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Combined effects of antibiotics and acidified nitrite on biofilm formation by beta-lactamase-producing uropathogenic bacteria

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Hosan Yousif Hassan[1*] Safaa Toma Hanna Aka[1] Salah Tofik Jalal Balaky[2] Aras Najmaddin Hamad [3]

Abstract

Background and objective: The increased prevalence of extended- spectrum beta-lactamase producing Enterobacteriaceae has increased the use of last-resort antimicrobial drugs like Carbapenems. An alternative idea is to use new combinations of common antibiotics. The aim of the present study is to examine whether acidified nitrite has the ability to enhance the activity of beta-lactam antibiotics against the biofilm formation and bacterial growth of beta-lactamase- producing uropathogenic isolates.

Methods: In this cross-sectional study, a total of 37 beta-lactamase- producing uropathogens were collected from patients at Urology Department at Rizgary Teaching Hospital in Erbil/Iraq. Biofilm formation was determined using a 96-well tissue culture plate assay. The ability to produce beta-lactamase production was detected by a phenotypic confirmatory combination disk diffusion test. The sub-minimal inhibitory concentration of antibiotics alone and in combination with each of acidified nitrite, ascorbic acid and sodium nitrite towards biofilm formation were observed.

Results: The combination of cefotaxime with each of acidified nitrite (P <0.001), ascorbic acid (P <0.001) and sodium nitrite (P = 0.003) significantly enhanced the effect of cefotaxime against the biofilm producing activity of beta-lactamase producing uropathogens. Furthermore, with ceftazidime identical synergistic results were obtained with ascorbic acid (P = 0.001) and acidified nitrite (P = 0.007).

Conclusion: Acidified nitrite significantly improved the activity of beta-lactam antibiotics against the biofilm mass of beta-lactamase producing uropathogens.

Keywords: Beta - lactumase; Acidified nitrite; Biofilm; UTI.

Introduction

Urinary tract infections (UTIs) are the second most common reason for the prescription of empirical antibiotics and therefore a leading cause for the increase in antibiotic usage and resistance. The majority of infections are caused by the gram-negative uropathogenic Enterobacteriaceae *Escherichia coli.*[1]

The increased prevalence of extended-spectrum beta-lactamase producing Enterobacteriaceae has increased the use of last-resort antimicrobial drugs such as carbapenems.[2]

Uropathogenic *E. coli* strains have many virulence factors, of which the ability to form biofilm is regarded as one of the most important factors. Biofilm in these strains are significant colonizers of medical devices like urinary, arterial and venous catheters. The main cause of catheter-associated and recurrent UTIs are due to the biofilm producing uropathogenic isolates.[3] Studies reported that about 80% of all infectious diseases are biofilm related and in addition are responsible for more than 60% of nosocomial infections.[4]

[1] Department of Pharmacognosy, College of Pharmacy, Hawler Medical University, Erbil, Iraq.
[2] Department of Medical Microbiology, College of Health Sciences, Hawler Medical University, Erbil, Iraq.
[3] Department of Pharmaceutical Chemistry, College of Pharmacy, Hawler Medical University, Erbil, Iraq.
Correspondence: hozan.yousif.hassan@gmail.com

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Empirical or antibiotic treatment based on antibiotic sensitivity test is usually inefficient towards infections caused by biofilm producing isolates.[4] It is indicated that biofilms are difficult to eliminate and usually resistant to antibiotic levels that are 10-1000 times greater than levels required to eradicate free living bacteria.[5-7]

Antibiotics fail to penetrate the polymeric matrix which surrounds the bacterial cells as a consequence the embedded bacterial cells have time enough to acquire resistance through horizontal gene transfer.[5,8]

An alternative idea is to use new combinations of common antibiotics. A number of studies have reported that activity of an antibiotic can be enhanced by combination with nitric oxide (NO) which is generated from acidified nitrite.[8-12] On one hand, various studies have described the beneficial antibacterial effect of acidified nitrite against the growth of pathogens like *E. coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Candida albicans.*[13,14] On the other hand, *in vitro* and *in vivo* tests described the ability of acidified nitrite in the reduction of biofilm formation in which nitric oxide performed directly or indirectly a significant role.[15-21]

Most of the plasma nitrate is excreted in urine and in case of a lower UTI with a member of the Enterobacteriaceae, nitrate within the urinary bladder is reduced to nitrite through the bacterial enzyme nitrite reductase. In acidic conditions with a pH value below 5.5, which can be achieved by the ingestion of acidifying agents like ascorbic acid, nitrite is reduced to a number of reactive nitrogen intermediates. One of the intermediates is nitric oxide which is a free radical gas and has a main role in innate immunity with a short half-time. Nitric acid produced by epithelial cells and phagocytes results in damage to bacterial cell membrane and its DNA.[9]

The increase of antimicrobial resistance in the recent years has led to the conclusion that antimicrobial drugs alone might not be enough for the treatment of mixed or severe infection. As a result, combination therapy might be an important method to enhance the effect of antibiotics. We hypothesize that acidified nitrite can improve the effect of beta-lactam antibiotics against the biofilm of Extended Spectrum Beta-Lactamase producing uropathogenic isolates.

Methods

Study Design and Specimen Collection

This cross-sectional study was carried out from September- December 2017. A total of 37 beta-lactamase producing uropathogenic isolates of *E. coli* and *K. pneumoniae* were collected from patients attending the Urology Department at Rizgary Teaching Hospital in Erbil city, Iraq. The isolates were stored at -20°C, using 20% glycerol in nutrient broth suspension. Bacterial strains were sub-cultured on MacConkeyagar (Lab M Limited Company/ United Kingdom).

2. 2. Detection of Extended-Spectrum Beta-Lactamases

Each isolate was tested using the VITEK- 2 system (Biomerieux/ France) with the ESBL test panel. A quality control strain (*E. coli* ATCC 25922) was included in each run. In addition, the Phenotypic Confirmatory Combination Disk Diffusion Test (PCDDT**)** was performed for ESBL production. The test was considered positive when an increase in the growth-inhibitory zone around either the cefotaxime (CTX) or the ceftazidime (CAZ) disk with clavulanic acid (CA) (Becton Dickinson Company/ USA) was 5 mm or greater in the diameter around the disk containing CTX or CAZalone (Bioanalyse/ Turkey).

The positive isolates from phenotypic tests were further confirmed genotypically using PCR for one of the three ESBL genes, the SHV gene. Primers (Integrated DNA Technology/ Canada) have been used in previous studies, bla_{SHV} forward primer 5'TTAACTCCCTGTTAGCCA 3' and reverse 5'GATTTGCTGATTTCGCCC 3'

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have been used with a product size 768 base pairs.[22] The cycling parameters were as follow: initial denaturation at 94 °C for 3 min followed by 30 cycles. Each denaturation at 94 °C for 30 seconds, annealing at 50 °C for 30 seconds, amplification at 72 °C for 2 min and final extension for 10 min.[23]

Biofilm Assay

Biofilm formation was determined using 96 well tissue culture plate (TCP) (Caplugs, Evergreen /USA) assay using Crystal violet (Siegma Aldrich GmBH/ Germany), as described in previous studies.[37] The optical density (OD) of the bio-film was measured at the wavelength 630 nm using an ELISA reader (DAR800 Diagnostic Automation Inc. /USA). The isolates were classified into four different categories according to their biofilm-forming ability depending on their 2 ODc (the average OD of the control negative at threefold its standard deviation) of bacteria.[24]The categories were classified as follow:

Not able to form a biofilm (OD ≤ ODc)

Weakly able to form a biofilm (ODc < OD ≤ 2 x ODc),

Moderately able to form a biofilm (2 x ODc < OD ≤ 4x ODc)

Strongly able to form a biofilm (4 x ODc < OD)

Determination of Fractional Inhibitory Concentration Index (FICI) and Minimal Inhibition Concentration (MIC)

The FICI was calculated for each antibiotic in each combination to evaluate the effect of the combination described by previous studies.[25] Both beta-lactam antibiotics ceftazidime (Wockhardt Company/UK) and cefotaxime (Normon Company/ Spain) were used. The formula used to calculate the FICI was as follow:

FICI of drug A = (MIC of drug A in combination) / (MIC of drug A alone)

Synergy was defined as FICI ≤ 0.5, Indifference was defined as 1 < FICI ≤ 2, Antagonism was defined as FICI > 2.[26] For the assessment of MIC serial two-fold dilutions was performed for both antibiotics cefotaxime and ceftazidime. The concentration ranged from ¼ to 16-fold the MIC concentration within 200 µL LB broth. Drug-free medium was used in control wells. After incubation for 24 hours at 37 ºC. The bacterial growth was measured by ELISA-reader at a wavelength of OD_{490}.[27]

***In Vitro* Bacterial Growth Experiments**

Flat bottom 96-well microtiter plates with a final volume of 300 µL were filled with Luria Bertani broth and inoculated with 0.5 McFarland bacterial suspension per well. Ascorbic acid (Scharlau/ Spain) treatment was added at a fixed concentration of 10 mM, sodium nitrite (Scharlau/ Spain) was added in different concentrations of 0.5, 1, 2 mM and acidified nitrite with a fixed concentration of 10 mM ascorbic acid with 2 mM sodium nitrite. All chemicals used were prepared within LB broth. Sodium nitrite and both antibiotics were prepared once and stored in refrigerator while acidified nitrite and ascorbic acid were prepared daily fresh for the experiments. In addition, the combined effects of the antibiotics with each ascorbic acid, sodium nitrite and acidified nitrite were determined as described previously.[8]

Effect of Sub Minimal Inhibitory Concentration (sub-MIC) on Biofilm Formation

The beta-lactamase producing bacterial isolates which showed to be biofilm producers were chosen to detect the sub-MIC effects of antibiotics alone and in combination with each of acidified nitrite, ascorbic acid and sodium nitrite.

The bacterial suspensions were tested to 1/2 MIC to – 1/128 MIC within 100 µL LB broth at 37 °C for 48 hours. In addition, a row of wells was filled only with LB broth and classified as negative control. Following incubation, the sub-MIC was observed by staining with crystal violet and measured by ELISA-reader at OD_{630}.[27,28]

Ethical Approval

Approval was obtained from the Ethical Committee at College of Pharmacy, Hawler Medical University in Erbil, Iraq. In addition, prior to data collection, verbal consent was

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obtained from all patients.

Statistical Analysis

Data analysis was performed using the Statistical Package for Social Sciences (SPSS) version 23. The Chi square and Fischer's exact test was used to evaluate the association between two variables. Paired T-test was performed to compare the means of two variables. *P* value equal to or less than 0.05 was considered statistically significant.

Results

The Combined Effects of Acidified Nitrite, Ascorbic Acid and Sodium Nitrite with CTX and CAZ

The clinical isolates used for the present experiments were beta-lactamase producing uropathogens (*E. coli* and *K. pneumoniae*) and therefore resistant to third generation cephalosporins like CTX and CAZ. Most of the synergistic effects with CTX were achieved with the combination acidified nitrite of which about 45.9% were synergistic, followed by ascorbic acid with 35.1% and sodium nitrite with only 16.2%. Therefore, a significant association is present between the combination treatments with CTX and their effects ($P = 0.025$). Another antibiotic used in the study was ceftazidime, most synergistic effects were reported with acidified nitrite with 21.6% followed by ascorbic acid 16.2% and sodium nitrite with only 10.8%. Contrary to cefotaxime, there was no significant difference as illustrated in Tables 1 and 2.

Table 1 The FICI of the different combinations with cefotaxime and their effects against bacterial isolates

Combination treatments	Synergistic	Indifferent	Total	*P*-value
	No. %	No. %	No. %	
CTX+$NaNO_2$	6 (16.2)	31 (83.3)	37 (100)	
CTX+AA	13 (35.1)	24 (64.9)	37 (100)	0.025
CTX+AN	17 (45.9)	20 (54.1)	37 (100)	
Total	**36 (32.4)**	**75 (67.6)**	**111 (100)**	

Table 2 The FICI of the different combinations with ceftazidime and their effects against bacterial isolates

Combination treatments	Synergistic	Indifferent	Antagonistic	Total	*P*-value
	No. %	No. %	No. %	No. %	
CAZ+$NaNO_2$	4 (10.8)	33 (89.2)	0 (0)	37 (100)	
CAZ+AA	6 (16.2)	30 (81.1)	1 (2.7)	37 (100)	0.579
CAZ+AN	8 (21.6)	28 (75.7)	1 (2.7)	37 (100)	
Total	**18 (16.2)**	**91 (82)**	**2 (1.8)**	**111 (100)**	

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The Combined Effects of Acidified Nitrite, Ascorbic Acid and Sodium nitrite with CTX and CAZ against Biofilm Formation

The results of the sub-MIC of CTX and CAZ alone and in different combinations against biofilm formation by ESBL isolates are presented in Table 3. The results showed that there were highly significant differences in the mean effect between CTX treatment alone and each of CTX combinations (P <0.001 and P = 0.003). Furthermore, the same synergistic results were observed with CAZ with each of ascorbic acid (P = 0.001) and acidified nitrite (P = 0.007) as shown in Table 3.

Discussion

The increase of antimicrobial resistance in the recent years has led to the conclusion that antimicrobial drugs alone might not be enough for the treatment of mixed or severe infection. Investigations have reported that antibiotic activity can be enhanced by combination with nitric oxide which is generated from acidified nitrite.[8,10–12,16] As a result, combination therapy might be an important method to enhance the effect of antibiotics like cefotaxime and ceftazidime.

This variance in significance between the two antibiotics might be related to the structural difference of cefotaxime and ceftazidime. On one hand, the antibiotics are within the same antibiotic group and harbor the beta-lactam ring which is the active side of the drug; however cefotaxime is less basic and therefore, less ionizable which enable the compound freer to interact its receptor and though the activity of cefotaxime can be enhanced as seen in Figure 1. On the other hand, the structure of ceftazidime has a pyridine moiety (with increased water solubility, stability, and hydrogen bond-forming ability) which impart more basicity and ionizability to the whole structure. Therefore, the antibacterial activity of ceftazidime is not enhanced in the same way as in cefotaxime (i.e., pyridine moiety versus methyl ester side chain) as seen in Figure 2.

Table 3 Combined treatments of cefotaxime and ceftazidime with either sodium nitrite, ascorbic acid, or acidified nitrite against the biofilm of beta-lactamase producing isolates

Combination treatment		Paired Differences					t	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference			
					Lower	Upper		
Pair 1	CTX - CTX+AN	0.05650	0.03865	0.01033	0.03418	0.07882	5.469	<0.001
Pair 2	CTX - CTX+AA	0.05150	0.03127	0.00836	0.03344	0.06956	6.162	<0.001
Pair 3	CTX - CTX+NaNo2	0.04686	0.04855	0.01297	0.01883	0.07489	3.611	0.003
Pair 4	CAZ - CAZ+AN	0.01857	0.02194	0.00586	0.00590	0.03124	3.167	0.007
Pair 5	CAZ - CAZ+AA	0.01729	0.01515	0.00405	0.00854	0.02603	4.270	0.001
Pair 6	CAZ - CAZ+NaNo2	0.00929	0.01937	0.00518	-0.00190	0.02047	1.793	0.096

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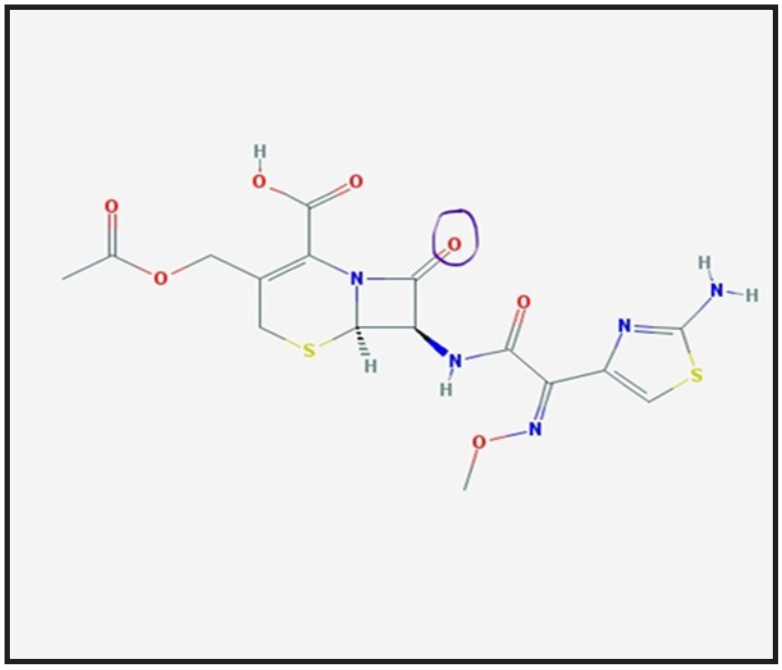

Figure 1 Chemical structure of Cefotaxime with the mark of the site of formation of its interactions and enhancement of its activity.

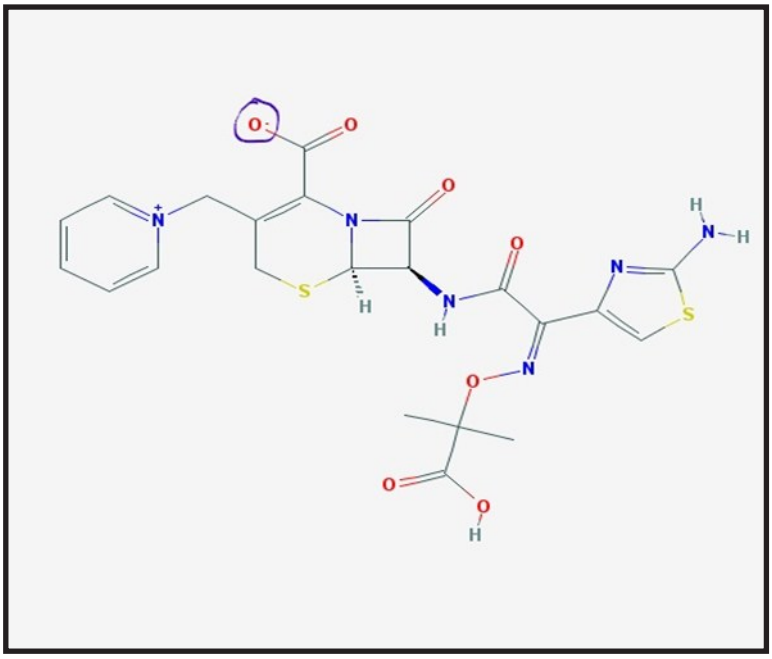

Figure 2 Chemical structure of Ceftazidime with the mark of the site of formation of its interactions and enhancement of its activity.

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Furthermore, previous *in vivo* investigations reported that the daily intake of more than 50 mg vitamin C alone can significantly decrease infection of the urinary tract.[29] Investigations described an opposite result of the present study, the combination treatment, ascorbic acid with the beta-lactam antibiotic (ampicillin) had an indifferent effect against the bacterial growth of *P. aeruginosa.*[25] However, the highest synergistic effect of cefotaxime and ceftazidime was reported with acidified nitrite which is similar to the results of the previous studies. Investigations stated a significant reduction of bacterial growth in combination of nitric oxide with aminoglycosides (tobramycin and gentamycin) against the bacterial growth of *P. aeruginosa* and *S. pneumoniae.*[8] Interestingly, identical synergistic results have been reported in an investigation which combined miconazole and nitric oxide. The combined effect led to prolonged growth inhibition in ESBL-producing uropathogenic isolate.[10]

Possible explanation for the synergistic effects with acidified nitrite is that sodium nitrite was ionized an enhanced antibacterial activity of the drugs and also ascorbic acid. As a result of the formation of a number of ion-dipoles interaction with their carbon oxygen atom of the beta-lactam ring in the structure of the drugs and in the structure of ascorbic acid. In the combination with acidified nitrite there were two sources with antimicrobial activity the drug and ascorbic acid. However, the synergistic effects in the combination with ascorbic acid was lower than with acidified nitrite because there was no factor to enhance the activity of the drug.

Our findings showed that synergistic effects with beta-lactam antibiotics were observed at least with sodium nitrite which is in agreement with other studies that detect the combined effect of aminoglycosides with sodium nitrite and concluded a decrease in eradicating of *P. aeruginosa*.[11] In the combination with $NaNO_2$, ion-dipoles interactions between the carbon oxygen atoms of beta lactam ring with sodium positive ion were formed, however, it was not as effective in comparison to acidified nitrite and ascorbic acid as mentioned earlier that the vitamin alone has antibacterial effects.

It is indicated that biofilms are difficult to eliminate and usually resistant to antibiotic levels that are 10-1000 times greater than levels required to bacteria in suspension.[5] Studies showed that about more than 60% of bacterial infections in developed countries are supposed to be biofilm producers and many of the recurrent UTIs are assumed to be caused by biofilm producing uropathogens.[5] The continuous development of MDR pathogens has changed the view of a researcher to rediscover common antibiotics with new combinations.[10] This study showed highly significant reduction of biofilm mass of ESBL producing isolates by the combination treatment of acidified nitrite with both CTX and CAZ. This enhancement of the activity of antibiotics with NO was in agreement with other studies in which NO improve the activity of amoxicillin-clavulanic acid against pneumococcal biofilm.[8] In addition, another study concluded that combination treatment of NO with antibiotic could augment the activity of tobramycin against the biofilm formation of *P. aeruginosa.*

An explanation for the synergistic effects with acidified nitrite treatment can be linked to NO which are small molecules, highly diffusible through lipid bilayer membranes like biofilm. NO showed to induce biofilm dispersal and led to a reduction in biofilm mass and increase in the same time the ability of antibiotics to penetrate the remaining biofilm and eradicate the distributed free-floating bacterial cells. Several previous *in vitro* and *in vivo* studies described the unaided ability of NO to reduce biofilm formation significantly in numerous bacterial species like *S. aureus, P. aeruginosa* and *E. coli* strains lacking the ability of ESBL formation.[17,18,30]

The outcomes of the study exhibited that

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the second combination treatment ascorbic acid with both antibiotics CTX and CAZ significantly reduced biofilm mass. Possible explanation might be due to the fact that biofilm mass is destabilized through acidity and ascorbic acid is one of the reducing agents. Previous studies reported an identical synergistic ascorbic acid effect in combination with Cold Atmospheric Plasma (CAP) which significantly decreased the biofilm mass of *S. aureus*, *E. coli* and *P. aeruginosa*.[31]

Several studies indicate that sodium nitrite alone can inhibit biofilm formation in *S. aureus* and *P. aeruginosa*.[11] We investigated the third combination treatment of sodium nitrite with antibiotic, and results showed a significant decline in biofilm formation only with cefotaxime (P = 0.003), which is in agreement with another study that detected a significant additional reduction of biofilm mass of *P. aeruginosa* through combination treatment of sodium nitrite with polymyxin (colistimethate).[11] Although the combined treatment of ceftazidime with sodium nitrite showed a reduction, it was not statistically significant (P = 0.096).

A possible explanation might be that the sodium nitrite effect might be linked with the antibiotic class used in combination. Studies described that sodium nitrite prevented the activity of aminoglycosides (tobramycin) towards *P. aeruginosa* biofilms.[32]

Notably, it is reported that bacteria developed a protective mechanism against NO through activation of the flavohemoglobin enzyme which can detoxify NO into non-toxic nitrite. In addition, it is indicated that gene and protein expression of flavohemoglobin increase in *E. coli* after exposure of NO. However, studies described that uroepithelial cells can be damaged by high concentrations of NO, therefore, it is important to regulate the NO levels within the urinary tract.[10,33]

A limitation of the study was that the biofilm experiments were performed *in vitro*. *In vitro* biofilms might be differently structured and react dissimilar in comparison with *in vivo* biofilms. Several factors might have an effect on biofilm formation within the human body, for instance, local pH and inflammation.[34] To our knowledge, this is the first study to investigate the combination treatment of beta-lactam antibiotics with each of acidified nitrite, ascorbic acid and sodium nitrite to enhance the antibiotic activity against ESBL producing uropathogens and their biofilm.

Conclusion

Ascorbic acid and acidified nitrite significantly improved the activity of beta-lactam antibiotics (ceftazidime and cefotaxime) in the reduction of biofilm mass of beta-lactamase producing uropathogens.

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Competing interests

The authors declare that they have no competing interests.

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