Antimicrobial Activity of Non-bond Colicin on Candida albicans Biofilm

Nayarah S. Hussain¹, Ismail I. Latif¹, Hind H. Obaid²
¹Microbiology Department, College of Medicine, Diyala University, Diyala, Iraq
²Biology Department, College of Science, University of Baghdad, Baghdad, Iraq

Received: 22/10/2019 Accepted: 17/12/2019

Abstract

Two hundred fifty mid-stream urine specimens were collected from Baqubah Teaching Hospital and Al-Batool Teaching Hospital from patients with urinary tract infections (UTI). Of these investigated urine specimens, 66 (26.4%) specimens showed positive growth culture of Gram-negative bacteria. From these, Escherichia coli was the most prevalent bacteria of the examined culture (41, 62.12%). Additionally, the cup assay was used to determine colicin producers while the most efficient colicin producers were estimated by the formation of larger inhibition zone. Approximately half of the investigated E. coli isolates (20, 49%) was colicin producers. Colicins was extracted after induction by mitomycin-C showed a concentration of 3020 μg/ml, as estimated utilizing the Lowry method, while its activity was 80 U/ml. Our study results showed that colicin had significant antibiofilm activity (P≤ 0.05) against Candida albicans and the effect seemed to be concentration dependent. However, the values of biofilm inhibition varied depending on the different tested isolates. The biofilm of isolate 5 showed the most significant inhibition (P≤ 0.05) by colicin with a value of 46%, while isolate 3 was less affected with an inhibition rate of 19% at the concentration of 2500 μl/ml.

Keywords: Escherichia coli, Colicin, Candida albicans, Antibiofilm.

الفعالية الضد مايكروبية للكولسين الغير مرتبط على الاغشية الحيوية للمبيضات البيضاء

نيره سامر حسن*, إسماعيل إبراهيم لطيف, هند حسين عبيد²
¹أفرع الاحياء المجهرية, كلية الطب, جامعة ديالى, ديالى, العراق
²قسم علوم الحياة, كلية العلوم, جامعة بغداد, بغداد, العراق

الخلاصة

تم عدل 250 عينة ادرار من الاشخاص المصابين بالتهاب المجاري البولية من مستشفى بعقوبة التعليمي و مستشفى البئول التعليمي. وجدت ان هناك 66 عينة ادرار (26.4%) من 250 عينة لديها نمو بكتيري للعزلات السالبة لصيغة كرام. الاشريكيه الفولونية كانت أكثر العزلات انتشارًا مقارنة بالعزلات الأخرى، حوالي 41 عزلة (62.12%) من العزلات السالبة لصيغة كرام. تم اختيار طرق الامتصاص الثابتة للكولينين من خلال قياس قطر منطقة تثبيط النمو. وجد أن العزلات المنتجة للكولينين كانت أقل قابلية من العزلات الغير منتجة له (ي) بنسبة 49%. بعد عملية استخدام الكولينين تم تحديد تركيز الريوتي الكولينين و بقيمة 3020 مايكروغرام/مل باستخدام طريقة لوري و كذلك تم تقييم فعاليه الكولينين و بقيمة 80 وحدة/مل باستخدام طريقة الخضر. من خلال هذه الدراسة وجد ان الكولينين الخام له تأثير مثبط لتكوين البايوفرم المنتج من قبل

*Email: nayarasamer@yahoo.com
Introduction

*Escherichia coli* is one of the most common pathogens, which causes a wide spectrum of diseases within and outside the intestinal tract [1]. Extraintestinal pathogenic *E. coli* (ExPEC) is the main causative agent of urinary tract infection, enteritis, septicemia and other infections such as neonatal meningitis [2]. One of its key pathogenicity features is the production of bacteriocins [3]. Bacteriocins are ribosomal synthesized antimicrobial peptides that have the ability to kill or inhibit the growth of other strains, without damaging the producing bacteria due to having specific immunity proteins [4]. These peptides are different in many features like molecular mass, the existence of post-translational modifications, mechanisms of bacteriocins release from producer cells, and others [5]. Genes of bacteriocin biosynthesis are clustered and encoded on plasmids, chromosome and/or transposons [6]. It is believed that the killing mechanism of colicins produced by *E. coli* can be accomplished by pore formation in the inner membrane of the target cell and degradation of intracellular components such as DNA and RNA [7].

*Candida albicans* is a dimorphic fungus that may be found as commensal in the oral cavity of healthy people, but it also causes recurrent, severe and even lethal systemic infections [8]. It is thought that the rising rate of immunocompromised patients could lead to increase the risk of candidiasis [9]. *C. albicans* can infect skin, mouth, throat and blood [10]. This may be attributed to the possession of many virulence factors that help *C. albicans* to infect the host. *C. albicans* virulence factors include polymorphism, adhesins and invasions, hydrolases, germ tube formation and biofilm [11]. Biofilm is a population of microorganisms attached to the solid surfaces and embedded in extracellular polymeric substances (EPS) that are composed of proteins, carbohydrates, and nucleic acids. Microorganisms usually produce these EPS matrix in a complex structure, which is comparable to honeycombs of the hive, that supports them as a mechanical defense and resistance against antimicrobials [12]. Patients can acquire infection due to the presence of biofilms on hospital equipment and medical devices, eventually leading to persistent infections [13].

There are several reasons to analyze the effect of colicin as antimicrobial against candida, such as the appearance of antimicrobial resistance candida and the side effects of these drugs as well as the spread of drug resistant biofilms.

Materials and methods

Isolation and identification of bacterial and fungal isolates

Mid-stream urine specimens were collected from Baqubah Teaching Hospital and Al-Batool Teaching Hospital from patients clinically diagnosed with urinary tract infections (UTI). The isolates were identified utilizing microscopic examination. Morphological features of the colonies and biochemical tests were conducted according to Brenner and Farmer [14] as well as by using chrome agar and Vitek-2 system.

*C. albicans* isolates were obtained from oral swabs from patients with renal impairment. *C. albicans* isolates detection was confirmed by forming germ tubes [15] and by Vitek-2 system.

Detection of colicin-producing isolates

Colicin-producing *E. coli* were detected using cup assay [16]. The most efficient producers showed the largest inhibition zone and the feature of the stability of bacteriocin production.

Extraction of crude non-bound colicin.

Previously incubated 2.5ml of nutrient broth with the selected colicin producer were added to sterile nutrient broth supplied with 5% of glycerol and then incubated for 14h at 37 °C. After the addition of 2μg/ml of mitomycin-C, they were incubated in an incubator shaker for 3 hrs then centrifuged at 5000 rpm for 30 min using refrigerated centrifuge. The non-bound colicin in the supernatant was separated from the cells. To eradicate the remaining cells, chloroform was added. To confirm the bacterial clearance, the supernatant was cultured on brain heart infusion. The activity of
coliicin was detected by using the well method [17] and colicin concentration was estimated by Lowry method [18,19].

Biofilm formation of C. albicans

After incubation of C. albicans isolates on sabouraud dextrose broth, they were diluted by sterile broth at the ratio of 1:20. Each well of the 96-well flat microtiter plates were filled with 200 μl of these isolate suspensions. Sabouraud dextrose broth was also used as negative control in separate wells of the 96-well flat microtiter plate. Experiments were performed in triplicates in which the plates were incubated for 48hrs at 37 °C. Following the incubation, the medium and the unbound cells were removed; the wells were washed by Phosphate Buffer Saline (PBS) then left to dry for 15 mins. 200 μl of Crystal Violet (CV) were added to each well and left for 20 mins. CV was removed and the plates were washed by PBS three times then left to dry at room temperature. 200 μl of solution of acetone: ethanol (20:80) was added to the wells and left for 10 min. Plate reader apparatus (Biotek/ USA) was used for reading the results at 450 nm. The optical density (OD) values were estimated, where the values > 0.320, 0.120 – 0.320, and < 0.120 were considered as reflecting strong, moderate, and weak reactions, respectively [20].

The inhibition of C. albicans biofilm formation

The suspensions of C. albicans were inoculated to 96- flat well microtiter plates previously inoculated with colicin at Minimum Inhibitory Concentration (MIC) concentrations. After 48hrs of incubation, the wells were washed by PBS then left to dry. 200 μl of CV was added for 20 min. CV was removed and the plates were washed by PBS and left to dry. 200 μl of acetone: ethanol solution (20:80) was added to the wells and left for 20 min, then the result was read by the plate reader [21, 22]. Biofilm inhibition was calculated using the below equation [23]:

\[
\text{Inhibition of biofilm formation}\% = \frac{\text{OD control} - \text{OD treatment}}{\text{OD control}} \times 100
\]

Results and discussion

Isolation and identification

Out of 250 urine samples, 66 (26.4%) isolates had bacterial growth, as shown in Figure 1. E. coli was the most prevalent Gram-negative bacteria in UTI samples, which was recorded in 40 (60.60%) of the 66 Gram-negative isolates (Figure 2).

![Figure 1](image1.png)  ![Figure 2](image2.png)

**Figure 1** - The percentage of bacterial growth with positive cultures from UTI isolated from UTI patients.

**Figure 2** - The percentage of E. coli isolates among other G- bacteria urine specimens

In this respect, a previous study reported that the prevalence of E. coli (38.90%) was higher than the other bacteria in UTI cases [24]. In this regard, several conditions may affect the prevalence of bacteria among patients. These may involve environmental, health, social, and cultural conditions of patients. In addition, the technical mistakes for isolation and identification of bacteria may give inconsistency in the reported findings[25].

Detection of colicin-producing E. coli by cup assay

By the detection of the inhibition zone using cup assay, we observed that 20 E. coli isolates (49%) were colicin producers (Figure 3) and the most effective isolate had the larger inhibition zone.
However, this result seems relatively different from the findings of a recent research [26] which illustrated that *E. coli* was the most colicin producing bacterial isolate which was produced by 36 (30.77%) out of 117 *E. coli* isolates. These differences occur due to several reasons such as the components of the used media [27] and the different methods used for the detection of colicin production [28]. Another study reported that the use of the cup assay and the addition of 5% glycerol gives robust results for the detection of bacteriocin producers [29].

![Graph of cup assay of colicin producing *E. coli* isolated from UTI patients.]

**Figure 3**-Percentage of cup assay of colicin producing *E. coli* isolated from UTI patients.

**Biofilm formation of *C. albicans***

Biofilm formation of *C. albicans* was assessed by the microtiter plate method [20]. The isolates 2 and 5 were found to form strong biofilms and their optical density values were 0.36 and 0.337, respectively, while moderate biofilms were found to be formed by the others isolates, with optical density ranged from 0.249 for isolate 1 to 0.313 for isolate 7 (Table 1).

**Table 1-**Values of Tissue Culture Plate (TCP) method of biofilm formation of *C. albicans* isolated from oral swabs of patients with renal impairment

| *C. albicans* isolates | OD   | Estimation of biofilm formation |
|------------------------|------|--------------------------------|
| C1                     | 0.249| Moderate                       |
| C2                     | 0.36 | Strong                         |
| C3                     | 0.269| Moderate                       |
| C4                     | 0.278| Moderate                       |
| C5                     | 0.337| Strong                         |
| C6                     | 0.276| Moderate                       |
| C7                     | 0.313| Moderate                       |
| C8                     | 0.312| Moderate                       |

An earlier study showed that *C. albicans* colonizing the oral cavity can form a biofilm on saliva coating areas [30]. Consistence with our finding, Udayalaxmi and Shenoy reported that 45.83% of Candida species were strong or moderate biofilm formers while the percentage of isolates that produce weak biofilm in their study was 54.16% [31].

**Inhibition of biofilm formation by non-bond colicin**

The results of biofilm inhibition were dependent on the concentration of colicin and the type of indicator *E. coli* isolate. This was evident when the higher concentrations of colicin led to significantly (P ≤ 0.05)increased inhibition of biofilm; . Thus, the relation between the extracted colicin concentration and the biofilm inhibition is inversely proportional in the tested *C. albicans isolates*.
The results demonstrated in Table-2 show that all C. albicans isolates were inhibited significantly ($P \leq 0.05$) by colicin, but their sensitivity was variant depending on the isolates. The isolates 1 and 5 were the most sensitive to colicin at the concentration 2500 µg/ml, with inhibition of biofilm values of 43% and 46%, respectively. At the same concentration, isolate 3 was the less sensitive isolate that showed a value of biofilm inhibition of 19%. There are some explanations of colicin action on the biofilm of microorganisms. Some bacteriocins act by disrupting the co-aggregation process of the membranes which is important for biofilm stability; thus it decreases biofilm development by reducing its biomass and thickness [32].

Other bacteriocins cause pore formation that results in an efflux of ATP from biofilm cells. The size of pores has to be larger than 1.5 in diameters which is enough to cause efflux of ATP [33]. Moreover, some bacteriocins have the ability to suppress biofilm genes such as atl (autolysin) and ica (intercellular adhesin) such as bacteriocin gallidermin [34].

Furthermore, ColA-43862 produced by Citrobacter freundii is known to have anti-biofilm activity, but it may not act as a limiting factor due to the complication of biofilm and its microenvironment that act as a barrier of colicins action [35].

An earlier study reported that a bacteriocin of Lactobacillus acidophilus had remarkably reduced biofilm cells of catheter-associated multidrug-resistance Pseudomonas aeruginosa. This bacteriocin can act as an alternative for antibiotics that hardly eliminate biofilm of P. aeruginosa [36]. A recent study reported that a bacteriocin of Bacillus subtilis (subtilocin) caused biofilm inhibition of Gardnerella vaginalis, with an inhibition value higher than 90%, however, it did not decrease the growth of planktonic cells. Also, it remarkably inhibited the biofilm of E. coli and L. monocytogenes. Inhibition of biofilm by these bacteria occurs because of their ability to inhibit the quorum sensing (QS) [37]. Bacteriocin EntV of Enterococcus faecalis has a reduction activity on virulence factors of C. albicans without affecting the viability of cells. It blocks hypha formation, which results in preventing biofilm formation as well as reducing inflammation and invasion of the epithelium by candida in marine models [38].

**Conclusions**

Biofilm of C. albicans was significantly inhibited ($P \leq 0.05$) by crude non-bond colicin and the inhibition effect was more evident at high concentrations of the extracted colicin. However, the biofilm inhibition effect of the E. coli extracted colicin seemed to be C. albicans isolates-specific as some isolates were more sensitive to colicin while others were less affected.

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Table 2-Inhibition of biofilm formation by non-bond colicin extracted from E. coli isolated from UTI patients.

| Concentration of colicin µg/ml | C1  | C2  | C3  | C4  | C5  | C6  | C7  | C8  | LSD value |
|-------------------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----------|
| 19.53                         | 30  | 3   | 1   | 21  | 12  | 6   | 6   | 5.72*   |
| 39.06                         | 33  | 7   | 16  | 27  | 20  | 6   | 6   | 5.36*   |
| 78.125                        | 33  | 5   | 16  | 34  | 22  | 7   | 9   | 5.09*   |
| 156.25                        | 35  | 18  | 17  | 36  | 25  | 18  | 19  | 4.66*   |
| 312.5                         | 39  | 21  | 10  | 36  | 29  | 23  | 20  | 5.83*   |
| 625                           | 39  | 29  | 16  | 30  | 43  | 33  | 29  | 6.01*   |
| 1250                          | 39  | 29  | 16  | 30  | 43  | 33  | 29  | 6.01*   |
| 2500                          | 39  | 29  | 16  | 30  | 43  | 33  | 29  | 6.01*   |

* (P<0.05).

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