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Sclerocarya birrea (A. Rich.) Hochst. and *Sterculia setigera* Del. extracts as a potential inhibitor of *Pseudomonas aeruginosa* PAO1 and *Chromobacterium violaceum* CV026 virulence factors to combat bacterial pathogenicity

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Abstract

The abuse of antibiotics has led to the widespread development of resistant bacterial strains, there is a need for the development of antimicrobial agents with new properties.

Aim of this study was to determine the anti-quorum sensing potential (expression of virulence factor inhibition) of ethanolic extracts of *S. birrea* and *S. setigera* on *Chromobacterium violaceum* CV026 and *Pseudomonas aeruginosa* PAO1.

The ability of the extracts to inhibit the production of violacein by *C. violaceum* and the biofilm formation of *Pseudomonas aeruginosa* was examined to determine anti-quorum sensing activity. The phytochemical screening and the determination of total polyphenols and total flavonoids were carried out. The antioxidant activity of the extracts was measured using anti-free radical DPPH model and the reducing of the ferric ion capacity.

For the inhibition of the production of violacein, *S. setigera* presented the best result with an inhibition of 70.19% compared to the negative control which is 1% DMSO. This inhibition is greater than that of ascorbic acid used as a reference. This species was also the most active on biofilm formation with an inhibition of 72.24% compared to the negative control, it thus showed greater activity than that of ascorbic acid used as a positive reference. The phytochemical study revealed the presence of tannin, flavonoids, triterpenes, saponosides, coumarin in our two extracts. *S. birrea* gave the best total polyphenol content with 326.05 ± 117.90 mg EAG / g of extract and showed the best result on the DPPH radical with an inhibition of 73.33 ± 1.46% which is statistically equal to that of gallic acid used as a reference.

These extracts showed good antibacterial activity by inhibiting the quorum sensing system. Thus, they could therefore be used for the manufacture of anti-quorum sensing phytomedicines without a bactericidal effect.

Keywords: Anti-quorum sensing; Violacein; Biofilm; *Sclerocarya birrea*; *Sterculia setigera*

1. Introduction

P. aeruginosa is an opportunistic bacterium with little or no virulence in healthy individuals but which can prove to be formidable in subjects with weakened immunity. The bacteria can activate a complex regulatory cascade leading to the simultaneous production of several virulence factors, when a certain population density is reached. This type of global

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regulation is called quorum sensing. The virulence of *P. aeruginosa* linked to quorum sensing. Furthermore, *P. aeruginosa* cells are also capable of associating with each other to form a heterogeneous structure called a biofilm. Bacteria in the biofilm are very resistant to antibiotics [1]. Over 80% of medical bacterial infections treated by physicians in developed countries are caused by organisms growing in biofilms [2].

The abuse of antibiotics creates selective pressure, leading to the widespread development of resistant bacterial strains [3]. Faced with the ineffectiveness of antibiotics in eliminating bacteria, there is a growing need for the discovery and development of antimicrobial agents which exhibit new or unexplored properties to effectively control and manage infectious bacterial diseases [4].

Inhibition of bacterial virulence and / or biofilm formation by targeting non-microbicidal mechanisms are examples of increasingly explored antipathogenic approaches [5]. Thus, medicinal plants inhibitors of quorum sensing have already been found. Okusa et al. [6] found that extracts of *Cordia gilletii* from wild (Boraginaceae) suppress the Quorum Sensing of *Pseudomonas aeruginosa* PAO1. The extract of *Forsythia suspense* inhibits the production of violacein in *C. violaceum* 12472 and also inhibits the production of virulence factors regulated by quorum sensing and biofilm formation in *P. aeruginosa* in a concentration-dependent manner [7].

Sclerorarya birrea and *Sterculia setigera* are two plants used in traditional medicine in Burkina Faso. According to Braca, A et al. [8].the stem bark, roots and leaves of *S. birrea* are used to treat hypertension, diabetes, dysentery and inflammation. The acetone extracts of *S. birrea* showed remarkable bactericidal activity against H. pylori killing more than 50% of the strains [9]. The bark of the species has also shown antibacterial activity against *Escherichia coli* and *Salmonella typhi*. [10].Several bioactive compounds have been isolated from *S. birrea* extract such as ellagic acid, Naringenin, Rhamnetine, quercetin [11]. Extracts of *sterculia setigera* have shown good antioxidant and anti-inflammatory activity in vivo and in vitro and weak cytotoxic effect on African green monkey kidney cells (Vero cells) [12, 13]. Akin-Osanaiye, B. C. et al., [14] showed that *S. setigera* species inhibited the growth of bacteria such as *P. aeruginosa, S. aureus* and *E. coli*. The phytochemical study revealed the presence of alkaloids, flavonoids, phenolic compounds and glycosides.

The objective of this study is to determine the inhibitory potential of virulence factors (quorum sensing system) of *Chromobacterium violaceum* CV026 and *Pseudomonas aeruginosa* PAO1 by *Sclerorarya birrea* and *sterculia setigera* extracts.

2. Material and methods

2.1. Plant material and extraction

The leaves of *Sclerocarya birrea* and *Sterculia setigera* were harvested in Kossodo (Ouagadougou, Burkina Faso) in June 2020. The species were authenticated and herbaria were deposited at the UFR / SVT herbarium. The samples were dried under laboratory conditions, out of the sun, then sprayed to obtain the vegetable powder.

The powder (50 g) of each plant material was placed in bottles containing 500 ml of absolute ethanol. The bottles were subjected to mechanical shaking for 24 h at laboratory room temperature. The macerates were filtered and then concentrated in an evaporator fitted with a vacuum pump and then evaporated to dryness. These extracts are kept and used for the various tests

2.2. Bacterial strains and growth conditions

P. aeruginosa PAO1 and *C. violaceum* CV026 strains used to assess anti-QS activity were provided from the Laboratoire de Biotechnologie Végétale (Université Libre de Bruxelles, Gosselies, Belgium). Both strains were grown in Luria-Bertani (LB) broth medium at 37°C for PAO1 and 30°C for CV026

2.3. Anti-Qorum Sensing activity

2.3.1. Inhibition of violacein production assay

Inhibition of violacein production in *C. violaceum* CV026 was tested according to Choo, J. H. and al. [15]. The production of violacein in the mutant C. violaceum CV026 was induced by adding exogenous N-hexanoyl-L-homoserine (HHL). An overnight culture of C. violaceum CV026 after dilution was added to plant extracts dissolved in DMSO (100 µg/mL final concentration) and supplemented with HHL (10 µM final concentration); and completed with LB broth (5 mL final volume). Tubes were incubated for 48 h at 30°C, with 175 rpm agitation. Bacterial turbidity (OD600nm) was measured

to assess bacterial growth. Violacein quantification was assessed after 48 h of growth. One mL of bacterial culture was centrifuged at 7000 rpm for 10 min and the supernatant was discarded. DMSO (1 mL) was added to the pellets and the solution was vortexed to dissolve violacein. After centrifugation (7000 rpm, 10 min), violacein was quantified by measured the absorbance at 575 nm.

2.3.2. Biofilm Formation and Quantification

The quantification of biofilm formation was assessed according to the method described by Vandeputte O. M.and al., [16]. An appropriately dilution (50 µL) of *P. aeruginosa* PAO1 overnight culture was added to LB medium supplemented with 10 µL of extract (100 µg/mL, final concentration) or DMSO in 12- well polystyrene plates. Plates were incubated for 24 h at 37°C. After incubation, planktonic bacteria were discarded with the supernatant, and the biofilms were gently washed three times with distilled water and then fixed with 1 mL of methanol (99%). After 15 min, the methanol is removed and the plates were dried at room temperature. In order to reveal the presence of biofilm, crystal violet (0.1% in water) was added to each tube (1mL) for 30 min at room temperature. Crystal violet was then discarded and biofilm were washed three times with 1 mL of water. Finally, in order to solubilize the crystal violet, 2 mL of acetic acid (33% in water) was added and the absorbance of the solution was read at 590 nm.

2.4. Phytochemical screening

The procedures described by Ciulei (1982) [17] were used for the detection of the different chemical groups. Thus: the reaction with iron trichloride ($FeCl_3$) is used for the detection of tannins and polyphenols, the Shibata test for flavonoids, the Feiggl-Frehden test for coumarins, the Liebermann / Buchard test for triterpenes / steroids,The foam test for saponosides.

2.5. Determination of phenolic compounds

2.5.1. Determination of total polyphenols

Total polyphenols were determined using Folin Ciocalteu reagent [18]. A volume of 125 µL of methanol extracts (100µg / mL) was mixed with 625 µL of Folin- Ciocalteu reagent (0.2 N). After 5 min, 500 µL of aqueous sodium carbonate (Na_2CO_3, 75 g / l) were added. After 2 h of incubation in the dark at room temperature, the absorbances were measured at 760 nm. A standard calibration curve was plotted using gallic acid (0-50 µg / mL). The results are expressed in milligrams of gallic acid equivalent per 1 g of dry extract (mg EAG / 1 g).

2.5.2. Dosage of flavonoids

The total flavonoid contents of the extract were determined by the colourimetric method [19]. A volume of 75 µl of 2% $AlCl_3$ in pure methanol was mixed with an equal volume of 1 mg/ml extract in methanol. The optical densities were read after 10 min at 415 nm using the spectrophotometer. A mixture of 75 µl of extract and 75 µl of methanol without $AlCl_3$ served as a blank. The results are expressed in milligrams equivalent quercetin for 1 g of dry extract (mg EQ / 1 g)

2.6. Antioxidant activities

2.6.1. Anti-radical activity DPPH

The anti-radical activity of the ethanolic extract (1 mg/ml) was evaluated by the DPPH (2,2 diphenyl-1-picrylhydrazyl) method [20]. This method is based on the reduction in absorbance at 517 nm of the stable free radical DPPH, in the presence of a hydrogen radical donor [26], three (03) tests were carried out by mixing 100 µl of the sample and 200 µl of DPPH (20 mg / l in methanol). After 15 minutes of incubation, the absorbance is read at 517 nm against a blank. The antiradical activity was expressed in percent inhibition.

2.6.2. Ferric ion reducing potential (FRAP)

The determination of the reducing power of the plant extract has been evaluated as described by Hinneburg et al., [21]. In test tube containing 0.5 ml of extract (1 mg / ml), 1.25 ml of phosphate buffer (0.2 M, pH 6.6) and 1.25 ml of potassium hexacyanoferrate (1% aqueous) were added. The mixture was heated at 50°C in a bain-marie for 30 minutes. After cooling, trichloroacetic acid (1.25 mL, 10%) was added, and the mixture was then centrifuged (2000 rpm for 10 minutes). Three aliquots (125 µl) of the supernatant were transferred in microplate to which 125 µl of distilled water and then 25 µl of $FeCl_3$ (0.1% aqueous) were added. The reductive power was evaluated at 700 nm against a standard curve of ascorbic acid. The experiment was carried out in triplicate (independent tests), and the reducing activity of the extract was expressed in mmol Equivalent Ascorbic acid per gram of extract (mmol EAA / g extract).

2.7. Statistical Analysis

All results were expressed as the mean value of several independent experiments (n = 3) ± standard deviation. For statistical analysis, Graph Pad Prism software (version 5.0) and MS Excel software were used to obtain standard curves and graphs, percentages of inhibition, averages and standard deviations. Anova one way followed by the Tukey test was used to measure the degree of statistical significance of the results. A significant difference was considered for P<0.05.

3. Results and discussion

3.1. Anti-quorum sensing activity of extracts

3.1.1. Inhibition of violacein production

The ability of *S. birrea* and *S. setigera* extracts to reduce the production of violacein produced by *C. violaceum* CV026 was determined as shown in Figure 1. This strain of bacteria is deficient in homoserine-lactone synthase and has was used to determine the anti-quorum sensing activity of the samples. At 100 mg / ml, extracts showed a good reduction in violacein. *S. setigera* presented the best result with an inhibition of 70.19% compared to the negative control which is DMSO 1%. This inhibition is greater than that of ascorbic acid used as a reference product. *S. birrea* presented the lowest value. Extracts of *S. birrea* and *S. setigera* do not inhibit bacterial growth of *C. violaceum* CV026 at a concentration of 100 mg / ml, so it does not affect cell viability (Figure 2)

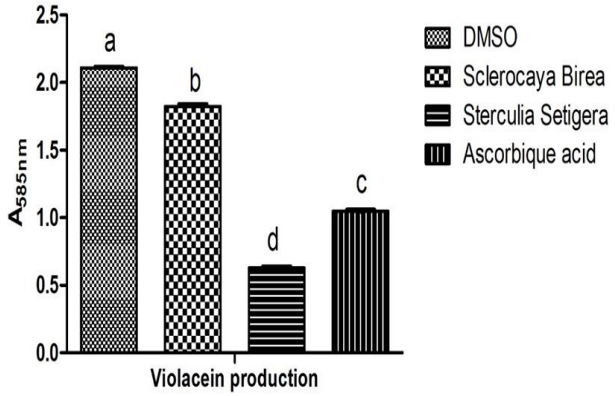


Figure 1 Inhibition of the production of violacein.
Results indicated by different letters are statistically distinct (p <0.05; Mean ± S.E.M = Mean values ± Standard error of means of three experiments)

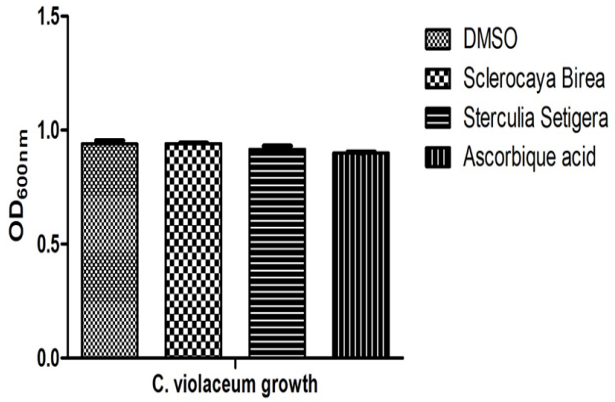


Figure 2 Effect of extracts on bacterial growth p <0.05; Mean ± S.E.M = Mean values ± Standard error of means of three experiments

3.1.2. Inhibition of bacterial biofilm formation

The formation of biofilm by the bacterium *P. aerusinosa* PAO1 is one of its virulence factors. The inhibitory potential of biofilm formation by extracts of *S. birrea* and *S. setigera* after 24 h of bacterial growth was determined (Figure 3). The extract of leaves of *S. setigera* was the most active with an inhibition of 72.24% compared to the negative control, thus showing a higher activity than that of ascorbic acid used as a positive reference. . Extracts of *S. birrea* and *S. setigera* do not inhibit the bacterial growth of *P. aerusinosa* PAO1 (Figure 4), so they do not have a bactericidal effect.

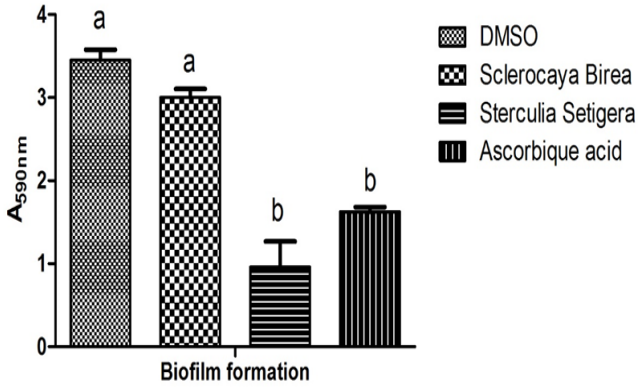


Figure 3 Inhibition of biofilm formation by extracts of *S. setigera* and *S. birrea.*
Results indicated by different letters are statistically distinct (p <0.05; Mean ± S.E.M = Mean values ± Standard error of means of three experiments)

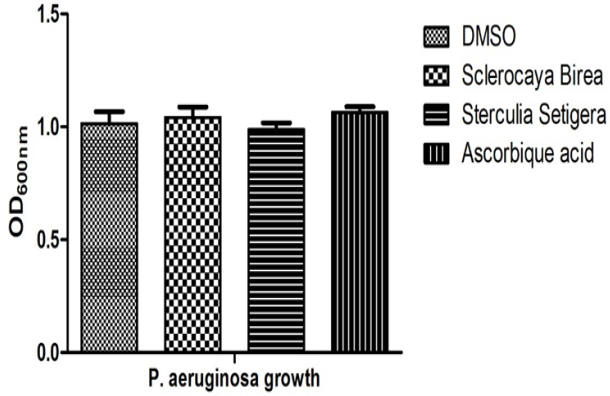


Figure 4 Effect of extracts on bacterial growth of *P. aerusinosa*

p <0.05; Mean ± S.E.M = Mean values ± Standard error of means of three experiments

3.2. Phytochemical screening

Phytochemical analysis revealed the presence of phenolic and terpene compounds in extracts of S. setigera and *S. birrea* (Table 1). Tannins and polyphenol, flavonoids, triterpene, coumarins were detected in both extracts. Saponosides were only detected in *S. birrea* extract

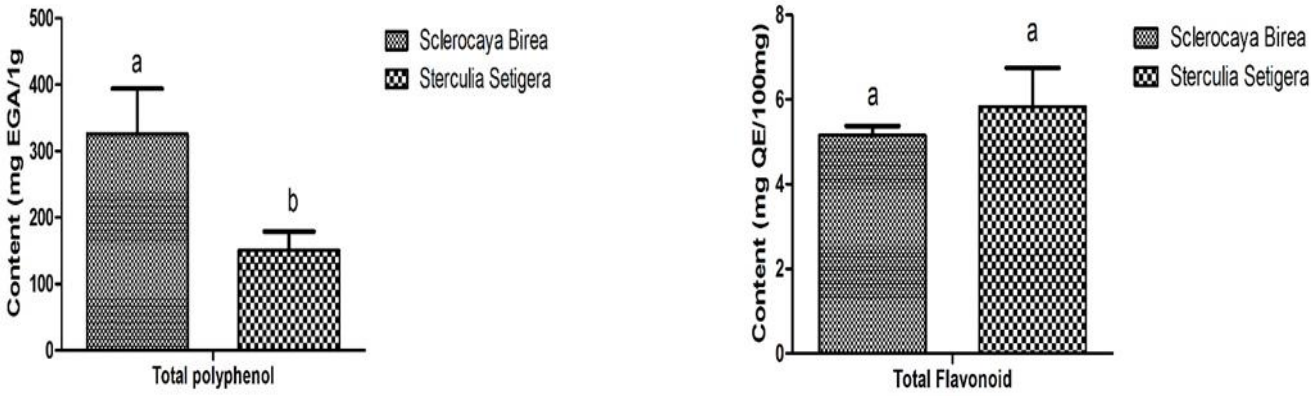
Table 1: Result of phytochemical screening

Plants	**Result of phytochemical screening**				
	Tannins and polyphenols	Flavonoïds	Saponosides	Sterols / triterpene	Coumarins
S. birrea	+	+	+	+	+
S. setigera	+	+	-	+	+

- = absence, + = presence

3.3. Determination of phenolic compounds

Figure 5 shows the contents of total polyphenols and total flavonoids in plant extracts. *S. setigera* and *S. birrea* gave total flavonoid contents of 5.83 ± 1.58 mg EQ / 100mg and 5.15 ± 0.38 mg EQ / 100mg, respectively, not statistically different (Figure 5B). *S. birrea* gave the best total polyphenol content with 326.05 ± 117.90 mg EAG / g extract against 150.51 ± 49.12 mg EAG / g extract for *S. setigera* (Figure 5 A).



A **B**

Figure 5 Content of total polyphenols and total flavonoids in plant extracts
Results indicated by different letters are statistically distinct ($p < 0.05$; Mean ± S.E.M = Mean values ± Standard error of means of three experiments)

3.4. Antioxidant potential of *S. birrea* and *S. setigera* extracts

The antioxidant capacity of our extracts have been evaluated by their anti-radical DPPH potential and their ability to reduce ferric ion to ferrous ion (FRAP method). The *S. birrea* extract showed the best result on the DPPH radical with an inhibition of 73.33 ± 1.46% which is statistically equal to that of the gallic acid used as a reference. *S. sétigera* presented the lowest result with 33.18 ± 0.21% as inhibition (Figure 6).

For the reducing activity of Fe^{3+} *S. birrea* showed a better result with an activity of 18.85 ± 0.40 mmol EAA / g of extract against 9.39 ± 0.27 mmol EAA / g of extract for *S. setigera*. (Figure 7).

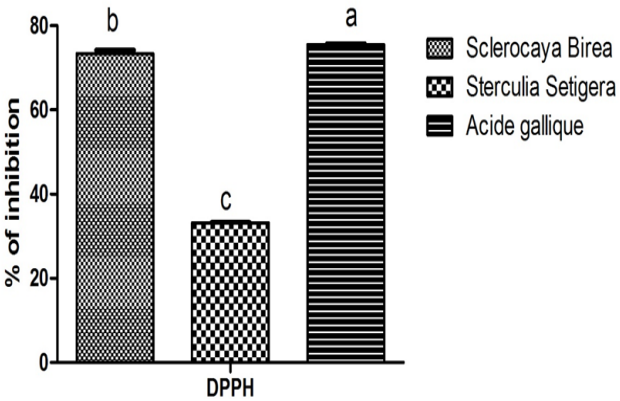


Figure 6 Anti-radical DPPH activity
Results indicated by different letters are statistically distinct ($p < 0.05$; Mean ± S.E.M = Mean values ± Standard error of means of three experiments)

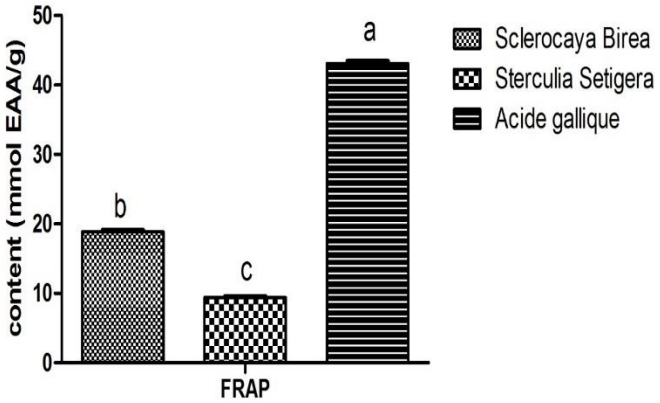


Figure 7 Reducing activity of ferric ion
Results indicated by different letters are statistically distinct ($p < 0.05$; Mean ± S.E.M = Mean values ± Standard error of means of three experiments)

4. Discussion

In bacteria such as *P. aeruginosa* and *C. violaceum*, virulence factors are regulated by quorum sensing, a global genetic regulatory mechanism dependent on cell density. Interference with quorum sensing circuits is considered a potential strategy to attenuate bacterial pathogenicity.

The production of violacein by *C. violaceum* is regulated by quorum sensing system dependent of the CviIR gene [22]. Thus a modification of the production of violacein by extracts can be considered as evidence of the anti quorum sensing activity thereof. *P. aeruginosa* PAO1, an opportunistic pathogen, uses two interrelated QS systems, LasI / R and RhlI / R, which regulate pyocyanin production, proteolytic, elastolytic activity and biofilm formation [23]. Biofilm formation allows *P. aeruginosa* to be protected against host defenses and antimicrobial agents [24].

This biofilm formation inside the host has enabled the successful establishment of pathogens and the development of chronic infections in humans [25].

Extracts of *S. setigera* and *S. birrea* showed good anti quorum sensing activity with a 70.19% inhibition of violacein formation for *S. setigera* greater than that of ascorbic acid used as a reference. These extracts also reduced the biofilm formation of *P. aeruginosa* without a bactericidal effect. Ratul Sarkara et al., [26] showed that *S. birrea* reduced the biofilm formation of *P. aeruginosa* with 75% inhibition, these results corroborate our results obtained. By inhibiting the production of violacein and biofilm, our samples could have acted on the regulatory genes involved in quorum sensing.

These plant extracts having shown a significant inhibition of the formation of biofilm mediated by quorum sensing, thus demonstrating the possible application of these plants in the management of persistent, chronic or recurrent infections (where the biofilms offer a persistent resistance to common antibiotics. used) [27].

Flavonoids, polyphenols, tannins, terpenes have been detected in our samples. Our results corroborate with those of Lawaly M. M., and al., and Akin-Osanaiye, B. C., and al.. [10, 14]. The extracts of *S. setigera* and *S. birrea* showed a good content of total flavonoids and total polyphenols. Studies have already demonstrated the anti quorum sensing activity of phenolic compounds.

Curcumin from *Curcurma longa* attenuates the biofilm formation of *P. aeruginosa* PAO1 [28]. Flavones have shown good anti quorum sensing activity by reducing the production of violacein by *Chromobacterium violaceum* [29]. Kaempferol showed anti-quorum sensing activity against *C. violaceum* and *P. aeruginosa* PAO1 at 100 µg / mL [30]. Elagic acid, tannic acid and epigallocatechin gallate inhibit biofilm production by interfering with the production signals of N-acyl-homoserine lactones which is a molecule involved in quorum sensing. Thus, the presence of phenolic compounds in extracts of *S. birrea* and *S. setigera* could explain the good anti-quorum sensing activity observed.

In this study the plant extracts showed good capacity for scavenging free radicals and reducing metal cations. Metal cations such as calcium, magnesium and iron have been implicated in maintaining and strengthening the integrity of the biofilm matrix [31]. Together with the antioxidant properties of the extracts, they could chelate or reduce the metal

cations involved in maintaining the integrity of the biofilm matrix and thus affect this integrity. This could explain the inhibition of biofilm formation by plant extracts.

S. birrea and *S. setigera* could therefore be used as an antimicrobial agent acting on the quorum sensing system without bactericidal effect to limit the development of resistance to infectious agents

5. Conclusion

From this study it emerges that the extracts of *S. birrea* and *S. setigera* exhibited anti-quorum sensing activities by inhibiting the production of violacein from *C. violaceum* and the formation of biofilm by *P. aeruginosa* PAO1 and also showed a potential interesting antioxidant. The phytochemical study revealed the presence of flavonoids, tannins, triterpenes, saponosides, coumarin in the extracts. These plant species can therefore be used in the treatment of pathologies resistant to antibiotics. For what follows, it would be interesting to determine the mechanism of action of the extracts on quorum sensing molecules, such as N-acyl-homoserine lactone.

Compliance with ethical standards

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Disclosure of conflict of interest

Authors have declared that no competing interests exist.

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