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ORIGINAL ARTICLE

In Vitro activity of EDTA, Kojic Acid and their combination against biofilm forming microorganisms causing Catheter associated Urinary tract Infections 2019

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ABSTRACT

Background*: It has been found that 80% of microbial infections are associated with biofilm formation. For example, urinary tract infections, middle-ear infections, formation of dental plaque, endocarditis and cystic fibrosis, biofilms are also found to be associated with indwelling medical devices such as contact lenses, central venous catheters and mechanical heart valves.* ***Objectives****: The aim of this study was to evaluate the ability of the isolated organisms causing CAUTIs to form biofilm, as well as the effect of EDTA, Kojic acid and their combination on inhibiting their biofilm formation.* ***Methodology****: The bacterial isolates were tested for their ability to form biofilm by Inoculum preparation, Washing, Fixation, Staining and Interpretation of the results by detecting their optical density (OD) of each well stained with crystal violet and measured at 620 nm using a Micro titre plate reader (Tecan. Infinit F50).* ***Results:*** *In this study the most frequently biofilm forming species was Pseudomonas 5(21.7%), while the least biofilm forming isolates were Enterococcus fecalis 2(8%). EDTA was the most powerful biofilm inhibitor as it inhibited biofilm by 78.6% with mean optical density reading 0.22 ± 0.15, EDTA and Kojic acid combination had nearly close results on biofilm as it decreased biofilm by 72.8% with mean optical density reading 0.28 ± 0.17, while Kojic acid decreased biofilm formation by 51.4% only with mean optical density reading 0.5 ± 0.31.* ***Conclusion:*** *EDTA was the most powerful biofilm inhibitor, EDTA and Kojic acid combination had nearly close results on biofilm to EDTA, while Kojic acid had the least effect on biofilm inhibition.*

INTRODUCTION

Device associated infections account for the majority of nosocomial infections, whereas, 95% of nosocomial urinary tract infections are catheter associated, 86% of nosocomial pneumonias are caused by mechanical ventilators, and 87% of nosocomial bloodstream infections are caused by intravascular devices [1].

Biofilm formation on the urinary catheter accounts for the persistence of CAUTIs, its formation starts with the deposition of naturally occurring urine components such as proteins and electrolytes [2].

Electrostatic charges on both bacterial and catheter surfaces contribute to the reversible attachment of aggregated microbial cells; these charges are influenced by the presence of divalent cations including Mg2+ and Ca2+. [3].

Hydrophobicity of the catheter surface has a great impact on the reversible cellular adhesion of microorganisms through Van der Waals forces [4].

Quorum sensing molecules produced by aggregated microorganisms when reach a threshold level, they stimulate extracellular matrix secretion forming the mature biofilm structure[5]. Changes in biofilm environment such as changes in urine Ph, electrolytes and nutrient concentration result in the detachment of Planktonic cells that become component of urine [6].

Freed Planktonic cells could further travel along with the blood stream or urinary system, then spread infections elsewhere in the body [7]. Control of Biofilm formation could be done by many ways as, limiting the use of urinary catheter only when needed and rapid removal of the catheter when it's not needed anymore [8]. Catheter material has a critical role in the frequency of biofilm formation [9]. Bacterial interference is defined as the use of nonpathogenic microorganisms to combat pathogenic microorganisms [10].

Quorum sensing inhibitors have been used to inhibit biofilms, as the pyrimidinone compound inhibits biofilm formation besides disrupting and removing deposited biofilm [11]. EDTA is a metal chelator used in the treatment of patients with heavy metal poisoning like mercury and lead, it is also used as an anticoagulant by chelating magnesium and calcium ions needed for coagulation enzymes [12]. It exerts its antimicrobial effect by chelating Mg^{2+} and Ca^{2+} ions from LPS in the outer cell wall of Gram-negative bacteria, which releases 50%

of the LPS, thus making the phospholipids of the inner membrane exposed, and enhancing the susceptibility to various antimicrobials [13].

EDTA has a wide range of antimicrobial inhibitory effect on various organisms including Gram positive bacteria, Gram negative bacteria, yeasts, amoeba and fungi. Metal ions chelation by EDTA causes weakening of the planktonic *Staphylococcus aureus* [14]. It has a great impact on biofilms produced by Uro pathogenic *E.coli*, as it inhibits the production of curli which is fundamental for bacterial adhesion and biofilm formation [15].It has been reported that EDTA can cause *Pseudomonas* biofilm dispersal [16]. EDTA inhibits the production of hyphae by candida albicans [17], and also inhibits Ca2+-calmodulin interaction and calmodulin-mediated protein phosphorylation which play a role in the development of hyphae and germ tube formation by *C. albicans* [18].

Kojic acid (KA) is a natural pyrone produced by certain filamentous fungi, mainly species of *Aspergillus* and *Penicillium*, and has no obvious side effects. It is produced by the fermentation of soy sauce, sake and rice wine, and acts as an effective antifungal agent, either in their original forms or when combined with other compounds producing highly efficient derivatives[19].

It is used as a natural antioxidant safely and used as a skin lightener without being toxic to the human genome [20]. It is a powerful iron chelator, as iron is an essential element for bacterial growth; its scavenging by iron chelating agents would control infection [21]. Various KA derivatives have been developed and also have an antimicrobial activity against many microorganisms[22]. Kojic acid is proved by an experiment to inhibit biofilm formation on glass slides, as it interferes with quorum sensing dependent upon the Lux system [23]. KA causes the leakage of the enzyme (β-galactosidase) which has an important role in maintenance of cell wall integrity, leading to the leakage of potassium from the cytosol and the loss of the zeta potential of cell, which is the electrical potential of the interface between the aqueous environment and the attached bacterial cells layer, causing distortion of the proteins concerned with cell division, hence leading to the bacterial inactivation [24].

METHODOLOGY

This study was conducted during the period from August 2017 till January 2018 on 84 patients from Intensive care units of Ain Shams University hospitals, from which 52 isolates causing CAUTIs were obtained. An informed oral consent was obtained from each patient and the committee of ethics has approved the research. Urine samples from catheters were collected from patients proved to suffer from catheter associated urinary tract infections according to Centers for Disease Control and Prevention[25]. Samples were collected aseptically below the connection junction of the catheter and drainage bag tubing by rubbing the catheter tube with alcohol 70% and urine samples were taken by a syringe and poured in a sterile urine sampling cups[26].Microbiological processing of urine samples was carried out according to Forbes et al [27]. Urine cultures were considered positive if there were $\geq 10^5$CFU/ml of pure isolates for maximum of two types of organisms, more than two organisms in the culture were considered to be contaminated .Pure colonies were identified by colonial appearance, microscopy, culture and biochemical techniques.

Testing of Biofilm formation:

The isolates were tested for their ability to form biofilm[28] utilizing the following steps:

Inoculum preparation

- Three to four colonies were suspended in tryptic soy broth and incubated without shaking for 18 hrs at 37^{o}C.
- After incubation, the turbidity of the bacterial suspension was adjusted to obtain turbidity optically comparable to that of the 0.5 McFarland standard (~10^8 CFU/ml) then it was diluted 1:100 in TSB + 1% glucose and inoculated into microtitre plate (200 µL per well).
- Every isolate was tested in 3 different adjacent wells. The wells which are not inoculated were filled with 200 µL of TSB + 1% glucose as negative control to be used as a spectrophotometric blank.
- The inoculated plate was covered with a lid and incubated aerobically for 24 hrs at 37°C under static conditions.

Washing

- After incubation, the contents of the wells were decanted into a discard container.
- Each well was washed three times with 300 µL of sterile saline.
- Following every washing step, the wells were emptied by flicking the plate.
- Prior to fixation of the biofilm, the plates were drained in an inverted position.

Fixation

- After washing, the remaining attached bacteria were heat fixed in oven at 60 degrees for one hour.

Staining

- The adherent biofilm layer formed in each MTP well was stained with 150 µL of crystal violet (2% Hucker crystal violet) for 15 min at room temperature.
- After staining, the stain was aspirated with a pipette and excess stain was rinsed off by placing the microtitre plate under running tap water. Washing was continued until the washings were free of the stain.
- After the microtitre plate was air dried at room temperature, the dye bound to the cells was resolubilized with 150 µL of 95% ethanol per well.

Ethanol was gently added and thereafter the microtitre plate covered with the lid (to minimize evaporation) and left at room temperature for 30 min without shaking (Figure 1).

Interpretation of the results

- The optical density (OD) of each well stained with crystal violet was measured at 620 nm using a MTP reader (Tecan. Infinit F50).
- The average OD values were calculated for all tested strains and negative controls. The cut-off value (ODc) was established. It is defined as three standard deviations (SD) above the mean OD of the negative control: ODc=average OD of negative controls + (3×SD of negative controls). ODc value was calculated for each MTP separately.
- Final OD value of a tested strain was expressed as average OD value of the strain reduced by ODc value (OD = average OD of a strain - ODc).
- For easier interpretation of the results, strains were divided into the following categories:
  - Non biofilm producer OD ≤ ODc.
  - Weak biofilm producer = ODc < OD ≤ 2×ODc.
  - Moderate biofilm producer = 2×ODc <OD ≤4×ODc.
  - Strong biofilm producer = 4×ODc < OD.

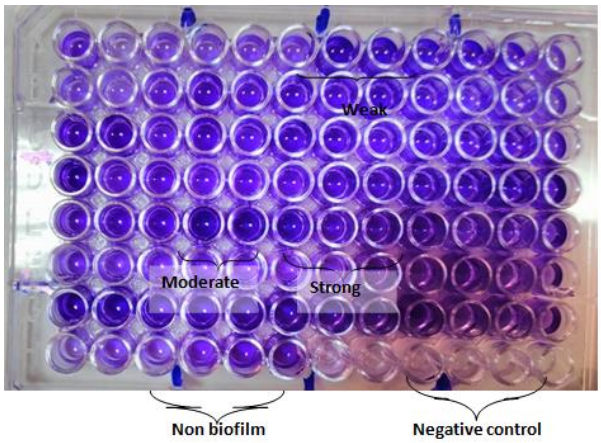

Fig. 1: Microtitreplate with different degrees of biofilm formation

Testing the effect of EDTA, kojic acid and their combination on the isolates detected to inhibit biofilm

- Aqueous solutions of EDTA, Kojic acid, provided by Techno Pharma India, and their combination were prepared, with final concentration of 8 mg/ml for EDTA [29] and 6.25 mg/ml for kojic acid concentration of 6.25 mg/ml [30].
- In order to reach the final required concentration for each anti-biofilm agent used after adding the inoculated broth, double the concentration was prepared for EDTA 16 mg/ml and for kojic acid 12.5mg/ml.
- 100 μl of inoculated broth was added with 100 μl of prepared aqueous solution of anti-biofilm agents, EDTA, kojic acid and their combination, each was tested in 3 wells in microtitre plate, the total volume of each well as made up to 200 μl.
- Each of the 23 isolates were inoculated in 6 separate wells, 2 wells with adding Kojic acid solution, 2 wells with adding EDTA solution and the last 2 wells with the combined solution of EDTA and kojic acid.
- The plates were incubated for 24 hours (Figure 2) at 37 degrees, then washed by isotonic saline, fixed by heating and stained with crystal violet (Figure3) as mentioned before then the amount of bound crystal violet in each well was measured with spectrophotometer at OD_{600}.

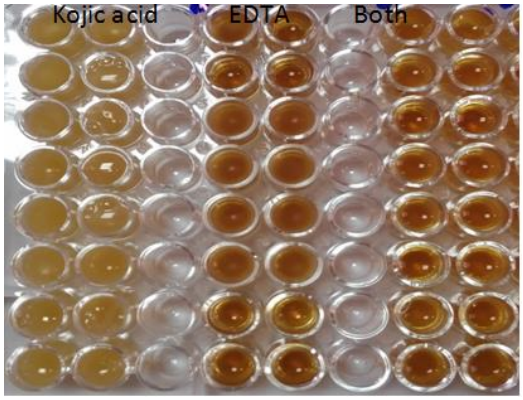

Fig. 2: Showing inoculated plate after 24 hours incubation

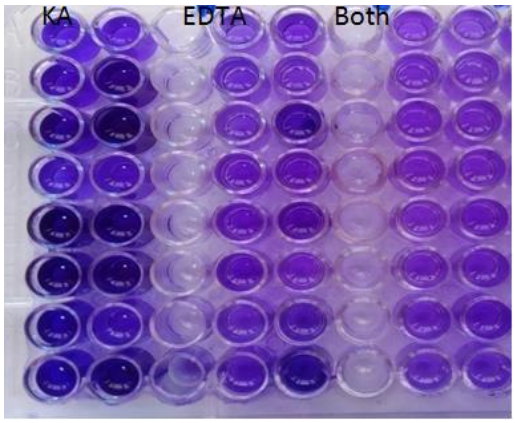

Fig. 3: Showing plate after staining with crystal violet

Statistical Analysis:

Statistical analysis was done on a personal computer using the Statistical Package for Social Sciences (SPSS) version 17 as follow:

- ***Descriptive statistics:***
  - Mean and SD for quantitative data.
  - Frequency number and percentage for qualitative data.
- ***Analytical statistics:***
  - Paired t-test used to compare between related samples (pre and post).
  - Level of significance considered at 0.05 i.e.:
    - P value >0.05 non-significant.
    - P value ≤0.05 significant.
    - P value ≤0.01 highly significant.

RESULTS

This study was conducted during the period from August 2017 till January 2018 on 84 patients from Intensive care units of Ain Shams University hospitals. 52 out of 84 had catheter associated urinary tract infections. The 52 CAUTI cases were included in this study. Most of the samples were collected from the surgical ICU 37(71.2%), followed by Internal Medicine ICU 11(21.2%) and the Geriatric ICU 4 (7.7%).The most common organism isolated was Candida 18(34.6%), followed by *E.coli* 9 (17.3%) and Pseudomonas 6(11.5%), while least isolates were Enterococcus fecalis 2(3.8%). Out of the 52 isolates, 23(44.2%) were biofilm forming while non-biofilm isolates were 29(55.8%).Among the 23 biofilm forming isolates 2 (8.7%) were weak biofilm forming, 4 (17.4%) were moderate biofilm forming while most of the isolates 17(73.9%) were strong biofilm forming.

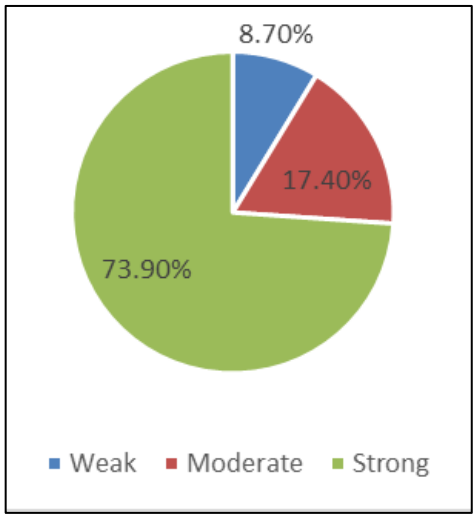

Fig. 4: Showing percentage of biofilm formation strength.

There was a statistically significant correlation between the duration of hospital stay and biofilm formation (P value <0.01), but there was no statistically significant correlation between the departments from which the samples were obtained and biofilm formation (P value: 0.8).The mean optical density reading OD± SD among biofilm forming isolates was 1.03±0.3. EDTA was the most powerful biofilm inhibitor as it inhibited biofilm by 78.6% with mean optical density reading 0.22 ± 0.15, EDTA and Kojic acid combination had nearly close results on biofilm as it decreased biofilm by 72.8% with mean optical density reading 0.28±0.17, while Kojic acid decreased biofilm formation by 51.4% only with mean optical density reading 0.5 ± 0.31. The tested biofilm inhibitors gave statistically relevant results (P value <0.001) in biofilm inhibition.

Table 1: The effect of EDTA, Kojic acid and their combination on the 23 biofilm forming isolates.

	Optical denisty Reading		
	Kojic acid	**EDTA**	**EDTA and Kojic acid combination**
Without	1.03±0.3		
With	0.5±0.31	0.22±0.15	0.28 ± 0.17
Percentage of inhibition	51.4%	78.6%	72.8%
P Value	<0.001	<0.001	< 0.001

DISCUSSION

Catheter associated urinary tract infections is considered one of the most serious infections among patients admitted in intensive care units. Pathogens encountered in CAUTIs can form biofilms that make them highly resistant to antibiotics. Novel strategies as catheter coating with antibiofilm agents were developed instead of using prolonged antibiotic treatment to avoid attachment of the microorganisms.

Metal ions such as iron, magnesium and calcium play an important role in biofilm formation and stabilization; thus, using metal chelators to coat urinary catheters inhibit biofilm formation on urinary catheter lumen, examples of these chelators are EDTA and Kojic acid. Our study showed that there was a statistically significant correlation between biofilm formation by organisms causing CAUTIs and the duration of catheterization, this was relevant in the study carried out by Ding et al[31] who stated that there is increased incidence of CAUTIs and biofilm formation with prolonged catheterization. Similar results were obtained from the study carried out by Patil et al,[32] where it was noted that after 7 days of catheterization 100% of patients included in the study had CAUTIs, by the 5th day 84% incidence of CAUTIs was 84.61%, on 3rd day catheterization the incidence was 61.54 while on one day catheterization the incidence dropped to 10.77%.

Regarding the isolated organisms the most frequently isolated organism was *Candida* 18(34.6%). Followed by *E.coli* 9(17.3%), *Staph aureus* 6(11.5%), *Pseudomonas spp.* 6(11.5%), Proteus 6(11.5%),

Klebsiella species (9.6%) and Enterococcus 2(3.8%). This was the same in the study done by Keten et al[33] where *Candida* isolates were 34.7%, followed by *E.coli* (20.6%), *Pseudomonas spp.* 14%, *Klebsiella* (9.9%).

On the other side Patil et al[31] reported different results where *E.coli* was the most isolated organism (30.76%) of the isolated organisms, followed by *Pseudomonas* (26.15%), *Klebsiella* (23.07%), Proteus (16.15%), Candida (3.07%), and enterococcus (7.61%) [31], another study carried out by Mahmoud et al[34] where the most common isolated organism causing CAUTI was *Staph aureus* (23%), followed by *Enterobacter cloacae* (18%), *E.coli* (18%), Klebsiella pnemonae (14.7%), other species (26.7%).In our study, (44.2%) of the isolates were non-biofilm forming, while biofilm formation occurred among (55.8%) of the isolates and most of them were strong biofilm forming 17(73.9%), (17.4%) were moderate biofilm forming and (8.7%) were weak biofilm forming.

Similar results were obtained from several studies performed on biofilm forming organisms isolated from urinary catheters; where Abdalla et al[35] reported higher percentage in biofilm formation among CAUTIs isolates which was (59.1%) from which (81.8%) were weak biofilm producers while (18.2%) were moderate biofilm producers, while Maharjan et al[36] reported less biofilm production among isolated organisms which was (46%) from which (23.3%) were strong biofilm forming, (30%) were moderate bioflm forming and (46.6%) were weak biofilm forming.

In our study we used EDTA which is a potent metal chelator used in a wide variety of medical purposes. It seemed to have the most effective antibiofilm activity, as it has been tested with the concentration of 8 mg/ml and it inhibited biofilm formation among CAUTI isolates by 78.6%, optical density readings of the 23 biofilm forming isolates were changed from 1.03±0.3 to 0.22 ± 0.15 (P value < 0.001).Other studies were done on central venous lines as the study carried out by Percival et al[37] who tested the effect of EDTA on organisms encountered in Central venous catheter (CVC)-related bloodstream infections; *Staphylococcus epidermidis*, methicillin-resistant *S. aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Candida albicans*, results revealed that the use of EDTA at concentration of 40-mg/mL−1 significantly reduced biofilm formation (P value<0.05)[37].

EDTA was most effective in inhibiting biofilms formed by *E.fecalis* as biofilm formation significantly decreased with the use of EDTA by 95% (P value 0.03). EDTA was tested in other studies for their ability to inhibit *E.fecalis* biofilms formed in root canals, the study carried out by Estreka et al[38] revealed that EDTA was capable of chelating iron, calcium and manganese ions disrupting the biofilm structure leading to the death of microbial cells within the biofilm [38], another study carried out by Rosen et al[39] performed a comparative study testing EDTA and other metal chelators like sodium hypochloride for their ability to inhibit *E.fecalis* biofilms. EDTA significantly inhibited both *E.fecalis* planktonic cells and biofilm formation.

In this study the antibiofilm action of Kojic acid wasn't as strong as EDTA; as Kojic acid when used with the concentration of 6.25 mg/ml, it decreased biofilms by 51.4%, optical density readings changed from 1.03±0.3 to 0.5 ± 0.31 and the results were statistically significant (P value < 0.001).

Kojic acid was most effective against biofilms formed by Klebsiella spp., as it inhibited biofilms by 86% (P value 0.02), *E.fecalis* biofilms decreased by 81% (P value :0.05) and, Kojic acid decreased biofilms formed by Pseudomonas by 58.3% which was statistically significant (P value: 0.007). Çevik et al[40] tested the effect of Kojic acid on Pseudomonas isolates with concentration of 2 mM which decreased Pseudomonas biofilms by 18-27%, while Banin et al[41] tested the effect of Kojic acid on Pseudomonas isolates and was found to decrease biofilms by 27%.

Kojic acid in combination with EDTA gave better results than using Kojic acid alone, but using EDTA alone gave better results than when combined with Kojic acid. EDTA and Kojic acid didn't seem to have a synergistic effect on inhibiting biofilms by most of the tested organisms except for Pseudomonas; as their combined effect inhibited Pseudomonas biofilms by 72.2% although statistically was insignificant (P value 0.12).Upon search no further studies were found to compare the effect of EDTA and kojic acid combination.

CONCLUSION

EDTA was the most powerful biofilm inhibitor, EDTA and Kojic acid combination had nearly close results on biofilm to EDTA, while Kojic acid had the least effect on biofilm inhibition.

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Conflict of interest:

- There has been no funding for this research, and there is no competing Personal financial interests in relation to the work described.
- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.
- The article is not published in other journal or a publisher

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