

*Full Length Research Paper*

# Anti-quorum sensing potential of *Ageratum conyzoides* L. (Asteraceae) extracts from Burkina Faso

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***Pseudomonas aeruginosa* causes infections in humans, particularly immune-compromised 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. The aim of this study was to assess the anti-quorum sensing activity of *Ageratum conyzoides* extracts. Chloroform fraction from hydro-methanolic extract at the sub-inhibitory concentration of 100 µg/mL reduced quorum sensing virulence factors production such as pyocyanin, elastases, and rhamnolipids in *P. aeruginosa* PAO1 after 8 and 18 h monitoring. Moreover, a significant inhibition in HSL-mediated violacein production on *C. violaceum* CV026 was recorded after 24 and 48 h monitoring without affecting the bacterial growth. The chloroform fraction was rich in polyphenols and triterpenes, and was found to interact with QS receptors. The regulatory genes (*rhIR* and *lasR*) and downstream genes (*RhIA* and *lasB*) were the most affected, while synthase genes (*lasI* and *rhII*) were the least affected. High-performance liquid chromatography with diode-array detection (HPLC-DAD) analysis allowed the identification and quantification of some compounds such as gallic acid, vanillic acid, ellagic acid, sinapic acid, and quercetin. Caffeic acid, rutin, and kaempferol were detected in trace amounts. The presence of these phytochemicals could be responsible for the observed anti-quorum activity. The present study is probably the first attempt to investigate the anti-QS potential of *A. conyzoides* against *P. aeruginosa*. These data provide additional scientific evidence to justify the wide use of *A. conyzoides* in traditional medicine in Burkina Faso.**

**Key words:** *Ageratum conyzoides*, *Chromobacterium violaceum*, *Pseudomonas aeruginosa*, quorum sensing.

## INTRODUCTION

Since the discovery of penicillin in 1928 by Alexander Fleming, antibiotics were the best ally of human and animals' immune system against bacteria (Gaynes,

2017). Unfortunately, the misuse and overuse of antibiotics in human health and agriculture resulted in a strong selection pressure and, consequently the

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emergence of multi-resistant bacterial strains to conventional antibiotics, a decade after their introduction (Andersson and Hughes, 2010; Jenkins et al., 2008; Randall et al., 2003). Additionally, the important international trade and migratory flows, as well as the transfer of antibiotic resistance to humans via food or contact with animals, have accelerated the proliferation of multi-resistant bacteria (OIE, 2012; Talbot et al., 2006; WHO, 2001). Hence, the effectiveness of antibiotics, which has so far saved millions of lives, is thus jeopardized. Bacterial resistance to antibiotics is a major concern for human health (Collignon and McEwen, 2019; Yang et al., 2021), with antimicrobial resistance classified by WHO as the third greatest threat to human health (Mancuso et al., 2021; WHO, 2017).

It is therefore more than urgent to renew the therapeutic arsenal and strategies against bacterial infections, particularly by developing:

- (i) New antibiotics with new mechanisms of action, with the high probability of selecting in the short or medium term some resistant strains to these new antibiotics (León-Buitimea et al., 2020)
- (ii) Alternatives to antibiotics (bacteriocins, bacteriophages, immunotherapies, antimicrobial peptides, predatory bacteria, inhibitors of bacterial virulence or resistance factors) with less impact on the selection of resistant strains (Hauser et al., 2016; León-Buitimea et al., 2020; Shao et al., 2020).

Inhibiting bacterial resistance factors/genes expression or mechanism seems to be a promising approach to restore bacterial susceptibility to already available antibiotics (Laws et al., 2019; Melander and Melander, 2017). On the other hand, reducing bacterial pathogenicity by inhibiting the production of virulence factors would benefit the infected organism by giving its immune system more time to fight the infection (Ruimy and Andremont, 2004). It is established that genes expression involved in the production of bacterial resistance and virulence factors is under the control of a cell-to-cell communication termed quorum sensing (QS), which is a mechanism used by bacteria to detect their critical cell numbers through producing and perceiving diffusible signal molecules in order to coordinate a common behaviour (Allegretta et al., 2017; Shao et al., 2020). As a result, QS inhibitors have been identified as a promising solution for controlling multi-drug resistant pathogens (Lamin et al., 2022).

*Pseudomonas aeruginosa*, is one of the multi-drug resistant pathogens listed by WHO and classified in the most critical pathogens group. In spite of the advances in antimicrobial therapy, *P. aeruginosa* infection remains associated with high mortality, from 18 to 61% (Kim et al., 2014; Zhang et al., 2020). *P. aeruginosa* was selected for investigation, as the mechanism that controls the transcription of genes involved in its virulence is well

known and is controlled by quorum sensing. In *P. aeruginosa*, QS coordinates AHL-mediated expression of virulence genes. It is a key opportunistic pathogen that leads to severe acute and chronic nosocomial infections in immune-compromised patients (Bielecki et al., 2008; Malgaonkar and Nair, 2019).

Numerous *P. aeruginosa* infections are marked by typical skin manifestations such as burn infections and over-infection of old wounds (Spernovasilis et al., 2021). Multi-resistant *P. aeruginosa* has posed a major challenge to conventional antibiotics and therapeutic approaches, which show low efficacy and cause serious side effects (Shao et al., 2020). Only Cefiderocol was listed in 2020 as an antibiotic with consistent clinical and microbiological efficacy against specific strains of *P. aeruginosa* (Bassetti et al., 2021). New medicines are thus required. Interfering with quorum sensing has been shown to be very effective in reducing *P. aeruginosa* pathogenicity (Mancuso et al., 2021). One of the recommended options involves natural antimicrobial drugs, such as natural plant compounds (Moradi et al., 2020). Medicinal plants are excellent chemical antimicrobial active agents (Mulat et al., 2020; Rasamiravaka et al., 2017). Plants produce various antimicrobial compounds, such as phenolics, terpenoids, flavanones, and quinones, due to their similarity in chemical structure to QS signals (acyl-homoserine lactone, AHL) and their ability to damage signal receptors (Chaudhry et al., 2021; Teplitski et al., 2021). Previous research has identified some plants as anti-QS (Moradi et al., 2020). But many others need to be further investigated for their ability to interfere with bacterial QS.

Burkina Faso's flora abounds with various plants from which anti-QS compounds can be sought (Tibiri et al., 2020). *Ageratum conyzoides* was selected according to previous studies which showed anti-microbial activity on specific Gram-positive and Gram-negative bacterial species such as *P. aeruginosa*, *Escherichia coli*, *Shigella dysentery*, and *Streptococcus aureus*, without any effect on the growth of these pathogens (Akinyemi et al., 2005; Pintong et al., 2020). Earlier studies reported a minimum bactericidal concentration of 160 mg/mL. Chah et al. (2006) reported a 90% wound healing rate with methanolic extract (6%), with no inhibition on the growth of the involved bacteria. *A. conyzoides* found in Burkina Faso, has long been used in folk medicine for infectious and skin diseases treatment (Nacoulma, 1996). The leaves were mainly used as poultices on wounds, burns, gastrointestinal pains, and anthrax (Nacoulma, 1996; Nébié et al., 2004). It is possible that the plant acts through another process that reduces the production of bacterial virulence factors.

The aim of the study is to provide a scientific justification for the application of *A. conyzoides* in traditional medicine for the treatment of skin infections, old wounds and burns with over-infection. Specifically, the objective was to highlight the anti-quorum potential

and the impact of *A. conyzoides* on the control of virulence factors associated with *P. aeruginosa* pathogenicity.

## MATERIALS AND METHODS

### Chemicals

p-Iodonitrotetrazolium, elastin congo red, acetic acid, hydrochloric acid, 3-(N-morpholino) propane sulfonic acid (MOPS), Folin-Ciocalteu reagent, perchloric acid, glacial acetic acid, vanillin-glacial acetic, aluminium trichloride, carbenicillin (antibiotic) hexanoyl homoserine lactone molecule (HHL), gallic acid, vanillic acid, ellagic acid, caffeic acid, sinapic acid, rutin, quercetin and kaempferol O-nitrophenyl- $\beta$ -D-galactopyranoside, nutrient agar, and Lauria-Bertani (LB) broth medium were obtained from Sigma-Aldrich (Germany). Solvents (n-hexane, chloroform, ethyl acetate, n-butanol) of analytical grade were provided from sigma Aldrich (Belgium).

### Bacterial strains, plasmids and growth conditions

*P. aeruginosa* PAO1 and *Chromobacterium violaceum* CV026 were provided from the Plant Biotechnology Laboratory (Université Libres de Bruxelles). Bacteria ( $10^6$  UFC/mL) were grown in LB broth (pH 7, 175 rpm) at 37°C for *P. aeruginosa* PAO1 and 30°C for *C. violaceum* CV026. *P. aeruginosa* derivatives harboring plasmid (pPCS1001, pPCS1002, p $\beta$ 03, pLPR1, p $\beta$ 02, p $\beta$ 01 and pTB4124) were streaked onto LB-MOPS broth (50 mM, pH 7.2, 175 rpm) supplemented with carbenicillin (300  $\mu$ g/mL).

### Plant material extraction

*A. conyzoides* L. (Asteraceae) samples were collected in Gampela in August 2014 (Ouagadougou, Burkina Faso) and formerly identified. Voucher specimen has been deposited at the national herbarium of Burkina Faso (HNBU) under code 8755. The whole plant material was washed and dried at room temperature. The dried material was reduced into powder and extracted by maceration for 24 h with methanol containing 20% water. The extract was concentrated in a vacuum evaporator and used for a liquid-liquid fractionation with n-hexane, chloroform, ethyl acetate and n-butanol successively. Collection and experimental research on this plant were in accordance with national guidelines in Burkina Faso.

### MIC and MBC assay

The minimal inhibitory concentrations (MIC) of the extract were determined by broth microdilution method (Eloff, 1988). An overnight bacterial culture was diluted with LB broth to obtain a starting inoculum (106 CFU/mL). Each inoculum (180  $\mu$ L) was incubated with a serial concentration of extracts ranging from 5 to 0.049 mg/mL. Bacteria growth was studied using p-iodonitrotetrazolium staining. After 18 h of incubation, 50  $\mu$ L of INT (0.2 mg/mL) was added to each well and incubated at 37°C for 30 min. A red colour indicated bacterial growth. To assess minimum bactericidal concentration (MBC), 20  $\mu$ L aliquots of all dilutions showing no bacterial growth were spread on LB agar plates (37°C, 24 h). The MIC were determined as the lowest concentration that inhibits bacterial growth, and the highest concentration that produces no bacterial colonies on a solid medium was chosen as the MBC (Ouedraogo and Kiendrebeogo, 2016).

### Bacteria kinetic growth assay

Kinetics growth was assessed for 48 h for CVO26 or 18 h for PAO1 as previously described (Rasamiravaka et al., 2018). A 5 mL volume of bacterial suspension was aliquoted into six or eight sterile tubes and grown (37°C for PAO1, 30°C for CV026) under continuous stirring (175 rpm). At regular time intervals (6 h for CVO26 and 3 h for PAO1), the turbidity or optical density of the bacterial culture at 600 nm is measured from the bacterial suspension. Bacterial cultures were then centrifuged at 3000G at 24°C for 5 min. The supernatant was removed and the bacterial pellet re-suspended with 5 ml of a sterile NaCl solution (9‰). Briefly, a range of bacterial dilutions ( $10^{-5}$  to  $10^{-8}$ ) was generated with sterile NaCl solution (9‰). 100  $\mu$ L of each dilution was spread on LB agar plate and incubated for 18 h (Rasamiravaka et al., 2018). Bacterial growth was assessed by determination of colony forming units (CFU/mL) according to the formula:

$$\text{CFU} = \text{Number of colonies} / (\text{Dilution} \times \text{volume}).$$

### Violacein production in *C. violaceum* CV026 assay

Violacein production induced by hexanoyl-L-homoserine lactone (HHL) was evaluated during 48 h. Violacein was extracted from the supernatant of the culture using the previously described method of Saqr et al. (2021), with some modifications. CV026 inoculum (100  $\mu$ L) was incubated for 24 and 48 h with 1.880 mL of LB broth supplemented with HHL and samples (20  $\mu$ L). Salicylic acid was used as a positive control. 1 mL of culture from each tube was centrifuged at 7000 rpm for 10 min in order to precipitate the insoluble violacein. The supernatant was removed and 1 ml of DMSO was added to the pellet. The solution was vortexed for 30 s to solubilise the violacein and then centrifuged again at 7000 rpm for 10 min. 200  $\mu$ L of the supernatant containing the violacein was introduced into 96-well microplates and the absorbance was measured at 585 nm. Violacein amount was assessed by using the absorbances ratio 585 nm/600 nm.

### Pyocyanin production in *P. aeruginosa* PAO1

The pyocyanin kinetic production was evaluated during 18 h (King et al., 2016). Briefly, 150  $\mu$ L of samples were inoculated with 750  $\mu$ L of PAO1 inoculum in LB medium for 8 and 18 h of growth. At each time point, the bacterial culture was centrifuged at 3000 G, 24°C for 5 min. 500  $\mu$ L of chloroform was added to 1 mL of bacterial supernatant and then vortexed to extract pyocyanin. To extract pyocyanin further, 400  $\mu$ L of the lower chloroform phase containing pyocyanin was added to 300  $\mu$ L of aqueous 0.2 N hydrochloric acid in Eppendorf tubes. The mixture was vortexed and then centrifuged to separate the two phases with pyocyanin in the upper aqueous phase. The absorbance at 380 nm of 200  $\mu$ L of aqueous phase was measured to assess the pyocyanin produced by the bacteria. Salicylic acid was used as a positive control. Pyocyanin amount was assessed by using the absorbance ratio 380 nm/600 nm.

### Elastase assay

Elastase production was detected using elastin Congo red (ECR) (Ahmed et al., 2019). The method consists of the degradation of elastin bound to Congo Red by a bacterial suspension containing elastase. *P. aeruginosa* PAO1 was grown with samples and control (DMSO 1% and salicylic acid) for 8 and 18 h at 37°C at 175 rpm in LB. The tubes were then centrifuged at 3000 g for 5 min at 24°C. 250  $\mu$ L of congo red elastin (5 mg/ml) dissolved in Tris-HCl buffer (0.1 M Tris-HCl pH 8; 1 mM CaCl<sub>2</sub>) was added to 750  $\mu$ L of

supernatant in each Eppendorf tube. The mixture was incubated at 37°C for 16 h at 200 rpm. The degradation of elastin causes solubilisation of Congo Red during this incubation phase, producing a red solution, whereas the undegraded Elastin-Red-Congo complex is insoluble in water. The reaction mixture was centrifuged (3000 g, 10 min) to separate the non-soluble part, and the absorbance of 200 µl of supernatant was read at 495 nm to estimate the elastin activity.

### Rhamnolipids assay

Rhamnolipid production was assessed by methylene Blue method (Rasamiravaka et al., 2016). This method relies on measuring the absorbance of the rhamnolipid-methylene blue complex moving through the chloroform phase. *P. aeruginosa* PAO1 were grown with samples and control (DMSO 1% and salicylic acid) for 8 and 18 h in 5 mL of LB medium (37°C, 175 rpm). All tubes were centrifuged (3000 g for 10 min) and 4 ml of supernatant recovered and filtered (0.1 µm Millipore filters). The pH of the supernatant was then adjusted to  $2.3 \pm 0.2$  with 1N hydrochloric acid. Rhamnolipids were extracted by stirring the supernatant with 4 ml ethyl acetate (centrifugation at 100 g for 1 min). The upper phase containing the rhamnolipids was collected in a 30 ml tube. The process was repeated three times and the extracts were collected and evaporated to dryness. The extracts of rhamnolipids were then dissolved in 4 mL of chloroform and mixed with 400 µl of freshly prepared methylene blue solution. The methylene blue solution was prepared by adding 200 µl of methylene blue reagent (1.4%, w/v in ethanol) and 4.8 mL of distilled water adjusted to pH  $8.6 \pm 0.2$  with 50 mM borate buffer. Tubes were vortexed for 5 min and incubated at room temperature for 15 min to allow complexation of the methylene blue to the rhamnolipids. 1 ml of the chloroform blue phase was added to a 2 ml Eppendorf tube and vortexed for 20 s with 500 µl of 0.2 N hydrochloric acid, allowing the complexed methylene blue to be transferred to the acid phase. Tubes are centrifuged at 100 g for 1 min and left at room temperature for 10 min. 200 µL of the acidic upper phase is transferred to a 96-well microplate well and the absorbance is measured at 638 nm.

### β-Galactosidase assay

All reporter strains of PAO1 were incubated in LB-MOPS-Carbenicillin for 8 and 18 h (50 µL, 37°C, 175 rpm) supplemented with samples (Extract and DMSO). In 12-well plates, 940 µL of LB-MOPS-Carbenicillin, 10 µL of samples (dissolved in DMSO), and 50 µL of PAO1 bacterial inoculum were incubated at 37°C. After incubation, the absorbances were read at 600 nm. 300 µL of permeabilization buffer was added to the initial mixture, then 75 µL+50 µL of buffer was incubated for 20 min at 37°C. The samples were then used to perform the β-galactosidase assay with O-nitrophenyl-β-D-galactopyranoside. 600 µL of ortho-nitrophenyl-β-D-galactoside (oNPG) substrate buffer was added and the tubes were allowed to incubate for 5 to 60 min at 37°C. The reaction was stopped with 700 µL of stop buffer (1 M Na<sub>2</sub>CO<sub>3</sub>). The absorbance of 100 µL of the supernatant was read at 420 nm. β-Galactosidase activity has been expressed in Miller units (Bonneau et al., 2020).

### Phytochemical analysis

#### Determination of total polyphenolic, total flavonoid, and total triterpenoid content

Total polyphenolic, flavonoids, and terpenoids contents in extract were determined using previous spectrometric method (Olech et

al., 2020). The polyphenol content was assessed with 25 µL of extract solution (0.1 mg/ml) and 125 µL of FCR solution (0.2N). After 5 min of incubation, 100 µL of sodium carbonate solution (75 g/L) was added. The absorbance was read after 1 h of incubation, at 760 nm against a standard curve of gallic acid (200 mg/L, R<sup>2</sup>=0.9952). Total polyphenolics were expressed as mg/g gallic acid equivalents (GAE). Flavonoid content analysis was performed with 100 µL of AlCl<sub>3</sub> (2% in methanol) and 100 µL of extract (1 mg/ml in methanol). Absorbance was read at 415 nm after 10 min from a quercetin standard curve (R<sup>2</sup>=0.9995). Total flavonoid was expressed as mg/g quercetin equivalent (QE). Triterpenoid content was assessed by the following process: 100 µL of sample (10 mg/mL in methanol), 150 µL of glacial acetic acid vanillin solution (5%) and 500 µL of perchloric acid were used as the reaction solution. The mixture was placed into a water bath (60°C) for 45 min, then cooled in an ice bath and 2.5 mL of glacial acetic acid was added. Absorbance was read at 548 nm against standard curve of ursolic acid (R<sup>2</sup>=0.9966). Total triterpenoids were expressed as mg/g ursolic acid equivalent (UAE).

### HPLC-DAD analyses

The chromatography analysis was conducted according to the protocol described by Meda et al. (2011). HPLC-DAD-UV-Visible chromatographic analysis of the phenolic compounds of the active fraction was carried out under isocratic conditions by using a C18 reverse phase column (4.6 mm × 250 mm) packed with 5 µm diameter particles. The mobile phase consisted of methanol, acetonitrile, water (40:15:45, v/v/v) containing 1.0% acetic acid. The mobile phase was filtered through a 0.45 µm membrane filter and degassed by ultrasound before use. Eight standards including five (05) phenolic acids (gallic, vanillic, ellagic, caffeic and sinapic acids) and three flavonoids (rutin, quercetin and kaempferol) were used. Each standard was dissolved in methanol to an initial concentration of 400 µg/mL and then cascade diluted to 6.25 µg/mL in 1 mL vials. The fraction was also dissolved in methanol. The injection rate was 0.5 mL/min and the injection volume was 10 µL. Chromatographic data were recorded over 210 to 400 nm, and integrated at 271 nm, 327 nm for phenolic acid and 365 nm for flavonoids. The used solvent was HPLC grades (Sigma-Aldrich, Belgium). Quantification of the compounds was done by peak area integration against the standard curves.

### Statistical analysis

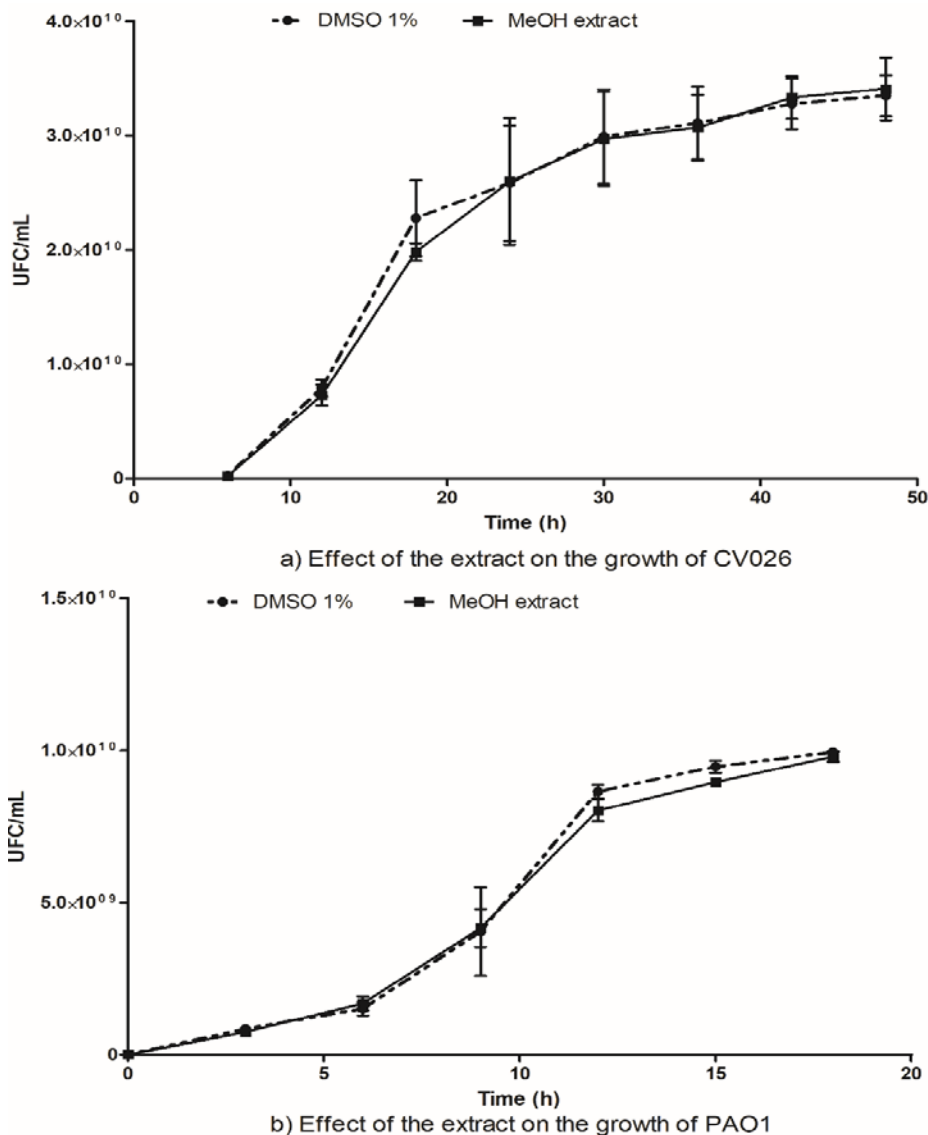
Experiment was performed in triplicate and data were expressed as mean ± SD. GraphPad prism Software was used for statistical analysis (GraphPad software Inc., San Diego, CA, USA); one-way or two-way ANOVA followed by the Tukey or Bonferonni test on GraphPad at the value ≤ 0.001 was considered significant.

## RESULTS

### Anti-QS effect

#### Effect on bacterial growth

The MICs for hydro-methanolic extract were 5.0 mg/mL for *P. aeruginosa* PAO1 and 2.5 mg/mL for *C. violaceum* CV026. For two bacteria the MBC values were 5 mg/mL (> 5 mg/mL for PAO1). MBC/MIC ratio (<4) for both strains indicated a bactericidal effect of extract. For



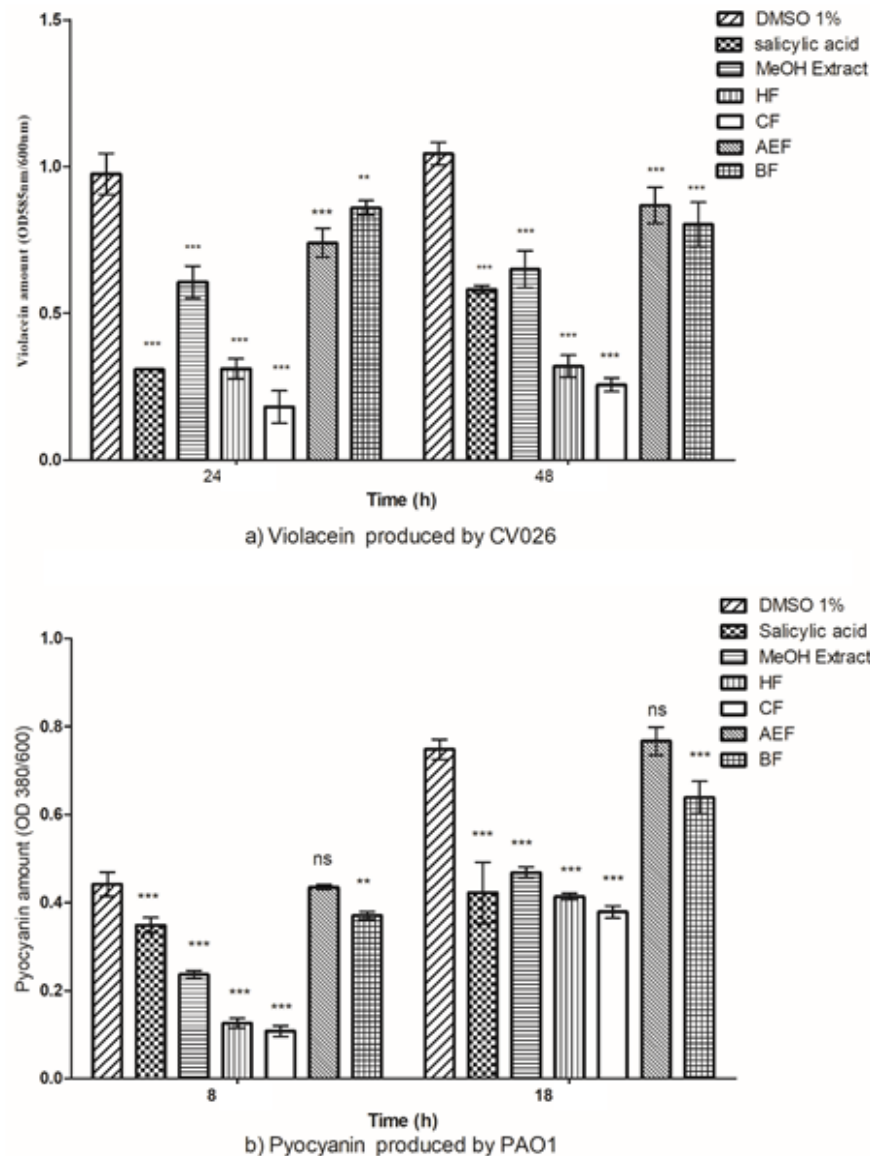
**Figure 1.** Effect on bacterial *C. violaceum* CV026 and *P. aeruginosa* PAO1 kinetics growth. Methanol extract (MeOH) at 100 µg/mL did not exhibit a significant effect on *C. violaceum* CVO26 kinetic growth during 48 h (a) and *P. aeruginosa* PAO1 kinetic growth during 18 h (b). Dimethyl sulfoxide (DMSO) was used as negative control. Mean values ± SD of triplicate independent experiments are shown.

concentrations below the MIC, *A. conyzoides* extract should not induce the bacteriostatic or bactericidal activity. So, at a minimum concentration of 100 µg/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.

#### **Effect of extract on bacterial kinetics growth**

Data of Figure 1a showed the *C. violaceum* kinetic in the presence of hydro methanolic extract in relation to DMSO 1% for 48 h. An exponential growth phase (0-24 h)

followed by a stationary phase (24-48 h) was observed. The exponential phase started immediately at the beginning of the incubation. In both phases, the same growth pattern of *C. violaceum* CV026 was observed. The results showed that the samples (methanolic extract (MeOH) and DMSO 1%) exhibited substantially similar growth kinetics. Figure 1b shows the *P. aeruginosa* PAO1 kinetic data in the presence of methanolic extract in relation to DMSO 1% during 18 h of growth. As shown in CV026 growth, as soon as the samples were added, the bacterial strains started immediately growing in two phases. After bacteria growth reached the exponential phase at 12 h, a stationary phase was followed until 18 h.



**Figure 2.** Chloroform fraction from *A. conyzoides* methanolic extract reduce violacéine production in CV026 (a) growth at 24 and 48 h and Pyocyanin (b) production in *P. aeruginosa* PAO1 growth at 8 and 18 h compared to DMSO 1% used as negative control. Salicylic acid was used as positive control. HF: n-Hexane fraction, CF: chloroform fraction; AEF: Ethyl acetate fraction, BF: n-Butanol fraction, \*\*\*Data that are statistically different ( $p < 0.001$ ), \*\*indicates  $p < 0.01$  ns: non-significant.

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 effects. Methanolic extract (100  $\mu\text{g/mL}$ ) did not significantly affect the cell viability according to CFU quantification.

#### **Effect on violacein production in *C. violaceum* CV026**

The methanol extract and its fractions were screened for

their effect on HHL-induced violacein production by CV026. As shown in Figure 2a, after treatment of sub-MIC concentration, the extract hexane (HF) and chloroform (CF) fractions induced a significant inhibition of production of violacein production (OD585 nm/600 nm) during 24 and 48 h. Compared to negative control, the violacein production inhibition >50% was found with the hexane and chloroform fractions. For the ethyl acetate (EAF) and butanol (BF) fractions, the inhibition was less noticeable (30%). CF was a best inhibitor of violacein production than methanolic extract and salicylic acid.

Importantly, the presence of the samples in the medium had no adverse effect on bacterial growth (Figure 1a). It is therefore conceivable that the negative effect on violacein production is not driven by growth inhibition but rather by disruption of the resistant quorum-sensing systems. On this basis, the impact of the extract and its fractions were investigated on the production of QS-dependent virulence factors, such as pyocyanin, elastase, rhamnolipid and selected genes involved in *P. aeruginosa* QS system.

### **Effect on pyocyanin production**

Figure 2b shows the pyocyanin amount produced per *P. aeruginosa* PAO1 in the presence of methanolic extract (MeOH) and fractions in relation to controls. As shown in Figure 2b, at 8 and 18 h, MeOH extract, hexane fraction (HF) and chloroform fraction (CF) have a significant impact ( $p < 0.001$ ) on pyocyanin production (OD380 nm/600 nm). This impact was more evident for the HF and CF fractions compared to salicylic acid used as positive control. These two fractions may contain similar or different compounds that express the same effects on pyocyanin production. The effect of butanol fraction (BF), was weaker than methanolic extract (MeOH extract), while no significant effect was found with the ethyl acetate fraction (EAF).

### **Effect on elastase and rhamnolipids production**

Rhamnolipids and lasB elastase are also important virulence factors in the virulence of *P. aeruginosa*. The results of this elastase and rhamnolipids assay were summarized in Figure 3. At 8 and 18 h, CF induced the lowest amount of elastase (Figure 3a) and rhamnolipids production (Figure 3b). The effect of CF fraction was more significant from 18 h onward, compared to salicylic acid. For the EAF and BF fractions, their effects were either similar to or less than that of MeOH extract. The effect of HF was similar to that of the stock extract and less than the CF fraction. It was suggested that a molecular difference in the composition of HF and CF. CF appears to contain compounds that are more effective than HF, and which effectively repress the production of these virulence factors. The CF fraction has therefore been studied for its ability to modulate the expression of virulence genes.

### **Effect on QS genes in *P. aeruginosa* PAO1**

If CF interfered with QS mechanisms, it should be reflected on the transcription of QS-regulated and QS-regulatory genes. In order to highlight any interference with the QS genes expression in *P. aeruginosa* PAO1, it was followed that the transcription rate over 8 and 18 h of

growth (Table 1). After 8 h of growth, the CF caused a significant decrease in the expression of the *rhIR* gene *lasI*, *lasR*, *lasB*, *rhIR* compared to the negative control. This effect was more pronounced after 18 h (except for *LasI*). Thereafter, the expression of the rhamnolipid synthesis genes, *rhIA* as well as the elastase synthesis gene *lasB* was significantly reduced at 18 h, whereas at 8 h no effect was evident. In contrast, salicylic acid reduced the expression of all QS-dependent genes after 8 h, but had a limited impact after 18 h. Indeed, only the *lasR* and *rhIR* genes were affected while the other had an equivalent expression to the DMSO condition. Compared to the extract and salicylic acid, the impact of CF was more noticeable on the *rhIR* gene both at 8 and 18 h ( $P < 0.001$ ) whereas on the *lasR* regulatory gene, no significant difference was observed after 18 h. In order to prove whether the decrease in  $\beta$ -galactosidase activity is really associated with a reduction in the expression of QS-related genes and not with a general effect on transcription/translation mechanisms, the promoter activity of the *aceA* gene was examined in *P. aeruginosa* PAO1. The addition of samples has no negative impact on *aceA* gene transcription, reflecting that they affect the expression of QS-related genes without affecting the transcriptional machinery of *P. aeruginosa* PAO1 (Table 1).

## **Phytochemical analyses**

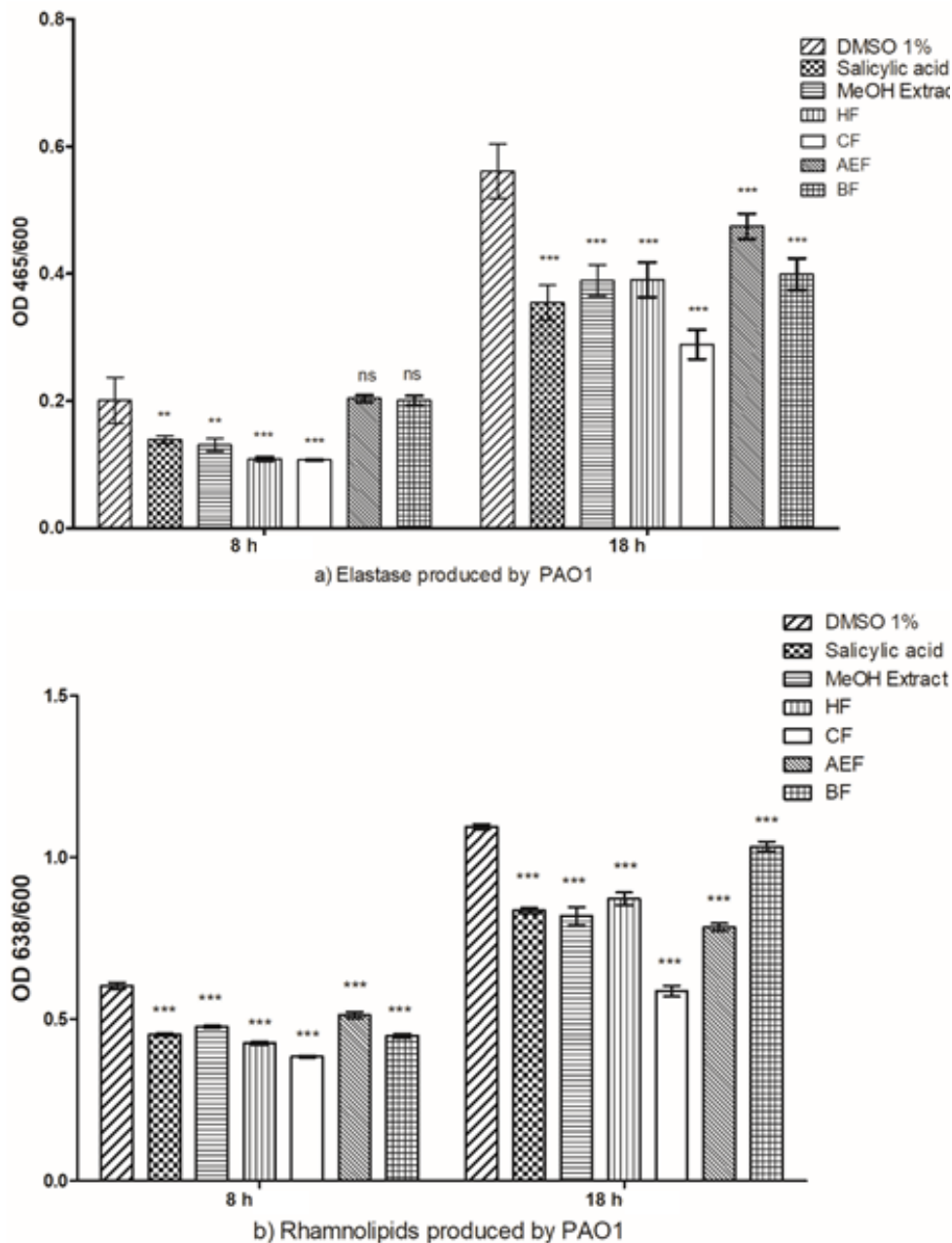
### **Polyphenolic, flavonoid and terpenoid contents**

Total polyphenol, flavonoid and terpenoid contents were analysed in the active fraction (CF) and the stock extract (MeOH extract). The amount of polyphenol, flavonoid and terpenoid compounds in the MeOH extract was  $123.33 \pm 5.6$  mg/g GAE,  $54.67 \pm 1.54$  mg/g QE and  $15.60 \pm 0.6$  mg/g UAE, respectively. For CF fraction, the values were  $683.14 \pm 60.39$  mg/g GAE,  $115.21 \pm 2.52$  mg/g QE and  $37.74 \pm 0.29$  mg/g UAE, in polyphenol, flavonoids and terpenoids, respectively. Mostly, high content of total polyphenol, flavonoids and terpenoids was found mainly in the active fraction (CF).

### **HPLC-DAD analysis**

The identification of compounds from the CF fraction was performed by injection on HPLC-DAD. The retention times of the different peaks were compared to the retention times of the different standards and identified (Figure 4). The summary information is listed in Table 2. Four (04) of the peaks were recognized, gallic acid ( $R_t=5.507$  min), vanillic acid ( $R_t=7.364$  min) and ellagic acid ( $R_t=8.279$  min), and sinapic acid ( $R_t=8.209$  min). Other compounds such as caffeic acid, quercetin, rutin and kaempferol, were also identified but their presence was revealed in trace form (except for quercetin) (Table





**Figure 3.** Chloroform fraction from *A. conyzoides* methanolic extract reduce elastase production (a) and rhamnolipids (b) production in *P. aeruginosa* PAO1 growth at 8 and 18 h compared to DMSO 1% used as negative control. Salicylic acid was used as positive control. HF: n-Hexane fraction, CF: Chloroform fraction; EAF: Ethyl acetate fraction, BF: n-Butanol fraction. \*\*\*Data that are statistically different ( $p < 0.001$ ).

2), while the rest of the peaks were not identified. The concentration of the identified compounds indicates a higher proportion of ellagic and sinapic acids (Table 2).

## DISCUSSION

*A. conyzoides* is an herbal medicine commonly used in traditional medicine to treat old wounds, infected burns

and dermatoses (Chahal et al., 2021; Igbinosa and Eribo, 2016). A great deal of *A. conyzoides*-oriented research has been carried out to discover compounds to control multidrug resistant pathogens (Kotta et al., 2020). Antibacterial activities of some extracts have been reported on several pathogenic strains such as *S. aureus*, *E. coli*, and *P. aeruginosa* (Igbinosa and Eribo, 2016; Odeleye et al., 2014). Beyond the bactericidal and bacteriostatic effect, bioactive compounds of the plant



**Table 1.** Effect of extract and CF fraction on the expression of *lasI*, *lasR*, *lasB*, *rhlI*, *rhlR*, *rhlA* and *aceA* genes in *P. aeruginosa* PAO1 after 8 and 18 h incubation.

Gene	8 h				18 h			
	DMSO	Salicylic acid	MeOH extract	CF	DMSO	Salicylic acid	MeOH extract	CF
<i>lasI</i>	740.1±85	533.1±135*	490.03±74**	444.4±63***	622.6±99	466.5±50	490.8±42	427.3±39*
<i>lasR</i>	907.0±260	657.88±43**	633.57±81***	536.1±20***	2117.32±58	1125±57***	1280±221***	791±43***
<i>lasB</i>	1157.3±117	747.24±24***	823.91±190***	912.9±20***	3000.3±143	2418.1±225	2400.0±137	1543.3±88**
<i>rhlI</i>	641.67±73	340±163**	410.88±126*	542.56±22	629.33±96	534.5±41	556.1±48	561.2±78
<i>rhlR</i>	4808.75±563	3514.39±313***	3212.21±582***	2637.71±248***	6016±903	4640.3±610***	4153±444***	3023.8±499***
<i>rhlA</i>	775.77±103	591.62±149**	672.07±170	780.35±63	1472.3±89	1225.7±89	1246.7±55	629.1±192*
<i>aceA</i>	1057.51±160	982.64±125	1086.10±115	965.03±80	2455.98±254	2277.50±47	1964.17±63	2455.59±446

CF: Chloroform fraction. The data are presented as mean ± SD for three replicates. Salicylic acid was used as positive control. \*\*\*Data that are statistically different ( $p < 0.001$ ).

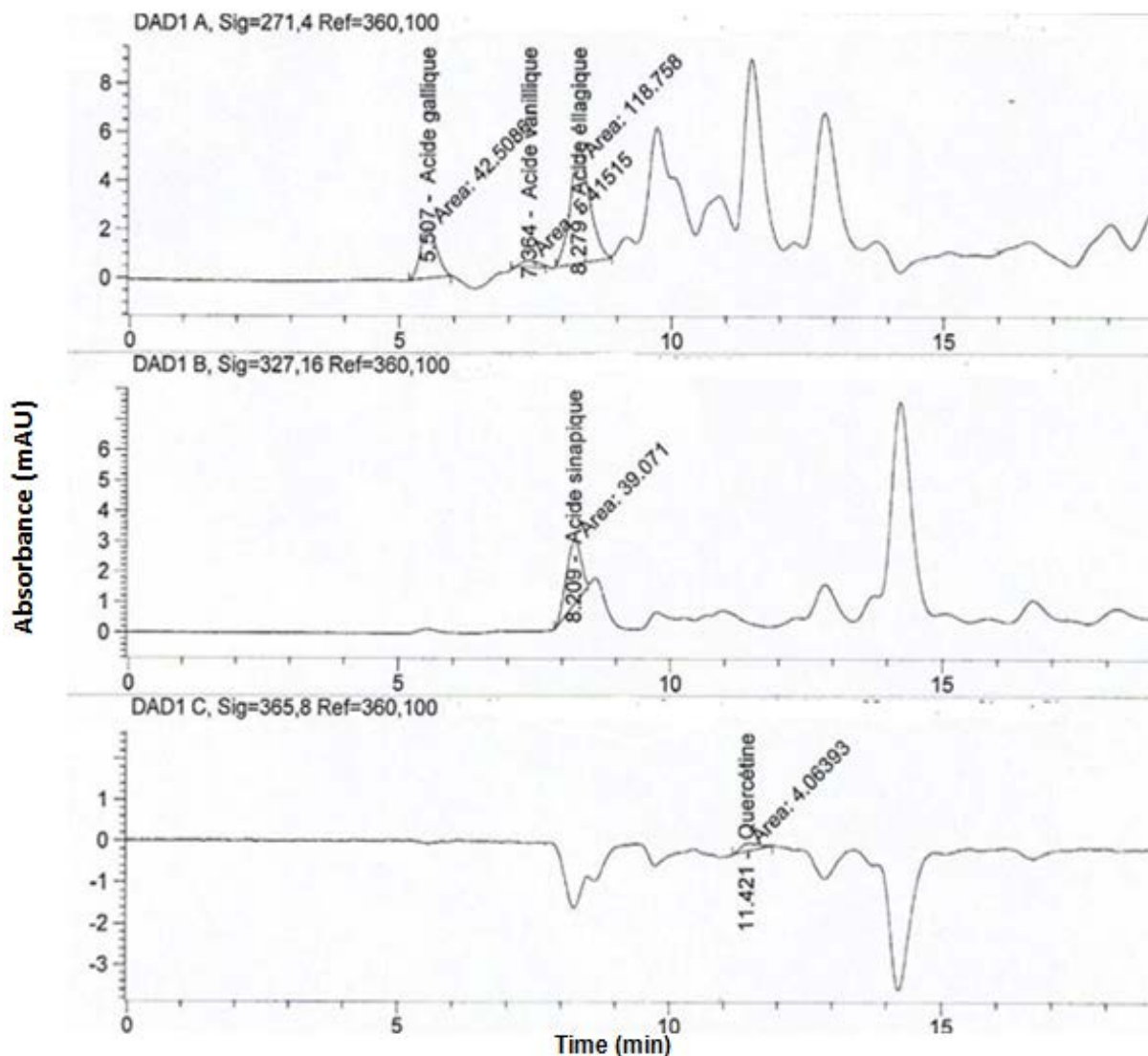
disruption of bacterial virulence. The study indicates that *A. conyzoides* exerts anti-virulence activities rather than a bactericidal effect against the Gram-negative opportunistic pathogen *P. aeruginosa*. The report is the first *in vitro* investigation of the anti-QS properties of *A. conyzoides* from Burkina Faso.

*C. violaceum* CV026 has a low human health impact, but is widely used as a reporter strain in QS screening (Zhu et al., 2011). As a result, chloroform fraction (CF) obtained from methanol (MeOH) extract reduces QS-mediated violacein production in *C. violaceum*, as well as pyocyanin, elastase, rhamnolipid production, and biofilm formation in *P. aeruginosa* PAO1 with no effect on bacterial growth. The effect of CF fraction was more significant from 18 h onwards, compared to salicylic acid, an inhibitor of quorum sensing (Ahmed et al., 2019; Prithiviraj et al., 2005). 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 et al. (2006) who reported the lack of inhibition on PAO1 growth by the methanolic extract, and those of Odeleye et al. (2014) whose results indicted a sensitivity of 160

mg/mL well above the MIC value recorded in this study. Virulence factors and the biofilm formation examined in this study are under QS control (Paluch et al., 2020); hence reduction/inhibition of these factors indicates possible QS antagonism, which may result either from a direct effect on gene expression and/or protein synthesis downstream of AHLs, or from an inhibition of AHL synthesis (Dekimpe and Déziel, 2009). In QS, the *rhl* system has been shown to directly control pyocyanin biosynthesis through the transcription factor *RhlR* and its autoinducer C4-HSL (Dekimpe and Déziel, 2009; Haque et al., 2018). C4-HSL forms a complex with *RhlR* to activate the transcription of *lasB* elastase genes and those involved in initiating pyocyanin (by *phz* operon) and rhamnolipids (*rhlAB* operon) biosynthesis (Cruz et al., 2020; Dekimpe and Déziel, 2009; Hendiani et al., 2019). According to the results, the CF significantly down-regulated the *RhlR* gene and caused the reduction of pyocyanin, rhamnolipids and *lasB*-elastase biosynthesis. This is evidence to support that CF may possess *RhlR* inhibitory *can act to control disease-causing bacteria through* activity against *P. aeruginosa* virulence.

Although the mechanism of action of CF is unclear, it is evident here that it acts upstream of the virulence genes (Hendiani et al., 2019).

Herein, the liquid-liquid fractionation of the *A. conyzoides* MeOH extract led to obtain a high content of polyphenol, flavonoids and terpenoids within the active fraction (CF). Recent works show evidence that several phenolic compounds, terpenoids and flavonoids have anti virulence effects (Asfour, 2018; Bouyahya et al., 2017; Santos et al., 2021). The HPLC results revealed high concentration of ellagic, sinapic, and gallic acids and low concentration of vanillic acid and quercetin. These compounds have been reported previously in *A. conyzoides* (Dang Xuam et al., 2004; Okunade, 2002). Most of them have been previously pointed out as QS inhibitors (Francesca et al., 2020; Manner and Fallarero, 2018; Onem et al., 2021; Quecan et al., 2019). Previous investigation has shown ellagic acid derivatives from *Terminalia chebula* Retz down-regulate the expression of QS genes, thereby attenuating the virulence of *P. aeruginosa* PAO1 (Sarabhai et al., 2013). A combination of ellagic acid and tetracycline was determined to effectively inhibit biofilm formation by *Propionibacterium acnes*



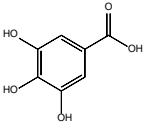
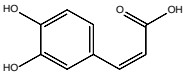
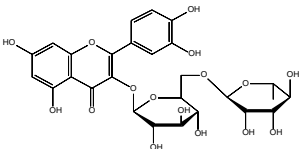
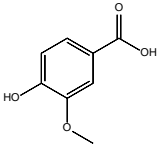
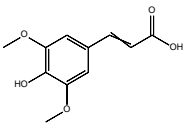
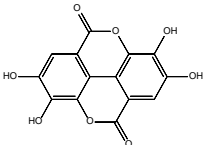
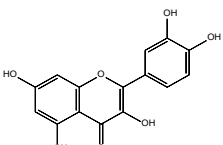
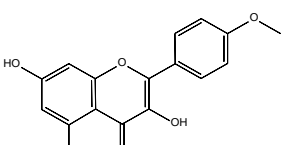
**Figure 4.** HPLC-DAD chromatograms of Chloroform fraction (CF) from *A. conyzoides* hydro methanolic extract. DAD 1A: signal 271 nm: gallic acid, vanillic acid, ellagic acid. DAD 1B: signal: 327.4 nm: sinapic acid. DAD 1C: signal: 365 nm: quercetin.

without affecting its growth (Sankar et al., 2016). Vanillic acid was identified as an active leader responsible for anti-quorum sensing activity in *Actinidia deliciosa* extract (Sethupathy et al., 2017). It also inhibited the violacein production in *C. violaceum* ATCC 12472 (Sivasankar et al., 2020). Ouyang et al. (2016) had shown that quercetin as an effective inhibitor of QS, and virulence factors including pyocyanin, protease and elastase in *P. aeruginosa* (Ouyang et al., 2016). Furthermore, the expression levels of *lasI*, *lasR*, *rhlI* and *rhlR* were significantly reduced, in response to 16  $\mu\text{g}/\text{mL}$  quercetin (Ouyang et al., 2016). Widely distributed in nature, gallic acid is a phenolic compound extensively studied for its numerous biological activities including anti-QS (Borges et al., 2014). Gallic acid had also been reported to be an

autoinducer-1 type QS inhibitor (Zhang et al., 2020). The reduced expression of virulence genes in the CF fraction could be attributed to the presence of these compounds (Nain et al., 2020), or/and to other unidentified phytochemicals.

Altogether, these non-bactericidal anti-virulence properties and the reported antimicrobial activities, provide additional evidence and support the long history use of this plant in traditional medicine for the treatment of skin infectious disease (Nacoulma, 1996). Indeed, the reduction of QS genes and the end-effect on virulence factors' productions allows to explain the historical use of *A. conyzoides* in Burkina Faso. These observations also provide an opportunity to extrapolate on how this plant could be used to control antibiotic resistant bacteria.

**Table 2.** Retention time, absorbance constants ( $\lambda$ ) and structure of all standards.

No.	Chromatographic peaks		Standards		Identification	Amount (mg/L)	Structure
	R <sub>t</sub> (min)	$\lambda_{\max}$	R <sub>t</sub> (min)	$\lambda_{\max}$			
1	5.507	234	5.503	235	Gallic acid	1.141	
2	6.622	N/A	6.622	260; 325	Caffeic acid	N/A	
3	6.869	N/A	6.869	255; 355	Rutin	N/A	
4	7.364	225; 288	7.350	220; 285	Vanillic acid	0.046	
5	8.209	235; 290	8.170	235; 290	Sinapic acid	9.92	
6	8.279	236; 280	8.356	235; 270	Ellagic acid	13.84	
7	11.421	255; 375	11.430	255; 380	Quercetin	0.194	
8	17.215	N/A	17.215	265; 365	Kaempferol	N/A	

N/A: Not available; R<sub>t</sub>: Retention time (min).

## Conclusion

The present study reports the anti-QS activity of *A. conyzoides* extract effectively inhibited QS genes expression, signal concentration and virulence factors in

*P. aeruginosa*. The promising properties may be due to the presence of various phytochemicals such as phenolic acid, flavonoids, and triterpenoids. These phytochemical compounds could be a factor that targets both the signals' molecules and the genes of QS.

Research is currently in progress to identify and isolate the bioactive compound.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

## ACKNOWLEDGEMENTS

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