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Evaluation of Synergistic Effect of Nicotinic Acid with Imipenem as Antibiofilm for Clinical *Pseudomonas Aeruginosa* Isolates

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Abstract

The present study aims to evaluate the synergistic activity of nicotinic acid (NIC) with the Imipenem (IMI) as an anti-biofilm for clinical isolated *Pseudomonas aeruginosa*. The values of minimum inhibitor concentration (MICs) for IMI and NIC (Separately) against *P. aeruginosa* were (16) ug/mL and (8) ug/ml respectively. Whereas, the concentration of NIC with IMI (as combined) for biofilm inhibition was 1 ug/ml for NIC and 4 ug/ml for IMI. The combining of NIC with IMI showed synergistic efficacy against formation of bacterial biofilm (at MIC levels). These results provide a conclusion that NIC combined with IMI is can be considered as a successful prospective treatment against the biofilm produced by *P. aeruginosa* and as antibacterial medication.

Keywords: Anti-biofilm, Imipenem, Nicotinic acid, *P.aurginosa*

تقييم التاثير التأزري لحامض النيكوتين مع Imipenem كمضاد للغشاء الحيوي لعزلات الزوائف الزنجارية السريرية

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الخلاصة

تهدف الدراسة الحالية الى تقييم الفعالية التآزرية لحامض النيكوتين (NIC) مع Imipenem (IMI) كمضاد للغشاء الحيوي للزوائف الزنجارية السريرية المعزولة. كانت قيم التركيز المثبط الأدنى (MIC) لحامض النيكوتين و Imipenem (وبشكل منفصل) ضد الزوائف الزنجارية هي (8) مايكروغرام/ مل و (16) مايكروغرام/ مل على التوالي ؛ في حين أن تركيز حامض النيكوتين مع Imipenem (كمركبات مرتبطة معا) لتثبيط الغشاء الحيوي كان 1 مايكروغرام/ مل للنيكوتين و 4 مايكروغرام/ مل لل Imipenem . اظهر الارتباط مابين حامض النيكوتين و Imipenem كفاءة تآزرية ضد تكوين الغشاء الحيوي البكتيري(عند مستويات التركيز المثبط الأدنى). هذه النتائج تؤدي الى الاستنتاج بأن Imipenem المرتبط مع حامض النيكوتين يعد علاج مستقبلي ناجح ضد الأغشية الحيوية المنتجة بوساطة الزوائف الزنجارية وكدواء مضاد للبكتيريا .

Introduction

Biofilm-associated with infections is creating havoc in healthcare facilities globally. Biofilms formed either on natural tissues (bones,skin, teeth, mucosa, etc.) ، or artificial devices (endotracheal tubes, central venous catheters, contact lenses, intrauterine devices, and urinary catheters, etc.) jointly

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contribute to 60–85% of all microbial infections [1]. *P.aeruginosa* is an important opportunistic pathogen for a human that can cause life-threatening contagion, especially in patients with expressing cystic fibrosis (CF) and arrangement attended with a compromised immune system. *P.aeruginosa* biofilms are managed formed on biotic and abiotic surfaces; this probable act given this microbe's the ability to causes diseases in analytic settings [2, 3]. Though there are competent antimicrobial agents that continue to produce the intended result against *P.aeruginosa* (i.e., carbapenem). In recent years an increasing of antibiotic resistance has been recounted for *P.aeruginosa* [4, 5]. Leaves in some plants act as a medical compounds that play a role in maintenance human health since ancient. Phytochemicals such as vitamin's (A, C, E,and K), tannins carotenoids, terpenoids, alkaloids, flavonoids, polyphenols, saponins, pigments, minerals and enzymes found to have antimicrobial antioxidant activity, the use of plant extracts and phytochemical elements with antimicrobial properties can be considered as great significance in therapeutic treatments for many purposes [6]. Alkaloids are natural products of diverse structurally group, possess antimicrobial activity such as (NIC). The present study aimed to identify synergy antibiotic activity of IMI, which is one of the most important drug against *P.aeruginosa*, when combined with the NIC is alkaloid, and this combination showed anti-biofilm formation *in vitro* against pathogenic *P.aeruginosa*.

Materials and methods

Bacterial Strains

Eighty medical samples were taken from patients in Al-Yarmouk Educational hospital/ Baghdad-Iraq. The specimens included Burn swab, wound swab, and Fractures swab.

Antimicrobial Susceptibility Testing

Clinical *P. aeruginosa* isolates collected from patients were subjected to the susceptibility test for different antibiotics by the Kirby–Bauer disk diffusion method. five ml of Brain heart broth medium was inoculated with bacterial isolate, and incubated at 37 ºC for 18 hours. transfer 100 ul (1.5×10^8 organism/ ml) of freshly bacterial growth monitored by McFarland tube No. 5 turbidity standard, which as equivalent to bacterial concentration for inoculum 1.5×10^8 organism / ml) wsa transferred to Mullar–Hinton agar plate and streaked by sterile cotton swab three times by rotating the plate approximately at an angle of 60 between streaking to ensure even distribution of the inoculum. The inoculated plates were placed at room temperature for 10 minutes to allow absorption of excess moisture. Then antibiotic disks were applied by sterile forceps on the surface of plates and incubated at 37 ºC for 18 hours in an inverted position. After incubation, the diameter of inhibition zone (clear area around disks) was measured by ruler which indicates the sensitivity of bacteria to that antibiotic and the result were compared with NCCLs (2012). The antibiotic group selected according to Clinical and Laboratory Standards Institute (CLSI) [7, 8]. The antibiotic selected were Imipenem, Cefepime, Aztreonam, Colistin, Piperacillin-tazobactam, and Ticarcillin-tazobactam from Mast Group Ltd, Merseyside, United Kingdom.

Preparation the Antibiotics and Active NIC Stock Solutions:

Stock solutions were prepared by dissolving the antibiotic powders in DW (Imipenem and Nicotinic acid) shown (Table-1).

Table 1- Preparation the antibiotics and active NIC stock solutions:

Antibiotics/active material	Dose	Amount of solvent	Primary Concentrations
Imipenem	500mg	10 ml (D.W)	50 mg /ml = 50000 µg /ml
Nicotinic acid	500m g	10 ml (D.W)	500mg /ml = 50000 µg /ml

Minimum Inhibitory Concentration (MIC)

Determination of minimum inhibitory concentrations (MICs) for IMI, and NIC against selected *P. aeruginosa* isolated (strongly biofilm formation than other isolate) were carried out by the micro titer technique for IMI and NIC. Micro titer technique often determined in 96-well microtiter plate format, bacteria are inoculated into a liquid growth medium in the presence of different concentrations of an antimicrobial agent. Growth was assessed after incubation for a defined period of time (18–24 hr) and the MIC value is read [7].

Biofilm Formation

Semi-quantitative of biofilm formation was estimated by using the Crystal Violet (CV) assay [9]. Briefly, 180µL of tryptone soy broth (TSB) and 20µL of bacterial suspension (10^7CFU/mL) of the

isolate were added to a microtiter plate (96 wells). A blank was used from TSB broth only, and the control was TSB broth with bacteria. Incubation of the microtiter plate at 37°C for 24 h, after incubation of the micro titer plate, media was removed by inversion and gently washing wells with phosphate buffer saline (PH 7.4). The adhered cells of the micro titer plate were blotted with 150µL of CV staining solution (2% w/v) for 30 min. the dye phosphate buffer Slain was discarded, and micro titer plates were desiccated for 15min at 40°C. Biofilm was quantified by adding 150µL of 96% of ethanol to each well and the color absorption was then measured at 575nm using Elisa reader (Lisa plus, Germany) after the adjustment to zero with the blank. Biofilm formation of isolate was considered as efficient when absorbance equal to or more than 0.15 at 575nm.

Inhibition of Biofilm Formation

Biofilm formation by *P. aeruginosa* (1×10^5 CFU/200µL) was inhibited after incubation for 24 h at 37◦C with IMI and NIC at (512) ug/ml concentration separately and as in the combination in the microtiter plates additionally. The OD of biofilm cells were measured at 630nm after staining with CV (2%, w/v). The controls were *P. aeruginosa* isolate in TSB-glucose without NIC or antibiotic [10].

Determination of the Fractional Inhibitory Concentration Index (FICI)

A checkerboard microdilution assay test was used for the determination of FICI, in order to estimate a possible interaction combination between IMI and NIC [11]. The concentration of individual compound and in the combination of NIC and IMI which prevented bacterial growth was recorded as the alone MIC. In the combination MIC of both drug divided on the alone MIC to fined FIC. The FICI value was then calculated as follows [11]:

FICI = FIC of NIC + FIC of IMI

Where

FIC = MIC in combination /MIC of alone.

Synergistic effect is defined as FICI of ≤0.5; indifference as; 0.5 < FICI ≤ 4; and antagonism as FICI of more than 4.

Statistical Data analysis

All Results were analyzed statistically by Statistical Package for the Social Sciences (SPSS) V23.

Results

Isolation of Bacteria

Eighty medical samples taken from patients in Al-Yarmouk Educational hospital/ Baghdad-Iraq, 59 sample(73.7%) gave growth on the culture media, and 21 sample (26.3%) without growth. Distribution of samples was depended on the type of sample, gender, and age as shows in Figure-1. Among 59 sample, 73.7% gave growth on the culture media. *P. aeruginosa* was 30 isolated (50.8%), while the distribution of *P. aeruginosa* was depended on the type of sample, gender, and age (Figure -2).

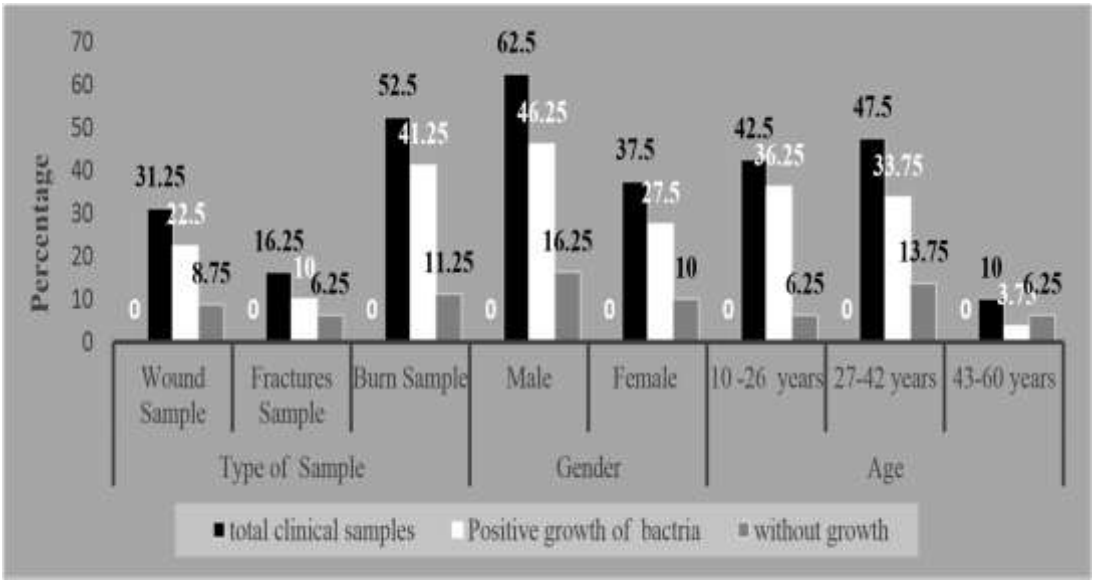

Figure 1-Distribution of samples depending on type of sample, gender, and age.

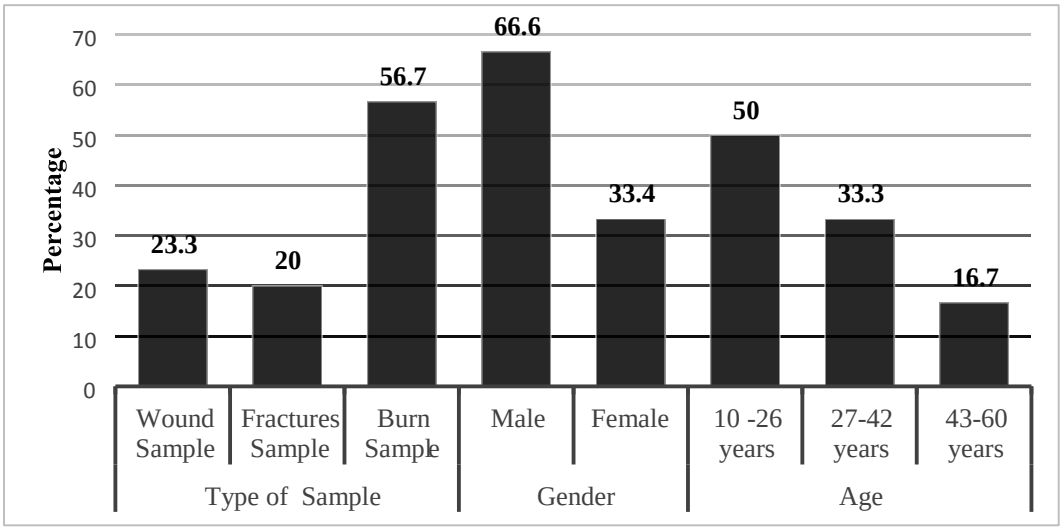

Figure 2-Distribution of *P.aeruginosa* depending on type of sample, gender, and age

Test of Susceptibility for the Bacteria

Abroad antibiotics arrayed, specific for *P. aeruginosa*, were chosen to study the susceptibility model of clinical isolate. Figure-3 and Table-2 show the susceptibility of the antimicrobial result for isolated bacteria.

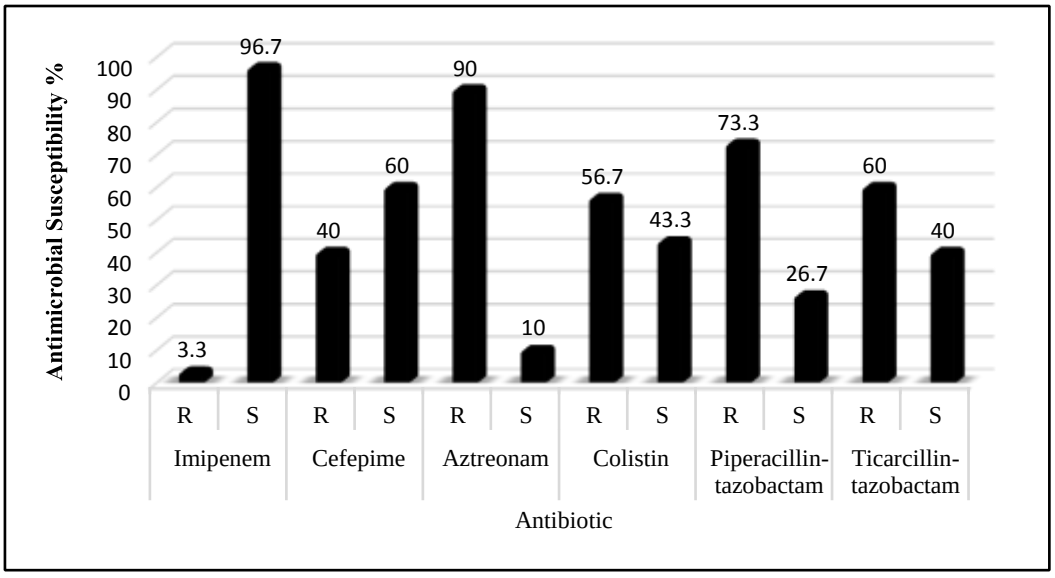

Figure 3-Test of susceptibility of antimicrobials against all *P.aeruginosa* isolates.
*R: Resisting, S: Sensitive.

Table 2-Test of susceptibility for selected *P.aeruginosa* isolates.

Antibiotics	Susceptibility	Antibiotics	Susceptibility
Imipenem	R	Colistin	R
Cefepime	R	Piperacillin-tazobactam	S
Aztreonam	R	Ticarcillin-tazobactam	R

Minimum Inhibitory Concentration (MIC)

The antimicrobial MIC test was performed by a micro titer technique according to CLSI guidelines [8]. The value of IMI and NIC alone were 16 ug/ml and 8 ug/ml respectively, and in combination were 4 ug/ml and 1 ug/ml respectively as shown in Table-3 and Figure-4.

Table 3-MIC of *P. aeruginosa*

Sample	MIC (ug/ml)
IMI alone	16
NIC alone	8
IMI in combination	4
NIC in combination	1

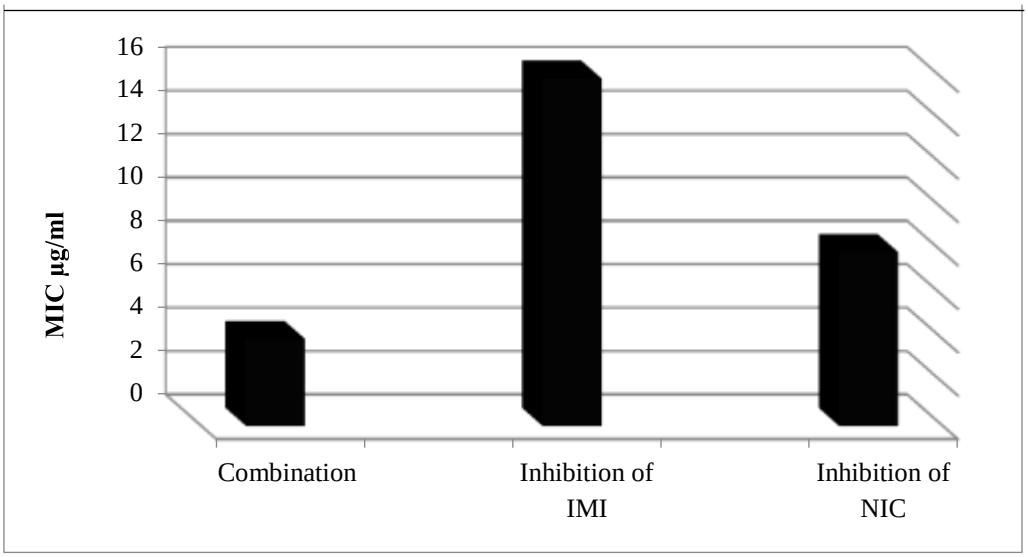

Figure 4-MIC values for Synergistic effect of nicotinic acid with the Imipenem on *P. aeruginosa* biofilm and FICI.

Fractional Inhibitory Concentration Index (FICI)

The result of FICI is presented in Table-4, depending on the FICI equations shown in determination of the fractional inhibitory concentration index (FICI) in present study.

Table 4-FICI values for combination of NIC with IMI on selected bacteria isolates.

Clear well	NIC		IMI		FICI (FIC of NIC + FIC of IMI)	Interpretation
	MIC Concn. (µg/ml)	FIC of drug NIC	MIC Concn. (µg/ml)	FIC of drug IMI		
A4	8	MIC NIC	0	MIC NIC	MIC NIC	MIC NIC
E8	0.5	0.0625	8	0.5	0.5625	Indifference
F7	1	0.125	4	0.25	0.375	Synergism
F6	2	0.25	4	0.25	0.5	Synergism
G5	4	0.5	2	0.125	0.625	Indifference
H5	4	0.5	2	0.125	0.625	Indifference
D1	0	MIC IMI	16	MIC IMI	MIC IMI	MIC IMI

Inhibition of Biofilm Formation

The strong's biofilm production by selected *P. aeruginosa* isolate that gives 1.413 at 575nm absorption was the strong biofilm from other isolates. The NIC and IMI inhibited *P. aeruginosa* biofilm formation at 24h. The best biofilm formation inhibition for *P. aeruginosa* is shown in Figure -5 for both IMI and NIC.

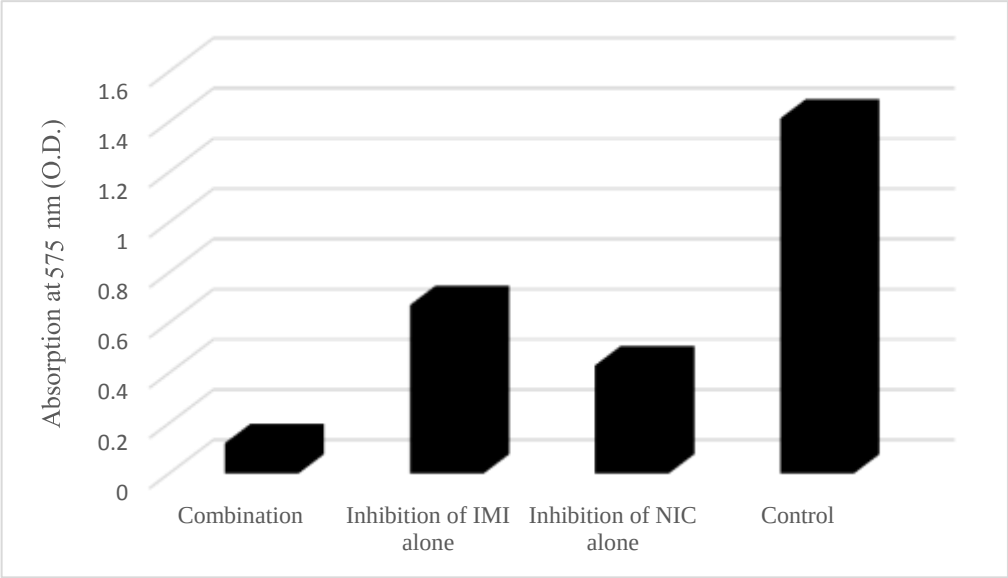

Figure 5-Inhibition of Biofilm Formation by IMI and NIC alone and in combination.

Discussion

The biofilm-associated infections have been well suited to a confrontation for doctors because of the insistence of biofilms that act of going up multidrug resistance. IMI is known for their effects as biofilm distraction antibiotic antimicrobial such destruction of biofilm, alteration of the bacterial outer membrane, and inhibition of expression of virulence factors [12]. IMI are inhibition the cell wall synthesis by effect on binding of penicillin-binding proteins (PBPs). IMI are made away through a porin in the across of outer membrane of *P. aeruginosa*, allows selective penetration of the drug with basic amino acids. The effect of alkaloid such as NIC on RNA polymerase, gyrase and topoisomerase IV enzyme. The current study suggests a useful outcome combination therapy of the IMI and NIC in burn, wound and fractures infected by *P. aeruginosa* that is remarkable in biofilm forming inhibition. We also identified a conventional and current anti-biofilm therapies target one bacterial species without considering that most biofilm-related chronic infections are due to the persistence of polymicrobial biofilms. Thus, there is no ideal solution to totally eradicate biofilm, but the key would be the simultaneous application of agents implementing mechanisms with synergic potential in order to disturb the biofilm structure and kill bacteria.

The use of computational tools to comprehensively understand anti-biofilm processes seems essential. They could be used for the analysis of the effects of anti-biofilm agents, in particular to assess their efficacy and to consider how they could impact the emergence of new classes of resistant microbes. This study concludes that synergistic activity of (NIC) extracts with commercial antimicrobials IMI showed promising results. IMI with alkaloid combination seems to be profitable of biofilm treatment generated from *P. aeruginosa* in contrast expressing the antibiotics only. To perfect of our knowledge, this report is citing first in-vitro state combination therapy of the IMI+NIC. The IMI and NIC *in vitro* results promising to choose the combination efficacy therapy. The combination of IMI (4 ug/mL) also NIC (1 ug/mL) resulted appear synergism in Chequerboard assay against planktonic cells (FICI < 0.5), depending on the result for IMI inhibition biofilm alone was (16ug/ml) and become (4 ug/ml) when combined, and for NIC was (8 ug/ml) /and become (1 ug/ml) when combined. Combination at inhibitory concentrations also appears benefit capacity to biofilm formation inhibition after 24 h.

Conclusion

Most of the studies for NICs and IMI revealed an ability of inhibiting antibacterial activities. In addition, such a synergistic combination of NIC and IMI in clinical situations is especially desired, thus in addition potency of the IMI with NIC joining in the treatment of biofilm can also be exploited. The study explained the alone and combinatorial impact of IMI and NIC *in vitro* generated *P. aeruginosa* biofilm cells.

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