Anti-quorum Sensing Potential of Ageratum Conyzoides L. (Asteraceae) Extracts

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Research Article

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Anti-quorum sensing potential of *Ageratum conyzoides* L. (Asteraceae) extracts.

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Abstract

***Background**: Pseudomonas aeruginosa* causes infections in human particularly immunocompromised patients with cystic fibrosis, severe burns and HIV, resulting in high morbidity and mortality. The pathogenic bacteria *P aeruginosa* produces virulence factors regulated by the mechanism called quorum sensing system.

Objective: The aim of this study was to assess the anti-quorum sensing activity of *Ageratum conyzoides* extracts

***Method**: Chromobacterium violaceum* reporter strain CV026 was used to highlight any interference with bacterium QS and strains derived from *P. aeruginosa* PAO1 were used to reveal any interference with the expression of quorum sensing genes, and to assess any impact of extract on the kinetics of the production of pyocyanin, elastases and biofilm formation.

Results: Hydro-methanolic extract at the sub-inhibitory concentration of 100 µg/mL reduced quorum sensing virulence factors production such as, pyocyanin, elastases, rhamnolipids and

biofilm formation in *P. aeruginosa* PAO1 after 18 hours monitoring. Extract showed significant inhibition in HSL-mediated violacein production on *C. violaceum* CV026 after 48 hours monitoring. Biofilm formation was inhibited up to 32%. It affected QS gene expression in PAO1. The regulatory genes *lasR / rhlR* and the *lasI* synthases were most affected. At 8hours, hydro-methanolic extract reduced both QS gene to more than 30% (*lasI/lasR* and *rhlI/R* respectively 33.8% /30.2% and 36% /33.2%). *RhlA* and *lasB* genes have been relatively affected (13.4% and 28.9%). After 18 h, this extract reduced significantly the expression of regulatory genes *lasR* (31%) and *rhlR* (39.6%) although synthases genes seemed to be less affected (*lasI*/21.2% and *rhlI*/11.6%). A limited impact was observed on the downstream genes (*lasB* /20.0% and *rhlA* /15.3%). No negative impact was observed on CV026 and PAO1 growth and cell viability. Our study also showed that *A. conyzoides* having ample amount of phenolics, flavonoids and triterpenoids. This phytochemical content could be one of the factors for showing anti quorum potential.

Conclusion: Results indicate that hydro methanol 80 % extract from *A. conyzoides* could be a source of potential QS inhibition compounds.

Keywords: *Ageratum conyzoides*; *Chromobacterium violaceum; Pseudomonas aeruginosa;* Quorum sensing, biofilm.

Introduction

Conventional infectious diseases treatment used antibiotics for killing or inhibiting the bacteria growth [1]. A major consequence was the development of drug resistance pathogens and the emergence of multi-resistant strains resulting from antibiotics overuse [2]. It has become essential to search for new effective antibacterial molecules. One of promoting strategies was the founding of compounds reducing the virulence of bacteria without killing them in quorum

47 sensing system [3]. Quorum sensing is a communication system depending on the bacterial
48 density [4].

49 In contrast to antibiotics, the quorum inhibition molecules do not have a direct effect on
50 bacterial growth but on the reduction of virulence. Quorum sensing inhibitors may therefore be
51 a new class of antibacterial agents [5, 6]. These compounds could be come from natural
52 products of medicinal plants.

53 *Ageratum conyzoides* found in Burkina Faso, has long been used in folk medicine for infectious
54 and skin diseases treatment [7]. This plant was reported to have significant antimicrobial
55 properties [8, 9]. The leaves were mainly used as poultices on wounds, burns, gastrointestinal
56 pains and anthrax. [7]. The ethanolic extract of *A. conyzoides* has shown antibacterial activity
57 against *Pseudomonas aeruginosa, Staphylococcus aureus*, *Giardia duodenalis*, *Escherichia*
58 *coli*, and *Shigella dysentery* strains [10, 11]. The minimum inhibitory concentration (MIC) and
59 the minimum bactericidal concentration (MBC) were 160 mg/mL for *P. aeruginosa* [10]. A
60 previous study was conducted for antibacterial and wound healing properties and revealed that
61 methanolic extract (6%) did not inhibit bacteria growth but more than 90% wounds healing was
62 recorded by the extract [12]. In Cameroon, reported anti-*Helicobacter pillory* activity of
63 hexanic and ethyl acetate fractions was reported [13]. From Ghana and Nigeria, *A. conyzoides*
64 had activity against the Gram-positive microorganism like *S. aureus* [14, 15] but no inhibition
65 of growth of *S. aureus* methicillin resistant were observed. Strong antibacterial properties of
66 the methanolic extract were evidence against *P. aeruginosa*, *S. aureus*, *Shigella ssp* and *Proteus*
67 *ssp* [16]. Recently, the essentials oils from *A. conyzoides* flowers stems showed moderate
68 activity against *S. aureus, E coli, Enterococcus faecalis* and *Citrobacter koseri* and the
69 hydroethanolic extract showed *S. aureus* MIC as 2% [9, 17]. However, according to the best
70 bibliographic knowledge there no study of this plant against pathogenic bacteria by using

quorum sensing pathway. Therefore, the present study, was initiated to investigate the anti-quorum sensing properties of hydro methanolic extract of *A. conyzoides* extracts.

Material and Methods

Material

Plant Material and Extraction

Ageratum. Conyzoides L. (Asteraceae) samples were collected in Gampela in August 2014 (Ouagadougou, Burkina Faso). This wild plant usually grows close to housing and crop fields and is generally considered as a weed by the local population. It has been collected in the fields after verbal permission from the owners. The plant was identified by Professor Amadé Ouedraogo, botanist of the Joseph Ki-Zerbo University. Plant Samples were washed and dried at room temperature. Voucher specimen of the material have been deposited at the national herbarium of Burkina Faso (HNBU) under code 8755. The dried material was reduced to powder and extracted by maceration with methanol (hydro-methanolic 20/80 v/v) at room temperature. The extracts were concentrated in vacuum evaporator. Extract solution (10 mg/ml in 100% DMSO) was obtained, after filtration (0.22 µm pore size) stored at 4^{o} C for future processing. Collection and experimental research on this plant were in accordance with national guidelines in Burkina Faso.

Bacteria and chemical reagents

p-iodonitrotetrazolium, elastin congo red, crystal violet, chloroform, acetic acid, hydrochloric acid, 3-(N-morpholino) propane sulfonic acid (MOPS), Folin-Cioralteun reagent, perchloric acid, glacial acetic acid, vanillin-glacial acetic, aluminium trichloride, carbenicillin (antibiotic), O-nitrophenyl- β-D-galactopyranoside, nutrient aga and Lauria-Bertani (LB) broth medium were obtained from Sigma-Aldrich (Germany). *P. aeruginosa* PAO1 and *C. violaceum* CV026 were provided from the Plant Biotechnology Laboratory (Free University of Belgium ULB).

Bacterial strains Plasmids and growth conditions

Pseudomonas aeruginosa PAO1 were grown at 37°C, stirring 175 rpm and *Chromobacterium violaceum* CV026 at 30°C, 175 rpm in LB broth (pH 7). The *P. aeruginosa* derivatives harboring plasmid pPCS1001, pPCS1002, pβ03, pLPR1, pβ02, pβ01 and pTB4124. were streaked onto LB-MOPS broth (50 mM, pH 7,2) supplemented with carbenicillin. All strains were from cryopreservation and were then incubate overnight.

Methods

Bacterial growth

MIC and MBC assay

The minimal inhibitory concentrations (MIC)of the extract were determined using 96 well microplates [18]. An overnight bacterial culture was diluted with LB broth to obtain a starting inoculum (10^6 CFU/mL). Each inoculum (180 µL) was incubated with a serial concentration of extracts ranged from 5 mg/mL to 0.049 mg/mL. Bacteria growth were studied by using p-iodonitroterazolium coloration and the MIC and MBC were determined.

Growth kinetics of *C. violaceum* CV026 and *P. aeruginosa* PAO1

The growth kinetics were assessed for 48 hours for CVO26 and 18 hours for PAO1. At regular time intervals (6h for CVO26, and 3h for PAO1), a bacterial colony count was performed to evaluate the impact of the plant extract on bacterial viability. The number of bacteria at baseline was 10^6 CFU m/L.

Virulence factors production

Violacein kinetic production in *C. violaceum* CV026 assay

The violacein kinetic production induced by hexonoyl-L-homoserine lactone was evaluated during 48 hours [19]. CV026 inoculum (100 µL) was incubated for 48h with1.880 mL of LB broth supplemented with HHL and samples (20 µL). Violacein amount was assessed by using the absorbances ratio 585nm / 600nm.

120 Pyocyanin kinetic production in *P aeruginosa* PAO1

121 The pyocyanin kinetic production was evaluated during 18 hours [20]. Briefly, 150 µL of
122 samples were inoculated with 750 µL of PAO1 inoculum in LB medium. From the supernatant,
123 pyocyanin was extracted with chloroform and re-extracted from chloroform with HCl for
124 evaluating the absorbance at 380 nm.

125 Elastase et rhamnolipids assay

126 Elastase production was detected using elastin Congo red (ECR) [19] , rhamnolipid production
127 by methylene Blue [21]. *P. aeruginosa* PAO1 were grown for 8hours and 18hours in 5mL of
128 LB medium (37°C,175 rpm). Elastase production was indicated by absorbance at 460 nm and
129 638 nm for rhamnolipids.

130 Biofilm formation

131 Biofilm formation in *P. aeruginosa* PAO1 was performed according to protocol described by
132 [22]. After 18 hours growth, the culture of PAO1 was washed three times, then fixed with
133 methanol 99% for 15 min. Crystal violet (0.1% in water) was added to each well after removing
134 of methanol and incubated for 30 min. Biofilm were stained coloured with crystal violet and
135 dissolved in acetic acid (33%) then the absorbance was read at 590 nm.

136 Genes Expression and β-Galactosidase assay

137 All the reporter strains of PAO1 were incubated in LB-MOPS-Carbenicillin for 8hours and
138 18hours (50 µL, 37 °C, 175 rpm) supplemented with samples (Extract and DMSO). After
139 incubation, the absorbances were read at 600 nm. Then samples were used to perform the β-
140 galactosidase assay with O-nitrophenyl- β-D-galactopyranoside. β-Galactosidase activity has
141 been expressed in Miller units [23].

Determination of total polyphenol, total flavonoid and total triterpenoid content

Plant extract (10mg/mL in methanol) was use for the quantitative tests. Total polyphenolic contents in extract were determined by spectrometric method as described by Singleton and al [24], total flavonoids as described by Arvouet-Grand and al [25] and total terpenoids as described by Fan and He [26] was carried out. total polyphenolics were expressed as mg/g gallic acid equivalents (GAE). Total flavonoid as mg/g quercetin equivalent (QE) and total triterpenoids as mg/g ursolic acid equivalent (UAE).

Statistical Analysis

Experiment was performed in triplicate and data were expressed as mean ± SD. GraphPad prism Software was used for statistical analysis (GraphPad software Inc., San Diego, CA, USA) the One-way or two-way ANOVA followed by the Tukey or Bonferonni test on GraphPad at the value ≤ 0.05 was considered significant.

Results

Effect on bacterial growth

MIC and MBC

The MICs for hydro-methanolic 80% extract were 5.0mg/mL for *P. aeruginosa* PAO1 and 2.5mg/mL for *C. Violaceum* CV026. For two bacteria the MBC values were 5mg/mL (> 5mg/mL for PAO1). MBC/MIC ratio (<4) for both strains indicated a bactericidal effect of extract. For concentrations below the MIC, *A conyzoides* extract should not induced the bacteriostatic or bactericidal activity. So, at a minimum concentration of 100µg/mL, corresponding to CMB/CMI >32 ratio, the strains should be tolerant to the plant extract which will allow evaluation of its intrinsic effect on QS-dependent bacterial factors. Thus, we used the sub-inhibitory concentration of 100 µg / mL for further processing.

Effect on bacterial kinetics growth

Data of Figure 1 (histogram) showed the *C. violaceum* kinetic in the presence of hydro methanolic extract in relation to DMSO 1% for 48 hours. The time-growth kinetic profile of PAO1showed two phases. An exponential growth phase (0-24h) followed by a stationary phase (24-48h). The exponential phase started immediately at the beginning of the incubation. In both phases, the same growth pattern of *C. violaceum* CV026 was observed. Bacteria cells have grown normally. The results showed that the samples (methanol 80% extract and DMSO1%) exhibited substantially similar growth kinetics. However, as shown in figure 2b, a negative impact was found on the time-dependent kinetics of violacein production.

Figures 2 (histogram) showed the *P. aeruginosa* PAO1 kinetic data in the presence of methanolic 80% extract in relation to DMSO 1% during 18 hours of growth. As shown in CV026 cells growth, as soon as the samples were added, the bacterial cells started immediately growing in two phases. After cell growth reached the exponential phase at 12hour, a stationary phase was followed until 18h (figure 3a). In both phases, the same growth pattern of PAO1 was observed. The results showed that the samples (methanol 80% extract and DMSO1%) exhibited substantially similar growth kinetics. Bacteria cells grown in the presence of extract showed normal growth. Methanolic 80% extract (100 µg / mL) did not significantly affect the cell viability according to CFU quantification (Histogram Figure 1 and 2).

Effect on violacein kinetic production

Significant inhibition of HHL-induced violacein production was recorded during the exponential phase ranging from 3% (6h) to 38% (24h) (figure 1 curve). In the stationary phase, there was a more or less constant reduction running from 35% to 32% for 30h-48h period. Highest rate was reached after 12h (42%). The results showed the time-dependent inhibitory effect of hydro-methanolic 80% extract on violacein kinetics production by *C. violaceum* CV026. The addition extract (100µg/mL) to the culture of CV026 without HHL did not induce

violacein production (data not shown). This was an indication that the extract did not contain any HHL-type compounds but contained some compounds with QS system deactivating ability. On this basis, *A. conyzoides* extract has been further investigates for its impact on the QS mechanism in *P. aeruginosa* PAO1.

Effect on pyocyanin kinetic production

As shown in figure 2 (curve), a negative impact was found on the time-dependent kinetics of pyocyanin production. A significant reduction at the exponential phase of PAO1 growth was induced by the extract. Thereafter, a lag phase in pyocyanin production was observed (about 3h). Pyocyanin production reduction was appeared at 9hour (exponential phase). The reported reduction rates were 45% and 42% at 9 hours and 12 hours respectively. In the stationary phase, there was a more or less constant reduction running from 40%, to 37% at 15h and 18h. Maximum reduction (45%) was reached after 9 hours of growth. The results clearly showed that pyocyanin production inhibition occurred during the exponential phase. The hydro-methanolic extract was further tested for its impact on elastase production and biofilm formation.

Effect on elastase production

The effect of methanolic 80 % extract (100 µg/mL) on the elastase production in PAO1 strain has been studied after 8h and 18h of growth according to previously described procedures in method section. Elastase amounts time depending for this experiment were given in figure 3a. As shown in figure 3a, no significant negative effect was observed on PAO1 growth at 8hours and 18 hours. However, hydro-methanolic extract decreased significantly ($P<0.05$) the activity of elastases as salicylic acid. At 8 hours, the effect of extract (35% inhibition) was relatively greater than salicylic acid (31% inhibition). In contrast after 18 hours, a low activity of the extract (31% inhibition) was observed compared to salicylic acid (37% inhibition), but

significant compared to DMSO. In both time points, no significant difference was found between the activity of extract and that of salicylic acid.

Effect on rhamnolipids production

Figure 3b shows the effect of the extract on the production of rhamnolipids. After 8h as at 18h, the amount of rhamnolipids was significantly reduced by 24% and 18% respectively compared to DMSO (negative control). After 8h of growth (exponential phase, figure 2 histogram) the effect of the extract was slightly increased over salicylic acid (21%) before settling at 18% (against 24% for salicylic acid).

Anti-biofilm formation in *P aeruginosa* PAO1

Given the link between QS and biofilm formation, methanolic 80% extract from *A. conyzoides* was assessed against QS-mediated biofilm formation by *P. aeruginosa* PAO1(Figure 4). Biofilm is a matrix of polysaccharides which protect *P. aeruginosa* from environment. As shown in figure 4, the number of cellular polysaccharides produced by *P. aeruginosa* PA01 over 18 h growth was reduced by the hydro-methanolic extract (100µg/mL) and salicylic acid. A moderate anti-biofilm activity was observed for extract (32%) and salicylic acid (44%). As shown in figure 1 and 2, the extract had no significant impact on *the P. aeruginosa* PAO1 growth. Based on these preliminary screenings, the effects of hydro-methanolic on QS regulation in PAO1 was further analysed.

Effect on QS genes in *P. aeruginosa* PAO1

In order to highlight any interference with the QS genes expression in *P. aeruginosa* PAO1, it was followed the transcription rate over 8h and 18h of growth (Table 1). The expression of synthetases genes (*lasI* and *rhlI*), regulatory genes (*lasR* and *rhlR*) and genes controlling virulence factors (*lasB* and *rhlA*) was investigated. Similarly, the expression of the aceA gene encoding isocitrate lyase (not related to QS) was also assessed. Salicylic acid was used as

positive control. With a final concentration of 100 µg/mL, no negative impact on PAO1end-point cell density was recorded but most of the QS genes were affected by extract when compared with salicylic acid and DMSO treatment (Table 1). As shown in this table, at 8h, hydro-methanolic 80% extract reduced both QS gene to more than 30% (*lasI/lasR* and *rhlI/R* respectively 33.8%/30.2% and 36% /33.2%). *RhlA* and *lasB* genes have been relatively affected (13.4% and 28.9%). After 18 h, hydro-methanolic 80% extract significantly reduced the expression of regulatory genes *lasR* (31%) and *rhlR* (39.6%) although synthetases genes seemed to be less affected (*lasI*/21.2% and *rhlI*/11.6%). A limited impact was observed on the downstream genes (*lasB* /20.0% and *rhlA* /15.3%). In general, for most of QS-related genes (i.e *RhlI*, rhlR and *lasI* and *lasB*) significant decreases were recorded after 8h. As shown in figure 2, no negative impact was observed on PAO1 viability; suggesting that the decrease in QS genes expression, were not due to a drop in cell viability. Moreover, it is interesting to point out that plant extract has no negative effect on *aceA* gene transcription. This is proof of its specific action on *rhlI/R*, *lasI/B* and las genes without disturbing the transcriptional machinery of PAO1.

Quantitative analysis of phytochemicals

The total phenolic compound is 123,33 ± 5,6 mg while the total flavonoid and total triterpenoid is respectively 112 ± 07 and 15,60 ± 0,6 mg. The presence of phenolic, flavonoids compound and triterpenoids provided the evidence that the plant may have antioxidant and antimicrobial activity.

Discussion

Breaking down bacterial resistance to antibiotics is one the major concerns in research for new drugs against infectious diseases. Traditional medicinal plants have long been a source of medicines [27, 28]. A great deal of plant-directed research has been carried out to discover compounds to control multidrug resistant pathogens [29, 30]. This study is the first in vitro

investigation on the anti-QS properties of *A. conyzoides* from Burkina Faso. Susceptibility test of hydro methanolic 80% extract showed an inhibition of quorum sensing rather than anti-bacterial effect.

Quorum sensing is a complex regulatory network that modulates the expression of multiple virulence factors such elastase, pyocyanin and rhamnolipids [31, 32]. *C. violaceum* CV026, has a low human health impact, but widely used as a reporter strain in QS screening [33]. *A. conyzoides* extract were found to affect significantly (time dependent) HHL-induced violacein production by CV026, and inhibits the production of pyocyanin. The extract also decreased the production of elastases as much as biofilm formation by wild type strain PAO1. Virulence factors and the biofilm formation examined in this study are under QS control [34] . As observed with *C. violaceum* CVO26, methanolic 80% extract had no negative impact on *P. aeruginosa* PAO1 growth and cell viability. Thus, at a concentration (100 µg/mL) below the MIC (5 mg/mL), no bacteriostatic or bactericidal effects were detected. This observation supports the findings of Chah and *al* [12] who reported the lack of inhibition of the PAO1 growth by the methanolic extract and those of Odeleye and *al* [10] whose results indicted a sensitivity of 160mg/mL well above the MIC value recorded in this study.

In *P. aeruginosa*, the QS regulates the expression of *lasI/R* and *rhlI/R* genes as well as the production of virulence factors such as elastase (*lasB*), *LasA* protease (*lasA*), alkaline protease (*aprA*), rhamnolipids (*rhlA/B*) and pyocyanin [35, 36]. Methanolic 80% plant extract used in this study showed significant effect on QS genes expression in *P. aeruginosa*. In general, the regulatory genes *lasR / rhlR* and the *lasI* synthase gene were most affected. Indeed. Plant extract caused inhibition of QS-controlled virulence factors genes (*rhlA* and *lasB*) which confirm the significant reduction in pyocyanin production, elastases production, rhamnolipids and biofilm formation [35]. The expression of the QS-independent gene, *aceA* gene (isocitrate lyase gene

expression regulator) was analysed. As shown in 1 the *A. conyzoides* extract has no impact on *aceA*.

Overall, the data indicated that the anti-QS activity of *A. conyzoides* extract could be caused by the interference with the target genes. The phytochemicals such as polyphenols, flavonoid and triterpenoids are able to inhibit the QS genes expression and the production of virulence factors [37, 38].

Altogether, these non-bactericidal anti-virulence properties, the ample amount of phenolic, flavonoid, triterpenoid and the reported antimicrobial activities, provides additional evidence and support the wide anti-infectious use of this plant in traditional medicine[7, 16] Indeed, the reduction of QS genes and the end-effect on virulence factors productions allow to explain the historical use of *A. conyzoides* and thus justify its ethnomedicinal use. These observations also provide an opportunity to extrapolate on how this plant could be used in the future. Focusing on anti-QS and ant-virulence, a new quorum quenching could be discovered from *A. conyzoides*.

Conclusion

The present study reports the anti-QS activity of *A. conyzoides*. Hydro methanolic 80% extract effectively inhibited QS genes expression, signal concentration and virulence factors in *P. aeruginosa*. The promising properties may be due the presence of various phytochemicals such as phenolic, flavonoid and triterpenoids. These phytochemicals compounds could a factor that target both the signals' molecules and the genes of QS. Thus, Research is currently underway to isolate the bioactive compounds.

Declaration

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this article.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

EC collected the plant sample, conducted the extraction, biological tests. VO and AR analysed and interpreted the data. MC contributed to the statistical analysis, graphing and plotting. He provided extensive input to the writing of the manuscript. MK is the research director and head of the laboratory. All authors have read and approved the manuscript.

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Figures and legend

Figure 1: Effect on bacterial *C. violaceum* CV026: Left y axis: kinetics growth UFC/ml histogram. Right y axis: Kinetics of violacein production curve graph.

*Figure 2:*Effect on bacterial *P. aeruginosa* PAO1: Left y axis: kinetics growth UFC/ml histogram. Right y axis: Kinetics of violacein production curve graph.

*Figure 3:*Methanol 80% extract from *A. conyzoides* reduce elastase (a) and rhamnolipids (b) production in *P. aeruginosa* PAO1 growth at 8 hours and 18 hours compared to DMSO 1% used as negative control. Salicylic acid was used as positive control. P value of $p<0.05$

*Figure 4:*Effect of methanol 80% extract from *A. conyzoides* on Biofilm formation in *P. aeruginosa* PAO1after 18 hours compared to DMSO 1% used as negative control. Salicylic acid was used as positive control. P value of $p<0.05$ was considered as significant. *** Data that are statistically different ($p<0.05$).

Figures

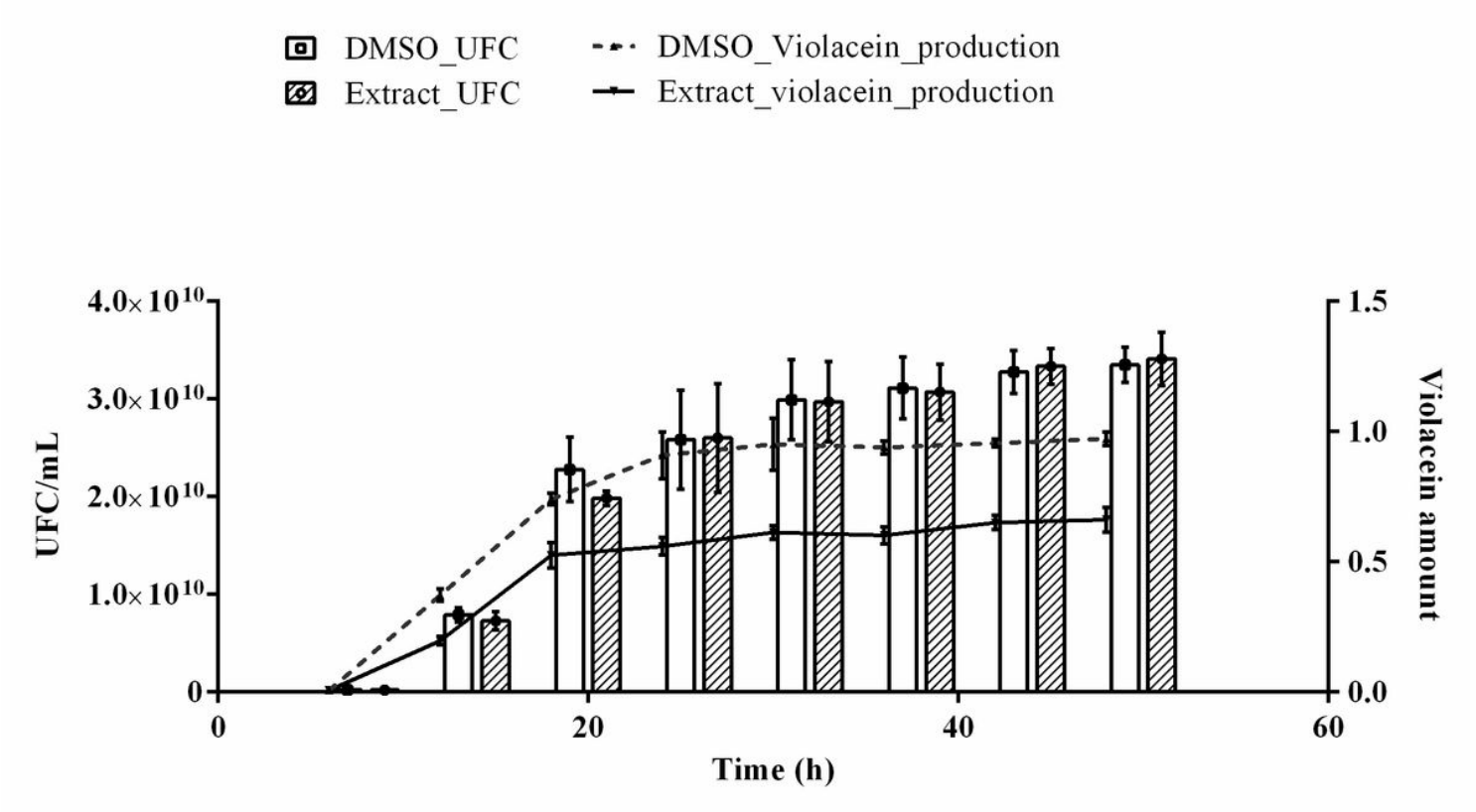


Figure 1

Effect on bacterial C. violaceum CV026: Left y axis: kinetics growth UFC/ml histogram. Right y axis: Kinetics of violacein production curve graph.

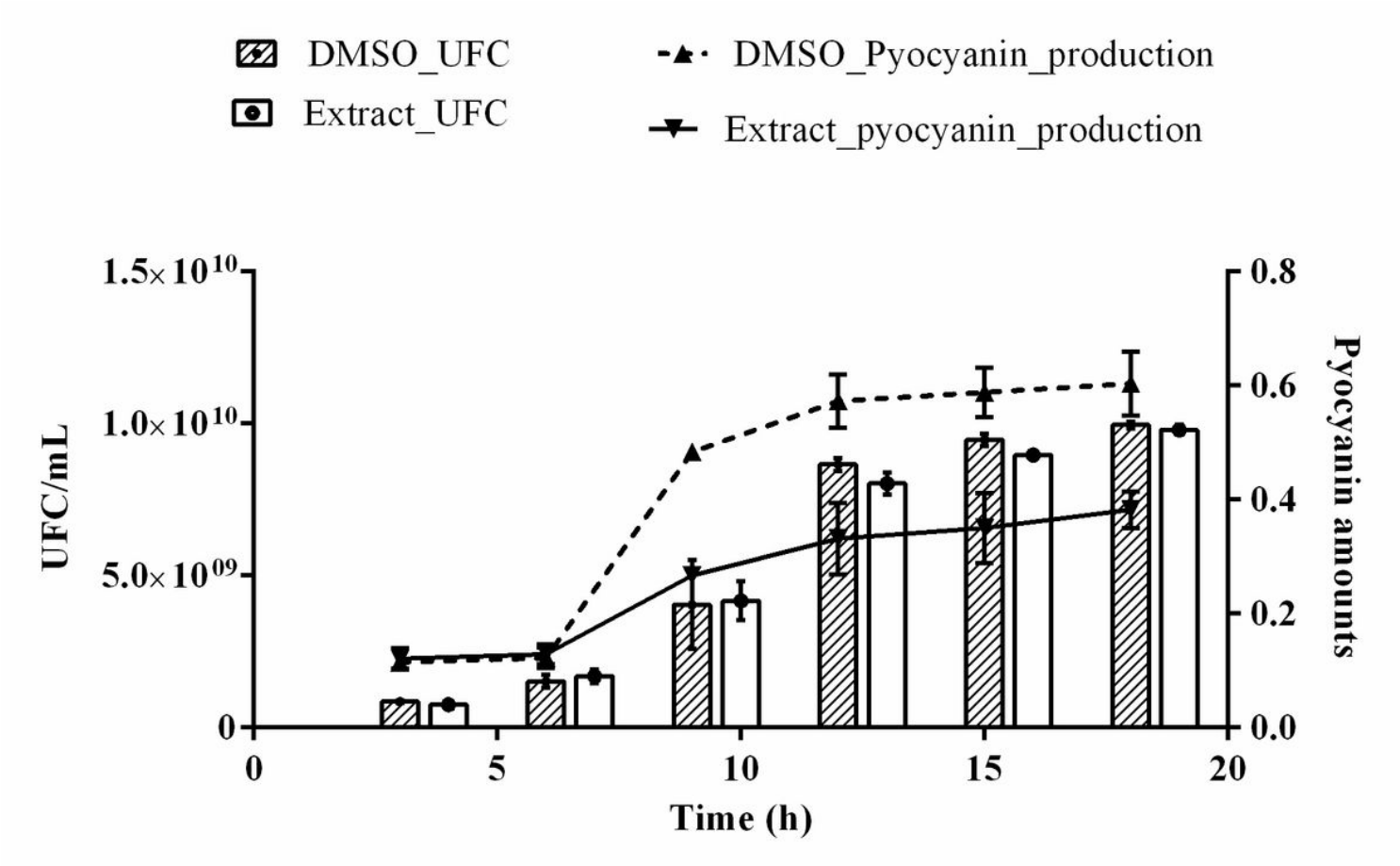


Figure 2

Effect on bacterial P. aeruginosa PAO1: Left y axis: kinetics growth UFC/ml histogram. Right y axis: Kinetics of violacein production curve graph.

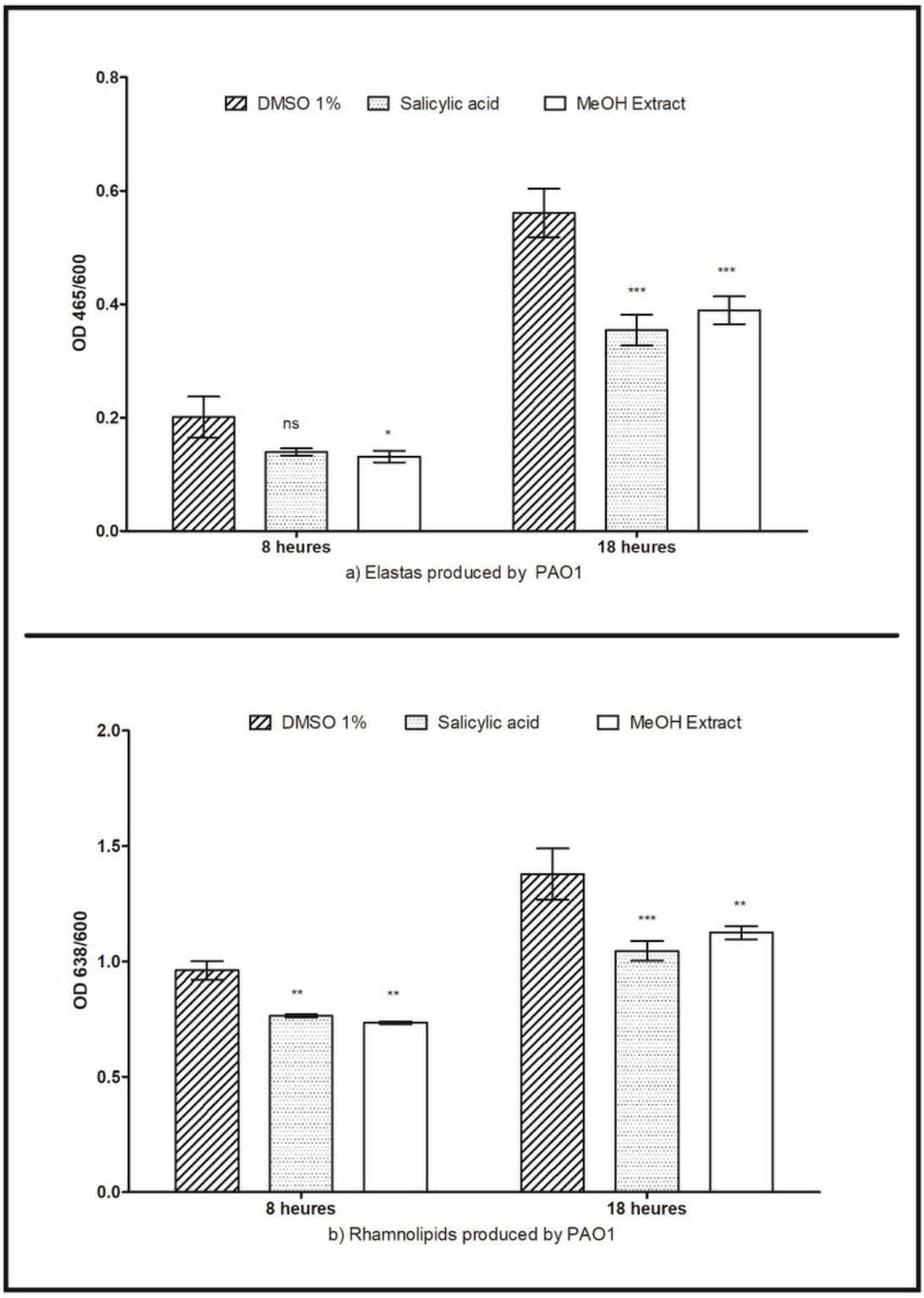
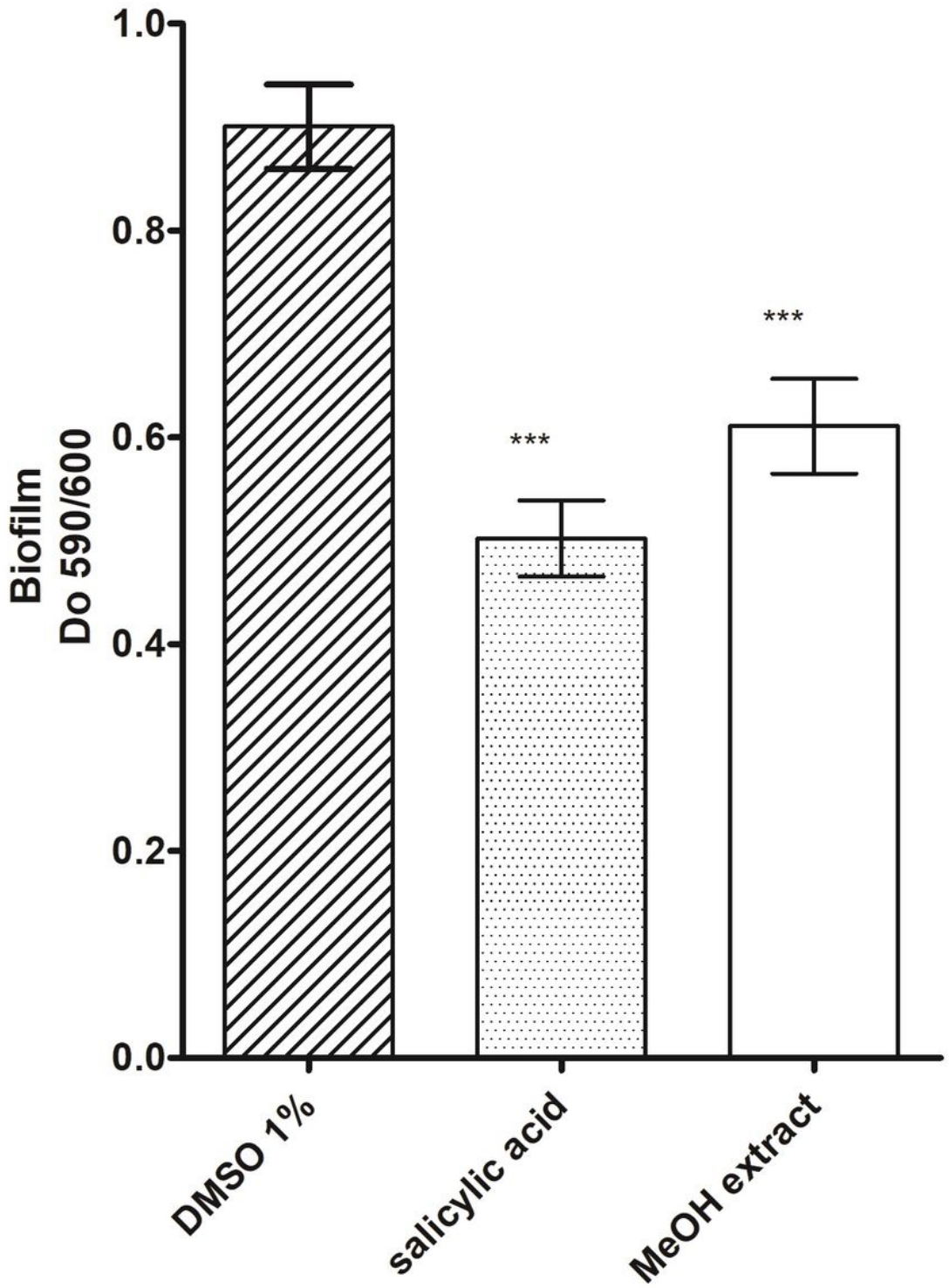


Figure 3

Methanol 80% extract from A. conyzoides reduce elastase (a) and rhamnolipids (b) production in P. aeruginosa PAO1 growth at 8 hours and 18 hours compared to DMSO 1% used as negative control. Salicylic acid was used as positive control. P value of $p<0.05$



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