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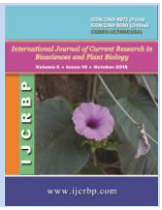

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Acacia seyal Del Bark Extract Reduces Quorum Sensing –Controlled Virulence Factor Production and Biofilm Formation in *Pseudomonas aeruginosa* PAO1

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ABSTRACT

Due to the various virulence factors produced and the growing resistance to antibiotics, *Pseudomonas aeruginosa* is the major cause of mortality in immunocompromised patients. Its pathogenicity regulated by the QS system lead to consider this mechanism of bacterial communication an opportunity for the control of infections caused by bacteria resistant to antibiotics. Thus, the ability of *Acacia seyal* bark extract to disrupt the cell-to-cell communication, to inhibit the production of virulence factors and biofilm formation was assessed in *Pseudomonas aeruginosa* PAO1. These investigations demonstrated that *A. seyal* bark extract inhibited significantly the production of QS-controlled virulence factors and biofilm formation without negatively affecting the growth of *P. aeruginosa* PAO1. These results suggest that *A. seyal* quenches the quorum sensing mechanism and could be a potent source for the research of new effective anti-QS drugs.

Introduction

The emergence of pathogenic bacteria resistance to antibiotics observe over the last decades has stimulated the search for news antibacterial drug with novel targets (Rasamiravaka et al., 2013). In recent years, the discovery of bacterial communication system which regulated the expression of virulence genes provided a new opportunity for the control of bacterial infections (Adonizio, 2008). This system known as QS is considered to be a key regulator mechanism in ecological adaptation and pathogenicity of bacteria (Bassler and Losick, 2006). This system is based, in

Gram negative bacteria, on the production of diffusible molecules, acylhomoserine lactones (acyl-HSL) (Vandeputte et al., 2010). These HSLs are synthesized by an HSL synthase. These molecules diffuse through the bacterial cell envelope and when their concentration reaches a critical threshold, they caused the activation of a transcriptional regulator which will then trigger the expression of target genes (Jimenez et al., 2012). Interfering with the QS system consists to inhibit the expression of virulence genes without affecting bacterial growth, while limiting selective pressure compared to antibiotics (Bjarnsholt and Givskov, 2007).

Pseudomonas aeruginosa, an opportunistic pathogen resistant to several classes of antibiotics, produces multiple virulence factors lead to chronic infections occurred in hospitals (Muller et al., 2009). To date, three QS systems have been characterized in *P. aeruginosa*, lasR/lasI, rhlR/rhlI and qscR. These systems are connected to within a cascade and control the expression of genes involved in virulence and biofilm formation (Jimenez et al., 2012). Biofilm is defined as a bacterial population adhered to a surface and coated with an exopolysaccharide matrix. *P. aeruginosa*, like some other Gram-negative bacteria, produces biofilms that form a physical barrier against antimicrobial agents. These biofilms can also form in catheters or implants and attack tissues such as teeth, eyes, lungs, ears (Adonizio, 2008). Biofilm formation allows *P. aeruginosa* to be protected against host defenses and antimicrobial agents (Rasamiravaka et al., 2015).

QS mechanisms and biofilm formation play important role in chronic infections and reduce sensitivity to antibiotics. Thus, anti-QS and antibiofilm agents could be an alternative for the control of infections caused by antibiotic-resistant pathogens (Rasamiravaka et al., 2015). Recently, many systems have been developed for the research of anti-QS agents in natural products. Medicinal plants has been reported to possess anti-QS and antibiofilm activities (Adonizio, 2008; Singh et al., 2012; Al-haidari et al., 2016). In our investigations, we demonstrated that *Anogeisuss leiocarpus* (DC) Guill. and Perr. reduced significantly QS-controlled virulence factors production and gene expression (Ouedraogo and Kiendrebeogo, 2016). These results lead to explore Burkina Faso flora for the research of medicinal plants which interfere with the QS sytems. *Acacia seyal* bark is traditionally used to treat toothache, dysentery, burns, syphilis (Tapsoba and Deschamps, 2006; Sereme et al., 2008) and showed potent antimicrobial activity (Musallam et al., 2018). This study aimed to assess its anti-QS and antibiofilm activities.

Materials and methods

Bacterial strains and growth conditions

Pseudomonas aeruginosa PAO1 and *Chromobacterium violaceum* CV026 used to assess anti-QS activity were provided from the Laboratoire de Biotechnologie Vegetale (Université Libre de Bruxelles, Belgium). Both strains were grown in Luria-Bertani (LB) broth medium at 37°C for PAO1and 30°C for CV026.

Plant material collection and extraction

Stem bark of *A. seyal* was collected in Loumbila region, Burkina Faso. The plant was identified by Dr Amade OUEDRAOGO from the Laboratoire de Biologie et Ecologie Vegetale (Université Ouaga 1 Pr JOSEPH-KI ZERBO, Burkina Faso). Plant material was dried, powdered and soaked during 24 h in methanol. After filtrated, extract was concentrated in a vacuum evaporator (Büchi Labortechnik AG, Postfach, Flawil, Switzerland) and dried.

Inhibition of violacein production in *C.violaceum* CV026

The anti-QS activity of *A. seyal* bark extract was assessed by evaluated its ability to inhibit violacein production in *C. violaceum* CV026 according to Choo et al. (2006). This mutant, *C. violaceum* CV026 is able to produce violacein when exogenous N-hexanoyl-L-homoserine lactone (C6-HSL; Sigma-Aldrich Chemie GmbH, Darmstadt,

Germany) is supplied in the growth medium. Overnight culture of *C. violaceum* CV026 was diluted and added to bark extract dissolved in DMSO (50-800 µg/mL final concentration) and supplied with C6-HSL (10 µM final concentration). After 24 h of incubation at 30 °C, 175 rpm, bacterial turbidity (OD_{600nm}) was measured to assess bacterial growth. For violacein quantification 1 mL of bacterial culture was centrifuged at 7000 rpm for 10 min. violacein contained in the pellets was dissolved in DMSO (1 mL). After centrifugation (7000 rpm, 10 min), the absorbance of the solution was measured at 575 nm to quantified the production of violacein.

Inhibition of pyocyanin and elastase production in *P. aeruginosa* PAO1

The ability of *A. seyal* bark extract to inhibit the production of pyocyanin was assessed according to the procedures described by Ouedraogo and Kiendrebeogo (2016). Overnight culture of *P. aeruginosa* PAO1 was diluted and added to bark extract dissolved in DMSO (50-800 µg/mL final concentration). After 18 h of incubation at 37 °C, 175 rpm, bacterial turbidity (OD_{600nm}) was measured to assess bacterial growth. Supernatant was used for pyocyanin determination (A_{380}). Elastase production was assessed according to Sarabhai et al. (2013). Briefly, 750 µL cell free supernatant was added to 250 µL elastin congo red solution (5 mg/mL in 0.1 M Tris-HCl pH 8; 1 mM $CaCl_2$) and the mixture was incubated at 37 °C for 16 h at 200 rpm. The mixture was centrifuged at 3000 g for 10 min and absorbance was read at 495 nm to estimate elastase activity.

Biofilm formation and quantification

Biofilm formation by *P. aeruginosa* PAO1 was assessed using 96-well round-bottom plate in polystyrene. Each well contained 200 µL of bacterial culture with different concentrations of bark extract (50-800 µg/mL). After 24 h of incubation at 37 °C the supernatant was discarded, and the biofilms were washed with distilled water and fixed with methanol (Vandeputte et al., 2010). Plates were dried after removing methanol, then crystal violet (0.1% in water) was added to each well and plates were incubated for 30 min at room temperature. Finally, the crystal violet stained, is dissolve in 200 µL of acetic acid (33% in water) and the absorbance of the solution was read at 590 nm.

Statistical analysis

One way analysis of variance (ANOVA) followed by Tukey test of GraphPad Prism software was used to determined statistical significance, p value ≤ 0.05 was considered significant (n=3).

Results

Anti-QS activity

We assessed the anti-QS activity of *A. seyal* bark using *C. violaceum* CV026. This strain is a mutant deficient in the homoserine-lactone synthase gene *cviI*. It produces violacein when homoserine-lactone is supplied in the growth medium. The production of violacein is controlled by QS system making *C. violaceum* CV026 an excellent strain for the research of anti-QS compounds. The effect of *A. seyal* bark extract at different concentration on violacein production was evaluated after 24 h of growth. As shown in Fig. 1A, *A. seyal* bark extract reduced violacein production by 25 % to 97 % compared to the control. These results indicate that the inhibitory effect is concentration-dependent (50-800 µg/mL). In presence of *A. seyal* bark extract *C. violaceum* CV026 growth was not affected negatively (Fig. 1B) confirming that *A. seyal* contains anti-QS compounds.

A. seyal bark extract affects QS-controlled extracellular virulence factors production

A. seyal bark extract significantly affects the QS system. So, we investigated the efficacy of this extract to inhibit QS-controlled virulence factors production in *P. aeruginosa* PAO1. The production of two virulence factors namely pyocyanin and elastase was evaluated. LasB elastase and pyocyanin are involved in the degradation of host

tissues during infection. Elastase (lasB) is a zinc metalloprotease that degrades immunological agents and elastin, a major component of the respiratory epithelium leading to permeabilization of the epithelium (Azghani et al., 2014). Pyocyanin is a blue-green phenazine pigment capable to alter the redox cycle on host cells leading to an increase of stress oxidative (Liu and Nizet, 2009). It also plays an important role in the virulence of *P. aeruginosa*, particularly by repressing the immune response of the host cell, inducing apoptosis of neutrophils (Denning et al., 2003; Kebir & Filep, 2013). At concentrations of 50 to 800 µg/mL *A. seyal* bark extract showed a pronounced inhibitory effect on pyocyanin production with a reduction ranging from 22 % to 86 % (Fig. 2A). Also, *A. seyal* bark extract reduced elastase activity by 8 % to 56% (Fig. 2B). As observe with *C. violaceum* CV026, the inhibitory effect of *A. seyal* bark extract is dose-dependent.

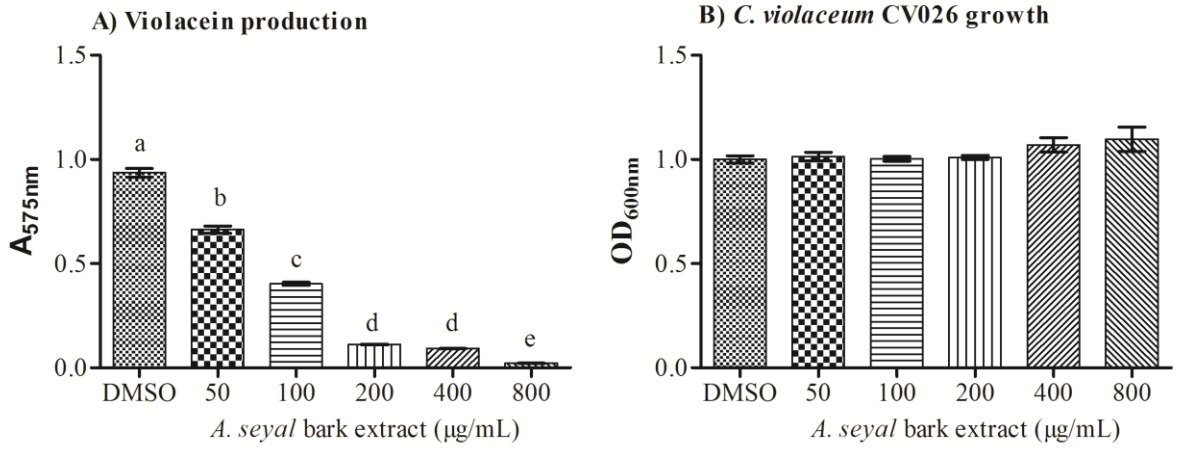

Fig. 1: Dose-dependent inhibitory effect of *A. seyal* bark extract on violacein production in *C. violaceum* CV026. A) Violacein was extracted as described in materials and methods and quantified by A_{575}. B) Growth of CV026 assessed at 600 nm. Dimethyl sulfoxide DMSO (1%) was used as negative control. Histogram with different letters in superscript are significantly different ($p < 0.05$).

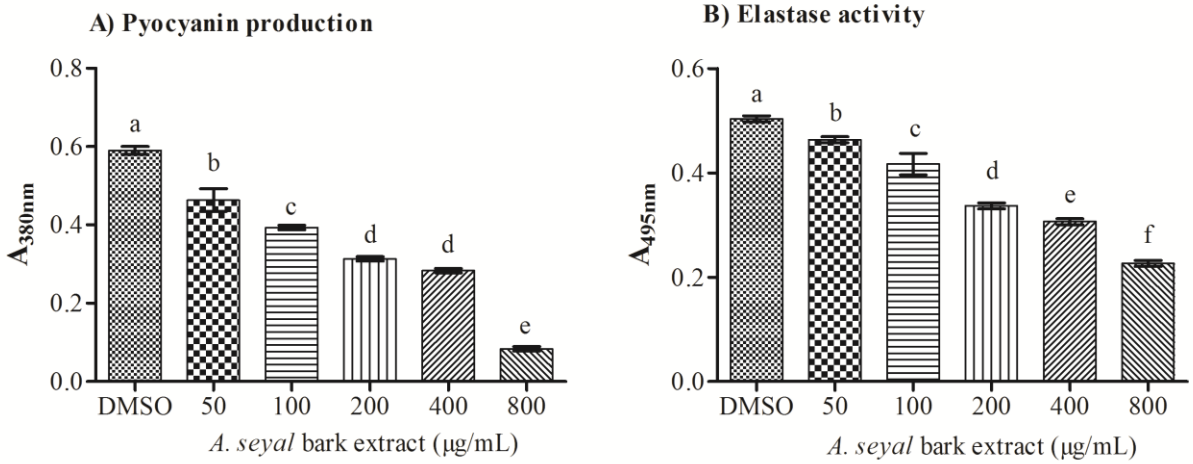

Fig. 2: Effect of *A. seyal* bark extract on *P. aeruginosa* PAO1 virulence factors. A) Pyocyanin production; B) elastase activity. Histogram with different letters are significantly different ($p < 0.05$).

A. seyal bark extract affect biofilm formation

Many studies demonstrated that QS interferes positively in the formation of biofilm of *P. aeruginosa* PAO1 (Jimenez et al., 2012). Since *A. seyal* bark extract showed inhibitory effect in the QS mechanism, its ability to inhibit biofilm formation in *P. aeruginosa* PAO1 was evaluated. Analysis of Fig. 3A indicates that *A. seyal* bark extract exhibited a significant inhibitory effect on PAO1 biofilm formation when concentration was higher than 50 µg/mL without affecting the growth of *P. aeruginosa* PAO1 (Fig. 3B). At the concentration of 800µg/mL the inhibitory effect recorded was 69%.

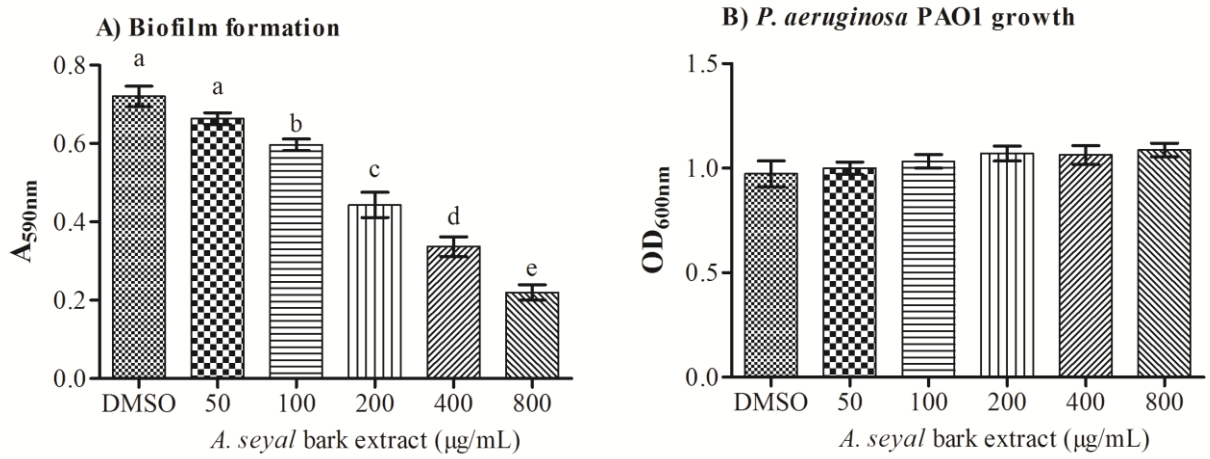

Fig. 3: Effect of *A. seyal* bark extract on biofilm formation (A) and *P. aeruginosa* PAO1 growth (B). Histogram with different letters in superscript are significantly different ($p < 0.05$).

Discussion

The pathogenicity of *P. aeruginosa* is due to its capacity to degrade host tissues by secreting proteases and toxins, and to be protected against antibiotics by forming biofilm (Muller et al., 2009). In this study, the two virulence factors namely lasB elastase and pyocyanin examined, and biofilm formation are controlled by QS mechanism (Jimenez et al., 2012). Natural products with anti-QS activity have the capacity to reduce the pathogenicity of bacteria resistant to antibiotics. Thus, *A. seyal* bark traditionally used to treat infectious diseases was investigated for its anti-QS activity. In this study we demonstrated that bark extract from *A. seyal* inhibits the production of pyocyanin and elastase as well as biofilm formation. Other studies demonstrated that medicinal plants inhibit lasB activity, pyocyanin production and biofilm formation (Adonizio, 2008; Rasamiravaka et al., 2013). Singh et al. (2009) demonstrated that extract from green pod of *Acacia nilotica* have an inhibitory effect on violacein production by up to 100% in a concentration-dependent manner. Fruit extract of *Lagerstroemia speciosa* inhibits significantly the production of violacein on *C. violaceum* CV026 and virulence factors of *P. aeruginosa* PAO1 in concentration-dependent manner (2 mg/mL-10 mg/mL). This extract also reduces the formation of biofilm by *P. aeruginosa* PAO 1 (Singh et al., 2012). The inhibitory effect of *A. seyal* bark extract on violacein production in *C. violaceum* CV026 observed in this study suggests that the structures of anti-QS compounds contained in *A. seyal* are similar to furanones from *Delisea pulchra*. Furanones with anti-QS activity are known as an antagonist of HSLs. The presence of some furanones in the bark of *A. seyal* have been reported (Eltayeb et al., 2017). Some compounds identified in the bark of *A. seyal* could also contribute to the anti-virulence and anti-biofilm

properties of this plant. Indeed, 4-ethylguaiacol identified in the bark by GC-MS analysis (Eltayeb et al., 2017) at 0.005 % has inhibitory effect on *Escherichia coli* O157 : H7 biofilm formation by >50% (Kim et al., 2016). Also, vanillin present in the stem inhibits *Aeromonas hydrophila* biofilm formation (Ponnusamy et al., 2009).

A. seyal like several medicinal plants is an important source for the research of anti-QS compounds. Catechin, isolated from *Combretum albiflorum* at 4 mM inhibits significantly pyocyanin and elastase produced by *P. aeruginosa*. This flavonoid also affects biofilm formation and QS-regulated genes expression (Vandeputte et al., 2010). Oleanolic aldehyde coumarate (at 200 µM) isolated from Bark of *Dalbergia trichocarpa* has been reported to inhibit pyocyanin and elastase production (respectively 64 % and 19 % of inhibition) and biofilm formation (44 % of inhibition) (Rasamiravaka et al., 2015). Two ellagitannins isolated from *Conocarpus erectus* namely vescalagin and castalagin showed potent anti-QS activity (Adonizio, 2008). Thus, *A. seyal* could be used in further investigations for the isolation and identification of compounds which interfere with the mechanism of QS.

Conclusion

We demonstrated that the extract of *A. seyal* bark exhibited strong anti-QS potential. It inhibits QS-regulated virulence factors production and biofilm formation in *P. aeruginosa* without affecting its growth. These biological properties can lead to the valorization of this plant in the treatment of diseases caused by pathogen resistant to antibiotics. Future investigations will be focused on the ability of the anti-QS molecules from *A. seyal* to interfere either with the mechanisms of perception or production of homoserine lactones (lasI/lasR, rhlI/rhlR QS systems).

Conflict of interest statement

Authors declare that they have no conflict of interest.

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