5.73	49.55 ± 25.77	154
DDAG + GEN	5.205 ± 0.739	6.717 ± 0.992	33.63 ± 6.6	60.8

Inhibition of extracellular polymeric substance

Establishment of reduced biofilm production by the drug treatment encouraged us to evaluate the status of extracellular polymeric substance, considered as major components of biofilm matrix produced by *P. aeruginosa.* This polymer contains carbohydrate polymers, nucleotide polymers, and amino acid polymers [41]. Firstly, DDAG in the presence of sub-MIC dose of either AZI or GEN significantly reduced the total carbohydrate content of biofilm, which was closed to 90%, but AG did not show any promising inhibition in comparison (Fig. 4a). Similarly, extracellular DNA (eDNA) content (Fig. 4b) and total protein content (Fig. 4c) were further estimated, and it was observed that DDAG significantly diminished the amount of total eDNA and total protein available in biofilm up to 90% in combination with AZI or GEN.

Attenuation of quorum sensing associated virulence factors

In order to understand the underlying mechanism of attenuation of biofilm formation by *P. aeruginosa* during treatment of DDAG, we examined the quorum sensing–associated factors such as exoprotease activity, LasB activity, and level of pyocyanin production by the bacteria (Figs. 5 and 6). In the present

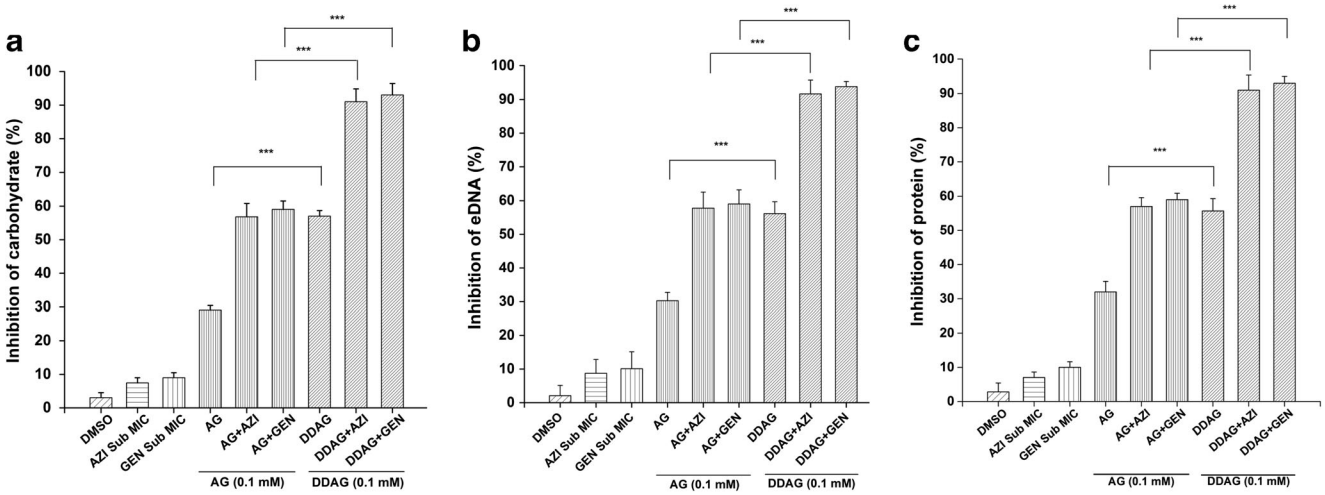

Fig. 4 Estimation of EPS of biofilm in the presence of andrographolide and 14-deoxy-11,12-didehydroandrographolide. Quantification of EPS such as carbohydrate, protein, and eDNA was estimated after 48 h of treatment in the presence of AG or DDAG with either AZI or GEN. **a** Percentage of carbohydrate polymers inhibition was calculated compared with untreated control. **b** Percentage of eDNA inhibition was calculated compared with untreated control. **c** Percentage of protein inhibition was calculated compared with untreated control. Each value represents as mean ± SD of five independent experiments. DDAG treatment results were compared with the outcomes of AG treatment, where ***$P < 0.001$; **$P < 0.01$; and NS, non-significant change

study, azocasein was used as substrate for determination of *P. aeruginosa* proteolytic activity. In this experiment, there was a significant inhibition of proteolytic activity of *P. aeruginosa*, which is up to 57% due to DDAG treatment, but inhibition was significantly less in AG-treated group. Furthermore, AZI or GEN synergistically reduced the bacterial exoprotease activity additionally up to 95% in the presence of DDAG (Fig. 5a). AZI or GEN did not show the similar pattern of exoprotease activity inhibition in combination with AG.

A significant decrease in activity of elastase enzyme produced by *P. aeruginosa* was achieved at 0.1 mM of DDAG (Fig. 5b), which was further augmented by 30% after addition of sub-MIC dose of AZI or GEN. This result interprets that the optimal conditions for LasB activity became hindered after treatment with DDAG.

Consequently, pyocyanin levels were measured in all treatment groups, demonstrated in Fig. 6a. Light green color represents the pyocyanin level in the pictorial illustration on petri plates after 48 h of drug treatment (inset of Fig. 6a). Under exposure of 0.1 mM concentration of DDAG, moderate reduction of pyocyanin production was noted, whereas AZI or GEN treatment with DDAG implemented synergistic decline

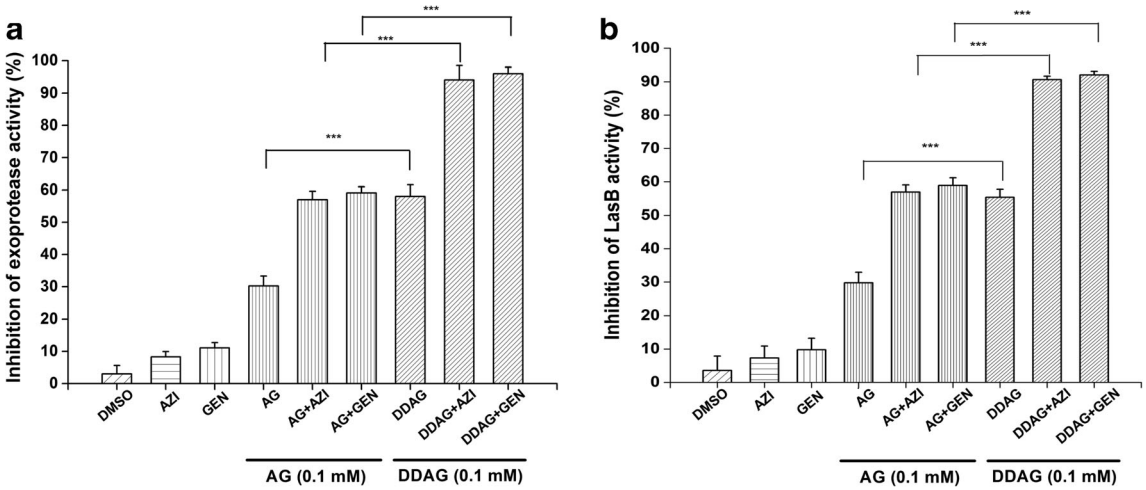

Fig. 5 Attenuation of quorum sensing–associated factors. Azocasein degrading protease activity and LasB activity were estimated after 48 h of treatment with AG or DDAG individually and combined with sub-MIC dose of AZI (6 μg/mL) or GEN (4 μg/mL). **a** Percentage of inhibition of azocasein degrading protease activity was represented compared with untreated control. **b** Percentage of inhibition of LasB activity was represented compared with untreated control. Each value represents as mean ± SD of five independent experiments; DDAG treatment results were compared with the outcomes of AG treatment, where ***$P < 0.001$

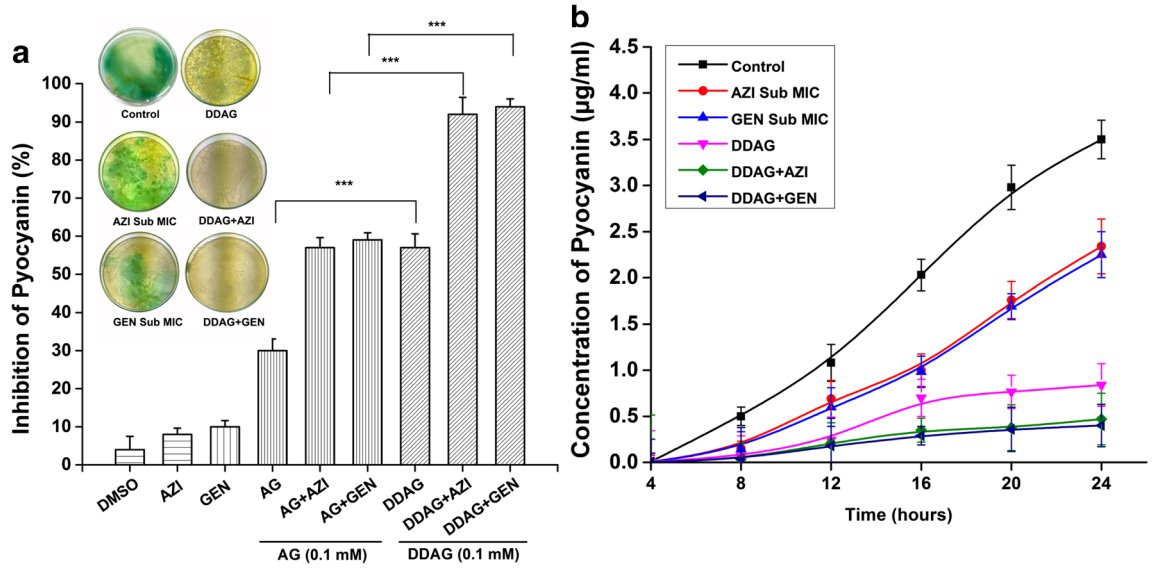

Fig. 6 Kinetics study of pyocyanin production through competitive inhibition by 14-deoxy-11,12-didehydroandrographolide with C4-HSL or C12-HSL. Secretion of virulent factor such as pyocyanin was estimated after 48 h of treatment with AG or DDAG individually and combined with sub-MIC dose of AZI (6 μg/mL) or GEN (4 μg/mL). **a** Percentage of inhibition of pyocyanin production was represented compared with untreated control. Inset represents the pyocyanin production on microbial culture petri plates after 48 h of treatment. **b** Pyocyanin produced by *P. aeruginosa* was measured after 4 h interval up to 24 h considering total eight groups, based on different drug treatments. In figure, black line represents control group without any drug treatment; red line represents bacteria treated with only AZI (6 μg/mL); light blue represents treated with only GEN (4 μg/mL); purple line represents only DDAG-treated group; green and dark blue lines represent DDAG with AZI or GEN, respectively. Each value represents as mean ± SD of three independent experiments

in the production of pyocyanin. This finding instigated us to evaluate time kinetics for 24-h duration of pyocyanin production considering different time intervals in *P. aeruginosa* growth culture in the presence of DDAG (Fig. 6b). Captivatingly, DDAG was quite capable to inhibit pyocyanin synthesis by *P. aeruginosa* from 8 h onwards to 24 h compared with AZI or GEN antibiotic treatment. In this context, we should consider our previous result (Fig. 6a), which was measured after 48 h but level of pyocyanin is still very less. While the study was carried out to check the efficacy of synergistic effects of DDAG with AZI or GEN, we found similar fashion of inhibition kinetics up to 24 h. This finding created the provision to focus on swarming motility of *P. aeruginosa*, which helps in rapid translocation for efficient colonization on a surface.

Studies of swarming motility of *P. aeruginosa*

We found that DDAG (0.1 mM) or sub-MIC dose of antibiotics (AZI or GEN) individually reduced the swarming motility compared with the untreated control group (Fig. 7). Furthermore, DDAG (0.1 mM) in combination with either AZI or GEN remarkably obstructed the swarming motility of *P. aeruginosa*, which indicates the synergistic efficacy of combined treatment against formation of biofilm and surface-attached bacterial colonies.

Discussion

Current knowledge about any particular natural bioactive compound from the herbal source on regulation of QS signaling and biofilm formation is quite limited. Therefore, it is very essential to know the role of any natural compound on regulatory mechanism of QS signaling as well as biofilm formation of bacteria because of their resistant nature towards conventional antibiotics. It is widely accepted that herbal products containing a variety of natural compounds could interact particularly with living system while they are administered in an animal body or human and thus treated as alternative medications for bacterial infections as well [42]. Herbal medicines are of great significance in the preliminary health concern of individuals, human communities, and healthcare professionals [43]. *A. paniculata* (family Acanthaceae) is a long-established herb used as a folk medication for a wide spectrum of sickness [44–46] because of having a very rich source of flavonoids and labdane diterpenoids [8, 47]. One research group demonstrated that the extract of *A. paniculata* has strong action against *P. aeruginosa*–mediated biofilm development along with reduction of virulence factor synthesis such as pyocyanin, elastase, protease, rhamnolipid, and hemolysin [48], but no compound has been identified for the action. This observation prompted us to hypothesize that any of the active compounds of *A. paniculata* might have strong action against biofilm development of bacteria that could be investigated.

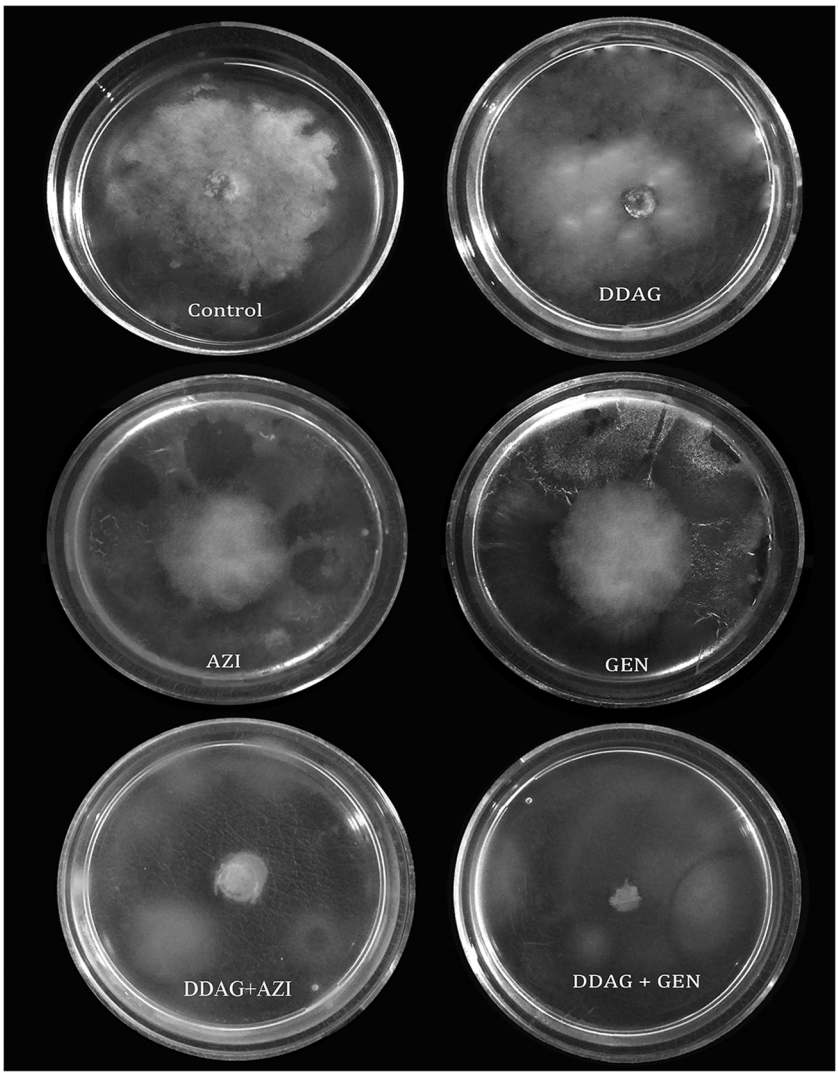

Fig. 7 Swarming motility assay of *P. aeruginosa* in biofilm. *P. aeruginosa* was cultivated for 48 h at 37 °C in polystyrene plates containing nutrient agar in the absence and presence of DDAG (0.1 mM) individually or combined with sub-MIC dose of AZI (6 μg/mL) or GEN (4 μg/mL). Each experiment was performed in three independent biological experiments and the demonstrated illustration is the representative of them

Andrographolide (AG) was identified as an inhibitor of QS system of *P. aeruginosa* [11], and its derivative 14-alpha-lipoyl andrographolide inhibits biofilm formation in a better way [49, 50]. Another report highlighted that andrographolide and its three derivatives such as 14-dehydroxy-11,12-didehydroandrographolide-3,19-bis (succinic acid) potassium sodium salt, 14-dehydroxyandrographolide-12-sulfonic acid sodium salt, and 14-dehydroxy-11,12-didehydroandrographolide-3,19-bis (succinic acid) potassium salt have been used clinically to get relief from inflammation, fever, and pain due to bacterial and viral infections for the last 40 years in China [7]. Dehydroandrographolide succinic acid monoester, a derivative of AG, also showed inhibition property against HIV infection [51]. The present investigation was carried out to evaluate the potential role of DDAG, major secondary metabolite (labdane diterpene lactone), isolated from the leaves of *A. paniculata*, against biofilm development and QS signaling pathway of *P. aeruginosa*. We have demonstrated a comparative study between DDAG and AG as inhibitor of biofilm formation.

This QS signaling pathway of *P. aeruginosa* is controlled by LuxI/R-type systems that mediate expression of virulent factors during infection to animal, which is known as pathogenic character of the infectious microbes to the host. *P. aeruginosa* possesses two canonical LuxI/R-type QS systems, LasI/R and RhlI/R, which produce and detect 3-oxo-C12-homoserine lactone (C12-HSL) and C4-homoserine lactone (C4-HSL), respectively [24, 25]. In brief, LasI primarily catalyzes the synthesis of 3-oxo-C12-homoserine lactone, and RhlI directs synthesis of C4-homoserine lactone in bacteria. At low bacterial population, LasI produces a basal level of 3-oxo-C12-HSL, followed by increment of bacterial cell density, 3-oxo-C12-HSL builds to a critical concentration, at which point it interacts with LasR. The second QS system (RhlI/R) consists of RhlI, which activates the synthesis of diffusible signal C4-HSL, and it binds with RhlR that activates

transcription of a number of genes, including *lasB*, *toxA*, and *lasI*, followed by the production of a variety of secondary pathogenic metabolites such as hydrogen cyanide and pyocyanin, those are responsible for persistent infection of host. We are aware of the fact that DDAG has lactone moiety in its structure which may create competitive binding with 3-oxo-C12-HSL and C4-HSL. To date, several natural compounds have been identified showing diminishing effects on virulence factor expression and antibiotic-resistant biofilm formation by *P. aeruginosa* without affecting its growth [52]. The furanones prevent acylated homoserine lactone (AHL) from binding to the LuxR homologs and eventually cause a rapid yield of these proteins [53, 54]. Baicalein is another one compound showing significant inhibition of the biofilm formation of *P. aeruginosa* at very low concentration without affecting its growth [55]. Its mode of action is to uphold the proteolysis of the signal receptor TraR [56], whereas PD12 and V-06-018 both help to inhibit LasR-dependent gene expression [57] but either of their low solubility or their elevated toxicity makes concern for further application.

In the present study, two major diterpenoids isolated from *A. paniculata*, 14-dehydroxy-11,12-didehydroandrographolide and andrographolide, were evaluated for their effects on inhibition of biofilm formation. For this purpose, AG or DDAG individually or along with either of any antibiotics AZI or GEN was used in vitro using *P. aeruginosa* model, which is completely established representation by the researchers for QS signaling pathway as well as biofilm study [7, 49, 50]. Here, we have selected 0.1 mM of DDAG with standard antibiotics (AZI or GEN) at sub-MIC dose as optimum concentration to stop more than 90% biofilm formation along with production of extracellular polymeric substances of biofilm, expression of virulence factor such as protease activity, LasB activity, and pyocyanin production. LasB activity of *P. aeruginosa* was determined by ECR assay method. The LasB gene is responsible for synthesis of LasB enzyme (elastase metalloprotease) that acts to degrade cells and tissue of the host via hydrolytic catalysis. Determination of LasB activity is useful to demonstrate the sequential phases of biofilm formation and its propagation. Significant inhibition on LasB elastase activity confirms the antagonist effect of DDAG on virulence factors. There was saturation point in inhibition process of biofilm while only DDAG was applied more than 0.1 mM because there might have a strong relation with the cell density of *P. aeruginosa* inoculation selected for this study, which was basically 10^6 CFU/mL of mid-log phase cell suspension of a 24-h culture at 37 °C. It was noted that DDAG treatment in same populated bacterial community has a higher inhibiting effect in consideration of similar time period compared with AG in a dose-dependent manner. As we know, as MIC dose completely eradicates the bacteria (*P. aeruginosa* here), there will be no possibility of biofilm formation, and the following studies of anti-biofilm activity will be worthless. To identify the minimum concentration of DDAG to eradicate the formation of biofilm by 48 h of treatment, we have used safranin for biofilm staining as per Gram staining theory (*P. aeruginosa* is a Gram-negative bacteria). Several research findings state that repeated exposure of any antibiotic against any bacteria may lead to develop multiple drug resistance [58]. AZI and GEN are well-known antibiotics used for treatment against *P. aeruginosa* infection [59, 60], but there is a chance to develop multiple drug resistance in *P. aeruginosa* against these antibiotics during repeated numbers of exposure. Atomic force microscopy study further revealed that there was no resistance against DDAG treatment by *P. aeruginosa*, which was established through height reduction of biofilm in DDAG-treated group and combined drug-treated group after comparison of roughness parameter with untreated control. Less roughness indicates the presence of lesser thickness in treated groups compared with the control group. This is also clearly noted that the average height of the biofilm was significantly decreased in the drug-treated group compared with the control group. This diminution of biofilm thickness deciphers the effectiveness of DDAG treatment to get the synergistic inhibitory effect in biofilm formation in the presence of antibiotics. The extracellular polymeric substances set up the functional and structural integrity of biofilm for survival of microbes in adverse situation. Quantification of these parameters is important to get the idea of disruption of maintenance of biofilm structural integrity during drug treatment. Estimation of total carbohydrate polymer, total protein, and total extracellular DNA of biofilm is a valuable approach to indirectly estimate the microbial biomass attached with the biofilm [61]. In our study, microbial biomass of biofilm was significantly reduced during treatment with DDAG (alone or in combination with AZI or GEN) compared with the untreated control bacterial population.

It is well reported that bacteria use a signal cascade, specifically QS, to manage certain activities including biofilm formation and virulence potentiality [62]. In our study, we observed DDAG has promising potential to inhibit QS signaling pathway. There are several diterpenoid compounds including modified molecules of AG, those have been reported to hamper the QS signal of microorganisms [7, 49, 50]. Both compounds, DDAG and AG, are structurally similar, although AG contains a hydroxyl group and two additional hydrogen atoms compared with DDAG. A research team tried to develop some synthesized AG analogs to investigate their mechanism of action for antibacterial activity [7]. In that study, they reported that alpha-lipoic acid acts as powerful antioxidant, which can nullify the free radicals, having capacity to chelate the transition metal ions like iron and copper [50]. AG conjugated with alpha-lipoic acid by an electron withdrawing group at C-14 resulted in an increase of compound's potency as per the objective of their study. As a result, this alpha-lipoic acid–conjugated AG molecule completely inhibited *P. aeruginosa* biofilm formation and exhibited synergistic activity with conventional antibiotics. Not only this, AG compounds have

shown to possess antimicrobial activity against several microorganisms. According to these reports, AG itself is not capable to stop biofilm production. Some other factors or modification of AG were associated with those observations, in which DDAG might have some role in inhibition study by using extract [24, 63].

These results further validated our hypothesis, i.e., competitive inhibition is taking place between DDAG and C12-HSL or C4-HSL in binding process with LasR and RhlR. The quorum sensing system is comprehensively investigated in *P. aeruginosa*, in which two acyl homoserine lactone–based systems, the *las* and the *rhl*, are involved to operate quinolone signaling system in a hierarchical fashion to regulate at around 4% of the genome expression [62]. This is also known that *lasI* and *rhlI* synthesize the auto-inducers C12-HSL and C4-HSL, respectively. LasR and RhlR bind DNA to modify transcription of target genes only after a threshold point of their respective auto-inducers is conquered [64]. This short of effective binding between LasR with C12-HSL to initiate further operon-related regulation might have affected in the presence of DDAG in our experimental model. As a result, the quorum sensing system was blocked and further regulation by RhlR binding with C4-HSL is being stopped. This observation was validated further through kinetics study. In the presence of DDAG, production of pyocyanin, a major downstream virulent factor was being reduced at high level. At present, we assume common lactone moiety of DDAG, and AG is the main responsible factor to create competitive inhibition with C12-HSL and C4-HSL molecule in the binding process of respective transcription factors. It might be concluded that a hindrance was generated in homeostasis of gene regulation by DDAG while biofilm was formed by the bacteria.

This shrinking of biofilm thickness interprets the effectiveness of DDAG treatment to get the synergistic inhibitory effect in biofilm formation in the presence of antibiotic. Attachment with surface is the early occurrence of microbial biofilm formation which is positively reflected by swarming motility [65]. Swarming motility is a particular fashion of flagella-mediated surface motility, which is exhibited by several bacteria including *P. aeruginosa* [66]. DDAG at 0.1 mM in combination with either AZI or GEN significantly reduced the swarming motility of *P. aeruginosa.* This outcome might have played an important role between rapid colonizing on a surface and biofilm formation for bacterial survival in adverse situation. This can be explained as DDAG acts strongly as quorum sensing inhibitor.

In conclusion, it can be inferred that 14-dehydroxy-11,12-didehydroandrographolide potentiates the inhibition of biofilm formation in the presence of the azithromycin and gentamicin. The combination of 14-dehydroxy-11,12-didehydroandrographolide with azithromycin or gentamicin does not allow the organism to develop multiple drug resistance because 14-dehydroxy-11,12-didehydroandrographolide additionally helps antibiotics to carry out their activity by inhibiting biofilm formation against *P. aeruginosa*. Furthermore, experimental outcomes suggest that 14-dehydroxy-11,12-didehydroandrographolide is a potentially useful agent in combination therapies with conventional antibiotics to combat biofilm-associated infections. We hope that 14-dehydroxy-11,12-didehydroandrographolide may be the pioneer in prospective drug development to combat worldwide problem of antibiotic resistance phenomenon by the microbes.

Acknowledgments MM is thankful to the National Institute of Technology, Agartala, for providing PhD fellowship. The authors are grateful to the Central Research Facility (CRF), National Institute of Technology, Agartala, for providing atomic force microscopy (AFM) facility.

Funding information This work is supported by project grant (File Number: YSS/2015/001965) to DN ROY from Science and Engineering Research Board (SERB), Department of Science and Technology (DST), Govt. of India.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Hancke J, Burgos R, Caceres D, Wikman G (1995) A double-blind study with a new monodrug Kan Jang: decrease of symptoms and improvement in the recovery from common colds. Phytother Res 9: 559–562
2. Melchior J, Palm S, Wikman G (1997) Controlled clinical study of standardized Andrographis paniculata extract in common cold—a pilot trial. Phytomedicine 3:315–318
3. Caceres D, Hancke J, Burgos R, Wikman G (1997) Prevention of common colds with Andrographis paniculata dried extract. A pilot double blind trial. Phytomedicine 4:101–104
4. Caceres D, Hancke J, Burgos R, Sandberg F, Wikman G (1999) Use of visual analogue scale measurements (VAS) to assess the effectiveness of standardized Andrographis paniculata extract SHA-10 in reducing the symptoms of common cold. A randomized double blind-placebo study. Phytomedicine 6:217–223
5. Gabrielian E, Shukarian A, Goukasova G, Chandanian G, Panossian A, Wikman G, Wagner H (2002) A double blind, placebo-controlled study of Andrographis paniculata fixed combination Kan Jang in the treatment of acute upper respiratory tract infections including sinusitis. Phytomedicine 9:589–597
6. Kumar RA, Sridevi K, Kumar NV, Nanduri S, Rajagopal S (2004) Anticancer and immunostimulatory compounds from Andrographis paniculata. J Ethnopharmacol 92:291–295
7. Jiang X, Yu P, Jiang J, Zhang Z, Wang Z, Yang Z, Tian Z, Wright SC, Larrick JW, Wang Y (2009) Synthesis and evaluation of antibacterial activities of andrographolide analogues. Eur J Med Chem 44:2936–2943

8. Roy DN, Mandal S, Sen G, Mukhopadhyay S, Biswas T (2010) 14-Deoxyandrographolide desensitizes hepatocytes to tumour necrosis factor-alpha-induced apoptosis through calcium-dependent tumour necrosis factor receptor superfamily member 1A release via the NO/cGMP pathway. Br J Pharmacol 160:1823–1843
9. Roy DN, Sen G, Chowdhury KD, Biswas T (2011) Combination therapy with andrographolide and d-penicillamine enhanced therapeutic advantage over monotherapy with d-penicillamine in attenuating fibrogenic response and cell death in the periportal zone of liver in rats during copper toxicosis. Toxicol Appl Pharmacol 250: 54–68
10. Singha PK, Roy S, Dey S (2003) Antimicrobial activity of Andrographis paniculata. Fitoterapia 74:692–694
11. Li H, Qin H, Wang W, Li G, Wu C, Song J (2006) Effect of andrographolide on QS regulating virulence factors production in Pseudomonas aeruginosa. Zhongguo Zhong yao za zhi= Zhongguo zhongyao zazhi=China J Chin Mater Med 31:1015–1017
12. Ooi JP, Kuroyanagi M, Sulaiman SF, Muhammad TST, Tan ML (2011) Andrographolide and 14-deoxy-11,12-didehydroandrographolide inhibit cytochrome P450s in HepG2 hepatoma cells. Life Sci 88:447–454
13. Donlan RM, Costerton JW (2002) Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev 15:167–193
14. Sretenovic S, Stojković B, Dogsa I, Poberaj I, Stopar D (2017) An early mechanical coupling of planktonic bacteria in dilute suspensions. Nat Commun 8:213
15. Khatoon Z, McTiernan CD, Suuronen EJ, Mah T-F, Alarcon EI (2018) Bacterial biofilm formation on implantable devices and approaches to its treatment and prevention. Heliyon 4:e01067
16. Barsoumian AE, Mende K, Sanchez CJ, Beckius ML, Wenke JC, Murray CK, Akers KS (2015) Clinical infectious outcomes associated with biofilm-related bacterial infections: a retrospective chart review. BMC Infect Dis 15:223
17. Crouzet M, Le Senechal C, Brözel VS, Costaglioli P, Barthe C, Bonneu M, Garbay B, Vilain S (2014) Exploring early steps in biofilm formation: set-up of an experimental system for molecular studies. BMC Microbiol 14:253
18. Sousa AM, Pereira MO (2014) Pseudomonas aeruginosa diversification during infection development in cystic fibrosis lungs—a review. Pathogens 3:680–703
19. Moradali MF, Ghods S, Rehm BH (2017) Pseudomonas aeruginosa lifestyle: a paradigm for adaptation, survival, and persistence. Front Cell Infect Microbiol 7:39
20. Déziel E, Lépine F, Milot S, He J, Mindrinos MN, Tompkins RG, Rahme LG (2004) Analysis of Pseudomonas aeruginosa 4-hydroxy-2-alkylquinolines (HAQs) reveals a role for 4-hydroxy-2-heptylquinoline in cell-to-cell communication. Proc Natl Acad Sci 101:1339–1344
21. Kirisits MJ, Parsek MR (2006) Does Pseudomonas aeruginosa use intercellular signalling to build biofilm communities? Cell Microbiol 8:1841–1849
22. Kaplan JA (2010) Biofilm dispersal: mechanisms, clinical implications, and potential therapeutic uses. J Dent Res 89:205–218
23. Bodelón G, Montes-García V, Pérez-Juste J, Pastoriza-Santos I (2018) Surface-enhanced Raman scattering spectroscopy for label-free analysis of P. aeruginosa quorum sensing. Front Cell Infect Microbiol 8
24. Mukherjee S, Moustafa D, Smith CD, Goldberg JB, Bassler BL (2017) The RhlR quorum-sensing receptor controls Pseudomonas aeruginosa pathogenesis and biofilm development independently of its canonical homoserine lactone autoinducer. PLoS Pathog 13: e1006504
25. Medina G, Juarez K, Díaz R, Soberón-Chávez G (2003) Transcriptional regulation of Pseudomonas aeruginosa rhlR, encoding a quorum-sensing regulatory protein. Microbiology 149:3073–3081
26. Das MC, Sandhu P, Gupta P, Rudrapaul P, De UC, Tribedi P, Akhter Y, Bhattacharjee S (2016) Attenuation of Pseudomonas aeruginosa biofilm formation by vitexin: a combinatorial study with azithromycin and gentamicin. Sci Rep 6:23347
27. Stepanović S, Vuković D, Hola V, BONAVENTURA GD, Djukić S, Ćirković I, Ruzicka F (2007) Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. Apmis 115:891–899
28. Schillaci D, Cusimano MG, Cunsolo V, Saletti R, Russo D, Vazzana M, Vitale M, Arizza V (2013) Immune mediators of sea-cucumber Holothuria tubulosa (Echinodermata) as source of novel antimicrobial and anti-staphylococcal biofilm agents. AMB Express 3:35
29. Majumdar M, Biswas SC, Choudhury R, Upadhyay P, Adhikary A, Roy DN, Misra TK (2019) Synthesis of gold nanoparticles using citrus macroptera fruit extract: anti-biofilm and anticancer activity. Chem Select 4:5714–5723
30. Patel JB (2017) Performance standards for antimicrobial susceptibility testing. Clinical and Laboratory Standards Institute
31. Dosler S, Karaaslan E (2014) Inhibition and destruction of Pseudomonas aeruginosa biofilms by antibiotics and antimicrobial peptides. Peptides 62:32–37
32. Hu Y, Ulstrup J, Zhang J (2012) Bacterial biofilms investigated by atomic force microscopy and electrochemistry. Kgs. Lyngby: DTU Chemistry
33. Combrouse T, Sadovskaya I, Faille C, Kol O, Guérardel Y, Midelet-Bourdin G (2013) Quantification of the extracellular matrix of the Listeria monocytogenes biofilms of different phylogenic lineages with optimization of culture conditions. J Appl Microbiol 114: 1120–1131
34. Essar D, Eberly L, Hadero A, Crawford I (1990) Identification and characterization of genes for a second anthranilate synthase in Pseudomonas aeruginosa: interchangeability of the two anthranilate synthases and evolutionary implications. J Bacteriol 172:884–900
35. Kurachi M (1958) Studies on the biosynthesis of pyocyanine.(II): isolation and determination of pyocyanine
36. Toder D, Gambello M, Iglewski B (1991) Pseudomonas aeruginosa LasA: a second elastase under the transcriptional control of lasR. Mol Microbiol 5:2003–2010
37. Kessler E, Safrin M (1994) The propeptide of Pseudomonas aeruginosa elastase acts an elastase inhibitor. J Biol Chem 269: 22726–22731
38. Low M, Khoo CS, Münch G, Govindaraghavan S, Sucher NJ (2015) An in vitro study of anti-inflammatory activity of standardised Andrographis paniculata extracts and pure andrographolide. BMC Complement Altern Med 15:18
39. Gadelmawla E, Koura M, Maksoud T, Elewa I, Soliman H (2002) Roughness parameters. J Mater Process Technol 123:133–145
40. Chatterjee S, Biswas N, Datta A, Dey R, Maiti P (2014) Atomic force microscopy in biofilm study. Microscopy 63:269–278
41. Jiao Y, Cody GD, Harding AK, Wilmes P, Schrenk M, Wheeler KE, Banfield JF, Thelen MP (2010) Characterization of extracellular polymeric substances from acidophilic microbial biofilms. Appl Environ Microbiol 76:2916–2922
42. Atanasov AG, Waltenberger B, Pferschy-Wenzig E-M, Linder T, Wawrosch C, Uhrin P, Temml V, Wang L, Schwaiger S, Heiss EH (2015) Discovery and resupply of pharmacologically active plant-derived natural products: a review. Biotechnol Adv 33:1582–1614
43. Firenzuoli F, Gori L (2007) Herbal medicine today: clinical and research issues. Evid Based Complement Alternat Med 4:37–40
44. Tang W, Eisenbrand G (1992) Panax ginseng CA Mey. In: Chinese drugs of plant origin. Springer, pp 711–737
45. Kapil A, Koul I, Banerjee S, Gupta B (1993) Antihepatotoxic effects of major diterpenoid constituents of Andrographis paniculata. Biochem Pharmacol 46:182–185

46. Jain D, Gupta M, Saxena S, Kumar S (2000) LC analysis of hepatoprotective diterpenoids from Andrographis paniculata. J Pharm Biomed Anal 22:705–709
47. Guan S-P, Kong L-R, Cheng C, Lim JC, Wong WF (2011) Protective role of 14-deoxy-11,12-didehydroandrographolide, a noncytotoxic analogue of andrographolide, in allergic airway inflammation. J Nat Prod 74:1484–1490
48. Banerjee M, Moulick S, Bhattacharya KK, Parai D, Chattopadhyay S, Mukherjee SK (2017) Attenuation of Pseudomonas aeruginosa quorum sensing, virulence and biofilm formation by extracts of Andrographis paniculata. Microb Pathog 113:85–93
49. Zeng X, Liu X, Bian J, Pei G, Dai H, Polyak SW, Song F, Ma L, Wang Y, Zhang L (2011) Synergistic effect of 14-alpha-lipoyl andrographolide and various antibiotics on the formation of biofilms and production of exopolysaccharide and pyocyanin by Pseudomonas aeruginosa. Antimicrob Agents Chemother 55: 3015–3017
50. Ma L, Liu X, Liang H, Che Y, Chen C, Dai H, Yu K, Liu M, Ma L, Yang C-H (2012) Effects of 14-alpha-lipoyl andrographolide on quorum sensing in Pseudomonas aeruginosa. Antimicrob Agents Chemother 56:6088–6094
51. Chang RS, Ding L, Gai-Qing C, Qi-Choa P, Ze-Lin Z, Smith KM (1991) Dehydroandrographolide succinic acid monoester as an inhibitor against the human immunodeficiency virus. Proc Soc Exp Biol Med 197:59–66
52. Asfour HZ (2017) Antiquorum sensing natural compounds. J Microsc Ultrastruct
53. Manefield M, de Nys R, Naresh K, Roger R, Givskov M, Peter S, Kjelleberg S (1999) Evidence that halogenated furanones from Delisea pulchra inhibit acylated homoserine lactone (AHL)-mediated gene expression by displacing the AHL signal from its receptor protein. Microbiology 145:283–291
54. Manefield M, Rasmussen TB, Henzter M, Andersen JB, Steinberg P, Kjelleberg S, Givskov M (2002) Halogenated furanones inhibit quorum sensing through accelerated LuxR turnover. Microbiology 148:1119–1127
55. Luo J, Dong B, Wang K, Cai S, Liu T, Cheng X, Lei D, Chen Y, Li Y, Kong J (2017) Baicalin inhibits biofilm formation, attenuates the quorum sensing-controlled virulence and enhances Pseudomonas aeruginosa clearance in a mouse peritoneal implant infection model. PLoS One 12:e0176883
56. Luo J, Kong J-l, Dong B-y, Huang H, Wang K, Wu L-h, Hou C-c, Liang Y, Li B, Chen Y-q (2016) Baicalein attenuates the quorum sensing-controlled virulence factors of Pseudomonas aeruginosa and relieves the inflammatory response in P. aeruginosa-infected macrophages by downregulating the MAPK and NFκB signal-transduction pathways. Drug Des Dev Ther 10:183
57. Müh U, Schuster M, Heim R, Singh A, Olson ER, Greenberg EP (2006) Novel Pseudomonas aeruginosa quorum-sensing inhibitors identified in an ultra-high-throughput screen. Antimicrob Agents Chemother 50:3674–3679
58. Karam G, Chastre J, Wilcox MH, Vincent J-L (2016) Antibiotic strategies in the era of multidrug resistance. Crit Care 20:136
59. Zelenitsky SA, Harding GK, Sun S, Ubhi K, Ariano RE (2003) Treatment and outcome of Pseudomonas aeruginosa bacteraemia: an antibiotic pharmacodynamic analysis. J Antimicrob Chemother 52:668–674
60. Mulet X, Maciá MD, Mena A, Juan C, Pérez JL, Oliver A (2009) Azithromycin in Pseudomonas aeruginosa biofilms: bactericidal activity and selection of nfxB mutants. Antimicrob Agents Chemother 53:1552–1560
61. Stewart PS (2014) Biophysics of biofilm infection. Pathog Dis 70: 212–218
62. Erickson DL, Endersby R, Kirkham A, Stuber K, Vollman DD, Rabin HR, Mitchell I, Storey DG (2002) Pseudomonas aeruginosa quorum-sensing systems may control virulence factor expression in the lungs of patients with cystic fibrosis. Infect Immun 70:1783–1790
63. Lau GW, Hassett DJ, Ran H, Kong F (2004) The role of pyocyanin in Pseudomonas aeruginosa infection. Trends Mol Med 10:599–606
64. Medina G, Juárez K, Valderrama B, Soberón-Chávez G (2003) Mechanism of Pseudomonas aeruginosa RhlR transcriptional regulation of the rhlAB promoter. J Bacteriol 185:5976–5983
65. O'Toole GA, Wong GC (2016) Sensational biofilms: surface sensing in bacteria. Curr Opin Microbiol 30:139–146
66. Kearns DB (2010) A field guide to bacterial swarming motility. Nat Rev Microbiol 8:634