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Effect of combination of doxycycline with natural products against planktonic cells, biofilm, and virulence factor of *Pseudomonas aeruginosa*

Larissa Yetendje Chimi[1], Armel Joseph Agokeng[2], Guy Sedar Singor Njateng[1]*, Jean Paul Dzoyem[1]**

Abstract

Background: *Pseudomonas aeruginosa* is an important environmental, opportunistic, and nosocomial pathogen with a significant threat to public health. Combination therapy has many advantages due to the simultaneous action of two drugs on two separate cellular targets. In the present study, the effect of combination of doxycycline and natural products against planktonic cells, biofilm, and virulence factors of *P. aeruginosa* was evaluated.

Methods: To perform this work, minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of doxycycline and natural products were determined by broth microdilution method. The microtiter plate assay method was used to determine the minimal biofilm inhibitory concentration (MBIC) and the minimum biofilm eradication concentration (MBEC). The effect of doxycycline and natural products against pyocyanin, swarming motility, and swimming motility was evaluated. The checkerboard method was used to evaluate the effect of combination of doxycycline with natural products against planktonic and biofilm cells.

Results: The MIC of doxycycline ranged between 2 µg/mL and 128 µg/mL with an average of 35.89 µg/mL. Sinapic acid showed the best inhibitory activity against planktonic cells with an average MIC of 27.79 µg/mL. At the sub-inhibitory concentrations, the pyocyanin production, swarming motility decrease, and swimming motility decrease. Out of the six combinations tested, combination formed by doxycycline and sinapic acid exhibited synergistic activity for the prevention of biofilm formation with a 6-fold reduction in MBIC of doxycycline.

Conclusion: This study revealed that doxycycline and sinapic acid combination could be considered as promising candidate for the development of therapy against *P. aeruginosa* infections.

Keywords: *Pseudomonas aeruginosa*, Biofilm, Virulence factors; combination, doxycycline; natural products.

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Background

Pseudomonas aeruginosa is a Gram-negative, aerobic, non-fermenting, and non-spore-forming bacillus of the Pseudomonadaceae bacterial family [1]. *P. aeruginosa* is a causative agent for 8% of all hospital-acquired infections, including pulmonary infections (tracheobronchitis and necrotizing bronchopneumonia), skin and soft-tissue infections (burn and surgical site infections), urinary tract infections, bacteremia, and endocarditis [1-2]. *P. aeruginosa* exhibits high levels of acquired and intrinsic resistance mechanisms to evade the action of antibiotics. In 2017, *P. aeruginosa* was recognized as one of the most life-threatening bacteria and listed as a priority pathogen for research and development of new antibiotics by the World Health Organization [3]. Furthermore, the eradication of *P. aeruginosa* is increasingly difficult to be performed due to the expression of virulence factors such as pyocyanin production, elastase, motility (swimming and swarming), and its remarkable ability to form biofilm [4].

Biofilm is characterized as a community of microorganisms that establish a connection between them and with their attached surface which can be biotic or abiotic. This connection is mediated by substances secreted by microorganisms present in the biofilm [5]. Biofilm formation is a strategy of microorganisms to successfully adapt and survive in hostile environments increasing its resistance to the effects of antimicrobial agents 10–1000 times [6]. Among the antibiotics indicated against Gram-negative bacteria, is doxycycline, the most recommended first-line single agent for empirical treatment of Hospital–acquired pneumonia (HAP) in United Kingdom. It is also recommended by national guidelines in the USA and Europe as first-line treatment of pneumonia [7].

Doxycycline is a second-generation tetracycline antibiotic. It is a bacteriostatic antibiotic broadly active against the community–acquired pneumonia (CAP) caused by *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, *Mycoplasma pneumoniae*, *Chlamydia pneumoniae,* and *Legionella* species [7]. However, on *P. aeruginosa* doxycycline is less active. The discovery and development of alternative therapeutic strategies against *P. aeruginosa* infections are increasing in demand and gaining more and more attention. Combinations of antibiotics and natural substances such as products of plant origin are often used in the treatment of bacterial and fungal infections with variable clinical success.

Several studies have shown that the use of products derived from natural plants has increased considerably all over the world, because of the innumerable advantages conferred on them by the secondary metabolites they contain and their low toxicity [8]. It has been shown that natural substances from plants can improve the performance of antibiotics [9]. Sinapic acid, is a natural herbal compound containing phenolic acid. It has been reported to be a major active component of Chinese traditional remedies [10]. Sinapic acid has been pharmacologically evaluated for its potent antioxidant, anti-inflammatory, anti-cancer, hepatoprotective, cardioprotective, renoprotective, neuroprotective, anti-diabetic, anxiolytic, and anti-bacterial activities [8]. Quercetin is a flavonoid compound widely present in plants and exhibits a variety of biological activities [11]. It has been shown to have a wide range of beneficial effects and biological activities, including anti-inflammation, anti-oxidation, and neuroprotection. [12]. Curcumin is a bright yellow chemical compound isolated from *Curcuma longa* L. (turmeric) plants (Zingiberaceae), a plant known for its medicinal use [13]. More than 3,000 studies have shown their numerous pharmacological effects, including antioxidant, antifungal, antiviral, anti-inflammatory, antiproliferative, proapoptotic, and especially antibacterial. Thymol is a naturally occurring phenol monoterpene derivative of cymene and isomer of carvacrol. Thymol is one of the major constituents of essential oils of thyme (*Thymus vulgaris L., Lamiaceae*). The interest in the formulation of pharmaceuticals, nutraceuticals, and cosmeceuticals based on thymol is due to the potential therapeutic uses of this compound for the treatment of disorders affecting the respiratory, nervous, and cardiovascular systems. Moreover, this compound also exhibits antimicrobial, antioxidant, anticarcinogenesis, anti-inflammatory, and antispasmodic activities, as well as a potential as a growth enhancer and immunomodulator [14]. Piperine is a type of amide alkaloid that exhibits pleiotropic properties like antioxidant, anticancer, anti-inflammatory, antihypertensive, hepatoprotective, neuroprotective, and enhancing bioavailability and fertility-related activities [15]. Plumbagin is the major active constituent in several plants including *Plumbago indica Linn.* (root). Plumbagin has been explored also for its anticancer activity. This compound has been shown to exhibit a wide spectrum of biological and pharmacological activities [16].

According to previous studies, antibiotics in combination with natural compounds are a novel strategy for developing therapies against bacterial infections. Natural products can potentiate the activity of antibiotics in the combination [17-18]. The use of plant extracts or pure natural compounds in combination with conventional antibiotics may hold greater promise for inhibiting and eradicating microbial biofilms [19]. However, few studies reported the interaction of doxycycline and natural product against planktonic and biofilm of *P. aeruginosa* reason why this study is carried out to fill that gap.

Methods

Thirty-seven clinical isolates of *P aeruginosa* collected from infected wounds of patients at the Dschang district hospital were used. This clinical isolate was identified using Maldi-Tof method (Unpublished data available upon request). The reference strain ATCC 27853 was purchased from American Type Culture Collection and was used as a control. All the isolates were classified as biofilm formers among which nineteen were classified as best biofilm formers. The antibiotic used in this work is doxycycline. Six natural products were used, namely: curcumin, piperine, plumbagin, thymol, quercetin, and sinapic acid. All the products and the antibiotic (Purity ≥ 98.5%) were purchased from Sigma-Aldrich. Dimethyl sulfoxide (DMSO), p-iodonitrotetrazolium chloride (INT), and 3 (4, 5-dimethylthiazole-2-yl)-2, were also purchased from Sigma-Aldrich. Muller Hinton Agar (MHB), Muller Hinton Broth (MHB), and Luria-Bertani medium (LB) were purchased from Dominique Dutscher SAS, France.

Antibacterial activity of doxycycline and natural product

The antibacterial activity of doxycycline and the antibacterial activity of natural products against the planktonic cells of the 38 isolates of *P. aeruginosa* were carried out by determining the MIC and MBC parameters. This was performed according to the broth microdilution method described by [17]. Doxycycline and natural products were respectively tested at the stock concentration of 1024 µg/mL and 4096 µg/mL. Results were expressed as MIC and MBC ranges and geometric means of the 38 isolates.

Antibiofilm activity of Doxycycline and natural products

The antibiofilm activity of doxycycline and natural products was evaluated against best biofilm-forming isolates (19 including the reference strain). It was carried out through the determination of the minimum biofilm-inhibited concentration (MBIC) and the minimum biofilm-eradicating concentration (MBEC) by the method described by Kirmusaoaglu and Kaşikçi [20]. Doxycycline and natural products were respectively tested at the stock concentration of 4096 µg/mL and 8192 µg/mL. Results were expressed as MBIC and MBEC ranges and geometric means of the 19 isolates.

Anti-virulence activity of doxycycline and natural products

The evaluation of the activity of doxycycline and natural products at sub-inhibitory concentrations ((MIC/2, MIC/4, and MIC/8)) was evaluated. For this purpose, the anti-pyocyanin activity and the effect of each substance on the swarming and swimming motility were evaluated.

Anti-pyocyanin production of doxycycline and natural product

The evaluation of the ability of doxycycline and natural substances to inhibit the production of pyocyanin by *P. aeruginosa* ATCC 27853 was carried out according to the method described by Kirmusaoaglu and Kaşikçi [20] with some modifications. Briefly, in 10 mL of bacterial inoculum with a concentration of 1.5×10^6 CFU/mL previously prepared in LB, solutions of doxycycline or natural substances of respective concentrations were added. The whole was placed in the incubator at 37°C for 72 h.
After 72 hours, the cultured broths were centrifuged at 8000 rpm for 10 min to separate the supernatant from the pellet. The supernatant was collected. To 5 ml of supernatant was added 3 ml of chloroform. The mixture was vigorously stirred. The chloroform layer was then collected, and 1 mL of 0.2 M hydrochloric acid was added to it. The mixture was centrifuged at 8000 rpm for 10 minutes. The optical density of the HCL layer was then measured at 520 nm using a spectrophotometer (Biobase BK-D590 Double Beam Scanning UV/Vis, China). The tests were carried out three times. The negative control (blank) consisted solely of 0.2 M hydrochloric acid. The concentration of pyocyanin in µg/mL was obtained according to the formula:

Pyocyanin concentration (µg/mL) = (Mean OD520nm – ODcontrol) x 17.072

Effect of doxycycline and natural products against motility of P. aeruginosa

The inhibition of *P. aeruginosa* ATCC 27853 motility was carried out at two levels, namely the inhibition of swarming in the semi-solid medium and the inhibition of swimming in the fluid medium.

Inhibition of swarming motility

The evaluation of the ability of doxycycline and natural substances to inhibit the movement of *P. aeruginosa* ATCC 27853 on semi-solid media was carried out according to the method described by O'May and Tufenkji [21] with some modifications. Briefly, the 1% agar LB plates were previously prepared and sterilized in the autoclave at 121°C for 15 minutes. Then, a concentration of antibiotic or natural substance was added to the culture media and well homogenized. Then, the media were poured into 82 mm non-compartmentalized Petri dishes. After solidification, 2.5 µL of bacterial suspension with a concentration of $1.5x\ 10^8$ CFU/mL was gently deposited on the surface of the agar (At the center). Petri dishes were sealed and placed in an incubator at 37°C for 72 hours. Following incubation, the motility area was measured. The test was carried out three times.

Inhibition of swimming motility

It was carried out following the method described by O'May and Tufenkji [21] with some modifications. LB 0.5% agar plates were prepared as previously described. Then, a concentration of doxycycline or natural substance was added to culture media and homogenized. The media were poured into non-compartmentalized 82 mm Petri dishes. After cooling, 2.5 µL of a bacterial suspension with a concentration of $1.5x\ 10^8$ CFU/mL was deposited in the agar (in the center). The dishes were sealed and placed in the 37°C incubator for 72 hours. At the end of the incubation period, the diameter of the motility zone was measured.

Checkerboard assay for combination studies

The checkerboard assay was designed to determine the effect of the combination of doxycycline and natural product against the nineteen best biofilm formers of *P. aeruginosa* isolates through determination of FICIs.

Assessment of interaction between doxycycline and natural products against planktonic cells

The effect of the combination of doxycycline with natural substances on planktonic cells was assessed by the checkerboard method as previously reported [19] Briefly, in two microtiter plates, 50 µL of MHB was introduced. In the first plate, 50 µL of the doxycycline solution was added to all the wells of the first column followed by a serial dilution. In the second plate, 50 µL of natural substance solution was added to all the wells of the first row, and dilutions were made as described for the first plate. At the end of the dilution, the contents of one of the plates were added to the second, with respect to the position of the wells. Thereafter, 100 µL of bacterial inoculum (1.5×10^6 CFU/mL) were introduced into the wells except for the neutral control wells. The plates were incubated at 37°C for 24 hours. The INT was used as an indicator of the bacterial growth and the fractional inhibitory concentration index (FICI) was calculated to evaluate the combination interaction as follows: FICI = (MIC of doxycycline in the combination/ MIC of doxycycline alone) + (MIC of natural product in the combination/MIC of natural products alone). The interaction was classified as follows: synergy when FICI ≤0.5, additivity when 0.5 < FICI ≤ 1, indifference when 1 < FICI ≤ 4, and antagonism when FICI > 4 [22]. When the MIC of a substance alone was higher than the highest tested concentration, this concentration was used to calculate the FIC.

Assessment of interaction between antibiotics and natural products against the biofilm formation

The effect of the combination of doxycycline and natural products to prevent biofilm formation was evaluated by the checkerboard method as previously described [19]. The experiment was carried out with MHB supplemented with 1% glucose. After incubation, the plates were emptied of their contents and washed three times with phosphate buffer saline (PBS) and the biomass of biofilm was

quantified using safranin by recording the optical density (OD) at 570 nm. The lowest concentration of antibiotics or natural products that reduces the biofilm biomass by 100% was considered as the minimal biofilm inhibitory concentration (MBIC). The well-containing MHB without bacteria was used as blank while the well-containing bacteria and MHB supplemented with 1% glucose were used as the positive control. The fractional inhibitory concentration index (FICI) was determined as described above.

Assessment of interaction between antibiotics and natural products against mature biofilm

The effect of the combination of antibiotics with natural products to eradicate the mature biofilm was carried out according to the checkerboard method described above except that the biofilm was formed before the antimicrobial treatment. The biofilm biomass was quantified with safranin (1%) and the biofilm-eradicating concentration (MBEC) of the antibiotics and substances was determined as described above. The effect of the association was determined after the calculation and interpretation of FICI values as described above.

Statistical analysis

Results were expressed as means ± standard deviations (SDs) of three independent experiments using Microsoft Excel 2013.

Results

MIC, MBC, MBIC, and MBEC determination of doxycycline and natural product

Table 1 below shows the effect of doxycycline and natural products both on the inhibition of planktonic cells and the biofilm of *P. aeruginosa* and on the eradication of its mature biofilm. It appears that the MICs of doxycycline range from 2 µg/mL to 128 µg/mL with an average of 35.89 µg/mL. These concentrations increase upon biofilm inhibition and eradication, reaching averages of MBIC of 256 µg/mL and MBEC of 1479 µg/mL, respectively. At the level of natural substances, sinapic acid showed better activity against the planktonic cells of *P. aeruginosa* with an average MIC of 27.79 µg/mL. These concentrations increase during antibiofilm activity and reach MBIC of 74.11 µg/mL and MBEC of 303.16 µg/mL for biofilm inhibition and eradication respectively. This activity is directly followed by that of quercetin.

Effect of doxycycline and natural product against expression of virulence factors

Anti-pyocyanin production of doxycycline and natural products

The ability of doxycycline and natural products to inhibit the production of pyocyanin from *P. aeruginosa* ATCC 27853 was evaluated at sub-inhibitory concentrations. It appears from Figure 1 that, in the presence of doxycycline at a concentration of MIC/2, the production of pyocyanin decreases considerably and goes from 4.85 ± 0.1µg/mL (control), to 0.45 ± 0.15 µg/mL. At the level of natural products, sinapic acid showed the best inhibitory activity on the production of pyocyanin at the concentration of MIC/2, the concentration of pyocyanin produced being equal to 0.32 ± 0.04 µg/mL. As the concentrations of doxycycline and natural

substances decrease, the concentration of pyocyanin produced increases.

The ability of doxycycline and natural products to inhibit the swarming motility of *P. aeruginosa* ATCC 27853 was evaluated. Figure 2 shows that doxycycline at a concentration of MIC/2 considerably decreases the diameter of the motility zone. This diameter goes from 41 ± 1 mm (control) to 6 ± 0.1 mm in the presence of doxycycline at the concentration of MIC/2. Sinapic acid also exhibits the best activity in inhibiting swarming motility. Indeed, in the presence of sinapic acid at a concentration of MIC/2, the diameter decreases to 3.66 ± 0.72 mm. Overall, as the concentration of doxycycline or natural substances decreases, the motility diameter increases.

The ability of doxycycline and natural products to inhibit the swimming motility of *P. aeruginosa* ATCC 27853 was evaluated. Figure 3 shows that, at concentrations of doxycycline and natural substances equal to MIC/2, the swimming motility diameter decreases considerably. The best activity was shown by sinapic acid. This diameter goes from 43.33 ±1 mm (control) to 6.67±0.53 mm in the presence of sinapic acid at the concentration of MIC/2. Overall, as the concentration of doxycycline or natural substances decreases, the diameter of swimming zone increases.

Effect of the combination of doxycycline with natural products

Effect of the combination of doxycycline with natural products against planktonic cells

Table 2 below shows the effect of the combination of doxycycline with natural products for inhibiting the growth of planktonic cells. It appears from this table that 4 out of 6 combinations showed additivity. The combination formed by doxycycline and quercetin presented the best activity with a FICI of 0.53. During this combination, the MIC of doxycycline was reduced 4-fold. This combination is followed by that formed by doxycycline and sinapic acid for which, the MIC of doxycycline was reduced by 3 times with a FICI of 0.6. No antagonism was observed.

Effect of the combination of doxycycline with natural products against biofilm formation

Table 3 below shows the effect of the combination of doxycycline with natural products on the formation of the biofilm of *P. aeruginosa*. It emerges from this table that out of the six combinations, one synergy was observed. This later was observed from the association formed by doxycycline and sinapic acid. During this combination, there is a 6-fold reduction in the MBIC of doxycycline. The FICI of this association is 0.48. 4 combinations showed additivity. No antagonism was observed.

Effect of the combination of antibiotics and natural product to eradicate mature biofilm

Table 4 below shows the effect of the combination of doxycycline with natural products for the eradication of the mature biofilm of *P. aeruginosa*. It appears from this table that 2 additivities and 4 indifferences were recorded. The best activity was shown by the combination formed by doxycycline and Sinapic acid with a FICI of 0.63 and a reduction of the MBEC of doxycycline by 3 times. This activity is followed by that of doxycycline and curcumin with a FICI of 0.99 and a reduction of the MBEC of doxycycline by 2 times. No antagonism was found.

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Discussion

The increasing prevalence of antibiotic resistance in *P. aeruginosa*, due to its arsenal of virulence factors involved, poses a real public health problem and has created an urgent need for suitable therapy. The possibilities of expanding the action spectrum of antibiotics and the suppression of emerging resistances are favored by the use of combination therapy [23]. During this work, the antibacterial, antibiofilm, and anti-virulence activity of doxycycline combined with natural products was evaluated against isolates of *Pseudomonas. Aeruginosa.*

Doxycycline exhibited MIC values between 2 µg/mL and 128 µg/mL. These values are similar to those of [24], who found MIC values against *P. aeruginosa* between 1 µg/mL and 32 µg/mL. Husain and Ahmad [25] found MIC values of doxycycline equal to 16 µg/mL against the *P. aeruginosa* strain PA01 and 64 µg/mL against the clinical isolate of *Pseudomonas aerugnosa* PAF-79. Petkova *et al* [26] found the MIC value equal to 4 µg/mL against clinical isolates of *P. aeruginosa.* The activity of doxycycline is thought to be because it inhibits bacterial protein synthesis by binding to the 30S subunit of the ribosome. For this purpose, it opposes the binding of amino acyl - tRNA to the acceptor consisting of the mRNA-ribosome complex, which stops protein synthesis [26]. The MBC values of doxycycline found by Moskowitz et al [24] are higher than 256 µg/mL. This result is different from that of Moskowitz et al [24]. Indeed, Moskowitz et al [24] found MBC values higher than 64 µg/mL. Indeed, the effectiveness of doxycycline is often altered, due to several mutations at the level of DNA and proteins and due to the hyperproduction of the efflux system [27]. *P. aeruginosa* has remarkable metabolic flexibility and an impressive ability to adapt to multiple conditions [28].
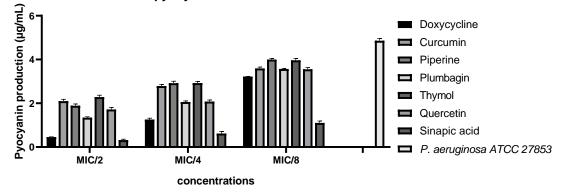
Doxycycline concentrations necessary for biofilm inhibition increased compared to its inhibitory concentrations against planktonic cells. This resistance of *P. aeruginosa* to the action of doxycycline could be because the formation of the biofilm constitutes a barrier to the action of antibiotics. Biofilm is notoriously resistant to antimicrobial agents in comparison with planktonic cells and withstand host immune defenses [29]. It is well-known that the MIC of an antimicrobial, effective on sessile bacteria, is 10 to 1,000 times more concentrated than the one that would be active on their planktonic version [30].

Sinapic acid showed excellent activity against planktonic cells with an average of MIC equal to 27.79 µg/mL according to the classification scale established by Tankeo and Kuete [31]. This activity is followed by that of quercetin and curcumin. Only Quercetin and sinapic acid showed the ability to eradicate the mature biofilm of *P. aeruginosa.* This effectiveness of sinapic acid would be due to the presence in its structure, of two methoxyl and hydroxyl groups involved in its antibacterial action [8]. These results are similar to those found by [18] who also showed that sinapic acid had excellent activity against planktonic cells of *P. aeruginosa.* Many studies also showed that quercetin inhibits biofilm formation and virulence factors in *P. aeruginosa* [32].

At sub-inhibitory concentrations, doxycycline exhibited inhibitory activities on pyocyanin production, swarming, and swimming motility of *P.s aeruginosa* ATCC 27853. This result is in agreement with that carried out by Husain and Ahmad [25] which showed that the expression of the virulence factors of *P. aeruginosa* was dependent on the concentration of doxycycline in the medium. Indeed, this author has shown that the production of pyocyanin and the motility of *P. aeruginosa* decreased maximally in the presence of doxycycline at concentrations of 4 µg/mL. For this purpose, at these concentrations, a decrease of 69.1 % in the production of pyocyanin, and 73.1% in the swarming motility of the strain of *P. aeruginosa* PA 01 was observed.

During this work, 6 combinations were tested on planktonic cell growth inhibition, *P. aeruginosa* biofilm inhibition, and biofilm eradication. The best activity was shown by the combination formed by doxycycline and sinapic acid which exhibited synergistic activity for prevention of biofilm formation with a 6-fold reduction in MBIC of doxycycline. No antagonism was observed. The combination of conventional drugs and natural product was shown to be more effective in combating planktonic cells and biofilm of *P. aeruginosa* [33]. The synergistic interaction between doxycycline and Sinapic acid, regardless of their mechanisms of action, suggested that it is not only one compound that is responsible for the observed synergistic effect but that each of the identified compounds contributes to this effect resulting in a pleiotropic effect of both compounds. This result is in agreement with those obtained by Oh and Jeon [34] which showed that synergistic activities were obtained after combinations of phenolic compounds and antibiotics against *C. jejuni.* Indeed, certain phenolic compounds such as sinapic acid significantly reduce the expression of efflux pumps. The phenolic compounds also substantially increased membrane permeability and antibiotic accumulation in bacteria to act better [35]. In addition, many studies have shown good activities after the combination of quercetin and antibiotics against multidrug-resistant isolates of *P. aeruginosa* [36].

Figure 1. inhibition of pyocyanin production by doxycycline and natural products

swarming inhibition

Figure 2. Inhibition of swarming motility by doxycycline and natural products
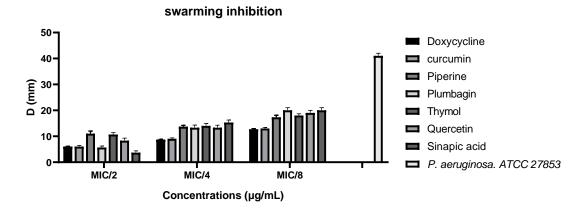
Swimming inhibition

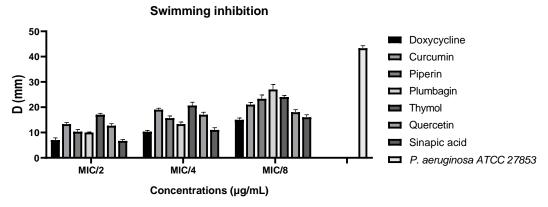**Figure 3.** Inhibition of swimming motility by doxycycline and natural products

Table 1. MIC, MBC, MBIC and MBEC values of doxycycline and natural products

Antimicrobial agents	MIC (µg/mL)		MBC (µg/mL)		MBIC (µg/mL)		MBEC (µg/mL)	
	Range	G-mean	Range	G-mean	Range	G-mean	Range	G-mean
	P=38				P=19			
Antibiotic								
Doxycycline	2-128	35.89	>256	-	128-512	256	1024-2048	1479.11
Natural Products								
Curcumin	64-1024	417.68	512-1024	905.85	128-2048	565.89	>2048	-
Piperine	>1024	-	>1024	-	>2048	-	>2048	-
Plumbagin	128-1024	644.92	>1024	-	1024-2048	-	>2048	-
Thymol	>1024	-	>1024	-	256-1024	464.84	>2048	-
Quercetin	32-128	77.47	128-1024	494.93	16-128	52.21	128-256	195.37
Sinapic Acid	16-64	27.79	128-1024	370.53	32-128	74.11	128-512	303.16

MIC: minimum inhibitory concentration, MBC: minimum bactericidal concentration; MBIC: minimum biofilm inhibitory concentration; MBEC: minimum biofilm eradication concentration. G-mean**:** Geometric mean value

Table 2. Mean of the minimum inhibitory concentration (MIC), fractional inhibitory concentration (FIC), and fractional inhibitory concentration index (FICI) of doxycycline and natural products (NPs) in combination against nineteen *P. aeruginosa* isolates.

Antimicrobial agents	MIC (µg/mL)				FIC		MIC reduction fold of ATB	FICI/ Interpretation
	Alone		Combined					
	Dox	NPs	Dox	NPs	Dox	NP		
Dox + Cur	56.42	640.00	26.32	229.05	0.47	0.36	2.14	0.82/A
Dox + Pip	56.42	>1024	36.21	592.84	0.64	0.58	1.56	1.22/I
Dox + Plu	56.42	>1024	26.95	687.16	0.48	0.67	2.09	1.15/I
Dox + Thy	56.42	>1024	16.63	485.05	0.29	0.47	3.39	0.77/A
Dox + Que	56.42	97.68	13.68	28.21	0.24	0.29	4.12	0.53/A
Dox + Sin A	56.42	35.37	15.00	11.79	0.27	0.33	3.76	0.60/A

Cur: curcumuin; Pip: Piperine; Plu: Plumbagin; Thy: Thymol; Que: Quercetin; Sin A : Sinapic Acid; Dox : Doxycycline; A : Additivity; I : Indifference; , NPs: Natural products; MIC: Minimal inhibitory concentration; FICI: fractional inhibitory concentration index; FIC: fractional inhibitory concentration

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Table 3. Mean of the minimum biofilm inhibitory concentration (MBIC), fractional inhibitory concentration (FIC), and fractional inhibitory concentration index (FICI) of doxycycline and natural products (NPs) in combination against nineteen *P. aeruginosa* isolates.

Antimicrobial agents	MBIC (µg/mL)				FIC		MIC reduction fold of ATB	FICI/ Interpretation
	Alone		Combined					
	Dox	NP	Dox	NPs	Dox	NP		
Dox + Cur	256	566.21	151.58	153.26	0.55	0.27	1.82	0.82/A
Dox + Pip	256	>2048	141.47	445.37	0.55	0.22	1.81	0.77/A
Dox + Plu	256	>1280	174.11	768.00	0.68	0.60	1.47	1.28/I
Dox + Ty	256	464.84	91.79	200.42	0.36	0.43	2.79	0.79/A
Dox + Que	256	52.21	84.21	17.89	0.33	0.34	3.04	0.67/A
Dox + Sin A	256	74.11	42.74	23.16	0.17	0.31	5.99	0.48/S

Cur: curcumuin; Pip: Piperine; Plu: Plumbagin; Thy: Thymol; Que: Quercetin; Sin A: Sinapic Acid; Dox: Doxycycline; A: Additivity; I : Indifference; S: Synergy; Natural products; MIC: Minimal inhibitory concentration; FICI: fractional inhibitory concentration index; MBIC: Minimal Biofilm inhibitory concentration; FIC: fractional inhibitory concentration

Tableau 4: Mean of the minimum biofilm eradication concentration (MBEC), fractional inhibitory concentration (FIC), and fractional inhibitory concentration index (FICI) of antibiotics (ATB) and natural products (NPs) in combination against nineteen *P. aeruginosa* isolates.

Antimicrobial agents	MBEC (µg/mL)				FIC		MIC reduction fold of ATB	FICI/ Interpretation
	Alone		Combined					
	Dox	NPs	Dox		Dox	NP		
Dox + Cur	1479.11	>2048	881.58	814.11	0.60	0.40	1.68	0.99/A
Dox + Pip	1479.11	>2048	633.26	1455.16	0.43	0.71	2.34	1.14/I
Dox + Plu	1479.11	>2048	1131.79	1562.95	0.77	0.76	1.31	1.53/I
Dox + Ty	1479.11	>2048	943.16	1185.68	0.64	0.58	1.57	1.22/I
Dox + Que	1479.11	195.37	983.58	75.79	0.66	0.39	1.50	1.05/I
Dox + Sin A	1479.11	303.16	592.84	69.89	0.40	0.23	2.49	0.63/A

Cur: curcumuin; Pip: Piperine; Plu: Plumbagin; Thy: Thymol; Que: Quercetin; Sin A: Sinapic Acid; Dox: Doxycycline; A: Additivity; I : Indifference; NPs: Natural products; MBEC: Minimal Biofilm eradication concentration; FICI: fractional inhibitory concentration index; FIC: fractional inhibitory concentration

Conclusion

It appears from this work doxycycline and sinapic acid inhibit the growth of planktonic cells of *P. aeruginosa*. At the sub-inhibitory concentration, doxycycline and natural products inhibit the production of pyocyanin, swarming, and swimming motility of *P. aeruginosa*. The combination formed by doxycycline and sinapic acid showed a synergistic activity against biofilm formation of *P. aeruginosa* with a 6-fold reduction of MBIC of doxycycline. This combination can be used for expanding the antimicrobial spectrum, preventing the emergence of antibiotic-resistant bacteria, and diminishing toxicity since a lower concentration of doxycycline can be used.

Abbreviations

ATCC: *American Type Culture Collection*
DMSO: Diméthylsulfoxyde
FICI: Fractional Inhibitory Concentration Index
INT: p-iodonitrotetrazolium chloride
LB: Luria-Bertani
MBC: Minimum Bactericidal Concentration
MBEC: Minimum Biofilm Eradication Concentration
MBIC: Minimum Biofilm Inhibitory Concentration
MHB: Muller Hinton Broth
MIC: Minimum Inhibitory Concentration
P. aeruginosa: *Pseudomonas aeruginosa*
PBS: Phosphate Buffer Saline

Authors' Contribution

JPD designed and supervised the study. LYC performed experiments, analyzed the data, and wrote the first draft of the manuscript. GSSN co-supervised the study and was involved in the manuscript correction. AJA performed the molecular identification of isolates. All authors read and approved the final manuscript.

Conflict of interest

The authors declare no conflict of interest.

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