

## Exploring new treatment strategies for *Pseudomonas aeruginosa* biofilm infections based on plant essential oils

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*Pseudomonas aeruginosa* is an important cause of nosocomial and biofilm-associated infections which is often difficult to eradicate, due to its intrinsic resistance and capacity to undergo mutations and adaptations. This study aimed to assess the anti-biofilm activity of three essential oils (EOs) (Cinammon (*Cinnamomum zeylanicum*), Tea tree (TTO) (*Melaleuca alternifolia*) and Palmarosa (*Cymbopogon martini*), and the existence of synergistic effects of TTO and its major component (Terpinen-4-ol (T4ol)) combined with Ciprofloxacin (CIP), against 24-hour-old *P. aeruginosa* biofilms. Results showed that the treatment of pre-established *P. aeruginosa* biofilms with the three plant EOs, T4ol and CIP, individually, promoted a significant decrease of biofilm biomass and the number of biofilm entrapped cells, mainly for higher concentrations. Nevertheless, the antimicrobial synergisms combining TTO or T4ol with CIP revealed to be more successful, since significant disturbance of biofilms occurs for lower concentrations of the products. T4ol associated with CIP was the combination that revealed the most promising synergistic effects. Therefore, T4ol-CIP association may be a useful therapeutic option to treat *P. aeruginosa* biofilm infections.

**Keywords** Plant essential oils; Biofilm-associated infections; *Pseudomonas aeruginosa*; Antimicrobial synergism

### 1. Introduction

*Pseudomonas aeruginosa* is an opportunistic human pathogen responsible for several different nosocomial infections and it is the main cause of severe and life-threatening infections, especially in immunocompromised patients [1-3].

This bacterium possesses high intrinsic resistance and an exceptional capacity to develop even more resistance to conventional antibiotics, through mutations and adaptations, as biofilm development, being thus difficult to control [1, 3-5]. In fact, when bacterial cells attach to a surface and undergo a biofilm lifestyle, they are protected by a self-produced adhesive matrix that confers a high level of additional intrinsic resistance that makes the biofilm-associated cells hard to eradicate towards common antibiotic treatments [2, 5].

Fluoroquinolones, a class of antibiotics, are commonly used to treat *P. aeruginosa* infections [2, 6]. However, a significant number of resistant *P. aeruginosa* strains showing multidrug resistance have been reported, including strains resistant to fluoroquinolones [1, 6-8]. It is believed that the indiscriminate and misuse of conventional antimicrobial drugs has promoted the increase of microbial drug resistance being, nowadays, a global health threat [7, 9, 10]. Overcome multidrug resistance became thus one of the major challenges for the development of ultimate therapies for *P. aeruginosa* infections [1].

An approach to defeat this problem, avoiding monotherapy and the undesirable toxic effects of high doses of drugs, can be based on the combination of conventional antibiotics with phytochemicals [1, 9]. An interesting group of these phytochemicals are plant essential oils (EOs) which are a complex mixture of secondary metabolites obtained from aromatic plants [11, 12]. In fact, it has been noticed that EOs and some of their constituents show interesting antimicrobial properties [9, 11]. There are even some studies pointing out that EOs and some of their components can have synergistic effect when combined with antibiotics [13-17]. Mechanisms of action, such as physicochemical interactions, interaction with multiple targets and inhibition of antibacterial-resistance mechanisms have been proposed to explain synergism between EOs components and antibiotics [12]. These works called the attention to the study of the effects of EOs, alone or in combination, against biofilm-associated infections.

Therefore, in this study the anti-biofilm activity of three different EOs (Cinammon (*C. zeylanicum*), Tea tree (TTO) (*M. alternifolia*) and Palmarosa (*C. martini*)) was assessed. Furthermore, TTO and its major component Terpinen-4-ol (T4ol) were combined with a conventional antibiotic belonging to the fluoroquinolones class (ciprofloxacin (CIP)) to infer a possible synergic activity against pre-formed *P. aeruginosa* biofilms.

### 2. Materials and Methods

#### 2.1. Bacterial strain and culture conditions

*Pseudomonas aeruginosa* ATCC 10145 was preserved in cryovials (Nalgene) at  $-80 \pm 2$  °C. Prior to each experiment, bacterial cells were grown on Tryptic Soy Agar (TSA; Merck) plates for 24 h at 37 °C.

To prepare the bacterial suspensions, one colony of *P. aeruginosa* was collected from the TSA plate and grown in Tryptic Soy Broth (TSB) for 24 h at 37 °C, in a horizontal shaker at 120 rpm. Subsequently, bacteria were washed twice with sterilized water. Standardized cell suspensions were prepared in TSB at cell density of  $1 \times 10^7$  cfu/ml.

## 2.2. Antimicrobial agents

Ciprofloxacin (CIP), Cinnamon oil (*Cinnamomum zeylanicum*), Tea tree oil (*Melaleuca alternifolia*) and Terpinen-4-ol (T4ol) were purchased from Sigma-Aldrich. Palmarosa (*Cymbopogon martini*) was gently provided by Dr. Alessandra Millezi, University Federal of Lavras, Brasil.

CIP stock solution was prepared at 160 µg/ml and 500 µl aliquots were stored at -70 °C. For the susceptibility testing, CIP concentrations ranged from 1.25 to 80 µg/ml.

EOs working solutions were prepared on the day of use at 3.84 % (v/v) in TSB supplemented with 2 % of DMSO. For the susceptibility testing, EOs concentrations ranged from 0.12 to 1.92 % (v/v).

The working solution of T4ol was prepared on the day of use at 0.96 % (v/v) in TSB, being 0.095, 0.19 and 0.38 % (v/v) the concentrations used on susceptibility tests.

## 2.3. Biofilm development and treatment

The methodology used to grow bacterial biofilms was based on the microtiter plate test developed by Stepanovic et al [18]. Cell suspension was diluted in TSB to obtain a final concentration of  $1 \times 10^7$  cfu/ml. Afterwards, 200 µl/well of bacterial suspension was transferred to sterile 96-well flat-bottom tissue culture plates (Orange Scientific, Braine-l'Alleud, Belgium). All plates were incubated aerobically on a horizontal shaker (120 rpm), at 37 °C, for 24 h.

After 24 h of biofilm growth, the supernatant of each well was removed and washed twice with 200 µl of sterilized water, being the well-attached biofilms (biofilms formed in the inner surfaces of each well of the microtiter plates) subsequently treated.

In a first approach, wells were re-filled with 200 µl of TTO, Cinnamon and Palmarosa essential oils, T4ol and CIP, individually, for 1 h. For synergism assessment, the well-attached biofilms were treated simultaneously with 100 µl of TTO (0.048 % (v/v)) or T4ol (0.19 % (v/v)) combined with 100 µl of CIP, for 1 h. A concentration of 0.19 % (v/v) of T4ol corresponds to a concentration of 0.48 % (v/v) of TTO [19].

In both experimental approaches, non-treated wells were filled with 200 µl of TSB for the same period of time. After that, the liquid content of each well was removed and biofilms were washed twice with sterilized water and reserved for further characterization.

## 2.4. Biofilm analysis

### 2.4.1. Biofilm biomass quantification

Biofilm mass of *P. aeruginosa* biofilms was quantified by crystal violet (CV) staining method, adapted from Stepanovic et al [18]. For that, the microtiter plates containing the biofilms were left to air dry for 30 min, and 200 µl of 98 % methanol were transferred to each well in order to fix the remaining attached cells, for 15 min. Afterwards, the plates were emptied and left to air dry. The fixed cells were stained with 200 µL of CV (Gram's staining; Merck) per well, for 5 min. After this staining step, the plates were washed with running tap water, air dried, and filled with 200 µl of 33 % (v/v) of glacial acetic acid (Merck) in order to resolubilise the CV bound to the adherent biofilm. After that, the quantitative analysis of biofilm mass was performed through the measurement of optical density at 570 nm of each well using a microtiter plate reader, being the results presented as OD<sub>570</sub> nm. Control experiments to avoid false results were also performed in order to determine whether the tested media and the plate material could adsorb CV and interfere with the biofilm biomass quantification. When the optical density was higher than 1.0 the sample was diluted with 33 % (v/v) of glacial acetic acid. For each condition tested, five different wells were used to perform biofilm analysis and the experiment was performed with three independent assays.

### 2.4.2. Biofilm cells enumeration

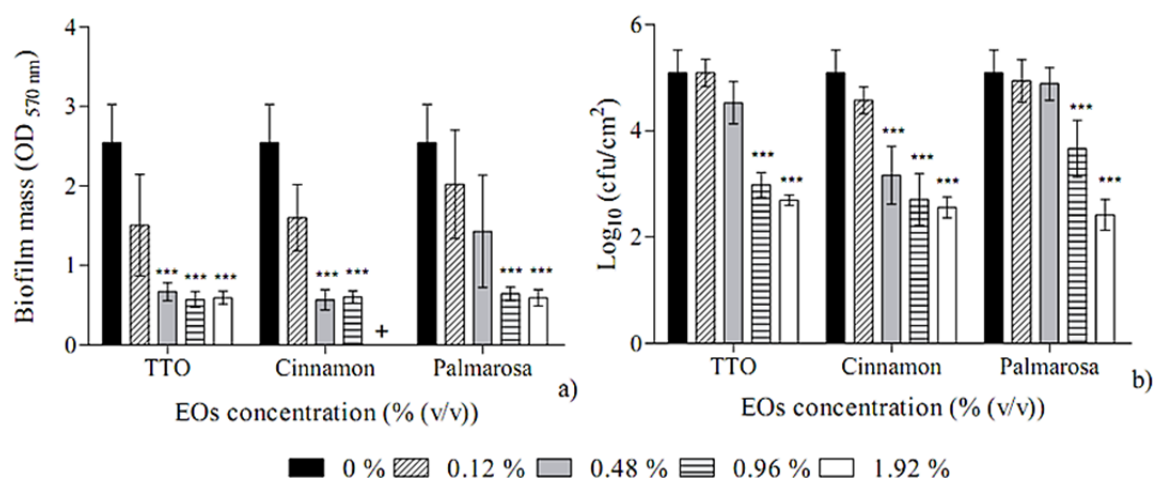
After each well has been filled with 200 µl of sterilized water, biofilms were detached by an ultrasonic bath with a Sonicor SC-52 (Sonicor Instruments, Copaque, NY, USA) operating at 50 kHz, during 6 min (these parameters were previously optimized in order to promote the removal of all the biofilm-attached cells without lysis). Subsequently, the biofilm suspensions of each three wells per condition were collected and gently vortexed for 1 min [20] to disrupt possible cell aggregates. In order to determine the number of cfu, biofilm suspensions removed by sonication were serially diluted, plated on TSA and incubated at 37 °C in an aerobic incubator for 24 h. After this time of growth, the number of cfu was enumerated and presented as cfu/cm<sup>2</sup>.

## 2.5. Statistical analysis

Statistical analyses were performed using GraphPad Prism, version 5.0. Statistical significance values of the groups' means of biofilm mass and cell number were evaluated using a one-way analysis of variance (ANOVA). Subsequently, Tukey's post hoc test was performed to compare pairs of columns. The results were presented as mean  $\pm$  standard deviation (SD). A  $P$ -value  $< 0.05$  was considered indicative of statistical significance.

## 3. Results

The susceptibility of pre-established biofilms to EOs, T4ol and CIP treatment can be observed in Figs. 1 and 2. In general, the three different EOs promoted a decrease of the biofilm biomass even for the lowest concentrations tested (Fig. 1a). However, only a maximum of 2.5 log cfu reduction was attained ( $P < 0.001$ ) regardless the EOs used (Fig. 1b).



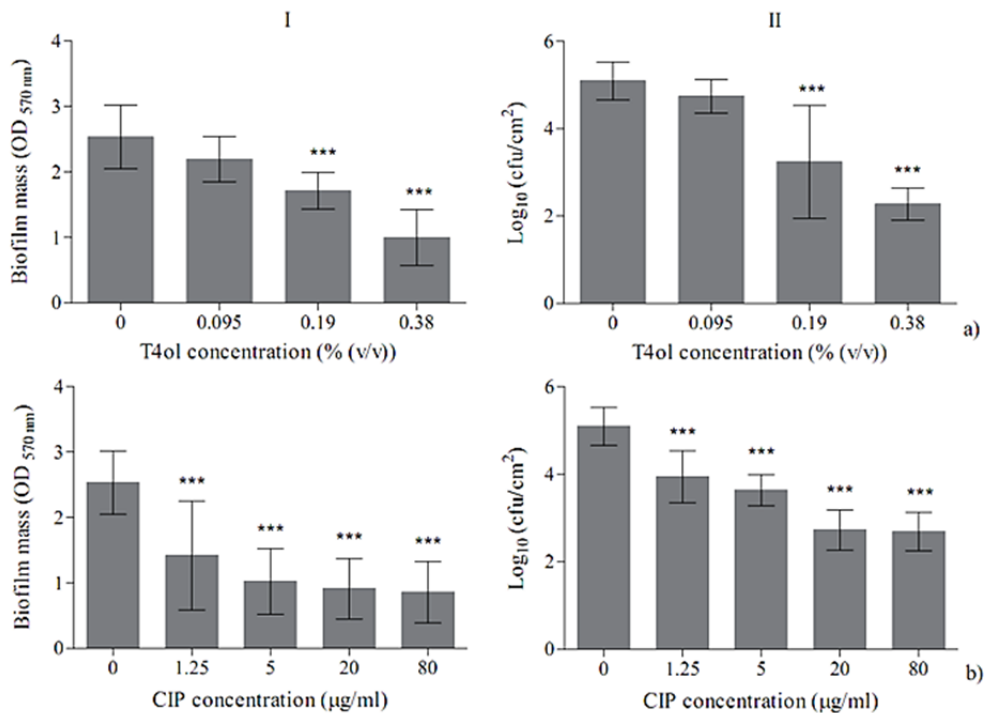
**Fig. 1** Biomass (OD<sub>570 nm</sub>) (a) and number of cultivable cells (b) of biofilms treated, during 1 hour, with EOs. Bars represent the average of 3 independent assays  $\pm$  SD. + Biofilm mass not determined for 1.92 % of Cinnamon. \*\*\*  $P < 0.001$  related to the control.

In summary, based on Fig. 1 it can be noticed that the three EOs tested had the capacity to interfere with pre-established *P.aeruginosa* biofilms, especially in reducing the amount of the attached biomass. Among the three EOs, TTO and Cinnamon were those that exhibited the best antimicrobial performance. Therefore, it was decided to choose TTO for the following synergism assays testing a concentration of 0.48 % (v/v). The selection of this TTO concentration was due to the fact that it caused a significant biofilm reduction (74 %) but not of the biofilm cell viability (0.56 log reduction).

For comparison purposes, the anti-biofilm effects of the TTO major component, T4ol, and a conventional antibiotic, CIP, which is commonly used to treat *P. aeruginosa* infections, were also evaluated.

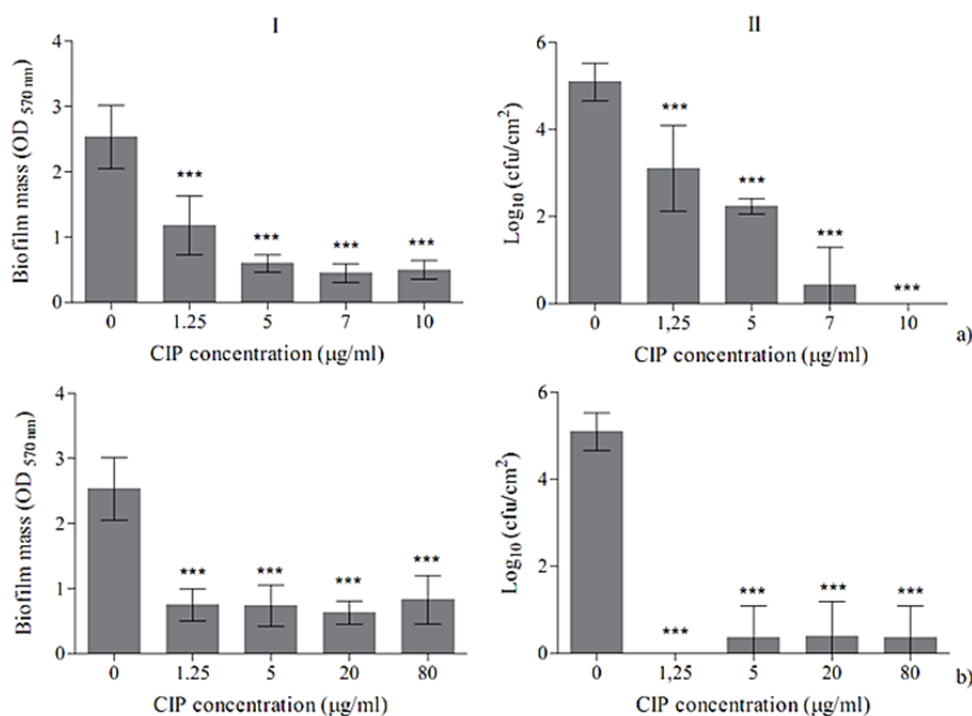
Figure 2a demonstrates that, similarly to TTO, T4ol was also able to interfere with 24-hour-old biofilms, since it promoted a reduction of both parameters analyzed (biofilm mass and number of viable cells) for concentrations above 0.095 % (v/v) ( $P < 0.001$ ).

Concerning the treatment with CIP (Fig. 2b), a disturbance of the biofilm mass and the number of biofilm viable cells was also observed, since there was a reduction for all concentrations tested. However, the maximum reduction of the number of biofilm viable cells was 2.4 log ( $P < 0.001$ ), even for the highest concentration tested.



**Fig. 2** Biomass (OD<sub>570 nm</sub>) (I) and number of cultivable cells (II) of biofilms treated during 1 hour with T4ol (a) and CIP (b). Bars represent the average of 3 independent assays ± SD. \*\*\*  $P < 0.001$  related to the control.

The association of natural products, in this case TTO (Fig. 3a) and its major component T4ol (Fig. 3b) with CIP, gave rise to a significant disturbance of the biofilm mass, right from the lowest concentration of CIP used. TTO (0.48 % (v/v)) combined with CIP promoted a high decrease of biofilm mass and a complete eradication of the biofilm viable cells for concentrations above 7 µg/ml of CIP ( $P < 0.001$ ). In the case of T4ol (0.19 % (v/v)) associated with CIP it was also observed a significant reduction of biofilm mass ( $P < 0.001$ ) and a decrease close to 100 % of the number of biofilm entrapped cells, even for the lowest concentration of CIP used ( $P < 0.001$ ).



**Fig. 3** Biomass (OD<sub>570 nm</sub>) (I) and number of cultivable cells (II) of biofilms treated during 1 hour with TTO (0.48 % (v/v)) combined with CIP (a) and T4ol (0.19 % (v/v)) combined with CIP (b). Bars represent the average of 3 independent assays  $\pm$  SD. \*\*\*  $P < 0.001$  related to the control.

#### 4. Discussion

Although the existence of several intrinsic mechanisms of resistance [4] on *P. aeruginosa*, all EOs and the TTO major component (T4ol) revealed ability to disrupt pre-established *P. aeruginosa* biofilms. In fact, they promoted a considerable reduction of biofilm mass and number of biofilm-entrapped viable cells, even just for the highest concentrations tested. The need of higher doses of antimicrobials to control *P. aeruginosa* biofilms can be due to the outer membrane of this Gram-negative bacterium, which contains hydrophilic lipopolysaccharides, that often acts as a barrier for macromolecules and hydrophobic compounds [21-23]. As a consequence, Gram-negative bacteria present high tolerance to antimicrobials, being more difficult to eradicate with low concentrations of EOs (hydrophobic antimicrobial compounds). The presence of an extracellular matrix and the physiological and phenotypic changes undergone by biofilm cells, among other mechanisms, can be responsible for resistance on biofilms, making biofilm-entrapped cells hard to eradicate compared to their planktonic counterparts [5, 24]. Ciprofloxacin belongs to Fluoroquinolones, a class of antibiotics which is commonly used to treat *P. aeruginosa* infections [2]. However, results revealed that, although CIP promoted a reduction of the biofilm mass, the number of biofilm viable cells did not suffer a high decrease, even for the highest concentration tested. *P. aeruginosa* has considerable defences beyond its outer membrane. One of them is the existence of efflux pump systems, as MexAB-OprM, which is responsible for the removal of fluoroquinolones and others antibiotics, dyes and detergents [4, 5] from the cells. Moreover, in the present study, the antimicrobial treatment was carried-out against pre-established *P. aeruginosa* biofilms developed for 24 h. This time of biofilm growth is often considered sufficient to attain a mature stage, in which biofilms show maximum resistance to antibiotics, making eradication a hard task [24]. Resistance to fluoroquinolones has been increasing in an alarming rate [6], being necessary to explore another alternatives to control pseudomonal infections. The combination of natural products with conventional antibiotics has been an approach increasingly exploited in order to slow down the emergence of microbial resistance [1, 9]. In order to assess the potential synergism activities of natural drugs (as EOs and its major components) with conventional antimicrobials, *P. aeruginosa* biofilms were treated simultaneously with TTO (0.48 % (v/v)) and CIP. The synergic effect was notorious since a high reduction of biofilm mass and the number of biofilm viable cells was observed even for the low concentrations of TTO and CIP. The enhancement of the anti-biofilm activity of CIP using TTO may be explained by the multi-target effect (cytoplasmic membrane [25, 26] and DNA synthesis inhibitors [12, 27]) or the reversal of antibiotic resistance, inhibiting active efflux pumps [28]. TTO is composed essentially by terpene hydrocarbons, mainly mono and sesquiterpenes and their related alcohols [29, 30]. It is known that monoterpenes are able to exert damages on membranes [25, 26]. In fact, increase of membrane

permeability, disruption of bacterial membranes, damage to membrane proteins, depletion of motive proton force, coagulation of the cytoplasm and cell content leakage, are common effects of most EOs [11, 12].

The antimicrobial activity of T4ol was similar to those displayed by TTO, corroborating that the biological activity of TTO can essentially be attributed to its major component (T4ol) [31]. The association of T4ol and CIP significantly improved the activity of the products when tested individually, promoting a high reduction of the number of viable cells even for the lowest concentration of CIP used. This combination reveals even better results than those attained with TTO-CIP association. T4ol possesses a hydrophobic/hydrophilic character having sufficient hydrophilicity to achieve bacterial cytoplasmic membrane and sufficient hydrophobicity to then diffuse through it [32]. The results obtained acquired additional importance since it is easiest to formulate an antimicrobial product containing a single component, than a product containing multiple components [12, 32]. Moreover, EOs composition varies with seasonal variation, climate, subspecies and oil-extraction method, having thus consequences for their application and antibacterial activity. Overall data highlighted the relevance of using natural products, as the major components of EOs or even EOs, to enhance the activity of conventional drugs, reducing the concentration of the drug needed to treat an infection, and consequently reducing its toxicity and side effects. Further, these data provides evidence that T4ol-CIP combinations may be a useful therapeutic option in the treatment of *P. aeruginosa* biofilm associated infections.

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