



Antioxidant, Antiquorum-Sensing and Antibiofilm Activities of *Balanites aegyptiaca* (L.) Del. (Balanitaceae) and *Terminalia macroptera* Guill. and Perr. (Combretaceae)

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Abstract: *Pseudomonas aeruginosa* like many pathogen bacteria produces various virulence factors and form biofilm that contribute to its pathogenicity and the growing resistance to antibiotics. The production of virulence factors in those multi-resistant bacteria is controlled by a mechanism of regulation termed quorum sensing. Interfering with this mechanism of bacterial communication constitute a strategy to attenuate bacterial pathogenicity. Our study aimed to assess the ability of *Balanites aegyptiaca* and *Terminalia macroptera* to interfere with the system of QS through inhibition of QS-controlled factors production and the formation of biofilm. Methanol extracts of galls, stem bark of *B. aegyptiaca*, and stem bark of *T. macroptera* were screened for anti-QS activity using *Chromobacterium violaceum* CV026 and *P. aeruginosa* PAO1. At a sub-inhibitory concentration of 100 µg/mL, galls and stem bark of *B. aegyptiaca* quenched the QS system by inhibiting violacein production on *C. violaceum* CV026 and pyocyanin production on *P. aeruginosa* PAO1. The bark of *T. macroptera* reduced significantly the production of violacein, pyocyanin and the formation of biofilm. Moreover, antioxidant activity of phenolic compounds contributes to reduce the oxidative stress induced by pyocyanin. Thus, *T. macroptera* is a potential candidate for the identification and isolation of news effective anti-QS compounds. This study introduces a possible validation for traditional use of *B. aegyptiaca* and *T. macroptera* and constitutes a new therapeutic approach for the treatment of infections caused by bacteria resistant to antibiotics.

Keywords: *Balanites Aegyptiaca*, *Terminalia Macroptera*, *Pseudomonas Aeruginosa* PAO1, Quorum Sensing

1. Introduction

The emergence of pathogenic bacteria resistance to antibiotics make crucial to find news antibacterial drug with novel target. In recent years, the discovery of bacterial communication system that regulates the expression of virulence genes of pathogenic bacteria has provided a new opportunity for the control of bacterial infections [1]. The quorum sensing inhibition has not a direct action on bacterial growth but on the reduction of virulence while limiting selective pressure compared to antibiotics [2].

P. aeruginosa, an opportunistic pathogen, have probably the best characterized QS system among Gram-negative

bacteria [3]. This pathogen is implicated in nosocomial infections and is the major cause of mortality in immunocompromised patients and burns patients infected [4]. The pathogenicity of *P. aeruginosa* is due to its capacity to secrete QS-controlled virulence factors including pyocyanin, elastase, LasA protease, alkaline protease, rhamnolipids, exotoxin A and hydrogen cyanide [5]. QS systems are ubiquitous among bacteria, and has been known to control a wide array of phenotypes in bacteria ranging from simple bacterial cell motility to complex communal behaviors such as biofilm formation, and the production of virulence factors [6]. Biofilm is a bacterial population attached to a surface and encapsulated with an exopolysaccharides matrix. The

ability of *P. aeruginosa* to form biofilm led to consider this mechanism as crucial in infectious processes [7]. Biofilm protects bacteria and increases their resistance to antibiotics and the immune system [8]. Thus, by disrupting the cell-to-cell communication of bacteria, it would be possible to repress the expression of QS-regulated phenotypes in relation to the treatment of bacterial infections caused by antibiotic-resistant strains [9, 10]. Simple assay systems have been developed recently for the research of anti-QS agents in natural products. Anti-QS activity has been reported in certain medicinal plants [11, 12]. In our previous *in vitro* investigations, we demonstrated that *Anogeisuss leiocarpus* (DC) Guill. and Perr. (Combretaceae) traditionally used to treat respiratory diseases and wound, reduces the production of QS-controlled factors and gene expression [13]. These preliminary results lead to select some medicinal plants of Burkina faso flora for screening their anti-QS activity. Ethnobotanical data indicate that *Balanites aegyptiaca* and *Terminalia macroptera* are used for the treatment of respiratory tract diseases, skin diseases and wound [14, 15]. In this study, these medicinal plants were investigated for its ability to interfere with the bacterial QS system. Total phenolic and flavonoid content and antioxidant activity of these plants were also assessed.

2. Material and Methods

2.1. Bacterial Strains and Growth Conditions

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

2.2. Plant Material Collection and Extraction

The galls and stem bark of *B. aegyptiaca* were collected in September 2017 in Loumbila (Burkina Faso); stem bark of *T. macroptera* was collected in Boussé (Burkina faso). The plants were identified by Dr Amade OUEDRAOGO from the Laboratoire de Biologie et Ecologie Vegetale (Université Ouaga I Pr Joseph KI-ZERBO, Burkina Faso). After dried at room temperature and powdered, plant materials were soaked during 24 h in methanol. After filtrated, extracts were concentrated in a vacuum evaporator (Büchi Labortechnik AG, Postfach, Flawil, Switzerland) and dried.

2.3. Determination of Total Polyphenol and Total Flavonoid Contents

Total polyphenol was determined by the Folin–Ciocalteu method [16]. Briefly, 25 µL of plant extract (100 µg/mL in methanol) was mixed with Folin–Ciocalteu Reagent (125 µL, 0.2 N) and 100 µL of sodium bicarbonate (75 g/L) were added to the mixture after 5 min. After 1 h of incubation, the absorbance was measured at 760 nm. A standard calibration curve ($Y = 0.005X + 0.00968$; $R^2 = 0.99$) was plotted using

gallic acid (0–200 mg/L). The results were expressed as mg of gallic acid equivalents (GAE)/100 mg of plant extract.

The total flavonoid content was determined using the Dowd method as adapted by [16]. Briefly, 100 µL of aluminium trichloride (2% in methanol) was mixed with the same volume of extract diluted in methanol. The absorbance was read at 415 nm after 10 min. Quercetin was used as reference compound for the establishment of standard curve ($y = 0.02891X + 0.0036$; $R^2 = 0.99$) and the average of three readings was used and expressed as mg of quercetin equivalents (QE)/100 mg of plant extract.

2.4. Determination of Antioxidant Activity

Antiradical activity was evaluated by using the DPPH (2, 2'-diphenyl-1-picrylhydrazyl) method. The DPPH assay was performed according to [16]. In microplate 96-well, 100 µL of methanol extract was mixed with 200 µL of DPPH solution (20 mg/mL in methanol). The absorbance was read at 517 nm after 15 min of incubation. A standard curve ($y = -27.94x + 8.15$; $R^2 > 0.99$) was generated with quercetin and the concentration of plant extract that reduces the DPPH radicals was expressed in mg equivalent of quercetin per gram.

2.5. Anti-QS Activity

2.5.1. Determination of Minimum Inhibitory Concentration (MIC)

The microdilution method was used to determine MIC values on *P. aeruginosa* PAO1 and *C. violaceum* CV026 according to [13]. An appropriately diluted bacterial culture was added to each well containing test extracts in order to obtain a final concentration range of 5 mg/mL to 0.15625 mg/mL. The microplate was incubated for 18 h at 37°C. To indicate bacterial growth, 50 µL of p-iodonitrotetrazolium (0.2 mg/mL) was added to each well and the microplate was incubated at 37°C for 30 min.

2.5.2. Quantitative Analysis of Violacein Production in *C. violaceum* CV026

Inhibition of violacein production in *C. violaceum* CV026 was tested according to [17]. The production of violacein in the mutant *C. violaceum* CV026 was induced by adding exogenous N-hexanoyl-L-homoserine lactone (HHL; Sigma-Aldrich Chemie GmbH, Darmstadt, Germany). 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, 175 rpm agitation. Bacterial turbidity (OD_{600nm}) was measured to assess bacterial growth. For colony counting (CFU/mL) at 48 h, 100 µL of bacterial culture were removed and diluted in LB broth to be plated onto LB agar and incubated (24 h, 30°C). 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.

2.5.3. Quantitative Analysis of Pyocyanin Production in *P. aeruginosa* PAO1

Inhibition of pyocyanin production was assessed according to the procedures described by [5]. After 18 h of growth in LB broth (37°C, 175 rpm agitation) *P. aeruginosa* PAO1 culture was washed in LB medium. Culture tubes containing appropriately diluted PAO1 cell suspension (250 µL), LB medium (4.7 mL) and plant extract (50 µL, 10 mg/mL in DMSO) or DMSO were incubated at 37°C, 175 rpm agitation. After 18h of incubation, bacterial turbidity was measured (OD_{600nm}) to assess bacterial growth. For colony counting (CFU/mL) at 18 h, 100 µL of bacterial culture were removed and diluted in LB broth to be plated onto LB agar and incubated (24 h, 37°C). After centrifugation of remaining bacterial culture (7000 rpm, 10 min, 24°C) pyocyanin was extracted from the supernatant (4 mL) with chloroform (2 mL) and re-extracted from chloroform with 0.2 M HCl (1 mL). The absorbance measured at 380 nm allows pyocyanin determination.

2.5.4. Biofilm Formation and Quantification

The quantification of biofilm formation was assessed according to the method described by [5]. 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.

2.5.5. Statistical Analysis

All experiments in this study were performed in triplicate (independent assays). One way ANOVA followed by Tukey test of Graph Pad Prism software was used to obtain graph and measuring the statistical difference.

3. Results

3.1. Total Polyphenol, Flavonoid Content, and Antioxidant Activity

Total polyphenol and total flavonoid quantification were assessed from methanol extracts of *B. aegyptiaca* and *T. macroptera*. The amount of total polyphenol of stem bark extract of *B. aegyptiaca* (53.21 ± 2.23 mg GAE/100 mg) was higher than those of galls extract (37.96 ± 1.14 mg GAE/100 mg); however the highest content of total flavonoid and polyphenol were obtained in bark extract of *T. macroptera* (Table 1).

The DPPH radical scavenging capacity was used to assess the antioxidant activity of methanol extracts of galls and stem bark. As shown in table 1, galls and stem bark extracts of both plants exhibited an interesting antioxidant potential.

Table 1. Antioxidant activities and phenolic contents.

Plant	DPPH (mg QE/10 g)	TP (mg GAE/100 mg)	TF (mg QE/100 mg)
B. aegyptiaca (galls)	46.03 ± 2.19 ^b	37.96 ± 1.14 ^c	3.77 ± 0.22 ^b
B. aegyptiaca (bark)	54.19 ± 1.85 ^a	53.21 ± 2.23 ^b	1.4 ± 0.02 ^c
T. macroptera (bark)	47.19 ± 0.67 ^b	81.02 ± 2.87 ^a	35.50 ± 1.14 ^a

Data in each column with different letters are statistically different (p<0.05). TP: Total polyphenol; TF: Total flavonoids; mg GAE: mg gallic acid equivalent; mg QE/: mg quercetin equivalent

3.2. Inhibition of QS-Regulated Violacein Production in *C. violaceum* CV026

Minimum inhibitory concentration values of galls (1.25 mg/mL), bark of *B. aegyptiaca* (0.62 mg/mL) and bark of *T. macroptera* (0.62 mg/mL) extracts were determined against *C. violaceum* CV026 by microdilution method. These results allowed for the selection of a sub-inhibitory concentration (100 µg/mL final concentration) for the anti-QS assay. The reporter strain *C. violaceum* CV026, deficient in the homoserine-lactone synthase gene *cviI*, was used to assess the anti-quorum sensing activity of the methanol extracts from galls, bark of *B. aegyptiaca*, and bark of *T. macroptera*

(Figure 1a). This strain is unable to produce by itself quorum sensing auto inducers (homoserine-lactones), but an external supply of homoserine-lactone in growth medium induced the production of violacein. The quantification of violacein production in the presence of galls and bark extracts showed that extracts at the sub-inhibitory concentration of 100 µg/mL reduced violacein production (Figure 1a) without affecting bacterial growth (Figure 1b). Bark extract of *T. macroptera* reduced significantly the production of violacein by up to 46%, while bark extract of *B. aegyptiaca* reduced violacein production up to 17%.

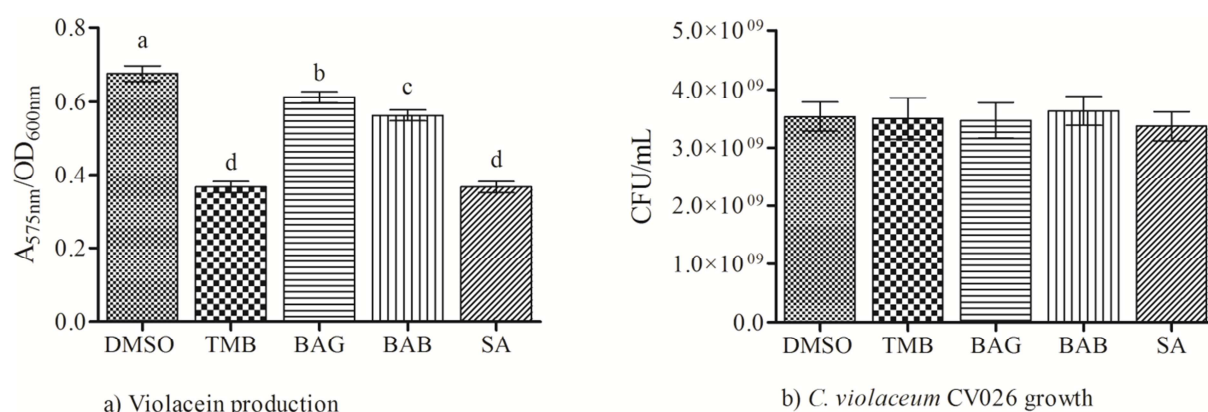


Figure 1. Effect of *B. aegyptiaca* and *T. macroptera* extracts on QS-controlled violacein production of *C. violaceum* CV026 after 48 h of growth. TMB: *T. macroptera* bark; BAG: *B. aegyptiaca* galls; BAB: *B. aegyptiaca* bark; SA: Salicylic acid was used as positive control. All samples were tested at 100 µg/mL final concentration. Histogramm with different letters are significantly different ($p < 0.05$). Dimethyl sulfoxide (DMSO 1%) was used as negative control.

3.3. Inhibition of Pyocyanin Production

Extracts of these plant species were also tested on *P. aeruginosa* PAO1. The MIC values (5 mg/mL, 2.5 mg/mL and 1.25 mg/mL respectively for galls, bark of *B. aegyptiaca* and bark of *T. macroptera*) evaluated allow for the selection of a sub-inhibitory concentration for the bioassay on *P. aeruginosa* PAO1. Interestingly, at the concentration of 100

µg/mL, galls and bark extracts reduced the production of pyocyanin of *P. aeruginosa* PAO1 compared to the control (Figure 2a). Maximum reduction of pyocyanin production was recorded in presence of bark extract of *T. macroptera* (55% of reduction). As observed with *C. violaceum* CV026, all extracts did not affect *P. aeruginosa* growth (Figure 2b).

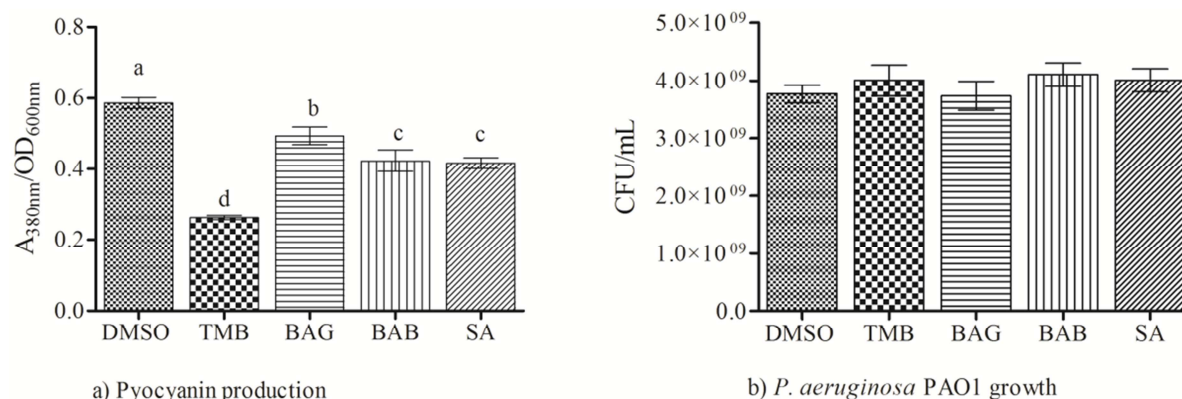


Figure 2. Effect of *B. aegyptiaca* and *T. macroptera* extracts on QS-controlled pyocyanin production of *P. aeruginosa* PAO1 after 18 h of growth. TMB: *T. macroptera* bark; BAG: *B. aegyptiaca* galls; BAB: *B. aegyptiaca* bark; SA: Salicylic acid was used as positive control. All samples were tested at 100 µg/mL final concentration. Histogramm with different letters are significantly different ($p < 0.05$).

3.4. Inhibition of Biofilm Formation

Many studies demonstrated that QS interferes positively in the formation of biofilm of *P. aeruginosa* PAO1 [6, 7]. Since *B. aegyptiaca* and *T. macroptera* extracts showed inhibition effect in the QS mechanism, their ability to inhibit biofilm formation in *P. aeruginosa* PAO1 was evaluated. The effect of galls and bark extracts on *P. aeruginosa* biofilm formation was assessed after 24h of growth. The bark extract of *T. macroptera* was the most active with a reduction up to 37% (Figure 3). The anti-QS assay on the two biomonitor indicated that bark of *T. macroptera* with the highest polyphenol content was the most active. These results suggest that polyphenol could be responsible for the anti-QS activity.

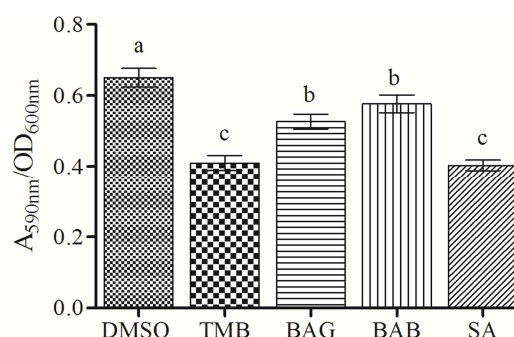


Figure 3. Effect of *B. aegyptiaca* and *T. macroptera* extracts on biofilm formation of *P. aeruginosa* PAO1 after 24 h of growth. TMB: *T. macroptera* bark; BAG: *B. aegyptiaca* galls; BAB: *B. aegyptiaca* bark; SA: Salicylic acid was used as positive control. All samples were tested at 100 µg/mL final concentration. Histogramm with different letters are significantly different ($p < 0.05$).

4. Discussion

B. aegyptiaca and *T. macroptera* are used in traditional medicine to treat infectious diseases and have shown antimicrobial and antioxidant activities [15, 18].

Our investigations show that methanol extracts from the galls, bark of *B. aegyptiaca*, and bark of *T. macroptera* exhibit anti-QS activity with a subsequent reduction of QS-controlled factors production. These extracts inhibit the production of virulence factors such as pyocyanin in *P. aeruginosa*. Indeed, pyocyanin alters the redox cycle of host cells, increases stress oxidative [19], inhibits wound repair by inducing premature cellular senescence, resulting in chronic inflammation in burn wounds infected by *P. aeruginosa* [20]. The antioxidant potential of phenolic compounds of *B. aegyptiaca* and *T. macroptera* is important in wound healing process by reducing the oxidative stress induced by pyocyanin. The ability of polyphenols to reduce the oxidative stress could permit the prevention of host cells senescence caused by reactive oxygen species. Additionally, the reduction of pyocyanin production protects host tissue against pathogens. Like many medicinal plants, *B. aegyptiaca* and *T. macroptera* are an important source of anti-virulence compounds which interfere with the QS mechanism of *P. aeruginosa*. The pathogenicity of *P. aeruginosa* is due to its ability to produce multiple virulence factors and to form biofilm. Thus, the effect of the galls, bark extracts of *B. aegyptiaca*, and bark extract of *T. macroptera* on the formation of the biofilm is tested. These extracts reduce significantly the formation of the biofilm. The QS system interferes with the different stages of biofilm formation, particularly the last phase of maturation [21]. Disruption of QS mechanism in the presence of the bark extract of *T. macroptera* could explain the disruption of biofilm formation. Polyphenols of these extracts could also be responsible for the QS inhibition. The presence of Kaempferol, caffeic acid, ferulic acid, p-coumaric acid, sinapic acid in galls and bark of *B. aegyptiaca* has been reported by [22]. P-coumaric acid is known to possess anti-QS properties on *Agrobacterium tumefaciens* and *Pseudomonas chlororaphis* [9]. Ferulic acid inhibits swarming and prevents biofilm formation by *P. aeruginosa* [23]. The highest anti-QS activity exhibited by *T. macroptera* bark could be due to the presence of tannins. This plant is one of the species rich in tannins. This group of secondary metabolites possesses anti-QS properties. *T. catappa* L. fraction rich in tannin at the concentration of 62.5 µg/mL inhibits the production of violacein in *C. violaceum*, LasA activity and biofilm formation in *P. aeruginosa* [24]. Two ellagitannins isolated from *Conocarpus erectus* reduce QS-controlled genes expression and virulence factors production in *P. aeruginosa* [1]. Other investigations demonstrated the anti-QS potential of ellagic acid, tannic acid and epigallocatechingallate [25]. Thus, *T. macroptera* could be use in further investigations for the isolation and identification of compounds which interfere with the mechanism of QS.

5. Conclusion

Natural products from Burkina faso flora possess anti-QS activity as shown in our in vitro investigations, suggesting that these products could be used for the development of drugs for the treatment of bacterial infections. The inhibitory effect of galls, bark extract of *B. aegyptiaca* and bark extract of *T. macroptera* on the production of virulence factors and the formation of biofilm contributes to reduce chronic infections. These biological properties could justify the use of these plants in traditional medicine against bacterial diseases. Future investigations will be focused on the capacity of anti-QS molecules from *T. macroptera* interfere with QS-regulated genes expression.

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Conflicts of Interest

The authors declare no conflict of interest.

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