Asian Pac J Trop Biomed 2016; 6(8): 698–701

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Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.elsevier.com/locate/apjtb

Original article http://dx.doi.org/10.1016/j.apjtb.2016.06.008

Phenolic compounds affect production of pyocyanin, swarming motility and biofilm formation of *Pseudomonas aeruginosa*

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ARTICLE INFO

Article history:
Received 21 Oct 2015
Received in revised form 5 Nov 2015, 2nd revised form 15 Feb, 3rd revised form 1 Mar, 4th revised form 4 Apr 2016
Accepted 10 May 2016
Available online 11 Jun 2016

Keywords:
Quorum sensing
Phenolic compounds
Pseudomonas aeruginosa
Biofilm
Swarming

ABSTRACT

Objective: To investigate the effects of plant-derived phenolic compounds (*i.e.* caffeic acid, cinnamic acid, ferulic acid and vanillic acid) on the production of quorum sensing regulated virulence factors such as pyocyanin, biofilm formation and swarming motility of *Pseudomonas aeruginosa* (*P. aeruginosa*) isolates.

Methods: Fourteen clinical *P. aeruginosa* isolates obtained from urine samples and *P. aeruginosa* PA01 strain were included in the study. The antibacterial effects of phenolic compounds were screened by well diffusion assay. Pyocyanin and biofilm activity were measured from culture supernatants and the absorbance values were measured using a spectrophotometer. Swarming plates supplemented with phenolic acids were point inoculated with *P. aeruginosa* strains and the ability to swarm was determined by measuring the distance of swarming from the central inoculation site.

Results: Tested phenolic compounds reduced the production of pyocyanin and biofilm formation without affecting growth compared to untreated cultures. Moreover, these compounds blocked about 50% of biofilm production and swarming motility in *P. aeruginosa* isolates.

Conclusions: We may suggest that if swarming and consecutive biofilm formation could be inhibited by the natural products as shown in our study, the bacteria could not attach to the surfaces and produce chronic infections. Antimicrobials and natural products could be combined and the dosage of antimicrobials could be reduced to overcome antimicrobial resistance and drug side effects.

1. Introduction

Antibiotics are commonly used for the treatment of bacterial infections. With the widespread appearance of multi antibiotic-resistant bacteria, it is becoming increasingly more difficult to treat bacterial infections with conventional antibiotics [1]. Thus, there is an increasing need for new strategies to cope with infectious diseases. The discovery that many pathogenic bacteria employ bacterial cell-to-cell communication or quorum sensing (QS) system to regulate their pathogenicity and virulence factor production makes this system an attractive target for the design and development of novel therapeutic agents [2]. QS is a communication system employed by a variety of Gram negative and Gram positive bacteria to co-ordinate group behaviors as function of cell density. QS system has been shown to control production of an array of extracellular virulence factors and the formation of biofilm in a variety of bacterial pathogens including *Pseudomonas aeruginosa* (*P. aeruginosa*) [3]. *P. aeruginosa* is an opportunistic human pathogen that preferentially infects patients with cancer or AIDS, patients immunocompromised by surgery, cytotoxic drugs or burn wounds, people with cystic fibrosis or blood, skin, eye and genitourinary tract infections. The pathogen produces many extracellular products, including elastase, LasA protease, alkaline protease, exotoxin A, rhamnolipids, pyocyanin and hydrogen cyanide [4]. All these

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The study protocol was performed according to the Helsinki declaration and approved by the Marmara University Ethics Committee.

Foundation Project: Supported by a grant from the Marmara University Scientific Research Fund (Project No.: SAG-B-110412-0079).

Peer review under the responsibility of Hainan Medical University. The journal implements double-blind peer review practiced by specially invited international editorial board members.

extracellular virulence factors are crucial for the competence of *P. aeruginosa* to establish and maintain infection. Production of these factors is controlled by QS system. *P. aeruginosa* also employs QS to control the formation of biofilms [5]. Biofilm formation is thought to protect the microorganisms from host defenses and provide increased resistance to antibiotics [6]. It has been suggested that inactivating the QS system of a pathogen can result in a significant decrease in virulence factor production [7]. Therefore, disruption of QS system offers a promising way to fight with multi antibiotic-resistant bacteria. Up to date, various types of screening have been carried out to find QS inhibitory molecules. Furanones, some synthetic compounds, certain recognized drugs and a wide range of natural substances, particularly extracts from plants and fungi have been shown to modulate QS-regulated phenotypes in Gram negative bacteria [8]. The majority of QS inhibitory compounds characterized so far are pharmaceutically unsuitable for human use due to toxicity, high reactivity and instability. Hence, attention has been focused on identification of non-toxic novel QS inhibitory molecules from natural sources. In recent years, plants like garlic, water lily, pea seedlings, vanilla, *Medicago sativa*, extracts of *Tremella fuciformis*, *Panax ginseng*, *Scorzonera sandrasica*, extracts of various medicinal plants from South Florida, clove oil and tannic acid are shown to modulate QS systems of Gram negative bacteria [9–11]. Plants have been used medicinally for thousands of years and can be a promising source for molecules that can potentially inhibit bacterial QS [12,13]. Phenolic compounds are plant secondary metabolites widely distributed in plant kingdom and have been long recognized for their antioxidant and antimicrobial activities [14]. However, the effect of their anti-QS activities of these compounds on microorganisms received less attention. Thus, in this study, we examined anti-QS properties of caffeic, cinnamic, ferulic and vanillic acids and their effects on swarming, biofilm formation, elastase and pyocyanin activity of *P. aeruginosa* PAO1 strain.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Fourteen clinical *P. aeruginosa* isolates obtained from urine samples from patients with urinary tract infection who admitted to Marmara University Hospital were included in the study.

All isolates were identified with mass spectrometry (Vitek MS, bioMérieux, France) and kept in freezer at −40 °C. Clinical isolates and standard *P. aeruginosa* PA01 strain were grown in Luria-Bertani broth at 37 °C overnight before experiments.

2.2. Phenolic acids

Vanillic acid, caffeic acid, cinnamic acid and ferulic acid were purchased from Sigma, St. Lois, MO, USA. The stock solutions of vanillic acid, caffeic acid, cinnamic acid and ferulic acid were prepared in ethanol/water mixture and added in final concentration of 4 mmol/L.

2.3. Antibacterial assay

The antibacterial effects of vanillic acid, caffeic acid, cinnamic acid and ferulic acid were screened by well diffusion assays. Bacterial turbidity was adjusted to McFarland standard No. 0.5 with sterile saline solution and 0.1 mL of suspension was immediately poured over the pre-warmed plates. Nearly 25 μL of each phenolic compound was pipetted onto the paper discs. The plates were incubated for 24–48 h at 30 °C. Antibacterial activity was determined by the diameter of inhibition zones (mm) around the wells. Ethanol was used as a negative control.

2.4. Pyocyanin assay

Pyocyanin was extracted from culture supernatants [15]. The cells were removed by centrifugation at 5 000 r/min and pyocyanin in the supernatant was extracted into chloroform by mixing 5 mL of supernatant with 3 mL of chloroform. Pyocyanin was then reextracted into 1 mL of acidified water (0.2 mol/L HCl) which gave a pink–red solution. For the quantitation of the pyocyanin within the solution, the absorbance was measured at 520 nm.

2.5. Biofilm formation

Overnight culture of *P. aeruginosa* strains was diluted to an optical density at 600 nm of 0.02. One milliliter aliquots of the diluted cultures were allocated in polystyrene tubes and incubated at 32 °C with gentle agitation for 10 h. Nonadherent cells were removed and their optical density at 600 nm was determined. After rinsing with distilled water, the biofilms were dyed with 1 mL of crystal violet (0.3%) and the excess dye was removed by washing with distilled water. For quantification of attached cells, the crystal violet was solubilized with 95% ethanol, and the absorbance was measured at 570 nm using a spectrophotometer [16].

2.6. Swarming motility assay

Swarming plates supplemented with phenolic acids were point inoculated with *P. aeruginosa* strains and incubated overnight at 37 °C [17]. Swarm plates consisted of 2 g of Bacto agar (Difco) and 3.2 g of nutrient broth (Oxoid) in 400 mL of distilled water. After autoclaving, filter-sterilized 10% (w/v) D-glucose in distilled water was added to a final concentration of 0.5% (w/v). The ability to swarm was determined by measuring the distance of swarming from the central inoculation site.

2.7. Statistical analysis

The statistical significance of each test condition was evaluated by student's *t*-test [Microsoft Excel 2007 software (Microsoft, Redmond, WA, USA)]; a P value of ≤ 0.01 was considered as significant difference.

2.8. Ethical issues

This retrospective study was conducted on previously isolated and archived clinical isolates, so there was no need for ethics approval for informed consent as indicated in National Code of Clinical Research published on 13th April 2013.

3. Results

3.1. The effect of vanillic acid, caffeic acid, cinnamic acid and ferulic acid on the growth of P. aeruginosa

QS inhibitory compounds that do not kill or inhibit microbial growth are less likely to impose a selective pressure for the development of resistant bacteria. Therefore, to eliminate bacteriostatic or bactericidal effects, *P. aeruginosa* PA01 was grown in the presence of different concentrations of vanillic acid, caffeic acid, cinnamic acid and ferulic acid. Concentrations up to 4 mmol/L had no effect on *P. aeruginosa* growth (data not shown). Therefore, a concentration of 4 mmol/L was used to determine the impact of vanillic acid, caffeic acid, cinnamic acid and ferulic acid on the production of pyocyanin, biofilm and swarming motility.

3.2. Influence of phenolic compounds on pyocyanin production

In order to assess the effect of tested phenolics on pyocyanin production, bacterial cultures were grown overnight in presence of 4 mmol/L vanillic acid, caffeic acid, cinnamic acid and ferulic acid. As shown in Figure 1, the addition of these compounds reduced the production of pyocyanin by 9–21% compared to the untreated cultures.

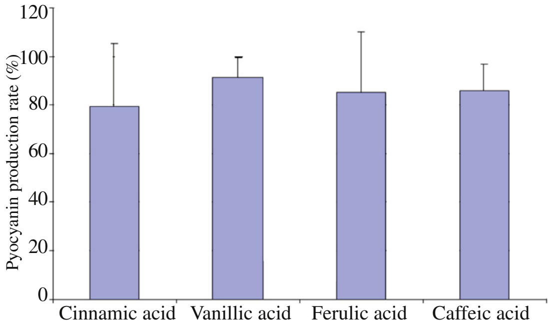

Figure 1. Effects of phenolic compounds on pyocyanin production of clinical *P. aeruginosa* isolates compared to control.

3.3. Effects of vanillic acid, caffeic acid, cinnamic acid and ferulic acid on biofilm formation and swarming motility

P. aeruginosa has the capacity to form biofilms which work as a penetration barrier for antimicrobials. Significant reductions in biofilm formation in clinical *P. aeruginosa* isolates were observed in the presence of tested phenolic compounds. The addition of cinnamic acid, ferulic acid and vanillic acid reduced biofilm production by 44%, 45% and 46%, respectively compared to the control (Figure 2). This was followed by caffeic acid which decreased biofilm production by 36% (Figure 2). Swarming motility is considered one of the virulence factors because it is required for the biofilm development and antibiotic resistance. All the tested phenolic compounds were shown significant influence on this virulence factor; the greatest inhibition of swarming motility was achieved by vanillic acid (67%) followed by caffeic acid (64%), cinnamic acid (54%) and ferulic acid (50%) (Figures 3 and 4).

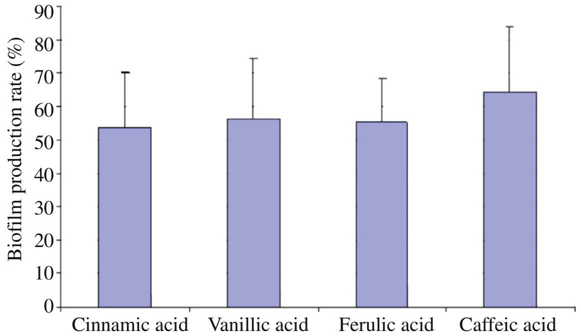

Figure 2. Effects of phenolic compounds on biofilm production of clinical *P. aeruginosa* isolates compared to control.

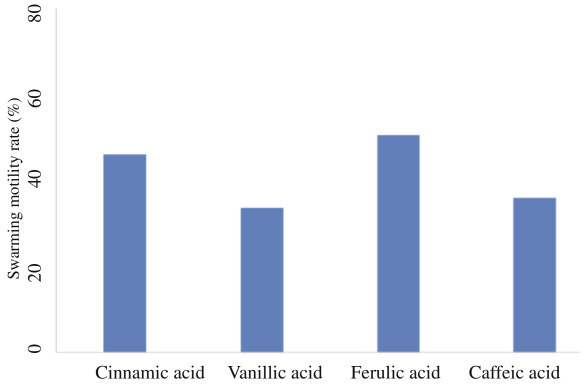

Figure 3. Effects of phenolic compounds on swarming motility of clinical *P. aeruginosa* isolates compared to control.

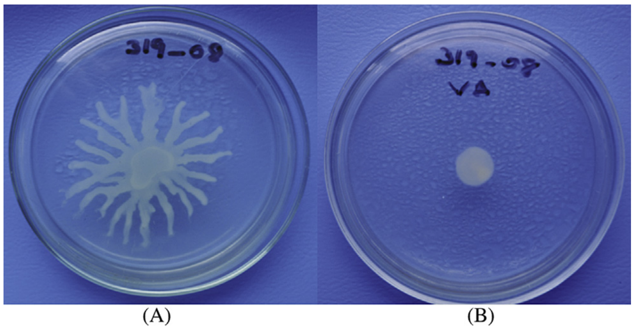

Figure 4. Testing for swarming motility inhibition.
A: Control; B: Vanillic acid added.

4. Discussion

High rate of infections among hospitalized patients and infection severity have persisted in the last decades and increased resistance rate to conventional antimicrobials raised a crucial need to explore new therapeutic options. Potential therapies targeting the virulence factor production could be an alternative therapeutic approach. Therefore, interfering with the QS system of pathogenic bacteria using QS inhibitor compounds constitutes a novel way for the management and the treatment of infectious diseases. Over the course of the last 15 years, a large number of structurally diverse QS inhibitors have been discovered. For example, synthetic acyl-homoserine lactone analogs, furanone derivatives, different heterocycles and a variety of recognized drugs such as some macrolide antibiotics, non-macrolide antibiotics, nifuroxazide, chlorzoxazone and salicylic acid have been reported to possess QS inhibitory activity [8,18,19]. Besides these molecules, some natural substances, particularly extracts from plants were shown to have a great potential to inhibit QS systems of Gram negative bacteria [9–11].

In this study, we tested the effects of phenolic compounds, vanillic, caffeic, cinnamic and ferulic acid on QS regulated virulence factor production of *P. aeruginosa* clinical isolates.

None of those products showed antibacterial activity at the tested concentrations but all had QS inhibitory effect. Production of pyocyanin was reduced, moreover, biofilm formation and swarming were around 50% blocked by those compounds. Swarming motility is considered as one of the virulence factors because it is involved in early biofilm development. Previous studies showed that *P. aeruginosa* mutants with altered swarming motility were also defective in biofilm formation [20,21]. It has also been reported that swarming strains of *P. aeruginosa* demonstrated an elevated resistance to multiple antibiotics [20]. In a study carried out by Borges *et al.*, QS regulated pigment production in *Chromobacterium violaceum* was not affected by phenolic compounds like gallic acid, ferulic acid, caffeic acid and epicatechin [22]. It is speculated that no positive result for QS inhibition by these phenolics can be due to the drawbacks of the QS inhibition assay used. Indeed, in accordance with our findings, Huber *et al.* demonstrated that some polyphenols [(−)-epigallocatechin gallate, (+)-catechin, ellagic acid and tannic acid] demonstrated anti-QS activity in *Escherichia coli* and *Pseudomonas putida* [23]. Extracts from *Moringa oleifera*, which contains phenolic compounds, also showed QS inhibitory activity [24].

In conclusion, the observation that phenolic compounds can inhibit QS-regulated virulence factors of *P. aeruginosa* may lead to the discovery of new non-toxic compounds from natural sources. Additionally, natural compounds that are capable of disrupting bacterial QS could potentially be used in combination with conventional antibiotics to increase the efficiency of current antimicrobials by reducing the minimal inhibitory concentration [25]. This approach may also help to overcome antimicrobial resistance and drug side effects.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

This work was supported by a grant (Project No.: SAG-B-110412-0079) from the Marmara University Scientific Research Fund.

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