Heliyon 7 (2021) e07002

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Quorum quenching activity of *Andrographis paniculata* (Burm f.) Nees andrographolide compounds on metallo-β-lactamase-producing clinical isolates of *Pseudomonas aeruginosa* PA22 and PA247 and their effect on *lasR* gene expression

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ARTICLE INFO

Keywords:
Andrographis paniculata
Pseudomonas aeruginosa
Quorum sensing
Biofilm
Protease
Swarming
Andrographolides

ABSTRACT

Andrographis paniculata (Burm f.) Nees is a tropical plant native to Southeast Asia that has been used as an effective remedy for a wide variety of illnesses in traditional Chinese and Ayurvedic medicine. The antimicrobial activity of its crude extract had been shown to be due to its quorum quenching activity. The study determined the effect of purified extracted compounds from the leaf of A. paniculata, namely: andrographolide, 14-deoxyandrographolide, 14-deoxy-12-hydroxyandrographolide and neoandrographolide on quorum sensing-mediated virulence mechanisms in clinical isolates of metallo-β-lactamase (MβL)-producing *Pseudomonas aeruginosa*. Their effect on the expression of the *lasR* gene, which codes for LasR, a transcription activator protein of the quorum sensing system in *P. aeruginosa* was also determined using RT-qPCR. All the pure compounds significantly decreased the biofilm formation, protease production and swarming motility of the *P. aeruginosa* isolates compared to the untreated controls ($p < 0.05$). Results of the RT-qPCR assay showed that all compounds significantly downregulated the expression of *lasR* compared to the untreated control ($p < 0.05$), supporting the position that the lower virulence activities of the treated group were due to quorum quenching activity of the pure compounds. Multiple comparisons using Tukey's HSD analysis revealed that the means of the relative expression of *lasR* of the isolates treated with the different compounds were not significantly different from each other ($p > 0.05$), suggesting equal potencies. Results show the potential of the isolated pure compounds from A. paniculata for use as antimicrobial agents as a result of their quorum quenching activities.

1. Introduction

Andrographis paniculata (Burm f.) Nees is an annual, branched and tropical herbaceous plant that is native to different regions in Southeast Asia. Phytochemical studies revealed that *A. paniculata* is comprised of diversified compounds which include labdane diterpenoid lactones, flavonoids and other miscellaneous compounds. Numerous studies have shown that *A. paniculata* possesses anti-cancer (Rajagopal et al., 2003), anti-bacterial (Pandey et al., 2019), immunostimulant (Kokate et al., 2002), and anti-viral therapeutic properties (Churiyah et al., 2015). Relative to this, there is an increasing use of bioactive compounds from plants as alternatives for antibiotics due to the rising prevalence of multiple drug resistant (MDR) bacteria caused by the selective pressure resulting from the widespread antimicrobial misuse worldwide.

Included among the three categories of drug resistant bacteria established by the Centers for Disease Control and Prevention (CDC) in 2020 is the MDR *P. aeruginosa*, which is classified under the category "serious threats". Among these MDR strains, metallo-β-lactamase (MβL)-producing strains are of major health concerns since they are resistant to carbapenems, which are the last resort drugs of choice for

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https://doi.org/10.1016/j.heliyon.2021.e07002
Received 3 July 2020; Received in revised form 3 August 2020; Accepted 30 April 2021

these opportunistic pathogens. The resistance genes are usually found in integrons which have the propensity to take up genes coding for resistance to other classes of drugs (Papagiannitsis et al., 2017). In addition, the integrons may be carried in mobile genetic elements such as plasmids that can be horizontally transferred to other bacteria belonging to the same or different genera, rendering recipient bacteria to become MDR strains (Pagano et al., 2016).

Pseudomonas aeruginosa is a Gram negative, non-fermentative, ubiquitous bacterium that is known for its increasing role in healthcare-associated or nosocomial infections, and its wide array of drug resistance and virulence factors (Jazayeri et al., 2016). These virulence factors include exotoxin and exoenzyme production and biofilm formation. A biofilm is comprised of an assemblage of surface-associated bacterial cells that are embedded in an extracellular polymeric substance matrix (EPS). The EPS renders the bacterial cells less permeable to antimicrobials, thereby conferring phenotypic resistance. The formation of biofilm is mediated by quorum sensing wherein the bacteria release chemical signaling molecules which allow them to communicate with each other and mount specific responses when the population has reached a certain level. There are two components of the QS systems of Gram negative bacteria: an autoinducer synthase enzyme and a transcriptional regulator (Rutherford and Bassler, 2012). An increase in the level of the autoinducers acyl homoserine lactones (AHLs), otherwise known as extracellular signaling molecules, may induce the binding and activation of transcriptional regulators (Williams et al., 2004). LasR is a transcriptional activator known to *P. aeruginosa* that upregulates the expression of genes involved in virulence-associated traits (Longo et al., 2013). This provides bacteria with advantages over host immunity. Although studies have shown the emergence of bacterial strains that were resistant to the activity of quorum quenching compounds (Maeda et al., 2012; Garcia--Contreras et al., 2013a, 2013b), many authors contend that this mechanism is less susceptible to the development of resistance citing that quorum sensing is not an integral part of bacterial growth and is not bactericidal, thereby imposing less selective pressure to the bacteria (LaSarre and Federle, 2013; Tang and Zhang, 2014; Ahmed et al., 2019; Borges and Simoes, 2019; Zhang et al., 2019).

1.1. Specific objectives

The study determined the quorum quenching activity of isolated pure compounds from *A. paniculata* on MβL-producing clinical isolates of *P. aeruginosa* as shown by their effects on biofilm formation, protease production, swarming motility and *lasR* gene expression.

2. Materials and methods

2.1. Bacterial isolates

Two clinical isolates of MβL-producing *P. aeruginosa* previously isolated from two tertiary hospitals in Metro Manila, Philippines were investigated in this study. PA22 was from a tracheal aspirate, while PA247 was from a surgical lesion. The isolates were identified in the hospital laboratory using the standard phenotypic assays comprised of colonial and microscopic morphology after Gram staining, pigment production, biochemical tests comprised of catalase test, oxidase tests, reactions in triple sugar iron agar, indole, methyl red, Voges Proskauer, citrate utilization, urease production and gelatin liquefaction. Before the study, the purity of the bacterial cultures was determined, and their identities were confirmed using *16SrDNA* sequencing.

2.2. Purified compounds from A. paniculata tested

The purified compounds from *A. paniculata* namely: andrographolide (L5), 14-deoxyandrographolide (L6), 14-deoxy-12-hydroxyandrographolide (L11) and neoandrographolide (L12) were kindly provided by the research group of Dr. Consolacion Ragasa of the Chemistry Department, De La Salle University, Manila (Ragasa et al., 2016). Andrographolide is the major constituent of *A. paniculata,* and 14-deoxyandrographolide, neoandrographolide and 14-deoxy-12-hydroxyandrographolide are its analogues. The chemical structures of these compounds are shown in Figure 1.

2.3. Violacein inhibition using the agar well diffusion method

18-hr trypticase soy broth (TSB) culture of the monitor strain *Chromobacterium violaceum* ATCC 12472 was inoculated on trypticase soy agar plates using the spread plate method. Two hundred microliters (200 μL) of 1.25 mg/mL of each compound were introduced into wells created in the agar. Sterile distilled water served as the negative control. Plates were incubated at 37 °C for 24 h.

Inhibition of quorum sensing activity was indicated by the absence of the violet pigmentation in colonies growing around the well containing the test compounds compared to the growth away from the wells.

2.4. Biofilm formation of clinical isolates of P. aeruginosa using crystal violet assay

A modified crystal violet assay for biofilm formation of O'Toole et al. (2000) and Banarjee et al. (2017) was used. The turbidity of overnight TSB cultures of the clinical isolates was adjusted to that of 0.5 McFarland standard to approximate 1.5×10^8 cells/mL. One hundred microliters (100 μL) of each isolate were inoculated into each well of a 96-well plate and incubated without agitation for 24 h at 37 °C. *Acinetobacter baumannii* ABL2*, A. baumannii* NBF1 and TSB served as positive control, non-biofilm former control and broth control, respectively. The assay was done in triplicate.

After incubation, wells were washed three times with phosphate buffered solution (PBS). The biofilms that were still attached to the wells were fixed with 100 μL absolute methanol, dried and stained with 125 μL 0.1% crystal violet for 15 min. Excess crystal violet was removed from each well, and the plate was washed three times with PBS. The remaining attached crystal violet in the well was solubilized by adding 125 μL of 95% ethanol and OD_{590} was measured using plate reader (BioTek ELx800, BioTek Instruments, Inc. VT, USA).

2.5. Activity of A. paniculata leaf compounds on P. aeruginosa biofilm formation using crystal violet assay

Similar procedure as described above was followed. In the assay, 100 μL of the different concentrations (0.31, 0.62, 1.25, 2.5, 5 mg/mL) of each compound were added to the wells together with the turbidity-adjusted clinical isolates. Wells inoculated with the test organisms but not exposed to the compounds served as the untreated controls. As above, the plates were incubated without agitation for 24 h at 37 °C to allow biofilm to form. Before further processing, the plates were observed for the presence of turbidity in all the treated wells and were compared to the turbidity of the untreated control wells to ensure that the compounds were not inhibitory to the test isolates. Percent inhibition of biofilm formation was calculated using the formula below (Eq. (1)) (Al-Gamal et al., 2019). Similarly, the minimum biofilm inhibitory concentration (MBIC), which is defined as the lowest concentration that resulted in the inhibition of biofilm formation was determined. The lowest concentration of the compound that produced an absorbance which was significantly lower than the absorbance of the cultures not exposed to the compounds at $p < 0.05$ was considered as the MBIC. The MBIC values obtained were used in the determination of the effects of the *A. paniculata* compounds on the protease and swarming assays, and on the *lasR* gene expression.

$$\%\ inhibition = \left(1 - \left(\frac{XOD_t - XOD_{mc}}{XOD_{ut}}\right)\right) \times 100 \quad (1)$$

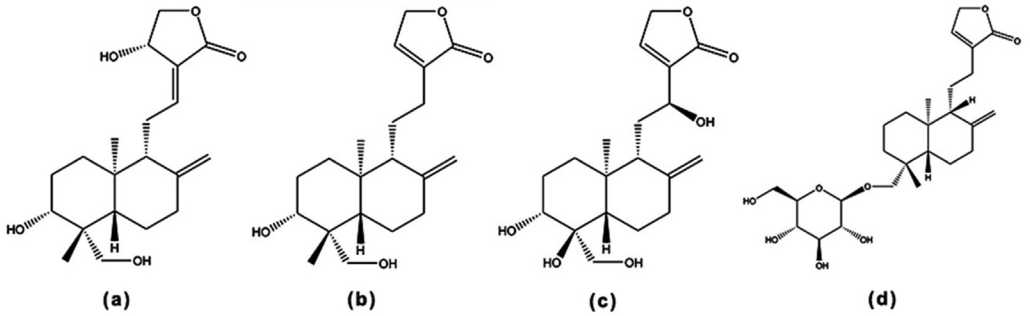

Figure 1. Chemical structures of the *A. paniculata* leaf compounds (Ragasa et al., 2016) tested in the study: (a) L5 - andrographolide, (b) L6 - 14-deoxyandrographolide, (c) L11 - 14-deoxy-12-hydroxyandrographolide, (d) L12 - neoandrographolide.

X = mean; ODt = optical density (590nm) of the test well; $ODmc$ = optical density (590nm) of the media control well; $ODut$ = optical density (590nm) of the untreated

2.6. Protease assay

The sterile culture supernatants of *P. aeruginosa* PA22 and PA247 for the protease assay were prepared using a modification of the method of Chu et al. (2013) and El-Mowafy et al. (2014). The turbidity of the 18-hour nutrient broth (NB) culture of each isolate was adjusted to 0.4 at OD_{590}, after which 30 μL were inoculated into 3 mL of NB. Appropriate amounts of the L5, L6, L11 and L12 test compounds were added to the tubes to obtain final concentrations equal to their respective MBICs. NB cultures with 1.25 mg/mL bromofuranone and without the test compounds served as positive and untreated negative controls, respectively. The tubes were incubated at 37°C for 18 h. The OD_{590} of the 18-hour cultures were then read. In addition, 10 μL of each culture were inoculated onto nutrient agar and incubated at 37°C for 18 h. These were done to ensure that the isolates were not inhibited or killed by the compounds. The tubes were then centrifuged at 12,000 x g for 10 min, and the resulting supernatants were filter-sterilized using a 0.2 μm membrane filter (Acrodisc, Sigma-Aldrich, MO, USA). These sterile culture supernatants were used for the protease assay.

The protocol of the protease assay of Chu et al. (2013) and Fila et al. (2017) was followed in the study with modifications. One hundred microliters (100 μL) of the supernatant from the treated and untreated tubes were inoculated individually into wells of a 96-well microtiter plate. Two hundred microliters (200 μL) of 1.25% skim milk were added to each well and mixed well. The plate was incubated at 37°C for 18 h. The absorbance of the samples was read at 590nm. The assay was done in triplicate.

2.7. Swarming assay

The swarming assay was performed using the procedure of O'May and Tufenkji (2011), Chu et al. (2013) and Kazemian et al. (2015) with minor modifications. The appropriate amount of each pure compound was mixed well with 2 mL of the swarm agar to obtain a final concentration equal to its MBIC. The swarm agar-compound mixture was then overlayed on a nutrient agar plate and allowed to solidify for an hour. The swarm agar was composed of nutrient broth, 0.5% agar and supplemented with 0.5 g of D-glucose. The turbidity of the 18-hr cultures of PA22 and PA247 was adjusted to 0.4 OD_{590} and 1 μL was point-inoculated on the agar center, allowed to dry, and the plates were incubated at 37 °C for 18 h. The diameter of the swarming pattern was measured. Plates without the test compounds and with 1.25 mg/mL bromofuranone (Sigma-Aldrich, MO, USA) served as negative and positive controls, respectively. Each assay was done in triplicate.

2.8. Effect of A. paniculata leaf compounds on the expression of lasR gene in MβL-producing P. aeruginosa using RT-qPCR

2.8.1. RNA extraction

PA22 and PA247 were grown in TSB with 2% glucose at 37 °C for 18 h. The turbidity of the broth cultures was adjusted to equal 0.5 McFarland standard. One hundred microliters (100 μL) of the isolates were inoculated into each well of a 96-well microtiter plate together with appropriate amounts of the L5, L6, L11 and L12 test compounds to obtain final concentrations equal to their respective MBICs. Wells with TSB in place of the compounds served as the negative control. The plates were incubated without agitation at 37 °C for 18 h. RNA was extracted using Trizol isolation protocol according to the manufacturer's instructions (Invitrogen, Thermo Fisher Scientific, MA, USA).

2.8.2. RT-qPCR

For the gene expression assay, RT-qPCR was performed using 1X KAPA SYBR FAST One-step qRT-PCR master mix (containing PCR buffer, KAPA SYBR Fast DNA polymerase, KAPA SYBR Green 1 and $MgCl_2$) (Sigma-Aldrich, MO, USA), 0.3 μM *lasR* forward and 0.3 μM *lasR* reverse primers, 1X KAPA RT mix (containing Reverse Transcriptase and RNase inhibitor) and 1 μL of RNA template.

The primer sequences were: lasR Forward, 5′-ACCGTTTCATAGAGTCGGTC-3′ and *lasR* Reverse, 5′-ACCACTGCAACACTTCCTTC-3′ (O'Toole et al., 2000). For the quantification of transcripts of the housekeeping gene *16SrRNA, the primer sequences were: 16S* Forward, 5′-CAGGCCTAACACATGCAAGTC-3′ and *16S* Reverse, 5′-CGGCGGWG TGTACAAGGC-3′ (Al-Gamal et al., 2019).

The following conditions were applied: reverse transcription at 42 °C for 5 min, initial denaturation of 95 °C for 120 s, followed by 40 cycles of denaturation at 95 °C for 35 s, annealing at 60 °C for 35 s and elongation at 72 °C for 30 s, followed by melt ramp from 65° to 95 °C rising 1° per step. All measurements were conducted in duplicate.

2.9. Statistical analysis

The T-test and one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison HSD test were used to determine the significant differences of the means at an alpha of 0.05. All calculations were performed using GraphPad Prism version 8.4.2 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com).

3. Results

3.1. Inhibition of violacein pigment production of C. violaceum treated with A. paniculata compounds

All four compounds (andrographolide, 14-deoxyandrographolide, 14-deoxy-12-hydroxyandrographolide and neoandrographolide) tested

inhibited the violacein production, but not the growth of *C. violaceum* around the wells with the test compounds (Figure 2). Pigment production was not inhibited by the negative control, which was comprised of sterile distilled water. The quorum sensing-dependent pigment production of *C. violaceum* ATCC 12472 has been widely used as a biosensor for the detection of quorum quenching activities of numerous QS inhibitors found in different natural and medicinal compounds (Kothari et al., 2017; Harrison and Soby, 2020).

3.2. Biofilm formation

The clinical isolates of *P. aeruginosa* produced OD_{590} readings that were significantly higher compared to the non-biofilm producer *A. baumannii* NBF1 ($p < 0.05$). At the same time, their OD_{590} readings were not significantly different from those of the positive control *A. baumannii* ABL2 ($p > 0.05$) (Table 1). These isolates were used to determine the effect of the pure compounds from *A. paniculata* on the quorum-sensing-mediated biofilm formation.

3.3. Effect of the A. paniculata leaf compounds on the growth of P. aeruginosa PA22 and PA247

All concentrations of the different compounds tested for anti-biofilm formation activity did not inhibit the growth of PA22 and PA247. This was evidenced by the presence of comparable turbidity in all the treated and untreated control wells after incubation for 24 h at 37°C, before further processing for the determination of biofilm formation.

3.4. Anti-biofilm formation activity and the minimum biofilm inhibitory concentrations of the compounds (MBIC)

A. paniculata leaf compounds showed anti-biofilm activity in a concentration-dependent manner seen as a decrease in the OD_{590} of the treated clinical isolates compared to the untreated controls (Figures 3A and 4A). Biofilm formation of PA22 was significantly inhibited by all the compounds at all the concentrations tested, which ranged from 0.31 to 5 mg/mL compared to the untreated controls ($p < 0.05$). All the compounds likewise inhibited the biofilm formation of PA247, however, at higher MBICs that ranged from 1.25 to 2.5 mg/mL, compared to the MBICs of 0.31 mg/mL for all the compounds for PA22 (Tables 2A and 2B). The MBICs of the compounds were used to further determine their effect on the protease and swarming activities and on the expression of the *lasR* gene of the clinical isolates.

All the compounds were able to inhibit the biofilm formation of *P. aeruginosa* PA22 by 22–67% and PA247 by 6–12% at the lowest concentration tested which was 0.31 mg/mL, whereas the highest dose tested (5 mg/mL) resulted to inhibition in biofilm formation that ranged from 69-84% in PA22 and 23–56% in PA247 (Figures 3B and 4B). This shows the ability of *A. paniculata* to reduce the biofilm formation of *P. aeruginosa*.

3.5. Protease assay

The OD_{590} of the 18-hour broth cultures treated with the MBICs of the test compounds and bromofuranone used in the protease assay were not significantly different from the OD_{590} of the untreated control showing that the compounds were not inhibitory or bactericidal to the test isolates (Table 3). In addition, the broth cultures from these tubes also showed comparable amounts of growth when 1 μL of each culture was sub-cultured on nutrient agar plates (Figure 5).

Both PA22 and PA247 treated with the respective MBICs of the compounds exhibited significant decrease in protease activities when compared to the untreated controls (Table 4A and B; Figures 6A and B). Tukey's multiple comparison test showed that there were no significant differences in the results of the protease assay among the different compounds in both PA22 and PA247 isolates, indicating their equal quorum quenching activities.

3.6. Swarm assay

P. aeruginosa PA22 and PA247 cultured on nutrient agar plates treated with the MBICs of the compounds exhibited swarming diameters that were significantly smaller than those grown on agar without the compounds ($p < 0.05$) (Table 5A and B; Figure 7). This further shows the ability of *A. paniculata* for quorum quenching of *P. aeruginosa* virulence factors.

3.7. 16SrRNA and lasR gene expression quantification

The fold gene expression values or FGEV ($2^{-\Delta\Delta Ct}$) of the *16SrRNA* housekeeping gene did not differ significantly among the treated and untreated groups for PA22 and PA247 (Figures 8A and 8B) showing that the test compounds did not inhibit the growth of the bacterial isolates, but only inhibited the *lasR* gene expression in the treated but not in the untreated cells as discussed below.

Results for the effect of the pure compounds on the *lasR* gene expression are shown in Figures 9A and 9B. Tukey's multiple comparison

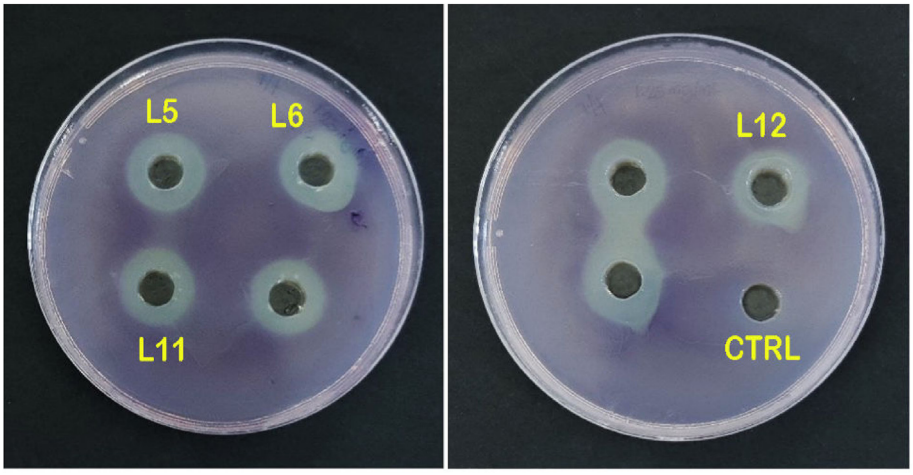

Figure 2. The quorum sensing inhibition activity of compounds from *A. paniculata on C. violaceum* shown by the inhibition of violacein pigment production in the growth zone around the well. andrographolide (L5), 14-deoxyandrographolide (L6), 14-deoxy-12-hydroxyandrographolide (L11), neoandrographolide (L12). The control well (CTRL) containing distilled water was negative for inhibition of violacein production. Unlabelled discs are not included in the study.

Table 1. Mean OD_{590} of *P. aeruginosa* clinical isolates and *A. baumannii* controls in the crystal violet biofilm assay.

Isolate ID	OD_{590}	p-value[a] *	p-value[b] *
P. aeruginosa PA22	3.12	9.099 e-0.5	0.061
P. aeruginosa PA247	0.75	<0.01	0.065
A. baumannii L2 (+) control	1.95		
A. baumannii NBF1 (-) *control*	0.15		
Trypticase soy broth	0.13		

*Significance level set at 0.05.
[a] PA clinical isolates were compared to the negative control *A. baumannii* NBF1.
[b] PA clinical isolates were compared to the positive control *A. baumannii* ABL2.

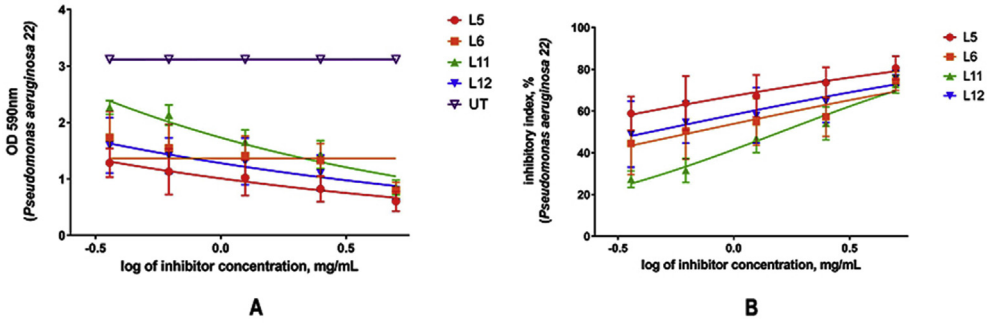

Figure 3. (A) Anti-biofilm formation assay of *P. aeruginosa* PA22 treated with compounds from *A. paniculata*. Values are the means of three independent experiments. (B) Percent inhibition of biofilm formation of *P. aeruginosa* PA22 treated with *A. paniculata* compounds: andrographolide (L5); 14-deoxyandrographolide (L6); 14-deoxy-12-hydroxyandrographolide (L11); neoandrographolide (L12); and untreated (UT).

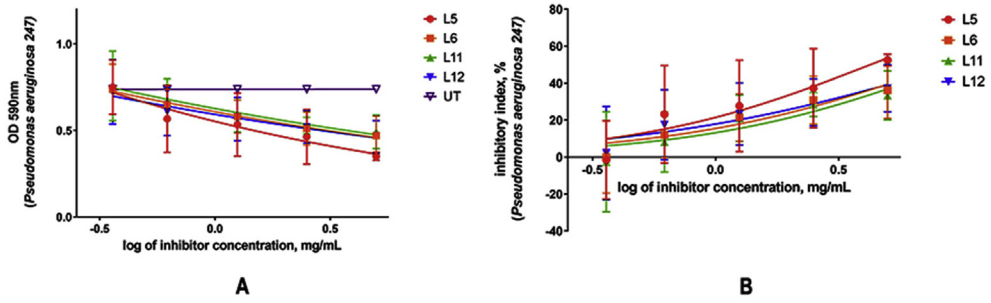

Figure 4. (A) Anti-biofilm formation assay of *P. aeruginosa* PA247 treated with compounds from *A. paniculata*. Values are the means of three independent experiments. (B) Percent inhibition of biofilm formation of *P. aeruginosa* PA247 treated with *A. paniculata* compounds: andrographolide (L5); 14-deoxyandrographolide (L6); 14-deoxy-12-hydroxyandrographolide (L11); neoandrographolide (L12); and untreated (UT).

Table 2. Mean minimum biofilm inhibitory concentrations (MBIC) of the compounds from *A. paniculata* for *P. aeruginosa* PA22 (A) and PA247 (B) and the corresponding OD_{590} in the crystal violet biofilm assay.

A	MBIC (mg/mL)	OD_{590} treated with MBIC	OD_{590} untreated	*p*-value*
L5	0.31	1.29	3.11	0.003
L6	0.31	1.73	3.11	0.006
L11	0.31	2.27	3.11	0.008
L12	0.31	1.59	3.11	0.01
B				
L5	2.5	0.46	0.82	0.04
L6	1.25	0.58	0.82	0.03
L11	1.25	0.58	0.82	0.04
L12	2.5	0.52	0.82	0.02

L5: andrographolide; L6:14-deoxyandrographolide;
L11: 14-deoxy-12-hydroxyandrographolide; L12: neoandrographolide;
*Significance level set at 0.05.

Table 3. Mean OD_{590} of *P. aeruginosa* PA22 (A) and PA247 (B) treated and not treated with minimum biofilm inhibitory concentration (MBIC) of compounds from *A. paniculata* and bromofuranone incubated for 18 h to check for the presence of bacterial growth inhibition.

A	MBIC (mg/mL)	OD_{590} of treated	OD_{590} of untreated	OD_{590} bromofuranone (+ control)	*p*-value*
L5	0.31	0.10	0.13	0.16	0.03
L6	0.31	0.14	0.13	0.16	0.31
L11	0.31	0.13	0.13	0.16	0.42
L12	0.31	0.12	0.13	0.16	0.07
B					
L5	2.5	0.19	0.21	0.17	0.19
L6	1.25	0.19	0.21	0.17	0.05
L11	1.25	0.18	0.21	0.17	0.01
L12	2.5	0.20	0.21	0.17	0.41

*PA clinical isolates were compared to untreated control.
L5: andrographolide; L6:14-deoxyandrographolide;
L11: 14-deoxy-12-hydroxyandrographolide; L12: neoandrographolide;
*Significance level set at 0.05.

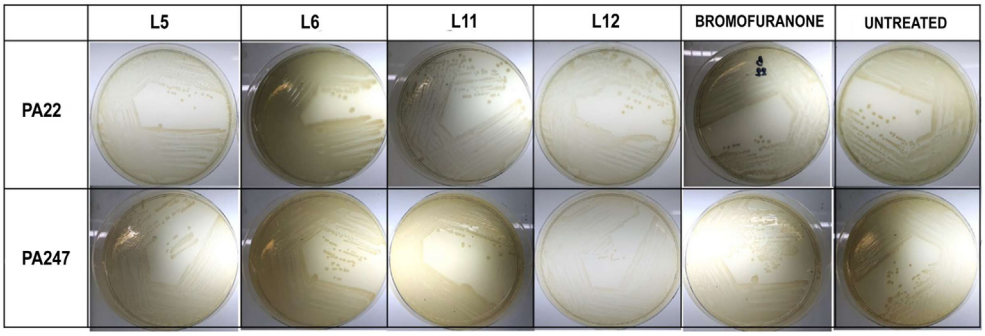

Figure 5. 18-hour cultures of *P. aeruginosa* PA22 and PA247 treated with the minimum biofilm inhibitory concentrations of the andrographolide (L5), 14-deoxyandrographolide (L6), 14-deoxy-12-hydroxyandrographolide (L11), and neoandrographolide (L12), showing absence of growth inhibition.

Table 4. Mean OD_{590} of *P. aeruginosa* PA22 (A) and PA247 (B) cultured in milk substrate treated and not treated with minimum biofilm inhibitory concentration (MBIC) of compounds from *A. paniculata* and bromofuranone in the protease assay.

A	MBIC (mg/mL)	OD_{590} of Treated	OD_{590} of untreated	OD_{590} bromofuranone (+ control)	*p*-value[a] *	*p*-value[b] *
L5	0.31	1.22	0.67	1.16	<0.01	0.23
L6	0.31	1.37	0.67	1.16	0.01	0.04
L11	0.31	1.38	0.67	1.16	0.01	0.05
L12	0.31	1.36	0.67	1.16	0.02	0.09
B						
L5	2.5	2.03	0.59	2.13	<0.01	0.14
L6	1.25	2.11	0.59	2.13	<0.01	0.31
L11	1.25	2.08	0.59	2.13	<0.01	0.18
L12	2.5	2.13	0.59	2.13	<0.01	0.43

L5: andrographolide; L6:14-deoxyandrographolide;
L11: 14-deoxy-12-hydroxyandrographolide; L12: neoandrographolide;
*Significance level set at 0.05.
[a] PA clinical isolates were compared to untreated control.
[b] PA clinical isolates were compared to the positive control bromofuranone.

test revealed that all compounds from *A. paniculata* significantly downregulated the expression of *lasR* in both PA22 and PA247 compared to untreated controls ($p < 0.05$). Meanwhile, there were no significant differences in the downregulated activities among the test compounds in both *P. aeruginosa* strains ($p > 0.05$). This connotes the similarity in the quorum quenching potencies of all compounds.

The significant downregulation of the FGEV of the *lasR* gene in the isolates treated with the compounds compared to the untreated controls concurred with the significant inhibition of the biofilm formation, protease and swarming activities in the test group.

4. Discussion

Andrographis paniculata is one of the most well-studied plants to date due to its therapeutic premise. In this study, the ability of the purified compounds from the leaves of *A. paniculata* to affect quorum sensing

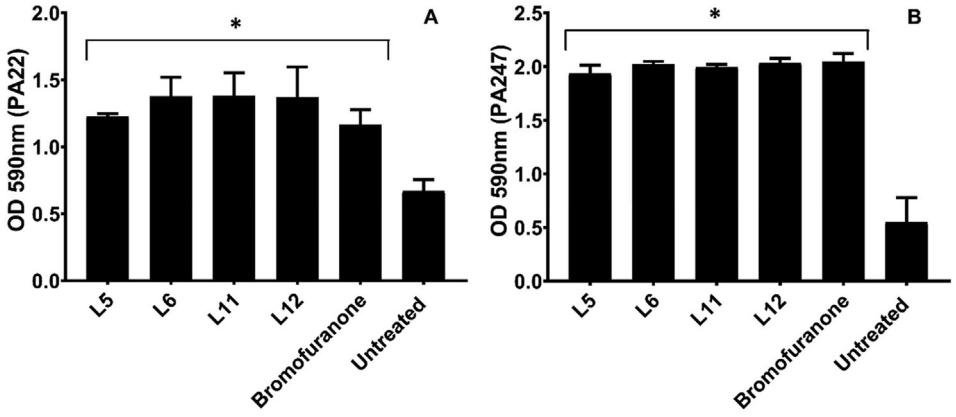

Figure 6. Mean OD_{590} of *P. aeruginosa* PA22 (A) and PA247 (B) cultured in milk substrate treated and not treated with minimum biofilm inhibitory concentration (MBIC) of compounds from *A. paniculata* and the positive control bromofuranone in the protease assay. Asterisks (*) indicate significant difference compared with the untreated control ($p < 0.05$).

Table 5. Mean diameters (in mm) of colonies of *P. aeruginosa* PA22 (A) and PA247 (B) treated with minimum inhibitory concentrations of *A. paniculata* compounds and bromofuranone in the swarming assay.

A	MEAN (mm)	SD	p-value[a] *	p-value[b] *
L5	10	0.81	<0.01	0.11
L6	9	0.81	<0.01	0.04
L11	11	0.81	<0.01	0.21
L12	9.3	1.24	<0.01	0.15
Bromofuranone	11	0.81	<0.01	
Untreated	18	0.81		
B				
L5	9.6	1.24	<0.01	0.09
L6	10.6	1.69	<0.01	0.43
L11	9	2.16	<0.01	0.9
L12	11	0.81	0.01	0.31
Bromofuranone	10.3	1.24	<0.01	
Untreated	19.6	1.24		

L5: andrographolide; L6:14-deoxyandrographolide;
L11: 14-deoxy-12-hydroxyandrographolide; L12: neoandrographolide;
*Significance level set at 0.05.
[a] PA clinical isolates were compared to untreated control.
[b] PA clinical isolates were compared to the positive control bromofuranone.

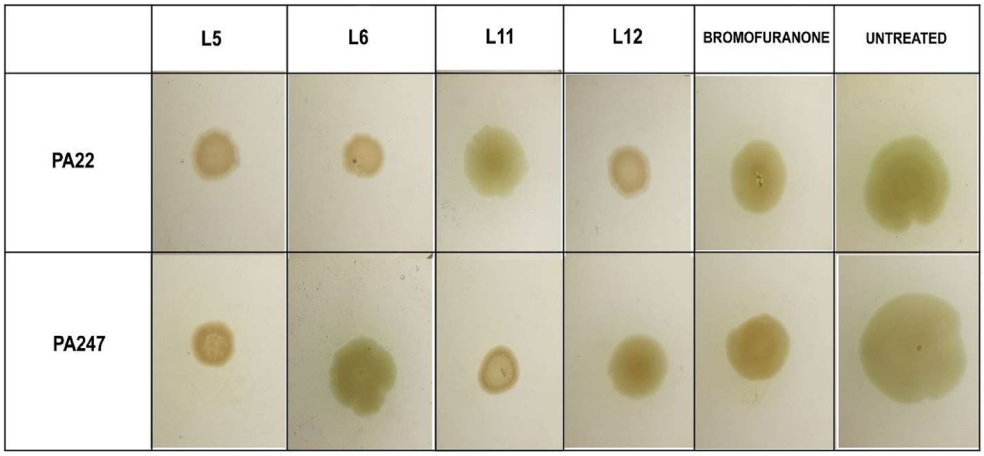

Figure 7. Swarming motility analysis of *P. aeruginosa PA22 and PA247* treated with the mean biofilm inhibitory concentrations of andrographolide (L5), 14-deoxy-andrographolide (L6), 14-deoxy-12-hydroxyandrographolide (L11), and neoandrographolide (L12).

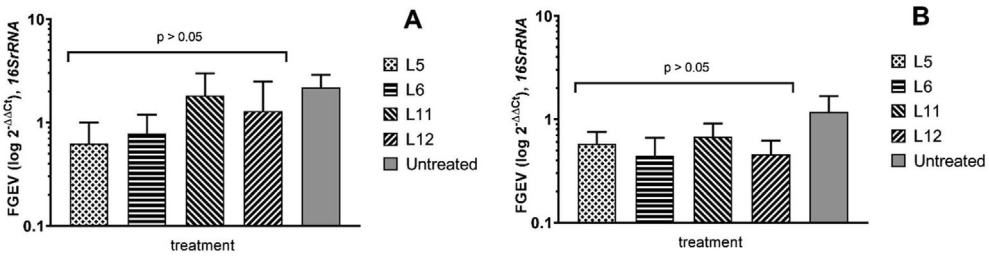

Figure 8. Relative fold gene expression values (FGEV) (log $2^{-\Delta\Delta Ct}$) of the *16SrRNA* of *P. aeruginosa* PA22 (A) and PA247 (B) treated with *A. paniculata* compounds with respective untreated controls: andrographolide (L5); 14-deoxyandrographolide (L6); 14-deoxy-12-hydroxyandrographolide (L11); neoandrographolide (L12); and untreated (UT). There were no significant differences between all the treated and untreated groups.

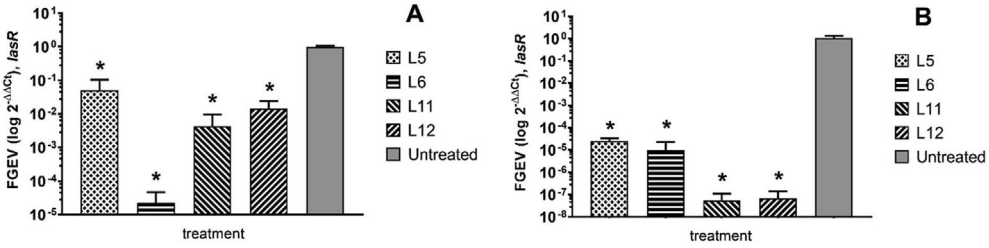

Figure 9. Relative fold gene expression values (FGEV) (log $2^{-\Delta\Delta Ct}$) of the *lasR* gene of *P. aeruginosa* PA22 (A) and PA247 (B) treated with *A. paniculata* compounds and respective untreated controls: andrographolide (L5); 14-deoxyandrographolide (L6); 14-deoxy-12-hydroxyandrographolide (L11); neoandrographolide (L12); and untreated (UT). Asterisks (*) indicate significant difference compared with the untreated control ($p < 0.05$).

activities was determined. The study showed that all of the four *A. paniculata* compounds tested had quorum quenching activities as seen by the inhibition of the violacein pigment production of *C. violaceum,* biofilm formation, protease production and swarming activities of the clinical isolates of MβL-producing *P. aeruginosa* PA22 and PA247. At the same time, the compounds downregulated the expression of the *lasR* gene, which codes for the LasR protein, a transcription activator protein of the quorum sensing system in *P. aeruginosa* that activates quorum sensing-dependent activities. The latter observation supports the contention that the inhibition of the tested virulence mechanisms was due to the quorum quenching activity of the pure compounds from *A. paniculata*.

The anti-biofilm activities of the compounds tested in the present study parallel the results of Murugan et al. (2013b), where between 80-90% of the biofilm formers were found to be inhibited by the methanolic extract of *A. paniculata.* The reduction of the biofilm by the extract was likewise shown to be concentration dependent. Sakuragi and Kolter (2007) showed that andrographolides inhibit the *las* QS system that is responsible for the transcription of the *pel* genes which encode proteins necessary for the production of the EPS. Disruption of the bacterial EPS synthesis halts biofilm formation (Murugan et al., 2013c) and allows bacteria to be susceptible to antimicrobials. LasR inhibitors similar to *A. paniculata* have also been reported to manifest changes in biofilm structure and a reduction in EPS matrix due to the repression of the QS system (Yang et al., 2009).

The study of Banarjee et al. (2017) demonstrated that in addition to the ability of *A. paniculata* to cause significant reduction in biofilm formation, it also inhibited QS-dependent virulence factors such as protease production, swarming motility, pyocyanin production and hemolysin without inhibiting bacterial growth. The swarming growth of PA22 and PA247 showed a significant decrease in motility when exposed to the MBICs of the leaf compounds. Swarming motility promotes bacterial adhesion to cell surface, which is part of the initial stages of biofilm formation. Additionally, the total proteolytic activity of *P. aeruginosa* in this study was inhibited in the presence of *A. paniculata* compounds. This observation is supported by the study of Li et al. (2006) which described the significant suppression of protease and swarming production of *P. aeruginosa* when treated with the andrographolide compound. Panche et al. (2019) characterized the inhibitory properties of the phenolic compounds found in *A. paniculata* in which certain proteases were inhibited without the problem of toxicity.

RT-qPCR results of *lasR* gene expression quantification showed that the *A. paniculata* purified leaf compounds were able to quench the quorum sensing activity of the bacteria by the downregulation of the *lasR* gene expression of PA22 and PA247 compared to the untreated controls. The FGEV of both PA22 and PA247 showed significant reduction in *lasR* gene expression ($p < 0.05$). A study by Algburi et al. (2017) reported that the transcriptional levels of the *las* and *rhl* quorum sensing-regulator genes in the QS system of *P. aeruginosa* were suppressed when exposed to 14-alpha-lipoyl andrographolide. The transcriptional regulator protein, LasI, directs the synthesis of the autoinducer 3-O-C12-HSL, which complexes with the transcriptional regulator LasR, thereby activating the expression of a number of genes involved in virulence.

QS inhibitors have been shown to effectively modulate transcriptional expression of QS-regulated and virulence-associated genes. Compound C30, a furanone derivative, attenuated QS-dependent expression of virulence factors through the suppression of the QS-regulated genes (Hentzer et al., 2003; Ahmed et al., 2019). Similarly, salicylic acid, nifuroxazide and chlorzoxazone were shown to downregulate the *lasR* gene expression, decrease biofilm formation while significantly decreasing the production of the virulence factors of *P. aeruginosa* in a dose dependent manner (Yang et al., 2009). In the same study, the potency of the salicylic acid and nifuroxazide were enhanced when *P. aeruginosa* were exposed to the compounds for an extended period. Ruffin et al. (2016) described that the presence of *lasR* mutants in their study were shown to exhibit markedly decrease virulence factors such as elastase and protease activities. This is reportedly due to the loss of LasR QS signaling function caused by the *lasR* mutations. Likewise, there was a delay in the activation of virulence factors in *lasR* mutants of *P. aeruginosa* (Smith et al., 2006). In the study of Ahmed et al. (2019), trans-cinnamaldehyde effectively downregulated the *las* and *rhl* QS systems, which caused a repression in the LasR and Rh1R proteins, thereby decreasing the expression of virulence-associated genes. Sub-inhibitory concentrations of trans-cinnamaldehyde produced a 22% and 65%

reduction in elastase and protease activities, respectively. Additionally, RT-qPCR data showed significant reduction in *lasA, lasB, rhlB* and *rhlC* expression after transcinnamaldehyde treatment which were correlated with the virulence factor results.

5. Conclusions

Results of the current study support the antibacterial property of purified leaf compounds from *A. paniculata* in terms of their quorum sensing quenching activity as shown by their ability to inhibit biofilm formation, decrease protease production and swarming motility of clinical isolates of MβL-producing *P. aeruginosa* through downregulation of the *lasR* gene expression. This opens the potential for their use as effective alternative treatments for MβL-producing bacterial pathogens.

Declarations

Author contribution statement

An Margarete Tan Lim, Glenn G. Oyong: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Maria Carmen S. Tan, Chien Chang Shen, Consolacion Y. Ragasa: Contributed reagents, materials, analysis tools or data.

Esperanza C. Cabrera: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Funding statement

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Data availability statement

Data associated with this study has been deposited at International Journal of Pharmacognosy and Phytochemical Research under the accession number ISSN: 0975-4873.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

Acknowledgements

The authors would like to thank the Molecular Science Unit Laboratory, Center for Natural Sciences and Environmental Research (CENSER), De La Salle University (DLSU), Manila for the use of equipment and facilities, and the DLSU Science Foundation, through the University Research Coordination Office for funding the laboratory materials and reagents.

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