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Anti-quorum quenching activity of methyl gallate isolated from galls of Guiera senegalensis J. F. Gmel (Combretaceae)
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Anti-quorum quenching activity of methyl gallate isolated from galls of *Guiera senegalensis* J. F. Gmel (Combretaceae)

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Many pathogenic bacteria produce virulence factors controlled by a mechanism of regulation named quorum sensing (QS). Inhibition of bacterial QS system is a more recent therapeutic approach to counterbalance the emergence of multi-drug resistant bacteria. This study aimed to assess the abilities of methanol extract from *Guiera senegalensis* galls and its isolated compound methyl gallate to quench the quorum sensing system. Methanol extract from galls of *G. senegalensis* at the concentration of 100 µg/ml demonstrated significant inhibitory effect on pyocyanin and violacein production respectively in *Pseudomonas aeruginosa* PAO1 and *Chromobacterium violaceum* CV026. Colum chromatography and recycling High Performance Liquid Chromatography (HPLC) of methanol extract from galls of *G. senegalensis* led to the isolation of one active quorum quenching compound. Different spectroscopic methods (MS and NMR) were used to elucidate the structure of this isolated compound as being the methyl gallate (MG). Methyl gallate at the final concentration of 12.5 µg/ml demonstrated good anti-QS activity by inhibiting violacein and pyocyanin production. Its low molecular weight and the capacity to interfere with the mechanism of QS make methyl gallate, an interesting candidate for development of drugs as an alternative to antibiotics to combat bacterial resistance.

**Key words:** *Guiera senegalensis*, methyl gallate, *Pseudomonas aeruginosa* PAO1, quorum sensing.

**INTRODUCTION**

*Pseudomonas aeruginosa* is a pathogenic bacteria able to infect insects, plants, animals, and humans (Rahme et al., 2000). This ubiquitous Gram-negative pathogen is a frequent cause of nosocomial infections and mortality in immunocompromised patients particularly in patients with cystic fibrosis, diffused panbronchitis, pulmonary deficiencies, major burn wounds, diabetes, cancer (Krcmery et al., 2006) and AIDS (Gomes et al., 2012).

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The pathogenicity of this opportunistic pathogen is due to its capacity to produce several virulence factors (elastase, exotoxin, pyocyanin, rhamnomipids) and to form biofilms (Jensen et al., 2007; Van Delden and Iglewski, 1998). The production of virulence factors by *P. aeruginosa*, like many other bacterial species is controlled by a cell-to-cell communication system dependent on bacterial density called quorum sensing (QS) (Castillo-juárez et al., 2015). This mechanism is based on the production of small molecules called acyl homoserine lactones (AHLS). These molecules diffuse through the bacterial cell envelope and when their concentration reaches a critical threshold, they cause the activation of transcriptional regulators which will then trigger the expression of virulence genes. Considering its central role in the pathogenicity, inhibition of the QS system is a therapeutic approach for the development of new drugs to counterbalance the emergence of antibiotic-resistant pathogens. Many systems to assess anti-QS activity have been recently developed for the research of active compounds in natural products. The production of a purple pigment, violacein, controlled by QS system in *Chromobacterium violaceum* making this strain an excellent biomonitor for the research of anti-QS compounds (Yong and Zhong, 2012). Thus, many plants have been widely screened for their anti-QS activity (Adonizio et al., 2008a; Ouedraogo and Kienderbeogo, 2016; Vandeputte et al., 2010). Phenolic compounds isolated from Combretaceae family already showed potent antiquorum activity (Sombié et al., 2012). Many species of Acharyya et al., 2015; Kamatham et al., 2015; Farhoosh et al., 2015) isolated e

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**MATERIALS AND METHODS**

**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).

**Plant material collection and extraction**

The collection of plant material consisted of galls of *G. senegalensis* J. F. Gmel (Combretaceae) were collected in Gampel (25 km, east of Ouagadougou, Burkina Faso). The plant was identified in the department of plant biology, University Ouaga I Pr Joseph Ki-ZERBO, Burkina Faso where a voucher specimen (ID: Lamien 01) was deposited. After drying at room temperature, the plant material was pulverized and stored at 4°C until used. The fine powder of plant material was soaked in methanol during 24 h. The extract obtained was filtered and concentrated in a vacuum evaporator (Büchi Labor technik AG, Postfach, Flawil, Switzerland).

**Isolation and structural elucidation of one of the major compounds**

The methanol extract (10 g) was fractioned by Vacuum Liquid Chromatography (VLC) with hexane, ethyl acetate and butanol. The ethyl acetate fraction (4740 mg) was dried and eluted with a gradient of hexane-ethyl acetate (from 0 to 100% ethyl acetate). One hundred and sixteen vials were collected and vials with the same phytochemical profiles in thin layer chromatography were assembled to give five fractions labeled A, B, C, D and E. Fraction D (750 mg) was further fractionated by gel filtration that was eluted with a gradient of hexane-dichloromethane (from 0 to 100% dichloromethane). Fifty-six vials were collected and assembled according to their phytochemical profile in thin layer chromatography (TLC) to give three sub-fractions D1, D2 and D3. Sub-fraction D3 was loaded on to Sephadex (Kieselgel 60; 70-230 mesh) column and eluted with methanol. Vial 10 to vial 70 which presented one spot on TLC plates were assembled (70 mg) and purified with high-pressure liquid chromatography (HPLC) recycling (LC-908W-C60 recycling preparative) eluted with methanol led to the isolation of SP14 (67 mg). The compound (SP14) was further analyzed with ESI-MS (Jeol JMS-HX 110) in order to determine its molecular weight. Structural elucidation of SP14 was done by using spectroscopic methods (MS (Jeol JMS-HX 110), 1HMR, 13C NMR (Bruker Avance 500 MHz), and 2D NMR (COSY, HMOC and HMBC)).

**Anti-QS activity**

**Determination of minimum inhibitory concentration (MIC)**

The determination of MIC values of SP14 on *P. aeruginosa* PAO1
Effect on extracellular factors regulated by QS

Inhibition of violacein production assay

Anti-QS activity of methanol extract (1 ml) or methyl gallate was assessed by the ability to inhibit the production of violacein in C. violaceum CV026 according to the method of Choo et al. (2006). This strain is a mutant deficient in the homoserine lactone synthase gene cvil, unable to produce homoserine-lactones. Exogenous N-hexanoyl-L-homoserine lactone (HHL; Sigma Aldrich Chemie GmbH, Darmstadt, Germany) at 10 mM final concentration was added to C. violaceum CV026 culture to induce the production of violacein. C. violaceum CV026 culture was diluted in LB broth (starting OD600nm ranged between 0.02 and 0.03) and incubated during 18 h of incubation at 37°C with agitation (175 rpm). The production of violacein was quantified spectrophotometrically at 575 nm.

Inhibition of pyocyanin production assay

The ability of methanol extract or methyl gallate to reduce the production of pyocyanin was assessed using the method described by Vandeputte et al. (2010). 250 µl of an appropriately diluted overnight culture of P. aeruginosa PAO1 were added to 4.7 ml of LB medium and supplemented with 50 µl of plant extract (10 mg/ml) or methyl gallate (1.25 µg/ml) dissolved in DMSO. The tubes were sampled to assess growth parameters (CFU/ml and OD600nm) and pyocyanin content after 18 h of incubation at 37°C with agitation (175 rpm). Pyocyanin was extracted from supernatant (4 ml) with chloroform (2 ml). Then, 1 ml of 0.2 M HCl was added to the chloroform layer. After centrifugation the absorbance of the top layer was measured at 600 nm for pyocyanin determination.

Statistical analysis

Experiments in this study were independently performed in triplicate. Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey test, using GraphPad Prism software (version 5.00 for window, GraphPad Software, San Diego, CA, USA). P value ≤ 0.05 was considered significant.

RESULTS

Compound identification

Chromatographic fractionation of the methanol extract of gall from G. senegalensis led to the isolation of a major compound designated SP14. SP14 was isolated as a white crystalline powder from the methanol extract. ESI-MS spectrum showed that the molecular weight of SP14 was m/z 184 [M]+ which corresponds to a molecular formula C_{12}H_{18}O_{5}. MS and NMR spectra led to the determination of SP14 structure as methyl 3,4,5-trihydroxybenzoate namely methyl gallate (Chaubal et al., 2005).

ESI-MS, 1H NMR and 13C NMR, 2D NMR (COSY, HMOC and HMBC) data are summarized: ESI-MS (70 eV) m/z 184 [M]+ (55), 153 (100), 125 (25), 107 (8), 79 (20), 51; 1H NMR (1Acetone, 125 MHz) δ 3.78 (3H, s, OCH3), δ 7.029 (2H, s, H-2, H-6); 13C NMR (Acetone, 125 MHz) δ 51.96 (OCH3), δ 109.82 (C-2, C-6), δ 121.79 (C-4), δ 138.79 (C-1), δ 146.11 (C-3, C-5), δ 167.24 (C=O). The 1H and 13C NMR spectra data give a total of five (5) protons attached to eight (8) carbons indicating that the compound is a relatively small molecule. The presence of two (2) aromatic protons [δ 7.11 (2H, s, H-2, H-6)], indicated a symmetrical molecule and have three (03) hydroxyl groups 5C 146.11 (C-3, C-5) and 5C 121.79 (C-4) and a methyl carboxylic acid δ 3.78 (3H, s, OCH3), δC 167.24. Comparing with the reported data, the 1H NMR and 13C NMR data are in agreement with those of literature (Ma et al., 2005; Ekaprasada et al., 2009).

Anti-QS activity of G. senegalensis galls extract

The MIC value of the plant methanol extract was determined by the microdilution method against C. violaceum. The value found was 2.5 mg/ml and this allowed for use of a sub-inhibitory concentration of 100 µg/ml for the anti-QS assay. Methanol extract from galls of G. senegalensis (100 µg/ml final concentration) significantly reduced violacein production up to 41% (Figure 1A) compared to the negative control culture. Galls extract did not inhibit bacterial growth at this concentration (Figure 1B). This result confirms the anti-quorum sensing activity of the galls extract. Salicylic acid was used as a positive control because of its known interference with the QS mechanism (Vandeputte et al., 2010).

The MIC value of G. senegalensis galls methanol extract on P. aeruginosa PAO1 was 5 mg/ml. The methanol extract of galls was tested at the sub-inhibitory concentration of 100 µg/ml on P. aeruginosa PAO1. The effect of the extract was evaluated on the production of pyocyanin, one of the virulence factors secreted by P. aeruginosa. Pyocyanin is an extracellular virulence factor which can be detected in the culture medium by its characteristic blue-green color. The production of pyocyanin is controlled by the mechanism of QS. As shown in Figure 1C the galls extract significantly reduced the production of pyocyanin up to 47% without affecting P. aeruginosa PAO1 growth when compared to the negative control medium (Figure 1D). The galls extract
exhibited a significant anti-QS activity compared to salicylic acid used as positive control. In summary, the galls extract from *G. senegalensis* significantly reduced the production of QS-controlled factors such as violacein and pyocyanin. The galls extract at the concentration of 100 µg/ml did not have an effect on the growth of *P. aeruginosa* and *C. violaceum*. The reduction of violacein and pyocyanin production is not attributed to a bactericidal effect, but is suggested to be an interference with the QS mechanism of bacteria. These results showed that galls of *G. senegalensis* contain anti-QS agents which can be isolated.

**Anti-QS activity of methyl gallate (MG) isolated from galls of *G. senegalensis***

The ability of MG to reduce the production of violacein and pyocyanin was assessed. As shown in Figure 2A, violacein production was significantly affected by MG at the concentrations of 100, 50 and 25 µg/ml. MG inhibited bacterial growth at these concentrations (Figure 2B). The growth of *C. violaceum* CV026 was highly reduced by MG at the concentrations of 100 and 50 µg/ml as indicated by the inhibition of violacein production in the medium.

MG at the low concentration of 12.5 µg/ml inhibited violacein production without affecting *C. violaceum* CV026 growth (Figure 2B) compared to 1% DMSO used as negative control. The growth parameters (density and colony forming unit) were evaluated in order to confirm that MG at the concentration of 12.5 µg/ml did not inhibit the growth of bacteria. As shown in Figure 2C, at the concentration of 12.5 µg/ml MG did not affect the growth of *C. violaceum CV026* during 48 h. MG (12.5 µg/ml) also did not have any effect on *C. violaceum* viability (CFU/ml) as shown in Figure 2D. These results indicate that the reduction of violacein production is not due to a bactericidal effect of MG on *C. violaceum* CV026. The concentrations of MG less than 12.5 µg/ml did not negatively affect the production of violacein (Figure 2A).

MG at the concentration of 12.5 µg/ml significantly reduced the production of pyocyanin (Figure 3A) up to 65% without affecting *P. aeruginosa* PAO1 growth during
Figure 2. Anti-quorum sensing activity of methyl gallate (MG). (A) Violacein production inhibition by different concentration of MG. (B) C. violaceum CV026 growth in presence of MG at different concentration (100-3.125 µg/ml). (C) Effect of MG (12.5 µg/ml) and salicylic acid (SA) on kinetic growth of C. violaceum CV02. (D) Viability of bacterial cell in culture medium. Histograms with the same letter (a-e) was no significant for p<0.05.

Figure 3. Effect of methyl gallate (MG) at 12.5 µg/ml on pyocyanin production in P. aeruginosa (A) pyocyanin production in P. aeruginosa PAO1. (B) Effect of MG and salicylic acid on kinetic growth of P. aeruginosa PAO1 (D) P. aeruginosa PAO1 viability. Histograms with the same latter (a-c) was no significant for p<0.05.

18 h (Figure 3B and C). As observed with the crude extract of galls from G. senegalensis, MG also exhibited a strong effect on the inhibition of pyocyanin production compared to salicylic acid (Figure 3A). MG showed
higher anti-QS activity against C. violaceum CV026 and P. aeruginosa PAO1 at lower concentration than the crude extract of G. senegalensis galls.

DISCUSSION

Medicinal plants such as Combretaceae species are an inestimable source of chemical compounds for the isolation, identification and the development of new drugs to treat microbial infections (Koh and Tham, 2011). The galls of G. senegalensis are employed in the traditional treatment of cough, dysentery, malaria and possess antibacterial activity against many bacteria. An antioxidant activity of the galls of G. senegalensis has been previously reported (Sombié et al., 2011). This study demonstrated that the methanol extract from the galls of G. senegalensis reduces the production of two QS-controlled factors namely violacein and pyocyanin respectively in C. violaceum CV026 and P. aeruginosa PAO1. The decrease in pyocyanin and violacein production observed at low concentrations of the methanol extract is not due to an effect on bacterial growth. P. aeruginosa secretes various virulence factors like pyocyanin which can cause damage to host cells and tissues. The significant inhibitory effect on violacein production suggests that galls may contain antagonists of AHLs. Pyocyanin affects the redox cycle and increases the oxidative stress on the host cell (Liu and Nizet, 2009). Indeed, pyocyanin capable of inducing oxidative stress, inhibits wound repair leading to premature cellular senescence. Pyocyanin at the concentration of 10 mM showed an inhibitory effect on normal primary diploid fibroblasts (Mueller et al., 2009). Taken together antioxidant and anti-QS activities inhibition of the galls could contribute to the efficiency for the treatment of bacterial infections.

Polyphenol are also known for their anti-QS activity. The galls of G. senegalensis are known to be rich in phenol compounds (Sombié et al., 2011). Methyl gallate is one of the active molecules responsible for anti-QS activity and was isolated in this study. An array of biological activities of this compound including antioxidant, anti-tyrosinase properties and its anti-QS property using C. violaceum and P. aeruginosa has been reported (Chaubal et al., 2005; Tan et al., 2015; Hossain et al., 2017). Hossain et al. (2017) demonstrated that MG reduces the expression of the HHL synthetases genes (lasI and rhlI) and the QS regulator genes (lasR and rhlR) in concentration dependent-manner (16~256 µg/ml). As a consequence, the production of virulence factors was significantly affected. The production of pyocyanin was inhibited (37~64%) after 24 h of incubation while in our investigation the production of pyocyanin was reduced by 65% at the concentration of 12.5 µg/ml after 18 h of incubation. This difference on the percent of reduction could be due to the time of incubation or the method used. Vandeputte et al. (2010) demonstrated that catechin reduced the expression of lasI by 40% after 8 h of incubation, while after 18h, this reduction was 26%. Many phenolic compounds identified in galls of G. senegalensis already demonstrated anti-QS activity. The presence of gallic acid, kaempferol, quercetin have been reported previously by Lamien et al. (2005) and the epigallocatechin gallate by Bouchet et al. (1996). Quercetin suppressed and kaempferol showed anti-QS activity against C. violaceum and P. aeruginosa PAO1 at 100 µg/mL (Vasavi et al., 2014). Gallic acid (GA) showed inhibition effect in many virulence factors production among bacteria (Munoz-Cazares et al., 2017). The epigallocatechin gallate have antibiofilm activity and exhibits anti-virulence in sublethal concentrations (Munoz-Cazares et al., 2017). Salicylic acid (SA) and related compounds such as gallic acid demonstrated anti-quorum quenching activities. SA demonstrated inhibitory activity in the motility and production of extracellular virulence factors in P. aeruginosa (Munoz-Cazares et al., 2017). It inhibited pyocyanin by approximately 80% and decreased the elastase and exoprotease production (Munoz-Cazares et al., 2017). SA and GA are benzoic acid derivatives that possess an aromatic ring bearing one and three hydroxyl groups respectively. MG is the methyl ester of gallic acid. In this study, MG demonstrated significant strong anti-quorum sensing activity compared to SA using the same concentration of 12.5 µg/ml. The basic skeleton of these three compounds (SA, GA and MG) remains the same and the differences are the number of the hydroxyl groups on the aromatic ring and the type of substituents. The structure of MG may be responsible for its strong anti-virulence activity. The isolation permitted the obtention of potent anti-virulence compound. Based on its significant antibacterial activity (Choi et al., 2008; Acharyya et al., 2015), its in vivo anti-inflammatory activity (Correa et al., 2016) and its anti-virulence activity associated to its low molecular weight, MG may be an active ingredient for pharmaceutical preparation used for treating infections caused by bacteria.

Conclusion

Methanol extract of G. senegalensis galls quenches the mechanism of QS by inhibiting the production of pyocyanin in P. aeruginosa PAO1 and violacein in C. violaceum CV026. The isolated compound, methyl gallate demonstrated potent anti-QS activity. Methyl gallate strongly reduced the production of the virulence factor pyocyanin in P. aeruginosa PAO1. Thus, methyl gallate could be a potential candidate to develop an efficient drug for the treatment of recalcitrant bacterial infections. Futures investigation will allow to determine the effect of methyl gallate on QS-controlled genes expression and the interference with the mechanisms of perception or
production of homoserine lactones.

**CONFLICT OF INTERESTS**

The authors have not declared any conflict of interests.

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