The Effect of New Hydantoin Derivative (Compound) on *Acinetobacter baumannii* Biofilm Formation Isolated from Clinical Sources

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Abstract

Completely 538 clinical specimens of blood, wounds, burns, sputum and urine were included in this study which was carried out in 4 hospitals. The isolation and diagnosis of fifty-two *Acinetobacter baumannii* isolate 36.54% from blood specimens, whereas wounds specimens constituted 23.08%, burns specimens achieved 17.31%, sputum specimens formed 13.46% and a low percentage was in urine specimens which accomplished 9.62 %. Totally isolates of bacteria were identified via the biochemical, cultural and microbial characteristics and confirmed via VITEK 2 Compact system. It was found that all (100%) of bacteria isolates were resistant to amoxicillin-clavulanic acid and Cefepime, while Imipenem and Meropenem were the most effective antibiotics. The isolates showed the ability of biofilm production by Microtitration plates method (M.T.P), from the total number (52) isolate tested for biofilm formation,(33) were producers, as strong biofilm producers were (15) 45.46 % and (18)54.54% moderate while (19) 36.53% isolates were measured as none or weak producers of biofilm. MIC for new hydantoin derivative ($C_{11}H_{11}N_3O_2S$) was determined against fifteen isolates were active in MIC values equal to 250μg/ml gave (9) *A. baumannii* isolates, while in MIC, values equal to 125μg/ml gave (6) isolates.

The results showed the inhibitory effect of the hydantoin compound on biofilm formation for 15 bacterial isolates used in this study with variable inhibitory effect on bacterial isolates ranged from 79.72% to 98.85%. The highest inhibitory effect percentage is (98.85%).

1-Introduction

Infectious diseases are stay a chief deaths cause especially in rising countries, for instance new infectious diseases ascend and a developing number of multi-drug resistant strains of microbial pathogens occur [1]. The latter hassle applies specifically to Gram-negative bacteria, such as *A. baumannii*. Multidrug resistance indicates a critical function in the letdown of remedy of cancer and infectious illnesses [2]. Microorganisms have developed various approaches to face up to the antibiotics poisonous effects and different capsules [3],[4].Discovery of chemotherapeutic marketers played a very necessary position in controlling and preventing such diseases. Chemotherapeutic dealers are remoted both from dwelling organisms recognized as antibiotics, or they are chemical compounds organized through chemist [5],[6]. Hydantoin derivatives have a diversity of biochemical and pharmacological traits and are used to deal with quite a few human ailments and extensive vary of other pharmacological characteristics, counting herbicidal, antitumor, anti-inflammatory, fungicidal, hypolipidemic, anti-HIV, antihypertensive and antiarrhythmic activities [7],[8]. *A cinetobacter baumannii* is a public causative agent of nosocomial infections and it has come to be a pathogen of enlarged clinical importance because of its first-rate functionality to reason outbreaks of infections and to obtain resistance to nearly definitely nowadays used antibiotics, involving the carbapenems [9],[10]. A. baumannii is characterised via its capability to spread, its capacity to continue to exist on an environmental surface [11]. And its tremendous easiness with which it obtains

antimicrobial multiple resistance. The past 15 years, many *A. baumanni*i nosocomial traces isolated worldwide are particularly resistant to nearly all available families of cutting-edge antibiotics [12],[13]. Biofilm-forming capability and antibiotic resistance are measured to be two serious elements for the accomplishment of *A. baumannii* as an ordinary nosocomial pathogen [14]. While, the formation of biofilm capability in spite of its vital function in the enchancment of resistance of antibiotic and pathogenesis of *A. baumannii* has been poorly examined [15],[16].

2-Aim of the Study

Synthesis and analyzing for new hydantoin derivative (compound) as a precise antimicrobial action which may be suitable to be used as chemotherapeutic means.

3- Materials and Methods

3.1Samples collection

A whole of 538 clinical samples of blood, wounds, burns, sputum and urine were included in present study which was carried out in 4 hospitals. Through the period ranging from 1 February 2017 until June 2017. Bacterial diagnosis initially diagnostic depending on Gram reaction and morphological characteristics of the colonies based on bacterial growth on MacConkey agar and Blood agar, as well as the number of biochemical test and Api 20 E strip reading . The second technique VITEK 2 compact system. *A. baumannii* was distinguished by the following methods; heamolysin test and IMVC test [17].

3.2 Susceptibility of Antibiotic

Experiment disk agar dissemination according to Kirby Baur standardized susceptibility of antimicrobial, single disk way was carried out toward twelve antibiotics.The antibiotic discs were as follows:, Ciprofloxacin (CIP, 5µg/disc) , Piperacillin (PIP, 100µg/disc) Tobramycin (TB, 10µg/disc), ceftriaxone(CTR, 30µg/disc) , Ceftazidime(CAZ, 30µg/disc), Cefepime (CFP ,30µg/disc), Aztreonam(ATM, 30µg/disc), Amoxicillin-Clavulanicacid (AUC, 20/10µg/disc), Amikacin (AK,30µg/disc) , Gentamicin(CN, 10µg/disc), Meropenem (MEM,10µg/disc) and Imipenem (IMP, 10µg/disc), Bioanalyse (Turkey) [18].

3.3Biofilm Formation

The bacterial activity for biofilm formation was measured by quantitation of biofilm by microtiter plate (M.T.P)**:** The current study screened the all medical isolates of *A. baumannii* for their capability to biofilm formation via microtitration plates approach as stated by way of [19]. With some modification, *A. baumannii* were inoculated in three ml of brain heart infusion (BHI) with 1% glucose [20]. The broth was incubated for 24 h at 37ºC. 200 µL aliquots of only BHI + 1% glucose previously prepared were distributed into 3 wells of the column one of microtitration plate to function as a control and 200 µL of the diluted cultures were added to the wells of the microtitration plate and incubation for 24 h at 37°C.Adherent micro-organisms forming biofilms were static with sodium acetate (2%) and marked with crystal violet (0.1% w/v), and permitted to incubate for 15 min at room temperature. After eliminating the crystal violet solution, wells were washed 3 times with distilled water to eliminate boundless dye. Lastly, totally wells were filled via 200 µL ethanol (95%) to discharge the dye from the cells and Optical density (OD) of biofilm was obtained by using ELISA reader at wavelength 550 nm.

The ability of biofilm formation=O.D of test sample - O.D of quality control.

Experimental reparation of 3- ((1-(4-hydroxyphenyl) ethylidene) amino) -2-thioxoimidazolidin - 4- one

New hydantion derivatives were prepared according to the literature procedure [21, 22]. In this study, new hydantion derivative was used compound 3-((1-(4-hydroxyphenyl) ethylidene) amino)-2-thioxoimidazolidin-4-one ($C_{11}H_{11}N_3O_2S$), and it melting point was detected (205-210˚C) and prepared as follow: carbonyl compounds (0.01mole) was dissolved in ethanol (30 ml) and (0.01mole) of thiosemicarbazide was added and then refluxed for three hrs. The resultant mixture was transferred into crushed ice and stirred for 15 min. Then the precipitated crystalline solids were filtered, laundered with water and were recrystallized from ethanol.Moreover, (0.01mole) of compounds2-(1-(4-hydroxyphenyl) ethylidene) hydrazine-1-carbothioamide were dissolved and their melting point were detected (185-1880˚C) $C_9H_{11}N_3OS$ and (0.01 mole) of chloroethylacetate in 30 ml ethanol to obtain a mixture. The mixtures were stirred for a few minutes, and then sodium acetate (0.02mole) was additional to the mixture. The mixture was refluxed for 6hrs. After cooling, the precipitate found which is filtered off and recrystallization from dioxane.

3.4 Determination of minimum inhibitory concentrations (MICs) of new hydantoin compounds for *A. baumannii* isolates

The antimicrobial efficiency of new hydantoin derivative (4-hydroxyphenyl) ethylidene) amino)-2-thioxoimidazolidin-4-one) against *A. baumannii* was tested by the standard broth dilution approach. By using serial two-fold dilutions of new hydantoin derivative in concentrations extending from (15.62 mg/ml to 500 mg/ml) the MIC was determined in BHI broth. In present study, wield the positive control contained BHI broth medium with experienced bacterial concentrations and negative control contained only BHI broth and incubated at 37^0C for 24hr. The (MIC) of the new hydantoin derivative for *A. baumannii* was measured at the lowest concentration of the new hydantoin compound required to inhibit the *A. baumannii* growth.

3.5The antimicrobial activity of the new hydantoin compounds for biofilm formation on *A. baumannii* isolates

The antimicrobial activity of the new hydantoin derivative against *A. baumannii* isolates used to be quantified according to the process described via [23], [24].

Briefly, wells of sterile ninety six well-flat bottom polystyrene tissue tradition dealt with plates had been stuffed with 200µL of the diluted cultures (180 µL BHI+ 1% glucose and20 µL bacterial culture) serve as a control .While the second well was filled with100 µl of the hydantoin compound in concentration of Sub MICs with 100 µl BHI containing bacterial culture and covering with parafilm. The plate was once incubated at 37°C for 24 h and later washed twice with distilled water and left to dry at room temperature for 15 min. Then we added 200µL of the crystal violet to all wells and left for twenty min. Later wells washed by sterile water and left to dry at room temperature for fifteen min. Subsequently for removing the crystal violet, wells were washed 3 times with D.W to eliminate boundless dye.

Lastly, totally wells were filled via 200 µL ethanol (95%) to discharge the dye from the cells and optical density of stained biofilm was gained through using micro ELISA auto reader at wavelength 595 nm.

% of inhibition of biofilm formation = 1- (Sample optical density /control optical density) ×100

4- Results and Discussion

4.1 Isolation and Identification of Bacterial Isolates

A total of 538 clinical specimens of blood, wounds, burns, sputum and urine were included in this study, 52 were positive to *A. baumannii* which was isolated in high percentage; 36.54% (n=19) from blood specimens; whereas, wounds specimens constituted 23.08% (n= 12), burns specimens achieved 17.31% (n= 9), sputum specimens formed 13.46% (n= 7) and low percentage was in urine specimens which accomplished 9.62 % (n= 5).

All bacterial samples expected to be an *A. baumannii* grown on MacConkey agar and Blood agar as enrichment media, then it occurs as Gram-negative coccobacilli and occasionally arranged in diplococcic under the microscope .Then, anumber of biochemical test were carried out and the results showed in table (1).

Table 1: Biochemical test consequences for *Acinetobacter baumannii*

ID	TEST	RESULT
1	Catalase production	+
2	Citrate utilization	+
3	Growth at 44ºC	+
4	Hemolysin production	– (γ hemolysis)
5	Motility	–
6	Urease production	–
7	Indole production	–
8	Oxidase production	–
9	Kliglar iron agar (KIA)	Alkaline slant / No change bottom, No gas , No H_2S
10	Lactose fermentation	-

All the above results were identification by Api 20 E strip reading and Vitek 2 compact system (17).

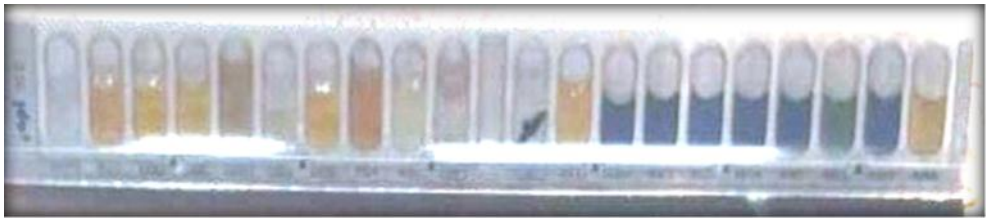

Figure 1: Results for *A. baumannii* by API 20 E system.

4.2 Antibiotics Susceptibility

The susceptibility tests toward (12) antibiotics were carried out and results are illustrated in Figure (٢) which showed that *A. baumannii* clinical isolates had 100% resistance to Amoxicillin-clavulanic acid and Cefepime. Also this study showed the highest resistance to Ceftriaxone (94.23%), Piperacillin (94.23%), Ceftazidime (90.38%), Aztreonam (88.46%), Gentamicin (86.53%), Ciprofloxacin (82.69%) and Amikacin (73.07%). Imipenem and Meropenem recorded moderate resistance; (55.76%) both them.It is worthy to remark that from 52 clinical isolates of *A. baumannii*, 21 isolates (40.38%) were resistant to Tobramycin.

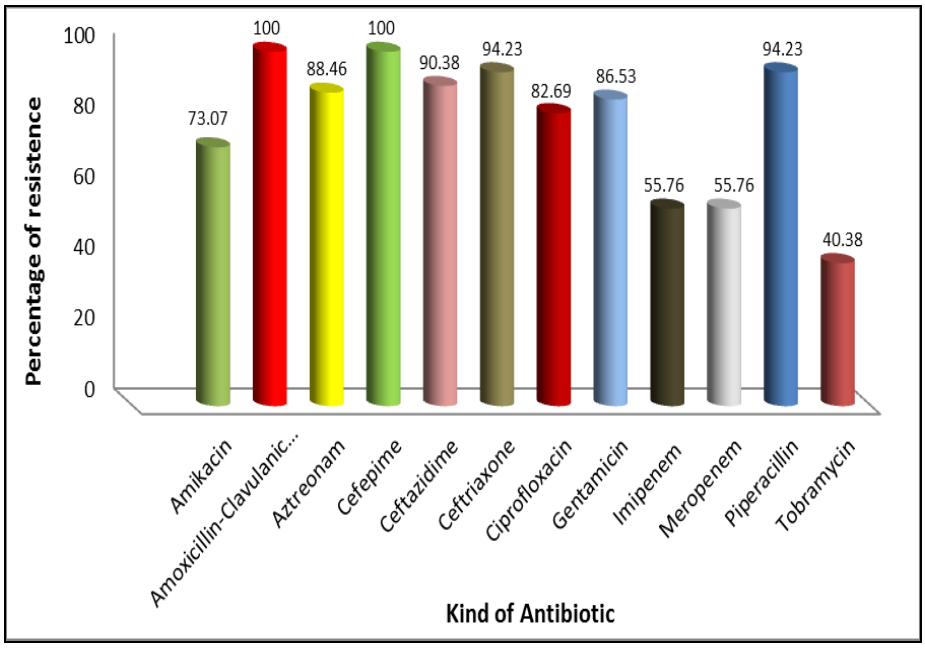

Figure 2: Antibiotic resistance of *Acinetobacter baumannii* isolates.

In a local study done via [25], reported that *A. baumannii* clinical isolates showed 100% resistance to Cefotaxime, Ceftazidime, Ceftriaxone, 95.45 % to Cefepime, Chloramphenicol, Aztronam and 40.90% to Imipenem. According to these local studies, can remark interestingly the increasing of resistance to Imipenem antibiotic in our hospitals.

[26] reported that >75% of their isolates were resistant to Imipenem, Meropenem and other antibiotics usually used for the handling of *A. baumannii*. Another study conducted by [27] reported a high level of resistance to Imipenem and Meropenem (55.6%) as well. [28] reported that the resistance percentage of Imipenem was (43.59%) for *A. baumannii* isolates.

4.3 Biofilm Formation by *A. baumannii*

The capacity of *A .baumannii* to the production of biofilm by all isolates from (blood, wound, burns swab and sputum as well as urine). MTP technique, from the whole number of (52) isolates verified for biofilm formation,(33) were producers, as strong biofilm producers were (15) 45.46 % and (18) 54.54% moderate while (19) 36.53% isolates were considered as none or weak biofilm producers figure 3.

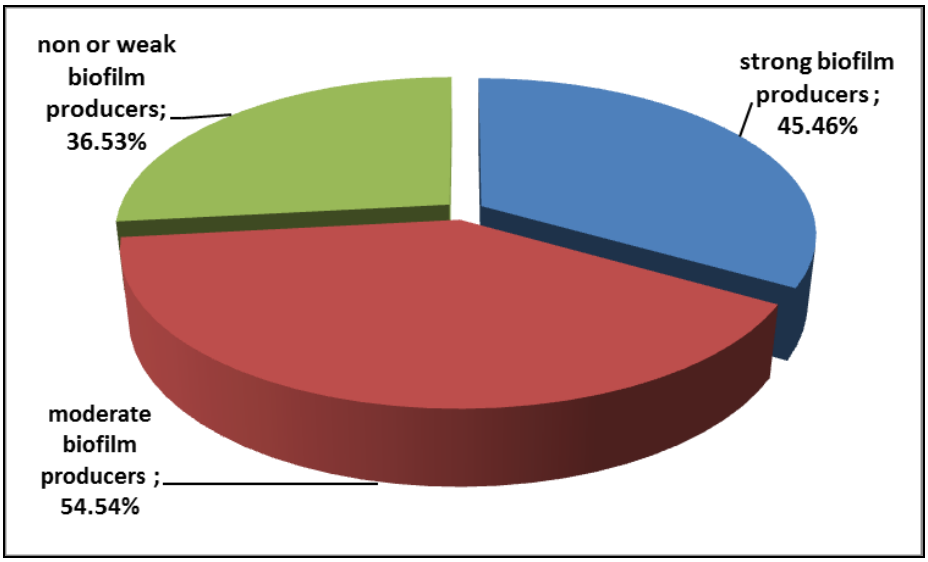

Figure 3: Biofilm producer MTP method by *A .baumannii* isolate

Many bacterial pathogens, which include *A. baumannii*, can develop either as dispersed (planktonic) cells or as matrix-enclosed communities referred to as biofilms. The potential to form biofilm is shared amongst scientific *A. baumannii* isolates, possibly because the biofilm mode of increase contributes to the ecological success of this pathogen in the hospital [29].

These consequences are disagreement with a study [29, 30] located that all *A. baumannii* isolates showed the ability to structure biofilms in MH broth.

Another study additionally in total, 249 (91%) isolate had been fine for biofilm formation, and 63 (23%) isolates exhibited extra strong biofilm formation [31]. The results of [32] are establish that most of the *A. baumannii* isolates from both blood and sputum were capable to form variable degrees of biofilm on polystyrene. In study of [33] exhibited of the ninety-two isolates studied, 56 (63%) made biofilm *in vitro*, 33 (36%) did not form biofilm. Consequently [34] are revealed strongly positive via microtitre plate assay in (n=32) *A. baumannii*, while the residual isolates were either one moderate adherent (n=18) *A. baumannii* or feeble producers (n=13) *A. baumannii* and non-biofilm producers (n=11).

4.4 Determination of Minimum Inhibitory Concentrations (MICs) of new hydantoin derivatives on *A.baumannii*

MIC for new hydantoin derivative ($C_{11}H_{11}N_3O_2S$) was determined against fifteen isolates of *A .baumannii* were multi-drug resistance and biofilm formation with the method (MTP) was the best and the consequences were interpreted after 16-18 hrs.of incubation at 37^{o}C according to the [35]. The (MIC) of the new compound for *A .baumannii* was measured at the lowermost concentration of the compound required to inhibit the growth of these micro-organisms, this plate containing different concentrations of the new hydantoin compound was incubated at 37^{0}C for 16-18hr.The compound ($C_{11}H_{11}N_3O_2S$) was screened in vitro for its ability to inhibit the progression of *A .baumannii*. The compound was active in MIC values equal to 250µg/ml gave (9) *A. baumannii* isolates; while in MIC values equal to 125µg/ml gave (6) isolates table 2.

4.5 The inhibition effect of new hydantoin derivative in Sub MICs concentration on *Acinetobacter baumannii* biofilm formation

The inhibition effect of the new hydantion derivative was studied in Sub MICs concentration on *A. baumannii* biofilm formation by using microtiter plate method (MTP) which considered more sensitive and the best method in detection of bacterial adherent and biofilm formation [20].

In Table (2) the results showed the inhibitory effect of the hydantoin compound on biofilm formation for 15 bacterial isolates used in this study with variable inhibitory effect on bacterial isolates ranged from 79.72% to 98.85%. The highest inhibitory effect percentage (98.85%) has appeared on the *A. baumannii* isolate number (14) was isolated from blood followed by 97.30%, 97.01%, 96.08% from Sputum, Wounds, Burns, respectively. While, the lowest inhibitory effect (79.72%) has appeared on the isolate number (36) was isolated from blood. Whereas, results showed that there were some differences in the values of the MICs of compound (1-1) in resistant of *A. baumannii* isolates ranged from 125µg/ml to 250µg/ml. Moreover, the results showed a significant difference ($P<0.05$) between the inhibition of biofilm rate and control.

Table 2: Biofilm formation and percentage of inhibition of the hydantoin compound on *A. baumannii* isolates according to source

No. of Isolates	Sources	MIC	Biofilm formation	Inhibition biofilm	% of inhibition
1	Blood	125	0.267	0.0186	93.03
3	Wounds	250	0.268	0.008	97.01
4	Burns	250	0.269	0.051	81.04
6	Burns	250	0.230	0.009	96.08
7	Wounds	250	0.192	0.006	96.87
13	Sputum	125	0.200	0.004	98.00
14	Blood	125	0.263	0.003	98.85
17	Sputum	250	0.240	0.017	92.91
28	Wounds	250	0.207	0.012	94.20
31	Sputum	125	0.223	0.006	97.30
32	Blood	250	0.222	0.045	79.72
36	Burns	250	0.247	0.020	91.94
39	Wounds	125	0.236	0.014	94.06
42	Blood	125	0.203	0.023	88.66
49	Wounds	250	0.284	0.016	94.36
LSD value	---	---	---	0.0193 *	---
* ($P<0.05$).					

Acinetobacter baumannii is a public causative agent of nosocomial infections and it has got a pathogen of augmented clinical importance because of its remarkable capability to cause outbreaks of infections and to gain resistance to nearly all presently used antibiotics [36].The results for the antimicrobial activity of hydantion derivatives are presented that it has potential as antimicrobial agent against bacteria[37].

5- Conclusions

The results of all *A. baumannii* clinical isolates showed multidrug resistance but the resistance among Beta – lactam were more than among other antibiotics and the antimicrobial activity for new hydantoin derivatives showed great improvement of activity of these compounds against *A. baumannii* biofilm formation.

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تأثير مشتق (مركب) الهدينتوينات الجديدة على بكتريا *Acinetobacter baumannii* المكونة للغشاء الحيوي والمعزولة من مصادر سريرية

الخلاصة

تضمنت هذه الدراسة حوالي ٥٣٨ عينة سريرية من الدم, الجروح, الحروق, القشع والادرار وقد جمعت في اربع مستشفيات. تم عزل وتشخيص (٥٢) عزلة من الـــ*Acinetobacter baumannii* وكانت ٣٦.٥٤% من عينات الدم, بينما شكلت عينات الجروح ٢٣.٠٨%, عينات الحروق ١٧.٣١%, عينات القشع ١٣.٤٦% واقل نسبة ظهرت كانت في عينات الادرار ٩.٦٢%. جميع العزلات البكتيرية شخصت بواسطة القحوصات الكيموحيوية, الخصائص الميكروبية والزرعية واكدت بواسطة نظام الفايتك. وجد ان جميع العزلات البكتيرية (١٠٠%) كانت مقاومة لمضادي amoxicillin-clavulanic acid وCefepime, بينما كانت مضادات Imipenem وMeropenem الاكثر فعالية. اظهرت العزلات القابلية على انتاج الغشاء الحيوي بواسطة استخدام طريقة اطباق العايرة الدقيقة Microtiter plate (M.T.P) , كان العدد الكلي للعزلات المنتجة للغشاء الحيوي بقوة (١٥) عزلة ٤٥.٤٦% والمتوسطة (١٨) عزلة ٥٤.٥٤% بينما كانت (١٩) عزلة ٣٦.٥٣% ضعيفة او غير منتجة للغشاء الحيوي. تم اجراء اختبار التركيز المثبط الادنى (MIC) لمشتقات الهدنيتوينات الجديدة ضد (١٥) عزلة وكانت قيمة MIC لتسعة عزلات من*A. baumannii* مساوية 250µg/ml, بينما كانت ستة عزلات قيمة الـــMIC لها 125µg/ml. اظهرت النتائج التأثير المثبط لمركب الهدنيتوينات على (١٠) عزلة بكتيرية منتجة للغشاء الحيوي المستخدمة في هذه الدراسة مع معدل تاثير تثبيطي متغاير على العزلات البكتيرية تراوح من 79.72% الى 98.85%, اعلى نسبة تأثير تثبيطي كانت 98.85%.

الكلمات الدالة: راكدة بومانية, مشتقات الهدنتوينات, الغشاء الحيوي, المضادات الحيوية.