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

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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 8 hours, 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
sensing system [3]. Quorum sensing is a communication system depending on the bacterial density [4].

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

*Ageratum conyzoides* found in Burkina Faso, has long been used in folk medicine for infectious and skin diseases treatment [7]. This plant was reported to have significant antimicrobial properties [8, 9]. The leaves were mainly used as poultices on wounds, burns, gastrointestinal pains and anthrax. [7]. The ethanolic extract of *A. conyzoides* has shown antibacterial activity against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Giardia duodenalis*, *Escherichia coli*, and *Shigella dysentery* strains [10, 11]. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were 160 mg/mL for *P. aeruginosa* [10]. A previous study was conducted for antibacterial and wound healing properties and revealed that methanolic extract (6%) did not inhibit bacteria growth but more than 90% wounds healing was recorded by the extract [12]. In Cameroon, reported anti-*Helicobacter pillory* activity of hexanic and ethyl acetate fractions was reported [13]. From Ghana and Nigeria, *A. conyzoides* had activity against the Gram-positive microorganism like *S. aureus* [14, 15] but no inhibition of growth of *S. aureus* methicillin resistant were observed. Strong antibacterial properties of the methanolic extract were evidence against *P. aeruginosa*, *S. aureus*, *Shigella ssp* and *Proteus ssp* [16]. Recently, the essentials oils from *A. conyzoides* flowers stems showed moderate activity against *S. aureus*, *E coli*, *Enterococcus faecalis* and *Citrobacter koseri* and the hydroethanolic extract showed *S. aureus* MIC as 2% [9, 17]. However, according to the best 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° 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-Ciorylateun 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 (10⁶ 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 piodonitroterazolium 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 10⁶ CFU mL⁻¹.

**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 with 1.880 mL of LB broth supplemented with HHL and samples (20 µL). Violacein amount was assessed by using the absorbances ratio 585nm / 600nm.
Pyocyanin kinetic production in *P. aeruginosa* PAO1

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

Elastase et rhamnolipids assay

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

Biofilm formation

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

Genes Expression and β-Galactosidase assay

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

Plant extract (10mg/mL in methanol) was used 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 induce 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.
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 PAO1 showed 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 12 hour, 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 investigated 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 9 hours (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 8 hours 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 \textit{P. aeruginosa} PAO1

Given the link between QS and biofilm formation, methanolic 80% extract from \textit{A. conyzoides} was assessed against QS-mediated biofilm formation by \textit{P. aeruginosa} PAO1(Figure 4). Biofilm is a matrix of polysaccharides which protect \textit{P. aeruginosa} from environment. As shown in figure 4, the number of cellular polysaccharides produced by \textit{P. aeruginosa} PAO1 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 \textit{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 \textit{P. aeruginosa} PAO1

In order to highlight any interference with the QS genes expression in \textit{P. aeruginosa} PAO1, it was followed the transcription rate over 8h and 18h of growth (Table 1). The expression of synthetases genes (\textit{lasI} and \textit{rhlI}), regulatory genes (\textit{lasR} and \textit{rhlR}) and genes controlling virulence factors (\textit{lasB} and \textit{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 PAO1-endpoint 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 antibacterial effect.

Quorum sensing is a complex regulatory network that modulates the expression of multiple virulence factors such as 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* PAO1 after 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 PA01: Left y axis: kinetics growth UFC/ml histogram. Right y axis: Kinetics of violacein production curve graph.
Methanol 80% extract from A. conyzoides reduce elastase (a) and rhamnolipids (b) production in P. aeruginosa PA01 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 PAO1 after 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).