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Original article

Anti-biofilm effect of copper oxide nanoparticles, EDTA, and Kojic acid on MDR-biofilm forming Gram negative superbugs in Sohag University Hospitals, Egypt

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ABSTRACT

Background: Multidrug-resistant (MDR) Gram negative superbugs are considered among the most important causes of healthcare associated infections and these resistant isolates are usually present in association with biofilms rather than planktonic existence. **Objectives:** This study aimed to assess the ability of EDTA, Kojic acid, and CuONPs to inhibit the phenomenon of biofilm formation by multidrug-resistant Gram-negative superbugs. **Methodology:** Gram negative bacilli were isolated on suitable media and identified biochemically by vitek-2 biochemical identification system. MDR isolates were tested for biofilm formation by tissue culture plate method, biofilm-producing isolates were exposed EDTA and Kojic acid by certain concentration and to copper oxide nanoparticle (CuONPs) by multiple gradual concentrations and re-tested for biofilm-production by the same method. **Results:** EDTA and Kojic acid reduced the biofilm-formation ability of the tested isolates by 85.4 % and 65.5% respectively with reduced mean optical density (OD) reading to (0.0267± 0.051) and (0.063± 0.108) respectively with a high statistically significant difference (p<0.0001). A concentration-dependent biofilm-inhibition effect was observed with different concentrations of CuO NPs (125-2000 µg/ml) for all the tested isolates regardless the genus and the species. **Conclusion:** there was a remarkable biofilm-inhibition effect for the three tested non-antibiotic substances; the use of these substances can provide a solution for the problem of difficulty in treatment of nosocomial infections due to MDR biofilm-forming Gram-negative superbugs.

Introduction

About 700,000 human deaths are attributed to multidrug-resistant (MDR) bacteria every year world-wide, it is expected to have10 million deaths by 2050 because of these infections [1]. Over the last years, there is a significant increase in the number hospital MDR bacteria, also called superbugs, particularly *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* spp [2]. More than 50% of these Gram negative bacilli (GNB) species that caused healthcare-associated infections (HAIs) have been reported to be MDR. Compared with infections due to the antibiotic-susceptible GNB counterparts, MDR-GNB infections frequently lead to poorer

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outcomes such as longer hospital stays, increased mortality, and higher hospitalization cost [3].

Antibiotic resistance is increasing in hospital settings, where a strong selective pressure encourages the selection and persistence of resistant, MDR and even pan-resistant strains [4]. Bacterial resistance to antibiotics is a consequence of a multiple phenomena from which the ability to form communities called biofilms; a matrix-enclosed aggregates of single or multiple strains of bacteria that adhere to biological or non-biological surfaces and communicate by secreting chemicals [5].

The number of antibiotics available to treat infections caused by MDR organisms is restricted and combined antibiotic therapy is recommended for the treatment of biofilm-associated infections caused by Gram-negative bacteria. These clinical issues emphasize the urgent need for new and more effective antibacterial methods [6].

Ethylene-diamine-tetra-acetic acid (EDTA) is a polyamine carboxylic acid used as a metal chelator and in low concentrations act as a food preservative and in combination with antibiotics [7]. EDTA was effective at eliminating Gram-positive, Gram-negative, and fungal species and represents a promising alternative to antibiotic treatment with less chance of the organisms developing resistance [8]. EDTA exerts its antimicrobial effect by chelating Mg2+ and Ca2+ ions from lipopolysaccharide (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 [9]. EDTA prevents curli production and inhibits bacterial adhesion which is required for the initiation of biofilm formation [10].

Kojic acid (KA) (5-hydroxy-2-hydroxymethyl-4H-pyran-4-one) is an organic acid produced by various species of fungi and bacteria; it has different biological activities such as antioxidant, anticancer, antimicrobial, and anti-inflammatory. It is a powerful iron chelator, which is an important element for bacterial growth [11]. Kojic acid is more active against Gram-negative bacteria than against Gram-positive ones, it was proved by an experiment to inhibit biofilm formation on glass slides, as it interferes with quorum sensing dependent upon the Lux system; a highly-conserved LuxR-type proteins that release many autoinducers which are compounds that accumulates in the surrounding environment during growth of bacteria to regulate cell densities under conditions of low cell or high cell density. The lux genes which encode these proteins are regulated during transcription [12].

Nanoparticles are the materials that have at least one dimension in the nanometer scale range (1–100 nm) and convey particular physical and chemical properties markedly different from those of bulk materials. The antimicrobial properties of nanoparticles made them a feasible solution to treat infectious diseases as they are able to target multiple sites in an organism makes them superior to conventional antibiotics [13]. Nano-particles can exert their antibacterial activity through many mechanisms, such as: (1) direct interaction with the bacterial cell wall; (2) inhibition of biofilm formation; (3) triggering the innate and adaptive host immune responses; (4) generation of reactive oxygen species (ROS); and (5) induction of intracellular effects leading to apotosis (e.g., interactions with DNA and/or proteins) [14].

Copper oxide (CuO) NPs generate ROS usually leads to chromosomal DNA degradation, which seems to be a “particle-specific” action [15]. When CuO NPs enter into bacterial cell metabolic functions are affected, such as active transport, electron transfer, and nitrogen metabolism [16]. The antimicrobial activity of CuONPs is comparable to that of silver NPs but at a lower cost [17].

Because the treatment of infections caused MDR biofilm- forming Garm negative bacteria becomes challenging and the need for non-antibiotic substances is mandatory as a last treatment option for infections by this category of organisms, this study was conducted to assess the ability of three different non-antibiotic chemical substances namely; EDTA, Kojic acid, and CuONPs to inhibit the phenomenon of biofilm-formation by multidrug-resistant Gram negative bacilli isolated from different types of healthcare-associated infections in Sohag University Hospitals.

Patients and Methods

This is a cross sectional study that was conducted at the Department of Medical Microbiology and Immunology, Sohag Faculty of Medicine and Sohag University Hospitals and extended along 2 years From January 2020 to January 2022. The study included 220 patients with healthcare associated infections suspected to be caused by multidrug resistant biofilm forming

organisms as catheter associated urinary tract infections, infected orthopedic implants, ventilator associated pneumonia (VAP), blood stream infections in patients have intravascular devices, infected burns, diabetic foot ulcers, infection in cystic fibrosis patients and surgical site infections (SSI).

- **Ethical approval:**

Informed consent was obtained from all patients included and the study was approved by the ethics committee of scientific research in sohag faculty of medicine.

- **Sample processing:**

Samples were obtained under complete aseptic conditions. Sterile cotton swabs were used for pus, dry sterile well-closed plastic cups for urine, sputum and endotracheal aspirate samples. Samples were centrifuged at 3000 rpm for 10 min, and the deposit was stained by Gram stain. Bacterial count was done for diagnosis of urinary tract infections by calibrated 10µl loop (presence of 10^5 CFUs per 1ml or more diagnoses UTI). All samples were enriched with nutrient broth for 24 hours at 37ºC before inoculation on MacConkey medium ***(Oxoid, UK)***, subcultures were done on eosin ethylene blue medium ***(Oxoid, UK)***, Triple Sugar Iron agar ***(Oxoid, UK)*** for differentiation of the *Enterobacteriaceae*, and Cetrimide agar: ***(Oxoid, UK)*** for identification for pseudomonas spp.

  - **Identification of the isolates at species level:**

Identification was done using automated identification system ***(Vitek-2 bioMérieux, France),*** sufficient number of colonies of a pure culture and to suspend the microorganism in 3.0 mL of sterile saline (aqueous 0.45% to 0.50% NaCl, pH 4.5 to 7.0). Identification cards were inoculated with microorganism suspensions using an integrated vacuum apparatus. All card types are *microbial identification using the bioMérieux VITEK®* incubated on-line at 35.5 + 1.0ºC. Test data from an unknown organism are compared to the respective database to determine a quantitative value for proximity to each of the database taxa. Each of the composite values is compared to the others to determine if the data are sufficiently unique or close to one or more of the other database taxa.

  - **Antibiotic susceptibility testing of isolates:**

Susceptibility of isolates to different antibiotics was tested by the disc diffusion method (Modified Kirby- Bauer method) according to the Clinical Laboratory Standards Institute (CLSI) guidelines *(CLSI, 2020)*. The used antibiotic discs were obtained from ***(Oxoid Ltd., Basingstoke UK)*** and included:

1- Enterobacteriaceae: Piperacillin (100µg), piperacillin/tazobactam (100/10µg), amoxicillin/clavulanate (20/10 µg), ceftazidime (30µg), cefotaxime (30µg), ceftriaxone (30µg), aztreonam (30µg), imipenem (10µg), meropenem (10µg), gentamicin (10µg), tetracycline (30µg), ciprofloxacin (5µg), levofloxacin (5µg), ofloxacin (5µg), norfloxacin (10µg), amikacin (30µg),trimethoprim-sulphamethoxazole 1.25/23.75µg), chloramphenicol (30µg), nitrofurantoin (300µg).

2- *Pseudomonas aeruginosa*: Piperacillin (100µg), piperacillin/tazobactam (100/10µg), ceftazidime (30µg), aztreonam (30µg), imipenem (10µg), meropenem (10µg), gentamicin (10µg), amikacin (30µg), ciprofloxacin (5µg), levofloxacin (5µg), ofloxacin (5µg), norfloxacin (10µg), colistin (10µg).

3-*Acinetobacter* spp: Piperacillin (100µg), piperacillin/tazobactam (100/10µg), ceftazidime (30µg), cefotaxime (30µg), ceftriaxone (30µg), aztreonam (30µg), imipenem (10µg), meropenem (10µg), gentamicin (10µg), amikacin (30µg), tetracycline (30µg), ciprofloxacin (5µg), levofloxacin (5µg), trimethoprim sulphamethoxazole (1.25/23.75µg).

4-*Burkholderia cepacia* complex: Ceftazidime (30µg), levofloxacin (5µg), trimethoprim-sulphamethoxazole (1.25/23.75µg), chloramphenicol (30µg), meropenem (10µg).

All Multidrug resistance (MDR), Extensive drug-resistance (XDR), and Pan drug-resistance (PDR) isolates were tested for biofilm formation by tissue culture plate method. MDR; resistance to at least one agent in three or more antimicrobial categories, XDR; resistance to at least one agent in all categories of antimicrobials except one or two categories, and PDR; resistance to all agents in all antimicrobial categories **(Magiorakos et al., 2012).**

- **Detection of biofilm forming strains by Tissue culture plate method (TCP):**

Three to four colonies were suspended in tryptic soy broth (TSB) with 1% glucose and incubated for 18 hrs at 37 ºC. After incubation, the turbidity of the bacterial suspension was adjusted to that of the 0.5 McFarland standard (~10^8 CFU/ml) then it was diluted 1:100 with fresh TSB medium. Two hundreds microliters of diluted cultures were used to fill the Individual wells of sterile 96 well- flat bottom polystyrene tissue culture treated plates ***(Oxoid, UK)***. Equal volume of TSB + 1% glucose was used as a spectrophotometric blank. The inoculated plates were covered and incubated aerobically for 24 hrs at 37°C. The wells were rinsed three times with 200 µL of phosphate buffered saline (pH 7.2). Prior to fixation of the biofilms, the plates were drained in an inverted position.

Biofilms formed by bacteria adherent to the wells were fixed by 2% sodium acetate for 20 minutes, stained with 200 µL of crystal violet (0.1%) for 15 min at room temperature, and the stain was aspirated. The microtitre plates were air dried for 20 mins, the dye bound to the cells was re-solubilized with 200 µL of 95% ethanol per for 30 mins [18].

Interpretation of the results: The optical density (OD) of each well stained with crystal violet was measured at 600 nm using a microtitre plate reader ***(Stat Fax 2100 auto reader)***. Final OD value of a tested strain was expressed as average OD value (OD of a strain – OD of the control):

- Non biofilm producer OD ≤ ODc (The optical density of the control).
- Weak biofilm producer = ODc < OD ≤ 2×ODc.
- Moderate biofilm producer = 2×ODc <OD ≤4×ODc.
- Strong biofilm producer = 4×ODc < OD. **Figure (1)**

- **Testing the effect of EDTA and KA on biofilm producing MDR-isolates:**

Aqueous solutions of EDTA and Kojic acid ***(Techno Pharma India)***, were prepared with final concentration of 8 mg/ml for EDTA **[19]** and 6.25 mg/ml for kojic acid [20]. The final required concentration for each agent was obtained by doubling the concentration 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 EDTA and kojic acid, the total volume of each well was made up to 200 μl. Each of the 76 isolates was inoculated in 6 separate wells, 3 wells for Kojic acid solution and 3 for EDTA. The plates were incubated for 24 hrs at 37 C^o, then washed by 200 µL of phosphate buffered saline (pH 7.2), fixed by 2% sodium acetate and stained with crystal violet as mentioned before then the amount of bound crystal violet in each well was measured with spectrophotometer at OD of 600 nm (**Figure 2)**.

- **Testing the effect of varying concentrations (125–2000 µg/mL) of copper oxide nanoparticles (CuO NPs) on biofilm producing isolates.**

The test isolates were freshly cultured in TSB medium at 37 °C. The working suspensions of CuO NPs (125–2000 µg/ml) **(Nano-Tech, Egypt)** were prepared in sterilized TSB medium and vortexed prior to use. One hundred microliters of test strain grown overnight in TSB culture medium (~10^7 cells/mL) were seeded the wells of TCP. Two hundred microliters of culture medium, containing varying concentrations (125–2000 μg/mL) of CuO NPs were added to the same wells. CuO NPs suspensions and untreated bacterial cells were used as negative and positive controls respectively. After incubation at 37 °C for 24 hrs, the co-existing freely suspended or loosely adhered cells were removed. Quantitative inhibition of biofilm formation was assessd using TCP ***(Oxoid, UK)***, Finally, the amount of strongly adhered biofilm was determined at OD OF 620nm using the microtiter plate reader ***(Stat Fax 2100 autoreader)***, after washing and drying for15 mins, crystal violet staining (0.25% for 30 min) and solubilization in 200 µl of 95% ethanol. The biofilm inhibition percentage was calculated by the following equation: Biofilm inhibition% = [(A − A_0 /A) × 100]; where A represents the absorbance of the positive control wells, and A_0 reveals the absorbance of the treated wells with an antimicrobial agent. Experiments were performed in triplicate. The data expressed as means ± SD [14].

Statistical analysis

The collected data was coded and verified prior to computerized data entry. The collected data was statistically analysed using Statistical Package for the Social Science (SPSS) version 23 program and expressed in tables. Microsoft 365 Excel was used to get graphs. The data were tested for

normality by Kolmogorov-Smirnov test. Chi-square and Fisher Exact tests were used for nominal data. paired-t test for parametric data and Wilcoxon Signed test for non-parametric numerical data. In all analyses, $p < 0.05$ indicated statistical significance.

Results

Our study was carried out at Medical Microbiology and Immunology Department, Faculty of Medicine and Sohag University Hospitals in the period from January 2020 to January 2022. The study included 220 patients with different types of health care-associated infections, from which 118 (53.6%) patients were infected by MDR and XDR, biofilm-forming GNB. The patient ages ranged from 6-85 years, the mean age ± SD was 39.14 ± 14.98. Males represented 55.08% and females represented 44.92% of cases.

The highest percentage of isolation was from SSIs, while the lowest percentage was from UTIs. Clinical isolates were distributed as follows; 28.8% of isolates were from SSIs with, 21.2% were infected burns, 11.9% from infective exacerbations of COPD, 10.2% VAP aspirates, 6.8% from diabetic foot ulcers, 5.9% from sputum samples of cystic fibrosis patients, 10.1% from infected orthopedic implants, and 5.1% from UTIs.

The most frequent isolate was *Pseudomonas aeruginosa* 31.4%, followed by *Klebsiella pneumoniae* ssp. 22.0%, *Escherichia. Coli* 17.8%, *Acinetobacter baumannii* complex 11.9%, *Enterobacter aerogenes* 9.3%, *Proteus mirabilis* 6.8%, while *Burkholderia cepacia* was isolated from one sample only (0.8%)

- **Antibiotic susceptibility profile of the isolates:**

1- Escherichia coli: All *E.coli* isolates were resistant to piperacillin, amoxicillin- clavulanate, piperacillin-tazobactam, ceftazedime, and cefotaxime. 4.8% were susceptible to ceftriaxone, 85.7% to imipenem, meropenem, and amikacin, and (95.2%) to nitrofurantoin.

2- Enterobacter aerogenes: the highest resistance was to piperacillin, amoxicillin- clavulanate, and piperacillin- tazobactam (9.1% only were susceptible), while the highest sensitivity was to chloramphenicol (81.8%).

***3- Acinetobacter baumannii*:** The highest resistance rate was to piperacillin of, cefotaxime**,** ceftriaxone and ceftazidime(14.3% isolates were susceptible) , while the highest sensitivity was to trimethoprim-sulfamethoxazole (71.4%)**.**

4- Klebsiella pneumonia: All *Klebsiella pneumonia* isolates were resistant to amoxicillin- clavulanate, piperacillin- tazobactam and ceftazidime, while the highest sensitivity was to imipenem and meropenem (92.3% were susceptible).

5- Proteus mirabilis: All isolates were resistant to nitrofurantoin, while the highest sensitivity was to imipenem and meropenem (62.5%). Fifty percent were sensitive to ceftazidime, while 37.5% were sensitive to cefotaxime and ceftriaxone.

6- Pseudomonas aeruginosa: 2.7% only were susceptible to piperacillin- tazobactam and ceftazidime, while the highest sensitivity was to meropenem (89.2%)**.**

7- ***Burkholderia cepacia:*** One isolate was detected and is found to be resistant to ceftazidime, trimethoprim- sulfamethoxazole and chloramphenicol, while sensitive to levofloxacin and meropenem **(Figure 3)**.

- **Biofilm formation:**

Biofilm-formation was detected in 64.4% of isolates while 35.6% of them where non-biofilm formers. From the biofilm-forming isolated; 19 (25%) were strong biofilm-producers, 24 (31.6%) were moderate while most of the isolates 33 (43.4%) were weak biofilm-producers. The biolfilm producing species were distributed as follows; 8 isolates (10.5%) belonged to *Acinetobacter baumannii* complex, one isolate (1.3%) was *Burkholderia cepacia* group, 13 (17.1%) were *E. Coli*, 6 (7.9%) were *Enterobacter aerogenes*, 19 (25%) were *Klebsiella pneumoniae*, 3 (3.9%) were *Proteus mirabilis* and 26 (34.2%) were *Pseudomonas aeruginosa* **(Table 1).**

- **Biofilm-inhibition effect of EDTA and Kojic acid:**

The mean optical density reading OD± SD among biofilm forming isolates was 0.183± 0.11. EDTA was more powerful than Kojic acid in inhibiting biofilm-formation as the use of EDTA was associates with 85.4 % ability in the tested strains, reduction in the biofilm-formation with mean optical density reading 0.0267± 0.051 while with Kojic acid there was a 65.5 % reduction with mean optical density reading 0.063± 0.108. This difference was of high statistical value ($p < 0.0001$). **Table (2)** and **Figure (4).**

- **Biofilm-inhibiotion effect of different concentrations (125-2000 µg/ml) of copper oxide nanoparticles (CuO NPs):**

A concentration-dependent biofilm-inhibition effect was observed with different concentrations of CuO

NPs **(125-2000 µg/ml)** for all the tested isolates regardless the genus and the species of it. For *Acinetobacter baumannii* complex isolates, their OD reading was (0.227± 0.108) and the percentage of biofilm-inhibition was (21.84%- 93.57%) with mean OD reading (0.1665± 0.18 - 0.0178± 0.043). For *Burkholderia cepacia* isolate, the OD was (0.311) and percentages of biofilm inhibition were (7.39%%- 97.1%) with mean OD of (0.288- 0.009). Regarding *E. coli* isolates, the mean OD was (0.1797± 0.125) and percentage of biofilm-inhibition was (28.21%- 87.75%) with mean OD reading (0.129± 0.0875- 0.022± 0.068).

F*or Pseudomonas aeruginosa* isolates, the mean OD reading was (0.1665 ± 0.111) and the percentage of biofilm inhibition was (29.12% - 83.78%) with mean OD reading (0.118 ± 0.909 - 0.027± 0.0308). For *Klebsiella pneumoniae* isolates, the mean OD reading is (0.198± 0.125) and the percentage of biofilm inhibition was (27.7%- 92.27%) with mean OD reading (0.143± 0.125- 0.0153± 0.057). Regarding *Proteus mirabilis* isolates, their mean OD reading was (0.208± 0.2016) and the percentages of biofilm-inhibition was (25%- 89.42%) with mean OD reading (0.156± 0.133- 0.022± 0.036). finally, the mean OD of biofilm-forming Enterobacter *aerogenes* was (0.125± 0.047 and the percentage of biofilm inhibition was (24.8%- 83.2%) with mean OD density reading (0.094± 0.047- 0.021± 0.044). The difference in all the readings of optical density before and after exposure to CuONPs in all isolates was of a statistical significance (p-value < 0.05). (**Table 3) and (Figure 5).**

Table 1. Strength of biofilm-production in different bacterial species**.**

Organism	**Degree of biofilm formation**			**Total NO. (%)**
	Weak NO. (%)	**Moderate NO. (%)**	**Strong NO. (%)**	
***Acinetobacter baumannii* complex**	1 (12.5%)	4 (50%)	3 (37.5%)	8 (100%)
***Burkholderia cepacia* group**	0	0	1 (100%)	1 (100%)
E. coli	7 (53.8%)	3 (23.1%)	3 (23.1%)	13 (100%)
Enterobacter aerogenes	3 (50%)	3 (50%)	0	6 (100%)
Klebsiella pneumonia	7 (36.8%)	7 (36.8%)	5 (26.3%)	19 (100%)
Proteus mirabilis	2 (66.7%)	0	1 (33.3%)	3 (100%)
Pseudomonas aeruginosa	13 (50%)	7 (23.1%)	6 (26.9%)	26 (100%)
Total	33 (43.4%)	24 (31.6%)	19 (25%)	76 (100%)

Table 2. Effect of EDTA and Kojic acid on different biofilm-forming species.

Organism		**OD before**	**OD after EDTA**	**OD after Kojic acid**
***Acinetobacter baumannii* complex**	***Mean± SD***	0.227± 0.108	0.056± 0.1052	0.091± 0.130
	% of inhibition		**75.06%**	**59.91%**
	p-value		0.001**	0.004**
***Burkholderia cepacia* group**	***Mean± SD***	0.311	0.105	0.043
	% of inhibition		**66.23%**	**86.1%**
	p-value		---	---
E. coli	***Mean± SD***	0.1797± 0.125	0.040 ± 0.601	0.104± 0.145
	% of inhibition		**77.7%**	**42.12%**
	p-value		< 0.001***	< 0.001***
Enterobacter aerogenes	***Mean± SD***	0.125± 0.047	0.0245± 0.0580	0.05258± 0.0524
	% of inhibition		**79.7%**	**56.36%**
	p-value		0.012*	0.039*
Klebsiella pneumoniae	***Mean± SD***	0.198± 0.125	0.009± 0.027	0.04315± 0.0957
	% of inhibition		**95.45%**	**78.2%**
	p-value		< 0.001***	< 0.001***
Proteus mirabilis	***Mean± SD***	0.208± 0.2016	0.0230± 0.0363	0.0437± 0.0377
	% of inhibition		**88.94%**	**78.99%**
	p-value		0.192 (NS)	0.270 (NS)
Pseudomonas aeruginosa	***Mean± SD***	0.1665± 0.1112	0.042± 0.0338	0.0842± 0.1234
	% of inhibition		**74.77%**	**49.42%**
	p-value		*< 0.001****	*< 0.001****

p- value was calculated by Wilcoxon Signed test. * Statistically significant, ** moderate statistical significance, and *** Statistically very highly significant.

Table 3. Effect of different concentrations (125-2000 µg/ml) of CuO NPs on biofilm-forming isolates.

Organism		Optical density reading				
		CuO 125 µg/ml	**CuO 250 µg/ml**	**CuO 500 µg/ml**	**CuO 1000 µg/ml**	**CuO 2000 µg/ml**
***Acinetobacter baumannii* complex**	**Before**	0.227± 0.108				
	After	0.1665± 0.18	0.1316± 0.093	0.0838± 0.0814	0.0442± 0.0791	0.0178± 0.043
	***p*-value**	0.001	0.001	0.001	0.003	0.001
	% of inhibition	**21.84%**	**52.49%**	**69.74%**	**84.04%**	**93.57%**
***Burkholderia cepacia* group**	**Before**	0.311				
	After	0.288	0.243	0.055	0.022	0.009
	***p*-value**	----------				
	% of inhibition	**7.39%**	**21.86%**	**82.31%**	**92.92%**	**97.1%**
E. Coli	**Before**	0.1797± 0.125				
	After	0.129± 0.0875	0.095± 0.853	0.062± 0.0766	0.0359± 0.069	0.022± 0.068
	***p*-value**	0.005	0.002	0.001	< 0.0001	< 0.0001
	% of inhibition	**28.21%**	**47.13%**	**65.49%**	**80.02%**	**87.75%**
Enterobacter aerogenes	**Before**	0.125± 0.047				
	After	0.094± 0.047	0.074± 0.047	0.0515± 0.043	0.037± 0.048	0.021± 0.044
	***p*-value**	0.005	0.001	< 0.0001	0.001	0.002
	% of inhibition	**24.8%**	**40.8%**	**58.8%**	**70.4%**	**83.2%**
Klebsiella pneumoniae	**Before**	0.198± 0.125				
	After	0.143± 0.125	0.0918± 0.060	0.062± 0.059	0.0293± 0.0580	0.0153± 0.057
	***p*-value**	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
	% of inhibition	**27.7%**	**53.63%**	**68.68%**	**85.2%**	**92.27%**
Proteus mirabilis	**Before**	0.208± 0.2016				
	After	0.156± 0.133	0.123± 0.119	0.090± 0.090	0.042± 0.044	0.022± 0.036
	p-value	0.321	0.222	0.220	0.212	0.191
	% of inhibition	25%	40.86%	56.73%	79.8%	89.42%
Pseudomonas aeruginosa	**Before**	0.1665± 0.111				
	After	0.118± 0.909	0.0707± 0.058	0.048± 0.041	0.036± 0.035	0.027± 0.0308
	***p*-value**	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
	% of inhibition	**29.12%**	**57.53%**	**71.17%**	**78.37%**	**83.78%**

Figure 1. TCP showing; (1) Strong, (2) moderate, (3) weak biofilm producing strains, and (4) Non biofilm forming strain.

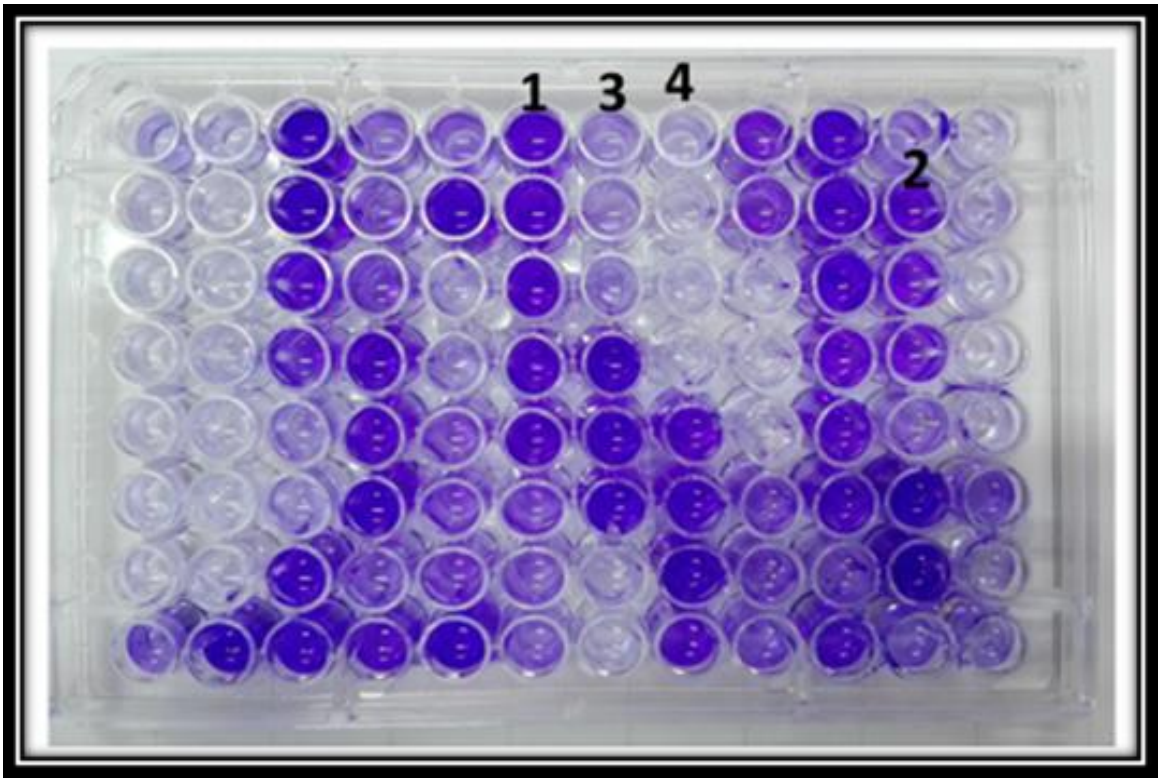


Figure 2. Transformation from strong biofilm former to moderate biofilm former and from biofilm forming to non-biofilm forming isolates under effect of EDTA and Kojic acid.

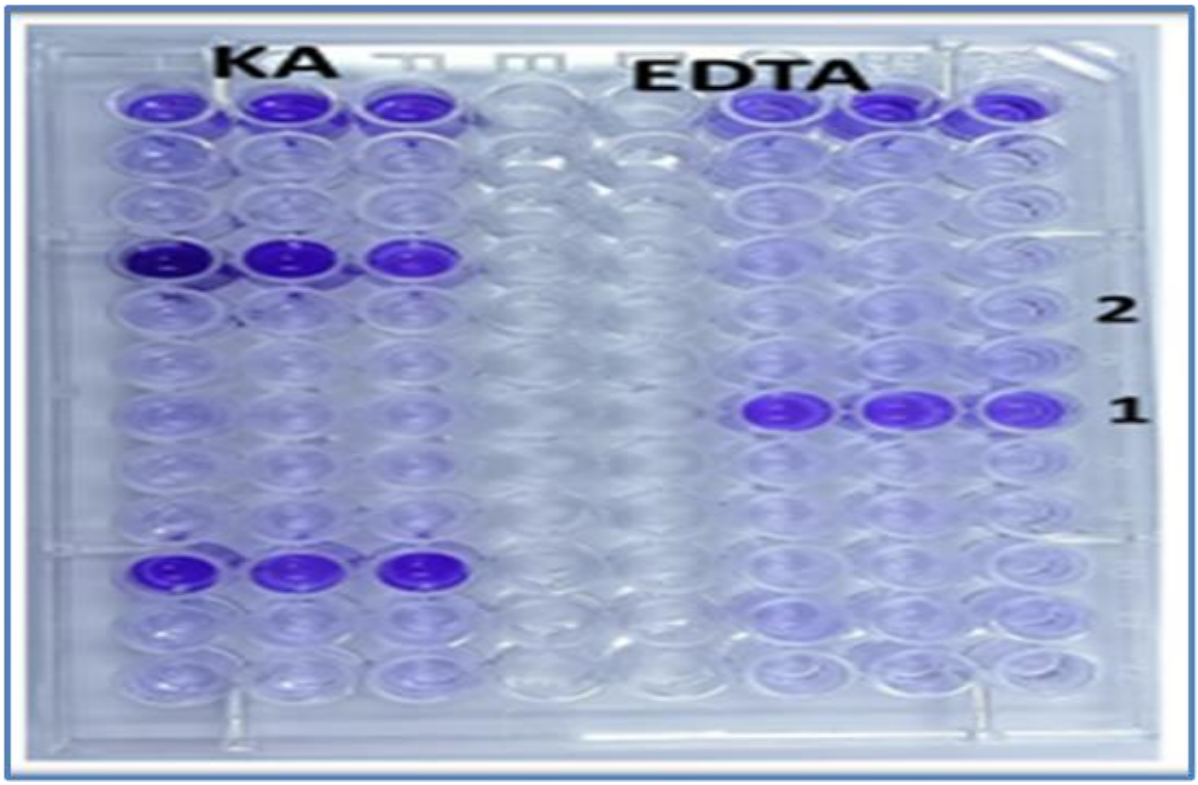
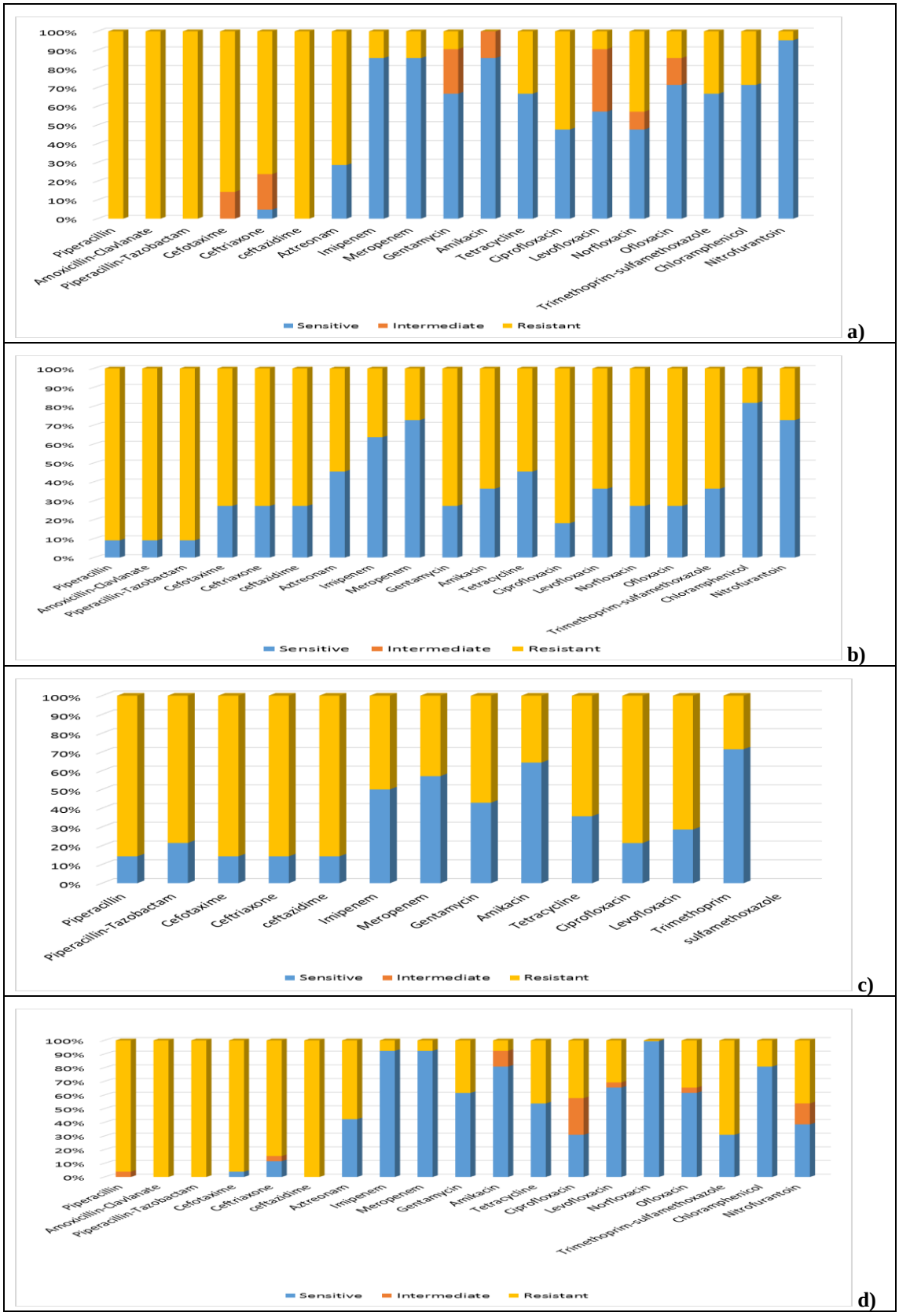


Figure 3. Antimicrobial susceptibility profile of; a) *Escherichia coli*, b) *Enterobacter aerogenes*, c) *Acinetobacter baumannii*, d) *Klebsiella pneumonia*, e) *Proteus mirabilis*, and f) *Pseudomonas aeruginosa*.



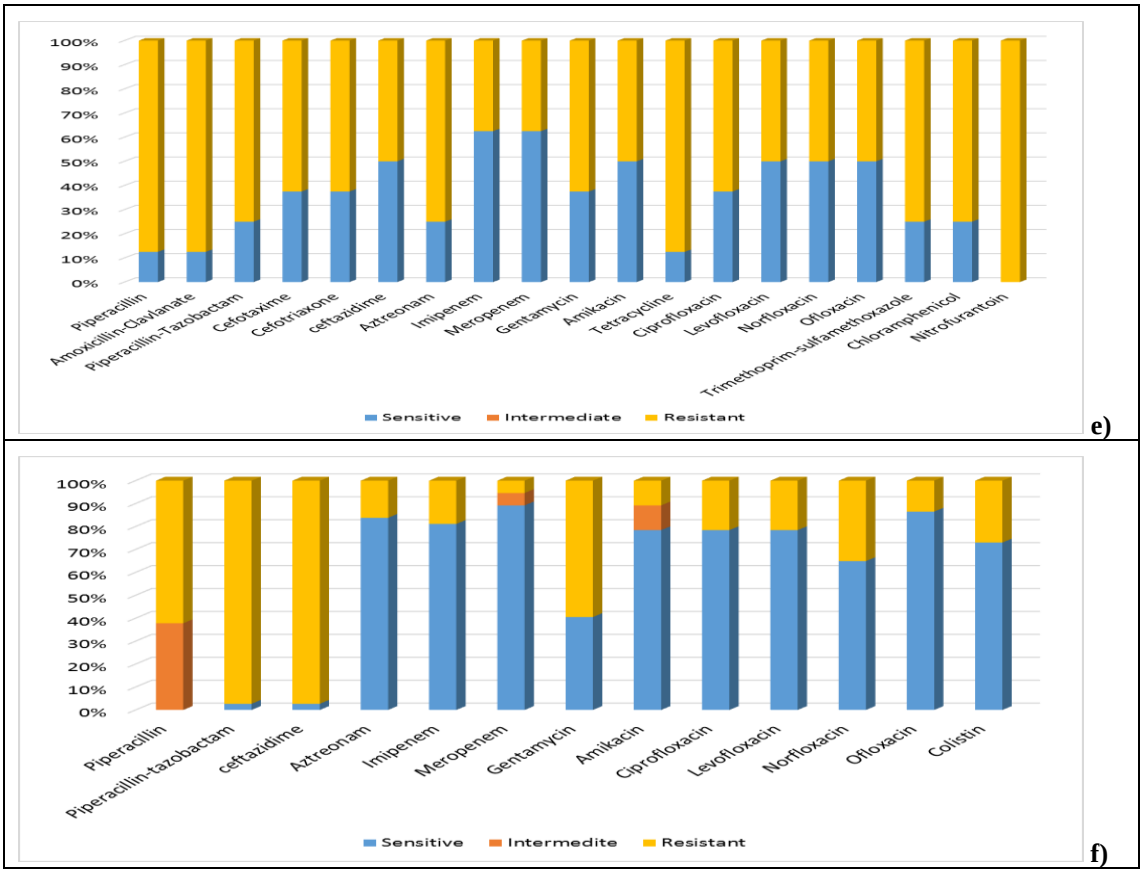


Figure 4. Optical density readings of biofilm-producers before and after exposure to EDTA and Kojic acid.

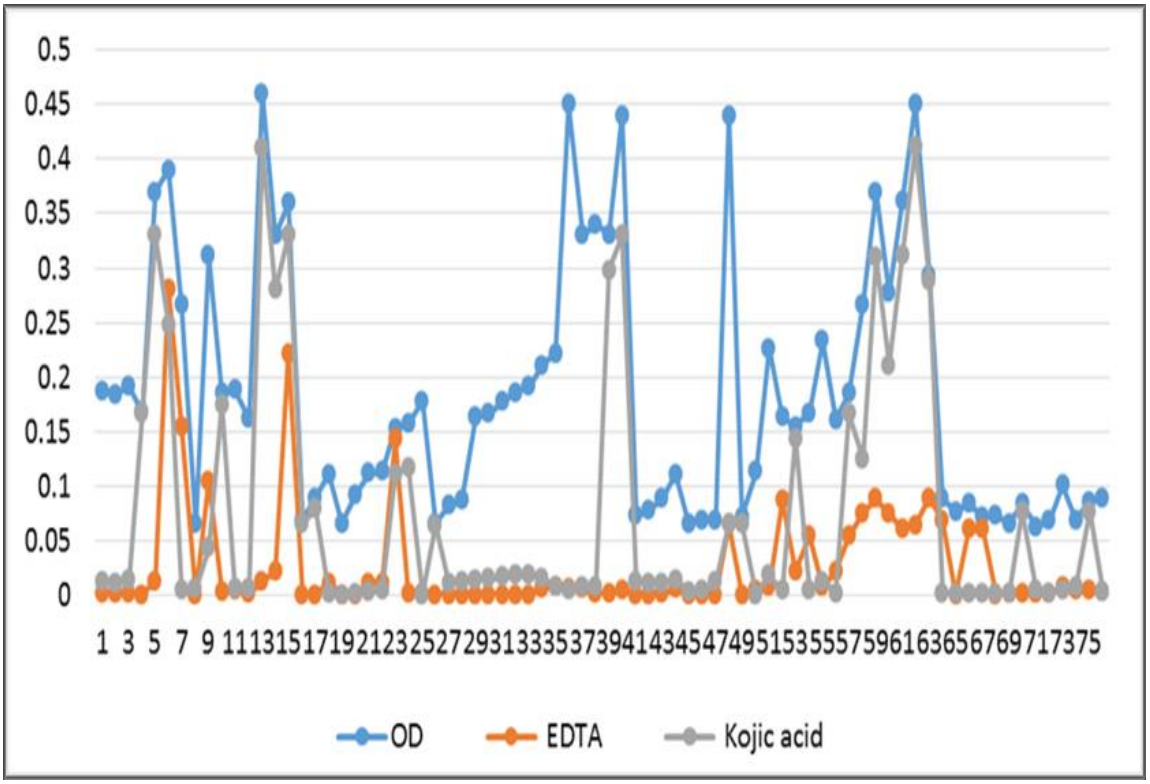


Figure 5. Optical density readings of biofilm-producers before and after exposure to CuO NPs; a) *Acinetobacter baumannii*, b) *E. Coli*, c) *Enterobacter aerogenes*, d) *Klebsiella pneumonia*, e) *Proteus mirabilis*, f) *Pseudomonas aeruginosa*.

Discussion

In this study 200 samples were collected from patients with different types of HAIs; gram negative bacilli were isolated from 118 samples. Seventy six samples (64.4%) were biofilm-formers, while non- biofilm forming isolates were 42 (35.6%), This was in agreement **Allam et al. (2017)** [21] and **Dumaru et al. (2019)** [22] studies where (64.28%) and (62.73%) of isolates were biofilm forming, respectively, **Baidya et al. (2021)** [23] reported biofilm-formation in 56.3% of their isolates, and **Macias-Valcayo et al. (2022)** [24] reported that most of Gram negative strains of HAIs were biofilm producers (97%).The results of **Almalki and Varghese (2020)** [25] where different from our results where they reported that (63.9%) of isolates were non-biofilm forming while biofilm forming isolates were (36.1%), which could be attributed to the difference in the species of Gram negative isolates and the single potential of every pathogen to form biofilms.

In our study, 43.4% of the isolates were weak biofilm formers, 31.6% moderate and 25% were strong biofilm forming. Nearly similar results reported by **Maharjan et al. (2018)** [26] where 23.3% of isolates were strong biofilm forming, 30% moderate and 46.6% were weak biofilm forming. **Netsanet et al. (2019)** [27] reported that 35.2% were

strong biofilm forming isolates, 16.7% were moderate biofilm forming and 27.8% were weak biofilm forming, the different methods used for assessment of the strength of biofilm formation could be a potential cause for this variation. **Macias-Valcayo et al. (2022)** [24] also reported different results where 41.3% of their isolates were strong producers, 34.8% were moderate and 21.8% were weak biofilm-producers.

In our study, the most frequent biofilm-producing isolates were *Pseudomonas aeruginosa* (34.2%) followed by *Klebsiella pneumoniae* (25%), *E. Coli* (17.1%), *Acinetobacter baumannii* complex (10.5%), *Enterobacter aerogenes* (7.9%), *Proteus mirabilis* (3.9%) and *Burkholderia cepacia* group (1.3%). A different finding was reported by **Maharjan et al. (2018)** [26] who reported that *E. coli* was found to be more biofilm-producer (33.3%) followed by *Klebsiella pneumoniae* (30%), *Pseudomonas aeroginosa* (20%), *Acinetobacter* spp. and *Enterobacter* spp. (3.33%), and **Netsanet et al. (2019)** [27] who reported that among biofilm-forming isolates the most frequently biofilm forming isolates were *E. Coli* (32.55%) followed by *Klebsiella spp.* (20.9%), *Proteus spp.* (9.3%), *Pseudomonas aeruginosa* (6.79%) *and Enterobacter* spp. (4.65%). This difference in the percentages of biofilm-forming species is attributed to the different rate of isolation of every spp. in different studies. Collectively the difference in biofilm-formation patterns among bacterial isolates in different studies may be due to differences in strain types, number of bacterial isolates, sample sizes, geographic locations, and methodological variations in assessment of biofilm formation.

In our study EDTA seemed to have an effective anti-biofilm activity where its use inhibited biofilm formation in 85.4% of the isolates, the OD were changed from 0.183± 0.11 to 0.0267± 0.051 (P- value < 0.0001). **Ali et al. (2019)** [28] tested EDTA with the same concentration we used against catheter-associated urinary tract infection **(**CAUTI) isolates. The results revealed that EDTA inhibited biofilm formation by 78.6%, OD readings were changed from 1.03±0.3 to 0.22 ± 0.15 (*p* value < 0.001). A study carried out by **Percival et al. (2005)** [29] **reported** that the use of EDTA on BSIs-associates Gram negative pathogens with a concentration of 40-mg/mL significantly reduced biofilm formation (*p* value <0.05). A study by **Chaudhary and Payas (2012)** [30] assessed the effect of EDTA disodium on curli production and biofilm formation by different concentrations including 1.25, 2.5, 4.0, 5.0, 10.0 and 20 mg/ml, The results showed that EDTA effectively inhibits curli formation and bacterial adhesion by *Klebsiella pneumoniae* at ≥ 5 mg/ml concentration.

Rahal et al. (2021) [31] tested the ability for *in vitro* biofilm formation by MDR *K. pneumoniae* isolates in the presence of eight concentrations of EDTA (4 to 512 µg/ml). The results showed that the highest antibiofilm activity by EDTA was demonstrated at the subinhibitory concentration (256 µg/ml) with biofilm inhibition percent (94.28%), while at very low concentration (8 µg/ml), it was found an obvious inhibitory effect on biofilm (82.11 %). The study suggests that EDTA plays an important role in the early stage of biofilm formation. All these findings were in accordance with our findings.

In our study, EDTA decreased *Proteus mirabilis* biofilm-formation ability by 88.94 % which agreed with the findings of **Percival et al. (2009)** [29**]** who examined the daily instillation of tetra sodium EDTA solution (80 mg/ml) as a method for reduction of the blockage rate of urinary catheters with encrustations of *Pr. Mirabilis*-biofilms. Our results agreed also with **Ali et al. (2019)** [28] who tested EDTA with the concentration of 8 mg/ml against CAUTI isolates, and the results revealed that EDTA inhibited biofilm formation among *Pr. mirabilis* isolates by 74.2%.

In our study, EDTA significantly inhibited *Enterobacter aerogenes* biofilm formation by 79.7 %, as far as our knowledge no studies tested the effect of EDTA on biofilms formed by *Enterobacter aerogenes*.

Regarding effect of EDTA on biofilms of *E. Coli* also, it was found that it significantly inhibited *E. Coli* biofilm by 77.7 %. **Gawad et al. (2017)**[7] investigated the effect of EDTA on the biofilm formation by MDR strong biofilm-producer Uropathogenic *E.Coli* and reported that EDTA with concentrations (10 and 20 mM) and Gelatin- EDTA coat inhibited biofilm-formation by strong- and moderate-biofilm formers 78.8% and 81.1%, respectively. **Ayyash et al. (2019)** [32] also stated that combination of triclosan (10 mg/mL) and EDTA (30 mg/mL) was significantly effective in eradicating biofilm formation among *E. coli* isolated including MDR strains on Foley catheters and combining triclosan/EDTA has a promising application as non-antibiotic catheter lock solution.

In this study, *Acinetobacter baumannii* complex biofilm-formation was inhibited by the use of EDTA by 75.06 %. Our results agreed with **Ramalingam and Lee. (2018)** [33] who tested the antibiofilm activity of an EDTA-containing nanoemulsion on multidrug-resistant *Acinetobacter baumannii*, their results revealed that the nanoemulsions inhibited colonization and subsequent biofilm-formation by from 61.8%, and with **Ahmad et al. (2019)** [34] who tested also the effect of EDTA in three concentrations (100, 125, 150 mg /L) on biofilm formation by *Acinetobacter baumannii* isolates with mean OD reading of 1.7410±0.53096, EDTA inhibited *Acinetobacter baumannii* biofilms with mean OD reading 0.9690±0.26539 (*p*-value: 0.001) at the concentration of 100 mg /L, 0.6140±0.14849 (*p*-value: 0.000) at the concentration of 125 mg /L , and 0.2730±0.43110 (*p*-value: 0.000) at the concentration of 150 mg /L.

Regarding effect of EDTA on biofilms of *Pseudomonas aeruginosa*; EDTA significantly inhibited the biofilms by 74.77% (*p*-value: < 0.001). **Colombari et al. (2021)** [35] investigated the effects of EDTA (concentrations of 2.5%, 0.75%, and 0.25%) on the ability of *Pseudomonas aeruginosa* to produce biofilms and stated that EDTA impaired both planktonic growth and biofilms significantly. *In our study the biofilms of Burkholderia cepacia* group isolates had a mean OD reading of 0.311, the use of EDTA inhibited biofilm by 66.23% with mean OD reading 0.105. To our knowledge no studies discussed the effect of EDTA on biofilms formed by *Burkholderia cepacia*.

In our study, the biofilm inhibitory activity of Kojic acid (KA) wasn't as strong as EDTA; as Kojic acid when used with the concentration of 6.25 mg/ml, it decreased biofilm formation by 65.5% in all isolates where the mean OD readings changed from 0.183± 0.11 to 0.063± 0.108 with a statistically significant difference (*p*- value: < 0.0001). Kojic acid was most effective against biofilms formed by *Burkholderia cepacia* group isolate as it inhibited biofilms by 86.1%. Kojic acid inhibited *Proteus mirabilis* biofilms by 78.99%. Different result was obtained from the study carried out by **Ali et al. (2019)** [28] who revealed that Kojic acid with concentration of 6.25 mg/ml decreased biofilm-formation by *Proteus* isolates by 55%. To our knowledge no studies were found testing the effect of kojic acid on biofilms formed by *Burkholderia cepacian*.

In this study, Kojic acid significantly inhibited biofilms formed by *Acinetobacter baumannii* complex isolates by 59.91% (*p*- value: 0.004). A study by **Li et al. (2022)** [36] who investigated the effect of Kojic acid with the concentrations of 1.5, 3, 6 mM on biofilm formation by *Acinetobacter baumannii*. The results revealed that Kojic acid could substantially suppress biofilm formation at concentrations over 3 mM, with a more efficient result at 6 mM, comparable to the positive control.

In this study, Kojic acid inhibited biofilms formed by *Enterobacter aerogenes* isolates by 56.36% (*p*- value: 0.039). To our knowledge no studies were found testing the effect of kojic acid on biofilms formed by *Enterobacter aerogenes*. In our study, Kojic acid inhibited biofilms formed by *Pseudomonas aeruginosa* isolates by 49.42% which was statistically significant (*p*- value < 0.001). **Ali et al. (2019)** [28] reported that Kojic acid with the concentration of 6.25 mg/ml decreased biofilms formed by *Pseudomonas* by 58.3% (*p*-value 0.007). **WU et al. (2018)** [37] tested the effect of Kojic acid on *Pseudomonas aeruginosa* isolates with concentration of 2 mM. The results revealed that Kojic acid inhibited biofilm formation between 5%-27% which was statistically significant (P-value < 0.05). All these findings agreed with our findings on the same species.

In our study, Kojic acid was least effective against biofilms formed by *E. coli* isolates as it inhibited biofilm formation by 42.12%. **WU et al. (2018)** [37] explored the effect of KA at sub-minimum inhibitory concentrations on biofilms of *E. coli* involved in food contamination. The results revealed that KA significantly inhibited biofilms formed by *E. coli*. Furthermore, the degree of inhibition was relatively concentration-dependent. Different results were obtained from the study carried out by **Ali et al. (2019)** [28] who reported that Kojic acid had little effect on biofilms formed by UPEC isolated from catheters and inhibited biofilm formation by only 11%. Collectively our study demonstrated that EDTA was more powerful than Kojic acid in biofilm inhibition as it inhibited biofilms by 85.4 %, in comparison to Kojic acid which decreased biofilm formation by 65.5 %. The tested biofilm inhibitors gave statistically relevant results (*p*-value < 0.0001).

Regarding effect of different concentrations of copper oxide nanoparticles on

biofilm forming *Acinetobacter baumannii* complex isolates; concentration dependent biofilm inhibition was observed with different concentrations of CuO NPs (125-2000 µg/ml), percentages of biofilm inhibition were (21.84%- 93.57%) with statistically significant reduction (*p*-value <0.05). **Boliang et al. (2022)** [38] tested the effect of biosynthesized CuO NPs at different concentration (100–1000 µg/mL) on biofilm formation by *Acinetobacter baumannii*. The results revealed that copper oxide nanoparticles were highle effective against biofilm forming *Acinetobacter baumannii*, in a concentration of 1000 µg/mL, bacteria exhibited a 95% biofilm inhibition. Regarding effect of different concentrations of copper oxide nanoparticles on *Burkholderia cepacia* biofilms; a concentration-dependent inhibition was observed with different concentrations of CuO NPs (125-2000 µg/ml), percentages of biofilm inhibition were (7.39%%-97.1%).

Regarding *E. coli* isolates; concentration dependent biofilm inhibition was observed with different concentrations of CuO NPs (125-2000 µg/ml), percentages of biofilm inhibition were (28.21%- 87.75%) with statistically significant reduction (*p*-value <0.05). **Zhao et al. (2022)** [39] tested the effect of CuO NPs at different concentration (100–1000 µg/mL) against biofilm producing *E. coli*. The results revealed that copper oxide nanoparticles were very efficient against biofilm forming *E. coli* and concentration dependent inhibition against the tested bacteria was observed. At 250 µg/mL concentration of CuO NPs bacteria exhibited 16% of biofilm inhibition; at 750 µg/mL bacteria exhibited 65% of biofilm inhibition and 96% was found at 1000 µg/mL concentration. **Cherian et al. (2020)** [40] also evaluated the anti-biofilm efficacy of CuO NPs against *E. coli* at varying concentrations (125–2000 µg/mL). A concentration depended biofilm inhibition was recorded, compared to the untreated controls. **Ali et al. (2019)** [28] tested the effect of bare nano-CuO at different concentration (125–2000 µg/mL) against biofilm producing *E. coli* and reported that CuO NPs in concentration range of 125-2000 µg/ml inhibited biofilm formation by 42.01 ± 7% to 58.45 ± 2%. Our results agreed with these studies.

Regarding *Enterobacter aerogenes*, a concentration dependent biofilm inhibition was observed, percentages of biofilm inhibition were (24.8%- 83.2%) with a statistically significant difference (*p*-value < 0.05). our findings agreed with **Cherian et al. (2020)** [40] who tested the effect of CuO NPs at concentration 100 µg/mL against biofilm producing *Enterobacter aerogenes* and stated that CuO NPs were successful in inhibiting biofilms by 70% (*p*- value < 0.05).

Regarding *Klebsiella pneumoniae* isolates; also concentration dependent biofilm inhibition was observed, percentages of biofilm inhibition were (27.7%- 92.27%) with statistically significant difference (*p*-value < 0.05). our results were in accordance with **Cherian et al. (2020)** [40] who tested the activity of the biosynthesized CuO NPs against *K. pneumonia* biofilms formation at concentrations of MIC, higher-MIC, and sub-MIC values (2, 1, 0.5, 0.25, 0.125, and 0.06 µg/ml**)** and revealed that the CuO NPs inhibited biofilms at the MIC value (1 µg/ml) by 99.8% **. Boliang et al. (2022)** [38] also tested the effect of biosynthesized CuO NPs at different concentration (100–1000 µg/mL) on biofilms of *K. pneumonia* and revealed that it had a potent effect against biofilm- formation in *K. pneumonia*, in 1000 µg/mL concentration, bacteria exhibited 94% inhibition. For *Proteus mirabilis* percentages of biofilm inhibition were (25%- 89.42%) along the different CuO NPs concentrations, and this was in accordance with **Zhao et al. (2022)** [39] where a very potent effect was observed against biofilm forming *Proteus mirabilis* at 250 µg/mL concentration with 18% of biofilm-inhibition.

For *Pseudomonas aeruginosa* isolates also a concentration depended biofilm inhibition was observed with (29.12%-83.78%) reduction and this also was in accordance with thefindings of **Sriyutha et al. (2011)** [41] and **Mohamed et al. (2021)** [42] who reported that CuO-NPs at 0.3, 0.15, 0.07, 0.03, and 0.01 mg/mL did not affect biofilm formations by *Pseudomonas aeruginosa*.

From these findings we highly recommend the use EDTA followed by CuO NPs, and finally kojic acid with the described concentrations for treatment of HALs infections caused by MDR-biofilm-producing Gram negative superbugs in different healthcare settings.

Conflict of interest: the authors state that they have no conflict of interest.

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