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

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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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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 5000 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