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Original Research Article

Enhanced Antimicrobial Efficacy of Leucine-Ciprofloxacin-Ceftazidime Combination against MDR *E. coli*

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Abstract: One of the greatest threats to public health is the multidrug resistant microorganisms (MD-resistant microorganisms), which also are referred to as the superbugs, and which manage to kill millions of people annually around the world. So, the purpose of the study is to have synergistic effect of antibacterial to give modern strategy to survive resistance bacteria to antibiotics. Clinical specimens of 60 patients were taken of those with burn burns and urinary tract infection of patients. Identification of bacterial isolates was done under cultural as well as by vitek-2 system and antibiotic susceptibility test was done to obtain the resistance of the isolates towards the antibiotics. Ciprofloxacin/leucine, Ceftazidime/leucine combination were tested against ten MDR Escherichia coli isolates in which the minimum inhibitory concentration (MIC) values were measured, gene on Gyrase A and Gyrase B was estimated using the real- time PCR on ten MDR E. coli isolates against the antibiotic alone and combination of each form of antibiotic and leucine. The MICs of isolates were also effectively reduced in isolates against CIP/leucine; and CAZ/leucine combination compared to their individual antibiotics (p 0.05). Results of Gyrase A and Gyrase B gene expression exhibited a major decline upon adding leucine to both antibiotics as compared with the control isolates (p<=0.05). Conclusively, it is notable to be able to notice the effect of leucine coupled with CIP and CAZ to the MDR isolates which is a plus to the ways of combating bacteria resistant to drugs, especially when combined with biological agents that are highly effective.

Keywords: *E. coli,* Antimicrobial Efficacy, Leucine-Ciprofloxacin-Ceftazidime.

INTRODUCTION

Multiple drug resistance (MDR) is the capability of microbe to be refractory to the impacts of antimicrobial medication, including antibiotics, a major dangerous threat to worldwide populace wellbeing. Antibiotic resistance occurs when bacteria produce defense mechanisms to withstand exposure to the substances (Bharadwaj, A *et al*., 2022). Intrinsic resistance intrinsic factors that lead to resistance and extrinsic factors, e.g. the notorious overuse and misuse of antibiotics, The term bacterial intrinsic resistance is used to describe intrinsic resistance as shown by some bacteria species to specific antimicrobial agents, which is coded in their genetic, structural or physiological characteristics. Unlike acquired resistance, which is due to mutation or horizontal gene transfer, because the outer barrier of many Gram-negative bacteria is impermeable, preventing the entry of the medications into the bacterial cell, they are naturally resistant to a number of antibiotic types. The multidrug resistance of Escherichia coli is a troubling issue which continues to emerge in veterinary and human health around the world. E. coli is highly susceptible to virtually all therapeutically significant antibiotic interventions and tends to gain resistance genes, largely through horizontal gene transfer. One of the most challenging mechanisms in E. coli is acquisition of genes encoding the extended-spectrum 8-lactamases, and they confer resistance to broad-spectrum cephalosporins (Wang C *et al*., 2018). Ciprofloxacin is a

bactericidal antibrief agent of fluoroquinolones category and it targets the DNA gyrase. Type II topoisomerases Escherichia coli has the enzyme called DNA gyrase which generates negative super coils in the DNA and thereby makes the replication, transcription and the repair process of DNA possible (Kaper JB *et al*., 2004) β-Lactam are notable examples such as ceftazidime which cause damage to the bacteria cell wall without killing the bacteria, a non-fatal implication that the β-Lactams bind with the PBP (Witz, G. & Stasiak, A., 2010).

Combination therapy is one of the possible successful strategies against the resistance to antibiotics exhibited by bacteria. This argument shows that combination therapy is a method of treatment against MDR infections that may be due to the added stress of the combination which may be in effect more than each one separately. These combinations can be seen in antibiotics and non-antibiotics combinations. The mixture of antibiotics and non-antibiotic substances is believed to be the most effective way (Worthington, R.J.; Melander, C., 2013). When mixed with antibiotics, non-antibiotic substance could many times enhance their effectiveness, but when used separately it produced very little or no antibacterial effect. Based on some of the studies, the generation of bacteria that are resistant or tolerant in different physiological conditions can be enhanced by metabolic activation of antibiotics. Added exogenous glutamate promotes the destruction of multidrug uropathogenic E. coli by multiple antibiotics in vitro (Debnath, S. K *et al*., 2022), (Tyers, M.; Wright, G.D., 2019). We have conducted a thorough research on an E. coli strain MDR. In vitro antimicrobial susceptibility testing was used to also obtain the minimum inhibitory concentrations (MIC) of the following combinations of antibiotics biological substances that do not appear to be antibiotics (leucine). Isolated mRNA was subsequently assessed in regards to its concentration and purity levels before we converted it into cDNA in order to compare the effects of the chosen mixtures. Gene expression was performed using RT-qPCR.

MATERIALS AND METHODS

Sampling

The sample (n=60) was collected among the patients similar to having urinary tract infection symptoms (with or without wound discharge) who signed the consent. By use of transport media, all the isolates were transferred under cooling conditions to make the diagnosis. Selective media was used to identify all the bacterial isolates and this included eosin methylene blue and MacConkey agar, in which the isolates were redone on nutrient broth to be identified further by using Vitek2 Compact System. Once identification of bacteria was done, bacteria susceptibility test was done on all the bacterial cultures in order to know the amount and kinds of bacteria that were resistant to antibiotics.

Measurement of Minimum Inhibition Concentration (MIC)

The assay was carried out as antimicrobial susceptibility test that entailed six categories of antibiotics on 30 samples of E. coli. Antibiotics, which have been used in this study, were (Imipenem, Azithromycin, Ciprofloxacin, Ceftazidime, Aztreonam and Gentamicin), out of these 6 varieties of antibiotics, resistance of bacterial isolates towards ciprofloxacin and ceftazidime were 100 percent. MIC was determined through a method of micro dilution broth (microwell method). Leucine was used in the following concentration: (2.5,5,10,20 mM) and each of the antibiotics: 30,40,50,60ug/ml were used. The incubation of microwell was done overnight under 370C (18hr) in incubator. The resulting readings were taken on Eliza reader at 260nm after they had been incubated.

Combination Activity: Combination Activity (E. coli) Molecular Study

RNA Extraction

Ten of the bacterial isolates would be used in the gene expression of the Gyrase A and Gyrase B, the total RNA of (non-treated, CIP treated, CAZ treated, CIP+leucine treated and CAZ+ leucine) treated bacterial cells would be extracted using (FAVORGEN Kit, Korea), The concentration and purity of RNA in the sample would be determined using a Nano drop device and the integrity of the extracted RNA by gel electrophoresis.

RT-PCR Method of Gene Expression

In order to determine the expression of Gyrase A and Gyrase B genes before and after treatment using combinations prepared as explained above, Real time - Polymerase chain reaction (RT-PCR) was adopted, The primers used to express Gyrase A and Gyrase B as well as the house keeping gene (GAPDH) are provided in Table1, In addition to that, the components of the quantitative qRT-PCR reaction are also provided in Table 2 and finally the General thermocycler program and the reaction conditions that we used in this study were as follows: (primary Expression of the Gyrase A and B gene was quantified as 2- Delta Delta CT and the fold of gene expression was arrived at.

Table 1: Oligonucleotide primer sequences, amplicon sizes, and references

Gene names	Primer Sequences (5'-3')	product Size (bp)	Reference of primer
GyrA	**F: GCCATGAACGTACTAGGC R: GGATATACACCTTGCCGC**	**180bp**	Yoshida, H *et al*.,1990
GyrB	**F: AGAAATTATCGTCACCATTCACGC R: GTACACCGTGTTCGTAGATCT**	**278bp**	Yamamoto, S., & Harayama, S..1995
GAPDH Housekeeping gene	**F: ACTTACGAGCAGATCAAAGC R: AGTTTCACGAAGTTGTCGTT**	**170bp**	(Barber, R. D *et al*.,2005)

Table 2: Reaction mixture of RT-PCR

Components	Sample volume in (ul)
2.5x Reaction Mix+ syber Green	10 ul
MgCl2	1.5 ul
Forward primer	1 ul
Reverse primer	1 ul
DNA sample	1.5ul
ddH2O	10 ul
Total volume	**25 ul**

Statistical Analysis

Collected data was statistically analyzed by SPSS 27 and graph pad prism 10 and the analysis carried out by t-test when there is a need to determine significance between two groups and ANOVA when there is the need to determine the significance between three or more groups and P-value was considered significant when was less than 0.05.

RESULTS

Isolated Bacteria

The entire samples of the bacteria were grown in Eosin methylene blue agar and MacConkey agar and left to incubate at a temperature of 37 o C within 24 hours so as to stimulate the growth of the bacteria. Once the bacterial isolate was determined by a Vitek2 compact system, the laboratory culture revealed that 30 out of all the samples were E. coli but the remainder were not. These findings were concurring with the outcomes of the culture identification which was carried out in our study and found that the samples were E. coli with 99 % of probability.

MICs Determination

In ten isolates, antibiotics were vulnerable to more than two drugs under multidrug resistance. the concentration of leucine was (2.51020)M table 3 and 4. Most of the pathogenic bacteria, which showed significant effect on the bacterial isolates (E. coli), results of each of the combinations obtained were highly significant, MIC of each combinations was 50ug/ml as observed in figures 1 and 2.

Table 3: Results of combination between Leucine and CIP

Cipro (µg/mL) Leucin (mM)	MIC (Mean±SD)				P-value
	30	40	50	60	
A- 2.5	0.280±0.14	0.192±0.12	0.188±0.12	0.165±0.07	**0.386**
B- 5	0.267±0.12	0.188±0.1	0.183±0.08	0.151±0.08	**0.325**
C- 10	0.197±0.08	0.142±0.08	0.109±0.08	0.112±0.08	**0.054**
D- 20	0.195±0.1	0.141±0.09	0.107±0.09	0.111±0.09	**0.134**
Control	0.580±0.12	0.580±0.12	0.580±0.12	0.580±0.12	
P-value	**<0.001**	**<0.001**	**<0.001**	**<0.001**	

Table 4: Results of combination between Leucine and CAZ

Cefta (µg/mL) Leucin (mM)	MIC (Mean±SD)				P-value
	30	40	50	60	
A- 2.5	0.255±0.03	0.247±0.03	0.233±0.03	0.234±0.03	0.330
B- 5	0.231±0.02	0.236±0.01	0.228±0.03	0.229±0.02	**0.898**
C- 10	0.123±0.03	0.114±0.02	0.176±0.04	0.181±0.04	**0.001**
D- 20	0.122±0.05	0.112±0.05	0.175±0.05	0.180±0.05	**<0.001**
Control	0.580±0.12	0.580±0.12	0.580±0.12	0.580±0.12	
P-value	**<0.001**	**<0.001**	**<0.001**	**<0.001**	

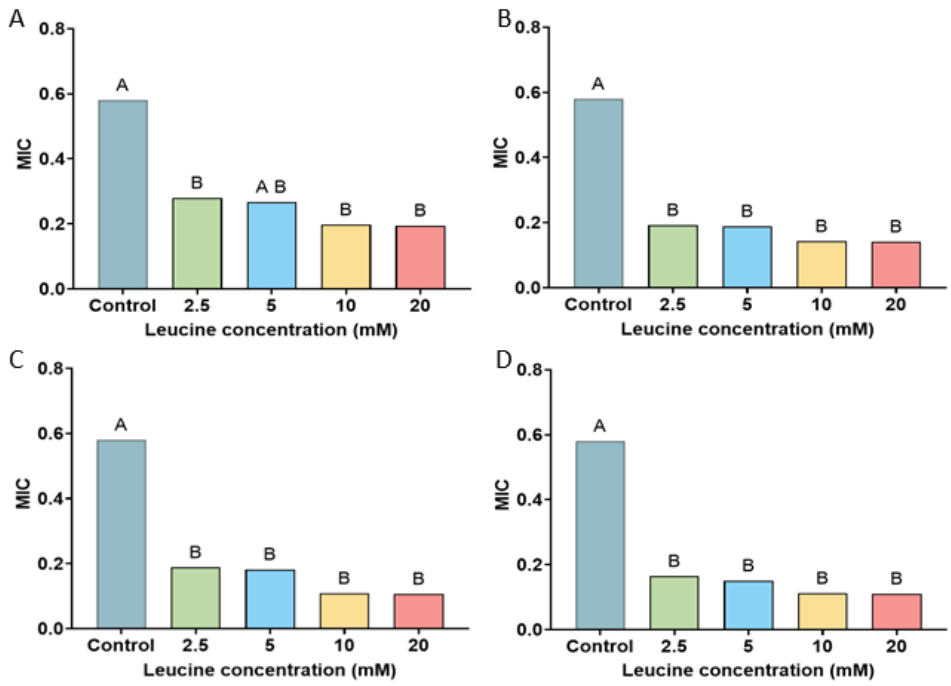

Figure 1: Results of combinations between Leucine and CIP at concentration (A) 30; (B) 40; (C) 50; (D) 60; Similar letters indicate no significance difference while different letters indicate significant difference.

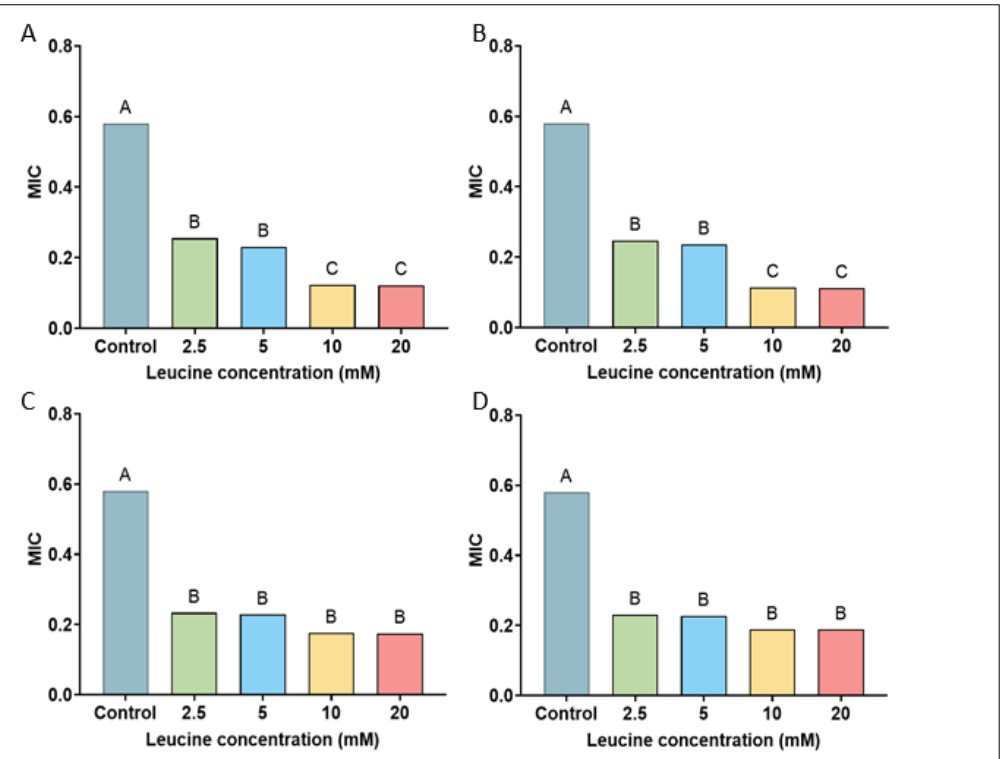

Figure 2. Results of combination between Leucin and CAZ at concentration (A) 30; (B) 40; (C) 50; (D) 60; Similar letters indicate no significance difference while different letters indicate significant difference.

***Gyrase A* and *Gyrase B* Gene Expression of Pathogenic Bacteria under Effect of Each Combination**

As seen in Figures 3 and 4, the fold of gene expression was computed for each bacterial isolate using the 2–ΔCt formula. For most pathogenic bacteria, the P value was highly statistically significant, indicating a significant effect of the combination on the expression rates of the *gyrase A* and *gyrase B* genes, respectively. Because of this, most bacterial isolates showed a drop-in expression rate following antibiotic treatment as compared to the rate prior to treatment.

Table 5: Comparison of Treatments with controls for *Gyrase A* gene

Treatment	$2^{-(\Delta\Delta Ct)}$ Mean±SD	P-value*	P-value**	P-value***
1A	1.05±0.47			
1B	1.07±0.51	0.869		
1C	1.33±0.85	0.222	0.280	
2-CIP/leu	0.03±0.03	0.005	0.007	0.006
3-CAZ/leu	0.05±0.03	0.006	0.008	0.006

*Comparison of each treatment with 1A; ** Comparison of each treatment with 1B; *** Comparison of each treatment with 1C;

Table 6: Comparison of Treatments with controls for *Gyrase B* gene

Treatment	$2^{-(\Delta\Delta Ct)}$ Mean±SD	P-value*	P-value**	P-value***
1A	1.24±0.66			
1B	1.28±0.58	0.872		
1C	1.4±0.9	0.205	0.120	
2-CIP/leu	0.06±0.06	0.002	<0.001	<0.001
3-CAZ/leu	0.07±0.07	0.003	<0.001	<0.001

*Comparison of each treatment with 1A; ** Comparison of each treatment with 1B; *** Comparison of each treatment with 1C;

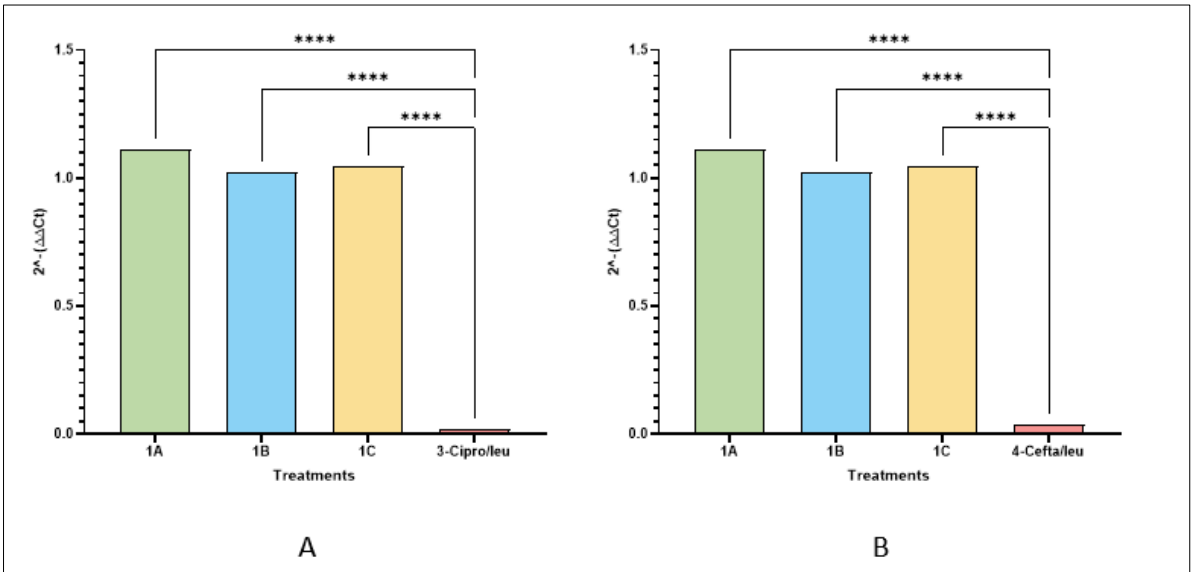

Figure 3: Multiple comparison of mean $2^{-(\Delta\Delta Ct)}$ for Gyrase A between control groups with Treatments (A) CIP/leu (B) CAZ/leu * indicate P-value <0.05; **** indicate P-value <0.001.**

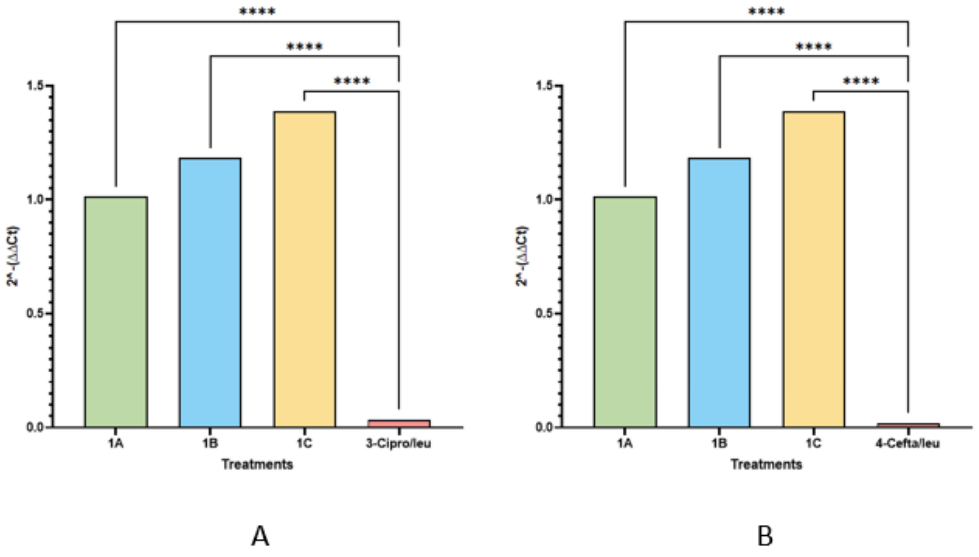

Figure 4: Multiple comparison of mean $2^{-(\Delta\Delta Ct)}$ for Gyrase B between control groups with Treatments (A) CIP/leu (B) CAZ/leu. * indicate P-value <0.05; **** indicate P-value <0.001.**

DISCUSSION

The multidrug resistant (MDR) Escherichia coli has posed a serious danger to the health of people, The great cause of the Multidrug resistance in E. coli is the evolvement of natural R plasmid genes (Parmanik, A *et al*., 2022). Interaction of leucine with the CAZ and CIP antibiotics has potential to treat MDR E. coli infections since it can enhance the effectiveness of antibiotics in the evasion of resistance and attack Gyrase A and Gyrase B genes. Synergistic effect against MDR E. coli was recorded in the present study on mixing leucine with the following concentrations (2.5,5,10,20 mM) with ceftazidime (50 ug/ml) and ciprofloxacin (50 ug/ml) separately. Further, the combination of ceftazidime 50 ug/ml with leucine (2.5,5,10 and 20 mM) and ciprofloxacin 50 ug/ml demonstrated an antagonistic effect against MDR E. coli. Thus, the composition of each of these antibiotics with leucine in the present work could have an even more significant effect on MDR E. coli than their separate application; the mixture of antibiotics and leucine led to a significant reduction ($p<=0.05$) in bacteria growth. Such findings are consistent with a past study outlined by (Mangal S *et al*., 2018) which indicated that ciprofloxacin Co-spray drying in L-leucine substantially improved the aerosol potential of ciprofloxacin and physical and aerosol stability of the (Dry Powder Inhaler) DPI formulation without producing any change in Fine Particle Fraction FPF when stored at 55 percent (Relative Humidity) RH. It is a known fact that co-spray drying of drugs with amino acids (amino acids like L- leucine) may improve aerosol performance, and that this may happen through L- leucine enrichment on the particle surface (Mangal *et al*., 2018). An earlier literature reported by (Li J, Zheng H, and Leung SSY., 2023) has reported that the L-leucine supplementation of the ceftazidime formulation increased its fine particle fraction by 1.2 folds. The study done above showed the reason to add L-leucine to enhance the sprays of spray-freeze-dried particles. The results of this work could in their turn justify the cause of successful improvement of the activity of the considered antibiotics in our research study and can be attributed to the process of the drop in MIC when Leucin is added to each antibiotic. At the genetic scale, Comparing the use of antibiotics each alone (CIP and CAZ) to the combination of each antibiotic with leucine showed a significant reduction ($p<=0.05$) in the in-gene expression of DNA gyrase A and B genes and this could be attributed to the fact that the combination of CIP/leucine and CAZ/leucine acted in response to both subunits associated with the DNA gyrase (GyrA and GyrB) and trapped the enzyme bound to the end of the DNA break inhibiting DNA re No prior literature is available to date that can inform of an interaction between ceftazidime or ciprofloxacin and L- leucine that influences either of the genes Gyr A or Gyr B. Thus, no study has ever disclosed such an association before and this study will bridge this gap. Any theory concerning the effect of leucine as adjuvants (activity enhancer) of ceftazidime or ciprofloxacin on gyrase genes is up to this moment, not experimentally proven.

CONCLUSION

Finally, it can be concluded that there is a potential ability to reclaim the effectiveness of antibiotics using synergistic combinations, particularly those which target key functions of the bacteria such as DNA gyrase activity, to overcome MDR in E. coli. These

combinations require further research in order to optimize and better comprehend the molecular mechanisms that lead to an increase in activity of the antimicrobials.

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