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Swarming Inhibitory Potential of Cinnamtannin B1 from *Cinnamomum tamala* T. Nees and Eberm on *Pseudomonas aeruginosa*

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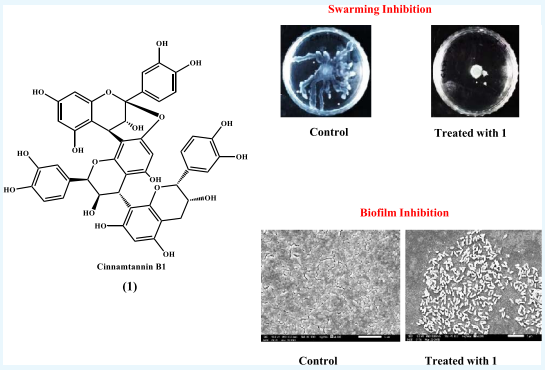
Supporting Information

ABSTRACT: In a preliminary screening, the methanol extract of *Cinnamomum tamala* leaves was found to inhibit the swarming motility of *Pseudomonas aeruginosa*. Bioassay-guided fractionation by silica gel column chromatography led to the identification of cinnamtannin B1 (**1**) as one of the active components of the extract. It inhibited the swarming motility (at 12.5 μg/mL) and biofilm formation (at 25 μg/mL) of*P. aeruginosa*. Comparative gene expression analysis revealed downregulation of *rhl*A and *fli*C genes upon treatment with the tannin. The tannin may be affecting rhamnolipid and flagellin production. Thus, cinnamtannin B1 is an active component of *C. tamala* responsible for inhibiting the swarming motility of *P. aeruginosa*.



INTRODUCTION

Pseudomonas aeruginosa is a gram-negative opportunistic pathogen responsible for numerous hospital acquired infections. It can infect a wide range of hosts including worms, plants, animals, and humans. In humans, it causes different infections in immunocompromised conditions. This is made possible because of the production of numerous virulence factors which facilitate the infection process. An important virulence property of *P. aeruginosa* is its motility. According to the environmental condition, it adapts to any of the three major kinds of motilities, namely, swimming, swarming, or twitching. Of these, swarming is a social behavior, enabling dissemination of bacteria over surfaces, and is regulated by quorum sensing regulatory circuits.[1] Nutrient limitation in medium triggered swarming motility, resulting in the hypothesis that it is an adaptation by bacteria to migrate to nutrient-rich regions.[2] It is also implicated in niche colonization prior to biofilm formation. Swarming bacteria are also known to be resistant to antibiotics and overexpress virulence factors.[3] Swarming has also been shown to have positive correlation to the clinical pathogenesis of ocular keratitis.[4] However, the factors inducing or suppressing swarming motility are not yet fully known. Thus, studying compounds inhibiting swarming motility offers an opportunity to get an insight into the mechanism, regulating this complex process.

Many plant-derived compounds are known to inhibit swarming motility and biofilm formation in *P. aeruginosa*. The most notable among them are citrus flavonoids such as naringenin,[5,6] tannins such as cranberry proanthocyanidins (PACs),[7] and green tea polyphenols.[8] In a preliminary study involving different plant extracts to identify inhibitors of swarming motility of *P. aeruginosa*, we found that the methanol extract of leaves of *Cinnamomum tamala* T. Nees and Eberm inhibited swarming motility at 5 μg/mL.[9] In order to find out the active component responsible for this inhibition, we carried out bioassay-guided purification of the active compound from the extract and attempted to study its possible mechanism of action. Here, we report the identification of cinnamtannin B1 as one of the active components of the extract and its effect on the expression of genes associated with swarming motility.

RESULTS AND DISCUSSION

Bioassay-Guided Purification of the Active Component. The active methanol extract of *C. tamala* (CtM) was subjected to silica gel column chromatography using chloroform, ethyl acetate, and methanol gradients. Bioassay of the resulting fractions revealed fraction F13 to be more active (Figure S1). F13 was further purified by successive fractionations on silica column using ethyl acetate and methanol (9:1 to 1:1) as eluting solvents, yielding compound (**1**). Subsequent bioassay of the subfractions revealed the subfraction F13.3 and **1** to be active (Figure 1).

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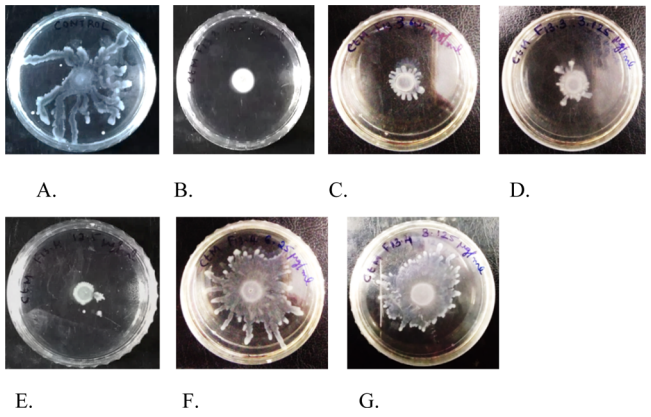


A. B. C. D.

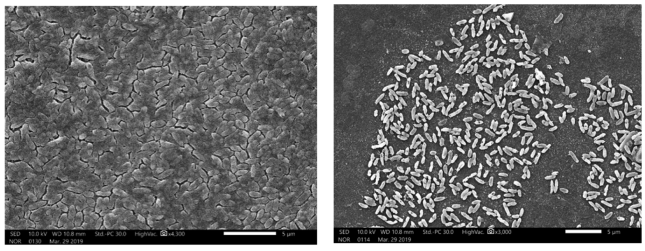
E. F. G.

Figure 1. Swarm assay of subfractions F13.3 and **1** on the swarming motility of *P. aeruginosa*. (A)—DMSO control; (B)—F13.3 at 25 μg/mL; (C)—F13.3 at 6.25 μg/mL; (D)—F13.3 at 3.125 μg/mL; (E)—**1** at 12. 5 μg/mL; (F)—**1** at 6.25 μg/mL; (G)—**1** at 3.125 μg/mL.

The subfraction F13.3 inhibited swarming at 3.125 μg/mL, while **1** inhibited swarming at 12.5 μg/mL (Figure 1). The activity was comparable to that of catechin, which inhibited swarming motility at 10 μg/mL in our previous screening[9] study. A thin-layer chromatogram analysis of F13.3 and **1** (F13.4 in Figure 2) revealed that F13.3 contained two closely located bands, while **1** contained the lower band of the two (Figure 2). A comparison of the subfractions with the compounds catechin and quercetin, both of which are reported to be present in the plant, revealed that the active compound was different from these two compounds (Figure 2). The R_f values for catechin and quercetin were 0.872 and 0.909, respectively. The R_f values for F13.3 a (lower band; Figure 2), F13.3 b (upper band; Figure 2), and **1** (Figure 2) subfractions were 0.5454, 0.6363, and 0.5636, respectively. The retention factors of F13.3 a (lower band) and **1** were similar, indicating that they were the same compound.

Comparing the proton and carbon NMR and mass spectroscopy data to available literature,[10] **1** was identified as cinnamtannin B1 (Text S1, Figures S1−S5).

Biofilm Assay. Biofilm formation is one of the key characteristics of *P. aeruginosa*, involving formation of an extracellular matrix containing polysaccharides, rhamnolipids, and extracellular DNA. Several natural compounds are known to interrupt the formation of biofilm, including furanone C-30[11] and malabaricone C.[12] We analyzed the effect of **1** on biofilm formation by *P. aeruginosa*. When viewed by a scanning electron microscope, it was observed that the treated cells were more dispersed and lacked the interconnecting matrix when compared to untreated control biofilm (Figure 3). When

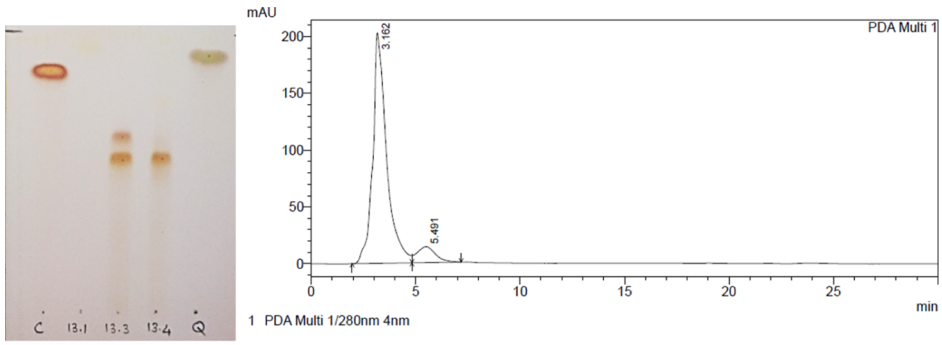


A. B.

Figure 3. Inhibition of biofilm formation by *P. aeruginosa* upon treatment with **1** as seen under a scanning electron microscope. (A)—biofilm formed by *P. aeruginosa* and (B)—dispersal of bacterial cells after treatment with **1**.

checked for its effect on growth, **1** did not inhibit the growth of the bacterium (Figure S2), indicating that the inhibition on swarming and biofilm was specific to these pathways and not because of growth inhibition.

Comparative Gene Expression Analysis of Swarm-Deficient and Proficient *P. aeruginosa*. In order to find the possible pathways affected by the active compound, we performed a comparison of gene expression levels between nonswarming, compound-treated bacteria and actively swarming untreated bacteria by real-time polymerase chain reaction (RT PCR). Gene expression analysis of swarm-deficient *P. aeruginosa* treated with **1** showed a downregulation of *rhl*A and *fli*C genes when compared to swarming competent bacteria (Figure 4), whereas no significant change in the extracellular polysaccharide genes *pel*A and *psl* was observed. *rhl*A and *fli*C genes encode proteins responsible for the synthesis of rhamnolipids and flagella protein flagellin, respectively. Rhamnolipids are known to contribute to swarming motility by acting as biosurfactants that reduce the surface tension over semisolid medium, facilitating rapid movement along the surface.[13] Another requirement for efficient swarming motility is the presence of functional flagella. In many bacteria, swarming is associated with cellular changes including cell elongation and hyperflagellation.[1] In *P. aeruginosa*, actively swarming cells were reported to be twice as long as the cells at the center and consisted of bipolar flagella.[14] *P. aeruginosa* mutants of *rhl*A and *fli*C genes are reported to be deficient in



A. B.

Figure 2. Thin-layer chromatogram (A) of F13.3 subfraction and **1** (F13.4) in comparison with that of catechin (C) and quercetin (Q). (B)—HPLC chromatogram of **1** at 280 nm.

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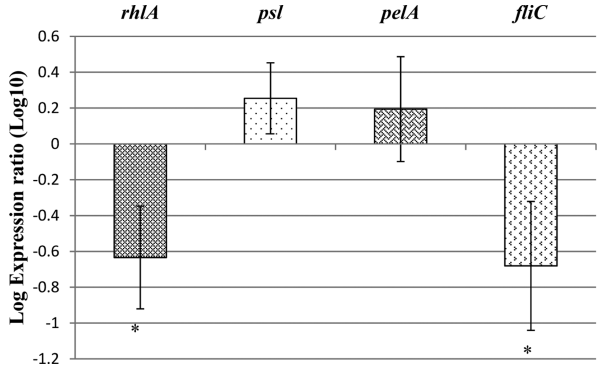

Figure 4. Comparative expression levels of different genes of *P. aeruginosa* upon treatment with **1** with that of untreated cells. The graph represents expression ratios in the $\log_{10}$ scale. The graph represents average values of two independent experiments performed in triplicates with SD. *Values are significantly different when compared to untreated control (P value < 0.001).

swarming.[13,14] A previous study using pomegranate tannins showed repression of flagellin production and motility in *Escherichia coli*.[15] Similarly, different tannins from cranberry, pomegranate, and green tea inhibited the swarming motility of *P. aeruginosa,* and it was partially restored by exogenous addition of rhamnolipids to the medium, indicating a role in rhamnolipid production.[7] The authors also proposed that the tannins could be interacting with flagellin.[16] However, studies on the effect of cinnamtannins on swarming motility have not been reported. The previous literature data on other tannins correlate with our observation of downregulation of rhamnolipid and flagellin genes upon treatment with cinnamtannin. Analysis of additional regulatory genes may reveal a clearer insight into the target of action.

C. tamala is reported to contain several tannins in addition to other flavonoids and eugenol.[17−19] The major among them are the A-type PACs also known as cinnamtannins.[10,20] Cinnamtannin B1 was isolated among other tannins from the bark of *C. tamala* and was found to contain immunosuppressive effects on splenocytes.[10] PACs from cranberry fruit juice have been known to interfere with the adhesion of gram-negative bacteria, especially *E. coli*.[21−23] A detailed report on tannins inhibiting virulence properties of bacteria is covered in the exhaustive review by Silva et al.[24] Other studies revealed that tannins from cranberry, pomegranate, and green tea blocked swarming without affecting swimming or twitching.[7,16] In another study, tannins from the plant *Anadenanthera colubrine* were found to be responsible for reduction in biofilm formation by *P. aeruginosa*.

Thus, our study has shown that cinnamtannin B1 from *C. tamala* is able to inhibit swarming motility and biofilm matrix formation by *P. aeruginosa*. Rhamnolipid and flagellar gene expression was lowered by the tannin based on RT PCR data. However, other possible mechanisms may also be responsible for the observed effect.

EXPERIMENTAL SECTION

General Experimental Procedures. NMR spectra of the sample were recorded on a Bruker AVANCE II 400 MHz NMR spectrometer using tetramethylsilane as an internal standard. High-resolution electron ionization mass spectrometry (HR EI-MS) spectra were recorded on an Agilent (Santa Clara, USA) 6530 Accurate Mass Q-TOF LC/MS system. Mobile phase used was water with 0.1% formic acid and acetonitrile (1:1) at a flow rate of 0.2 mL/min. Peaks were recorded in positive mode, and Agilent mass hunter workstation software was used for data analysis. Purity of the sample was analyzed by reverse phase high-performance liquid chromatography (HPLC) (Shimadzu, Japan). Compound **1** was dissolved in HPLC grade acetonitrile. From this, 20 μL of volume was injected to a RP-Capcell C18 MG II column (250 mm × 4.6 mm) with a particle size of 5 μm. The sample was run in an increasing gradient of acetonitrile (solvent B) in water (solvent A), starting from 0 to 80% B. The oven temperature was maintained at 30 °C, and sample detection was carried out using the photodiode array detector at 280 and 254 nm.

Plant Material. Dried leaves of *C. tamala* T. Nees and Eberm were bought from the local market in Pondicherry. The samples were identified by Professor N. Parthasarathy, Department of Ecology and Environmental Sciences, Pondicherry University. Specimens of the samples were deposited in the Department of Biochemistry and Molecular Biology, Pondicherry University [Voucher Specimen Number *C. tamala* (PUBMB 01)].

Extraction and Isolation. Approximately 90 g of powdered *C. tamala* leaves was sequentially extracted with 1800 mL of hexane, chloroform, and methanol. All the extracts were taken in separate round-bottomed glass flasks and were evaporated to dryness in a rotary evaporator (Buchi, Switzerland). The concentrated extracts were collected and stored in air-tight containers. From the methanol extract thus obtained, 2.4 g of extract was mixed with 230−400 silica, dried to a free flowing powder, and was loaded evenly onto a silica column (230−400 mesh size, Merck). The column was eluted using increasing gradients of chloroform, ethyl acetate, and methanol, starting from 100% chloroform to 100% ethyl acetate and 100% methanol. The column was finally washed with methanol/water (80:20). The eluted fractions were analyzed by thin-layer chromatography using the solvent systems: (i) chloroform/ethyl acetate/methanol (8:3:2) or (ii) ethyl acetate/methanol/water (10:1.65:1.35). Similar fractions were pooled together, and each pooled fraction was analyzed for swarming motility inhibition. The active F13 fraction (161 mg) was again fractionated through silica column (230−400 mesh) using ethyl acetate (from 100%) and gradually increasing gradient of methanol (till ethyl acetate/methanol 40:60) to yield F13.3. Subsequent column chromatography of F13.3 (14 mg) using ethyl acetate/methanol yielded **1** (18 mg).

The purity of **1** was analyzed by analytical reverse phase HPLC as described in General Experimental Procedures.

Swarming Motility Assay. The swarming motility assay for *P. aeruginosa* was performed using the column fractions (at a concentration of 200 μg/mL each) or the compound. The fractions or the compound dissolved in dimethyl sulfoxide (DMSO) to make stock solutions were added to 3 mL of molten swarm agar to the required concentrations and poured in Petriplates (60 × 15 mm). After solidification, 3 μL of overnight grown *P. aeruginosa* culture was inoculated in the center of the swarm plates. Two control plates were kept for the comparison of results, one containing swarm medium and the other containing DMSO with the medium. All the plates were incubated at 37 °C overnight.

To confirm the effect on swarming, the disk assay was performed using the fractions at a concentration of 200 μg/mL

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each. Five milliliters of the swarm agar media was poured in sterile plates and was allowed to set. After solidification, 5 μL of overnight grown *P. aeruginosa* culture was inoculated at the center of the plates. Sterile disks loaded with the required concentration of fractions and dried were placed on the agar a few millimeters away from the culture. A control disk was loaded with DMSO for comparison of the effect of the solvent. All the plates were incubated at 37 °C overnight.

Scanning Electron Microscopy (SEM) of Biofilm Formation. Biofilm assay was performed in a 12-well plate. Each well consisted of 1 mL of LB broth containing **1** at a concentration of 25 μg/mL. To the wells, 1 mL of 1/100 diluted overnight *P. aeruginosa* culture was added. Untreated control constituted 1 mL of LB broth and 1 mL of 1/100 diluted overnight culture of *P. aeruginosa*. The plate was incubated at 37 °C overnight. The coverslips were removed from the wells and were washed with phosphate-buffered saline (PBS). Fixation of the cover slips was done using 2.5% glutaraldehyde solution and osmium tetroxide and washed subsequently with PBS and water. The samples were dehydrated in a series of ethanol, dried, and analyzed by SEM at 10 kV at a magnification of 5 and 1 μm.

RT PCR Analysis. Total RNA was isolated from non-swarming cells and swarming cells taken from **1**-treated and untreated swarm agar plates. RNA isolation was done following the protocol of HiPurA Total RNA Isolation Kit from HiMedia (HiMedia, India). Dnase treatment was given to eliminate genomic DNA contamination. The quality and quantity of RNA were assessed by both agarose gel electrophoresis and nanodrop. cDNA was prepared using the Reverse Transcriptase Kit from Genei, Bangalore, India, using 600 ng of RNA of each sample. Comparative gene expression levels of the genes *pel*A, *psl*, *rhl*A, and *fli*C were analyzed by quantitative reverse transcription-PCR. Reactions were performed using KAPA SYBR Fast Universal master mix from Sigma according to the manufacturer's instructions, and program was run in Roche LightCycler 96. The program consisted of a preincubation at 95 °C for 600 s, followed by 45 cycles of a three-step amplification cycle at 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 10 s. After the reaction, the results were analyzed by the $2^{-\Delta\Delta Ct}$ method.[25] Expression levels were normalized against that of the *rpoD* constitutive gene.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b02471.

Swarm assay using the disk of column fractions of the *C. tamala* methanol extract; ST1.identification of CtM F13.4; effect of cinnamtannin B1 (**1**) on the growth of *P. aeruginosa*; ^{13}C NMR spectrum of cinnamtannin B1 (**1**); ^{1}H NMR spectrum of cinnamtannin B1 (**1**); HREI-MS of cinnamtannin B1 (**1**); and structure of cinnamtannin B1 (**1**) (PDF)

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Author Contributions

Author A.H. has contributed to elucidate the structure of the compound. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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