Research Article

Evaluation of Synergistic Antimicrobial Effect of Ascorbic Acid with Antibiotics Against *E. coli O157 In Vitro*

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Abstract | The present study was carried out to study the interaction between ascorbic acid and two antibiotic (ciprofloxacin and erythromycin) in-vitro and the in vitro. The research was included two experiments. The first one was to isolate and identify *E. coli O157*; *E. coli* sample was taken from a hospital, the sample was cultured on MacConkey agar, Sorbitol MacConkey agar, chrome agar, eosin methylene blue agar, then VITEK® 2 System was performed Data showed the sample was positive as a result of *E. coli O157*. The second experiment examined the pharmacodynamics of ascorbic acid in various states, including alone and in combination with ciprofloxacin and erythromycin. Results showed that the MIC of ascorbic acid (AA), ciprofloxacin (CIP), and erythromycin (ERY) against *E. coli O157* was 16 mg/ml, 2 g/ml, and 256 g/ml, respectively, and the values of MBC by utilizing of Time kill curve were 32 mg/ml, 4 g/ml, and 512 g/ml for each of them, respectively. The pharmacodynamics-interactions within double combinations of ascorbic acid (AA) with ciprofloxacin (CIP) and erythromycin (ERY) against *E. coli O157* isolate by micro dilution checkerboard assay showed that the combination of ascorbic acid with ciprofloxacin exhibited synergistic antibacterial effects through fractional inhibitory concentration (FIC index < 1) against tested isolate, while the combination of ascorbic acid with erythromycin exhibited antagonistic effects. Furthermore, time-kill analysis showed that ASCORBIC/CIP combination exhibited highest synergistic and bactericidal effect. It was determined that in vitro study against *E. coli O157* in the treatment of urinary tract infection, the combination of ascorbic acid and ciprofloxacin was both more potent and safer than the combination of ascorbic acid and erythromycin. From this study concluded the combination of ciprofloxacin, and erythromycin against isolated *E. coli* showed that this microorganism is highly sensitive to ciprofloxacin and resistance to erythromycin.

Keywords | Ascorbic acid, *E. coli O157*, Minimum inhibitory concentration, Time killing curve, Ciprofloxacin, Erythromycin

Received | May 26, 2023; **Accepted** | June 10, 2023; **Published** | July 22, 2023
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Email: nibras.z.yahya@gmail.com
Citation | Ahmed TF, Yahya NZ (2023). Evaluation of synergistic antimicrobial effect of ascorbic acid with antibiotics against *E. coli O157 in vitro*. Adv. Anim. Vet. Sci., 11(8):1391-1397.
DOI | https://dx.doi.org/10.17582/journal.aavs/2023/11.8.1391.1397
ISSN (Online) | 2307-8316

INTRODUCTION

Escherichia coli (or simply *E. coli*) is one of the many groups of bacteria that live in the intestines of healthy humans and most warm-blooded animals. *E. coli* bacteria help maintain the balance of normal intestinal flora (bacteria) against harmful bacteria and synthesize or produce some vitamins (Kliebert and Jindal, 2014). In human, approximately 86% of the patients whose infections were caused by *E. coli* were females (Mraidi and Lafta, 2021).

The infection with *E. coli O157* may cause changes in liver and kidney functions which were more sever at 15,30 days post infection (Al-Taii and Yousif, 2019). Even a low concentration of *E. coli O157* contamination with a small amount of infectious material could be harmful to human beings in nature (Mohammed, 2014). The microbiota may be regarded as the main source of UPEC bacteria, according to studies by (Ahmed et al., 2019).

Ascorbic acid is an essential nutrient for the body that cannot be synthesized by the body. This vitamin is an acid, unstable, easily soluble in water, so it does not accumulate in the body, also supplementation of the complementary ascorbic acid could maintain a normal immune system function (Al-Azawi and Alkenany, 2017).

Also (Diab, 2005) demonstrate that Ascorbic acid has a significant impact on decreasing and avoiding some of the oxidative stress changes in lipid profiles and atherosclerotic lesions of the aorta. Oxidative stress (generated by 0.5% H_2O_2) is thought to play a significant role in the etiology of atherosclerosis in rabbits.

Ciprofloxacin is an antibiotic agent in the fluoroquinolone class used to treat bacterial infections such as urinary tract infections and pneumonia (Zhang et al., 2017; Bartolomé and Solves, 2020).

While Erythromycin is a bacteriostatic antibiotic macrolide widely used in human medicine (principally in ambulatory care), as well as by farmers to control bacterial diseases and promote animal growth. With regard to environmental contamination, erythromycin A was more frequently detected in 139 United States stream sites than 21 other veterinary and human antibiotics (Kolpin et al., 2002).

The relationship between Ascorbic Acid and antibiotics is that Ascorbic acid produced slightly inhibitory effect on *E. coli* growth when used alone, but when combined with antibacterial or others antioxidants produced clear inhibitory effect. This may due to the effect of Ascorbic acid on growth of *E. coli* by reducing biosynthesis of extracellular polymer substances, especially the polysaccharide component of the matrix; therefore, once the extracellular polymer substances content is reduced, then the bacterial cells are more exposed to external medium and more susceptible to killing (Pandit et al., 2017). It was reported that Ascorbic acid potentiated the antibacterial activity of ciprofloxacin, this may be due to its pro-oxidant activity or by altering the membrane permeability (Srividya et al., 2017). The present study was designed to study the interaction between ascorbic acid and two antibiotic (ciprofloxacin and erythromycin) *in-vitro* and the *in vitro*.

MATERIALS AND METHODS

Collection and isolation

Urine samples were collected from Al-Yarmouk hospital in Baghdad and Al-Zahraa hospital in Wasit Hospital . One hundred samples were collected from human that were suffering from UTI signs. Urine samples were subjected to bacterial culturing on nutrient agar. The suspected colonies of *E. coli* were re-inoculated on selective media (Eosin Methylene blue, Sorbitol MacConkey agar and Chromogenic agar O157) for isolation and identification then incubated at 37°C for 24 hrs.

Sensitivity test

Commercially available discs (6 mm diameter) preloaded with amikacin (30 µg/disc), Chloramphenicol (30µg/disc), gentamicin (10µg/disc), erythromycin (15µg/disc), norfloxacin (10µg/disc), cefixime (5µg/disc), ciprofloxacin (5µg/disc), netillin (30 µg/disc), kanamycin (30 µg/disc), rifampicin (5µg/disc) and ascorbic acid tablet (500mg) were used. The agar disc diffusion method was adapted according to performance standards of CLSI (CLSI, 2018).

Determination of minimum inhibitory concentration

For this purpose, a stock solution of ciprofloxacin, erythromycin and ascorbic acid were prepared in Mueller-Hinton broth, then make serial dilution in different concentration that ranges between 0.0125-2048 µg/ml of these antibiotics and 4-1024 mg/ml for ascorbic acid were prepared, in 96 well microtiter plate, each well was inoculated with 100 µl of 106 CFU/ml *E. coli O157* and incubated on 37 °C for 24 hrs (CLSI, 2018). For colorimetric identification of bacterial growth, adding 20µl of TTC indicator (0.125% w/v) to each well of the test and re-incubated for 2 hrs (Viga et al., 2019).

In-vitro time kill curve

The time-kill curve assay of ciprofloxacin, erythromycin and ascorbic acid against *E. coli O157* isolate was based on the National Committee for Clinical Laboratory Standards. Briefly, bacterial suspension equivalent to 0.5 McFarland (1.5x108 CFU/ml) was prepared from overnight bacterial culture. 0.1 ml of the prepared bacterial suspension was diluted in 14.9 ml of Mueller-Hinton broth and incubated at 37 °C for 1 hr. to obtain 106 CFU/ml bacterial suspensions.

Ciprofloxacin, erythromycin and ascorbic acid had been dissolved in Mueller-Hinton broth to prepare the stock solution of antibiotic, after that ciprofloxacin, erythromycin and ascorbic acid concentrations from 4x MICs, 2x MICs, 1x MICs, 0.5x MICs and 0.25x MICs had been prepared in 5 McCartney's bottles in addition to controlling positive

tube and each bottle was inoculated with 0.1 ml of bacterial suspension and incubated on 37 ºC for 24 hrs.

Pharmacodynamics analysis of antibiotics combination

Minimum inhibitory concentration (MICS)

The antibiotic combinations of (erythromycin/ascorbic acid) and (ciprofloxacin/ascorbic acid) were evaluated on the bacterial isolate (*E. coli*). The microdilution assays were carried out in sterile stock solutions of all tested medications that were prepared in Mueller-Hinton broth, according to (Veiga et al., 2019). In a nutshell, the final concentrations of CIP, ERY, and ascorbic acid in 200 µl of MHB ranged from (16 to 0.03125 µg/ml), (2048 to 32 µg/ml), and (1024 to 4 mg/ml), respectively. From this broth, a two fold dilution was made from 100 µg in a U-shaped (400 µl well capacity) 96-well micro-titer plate. Un-inoculated and inoculated 200µl of MHB were used as the negative and positive controls, respectively, in the incubation process at 35 for 22 hrs. 20 µl of 0.125% TTC dye was added to each well for colorimetric determination of bacterial growth, and the wells were then incubated once more for two hours.

RESULTS AND DISCUSSION

The findings of our investigation demonstrated that the colonies of *E. coli O157* positive isolates on MacConkey agar were pink in color. This happens as a result of the bacteria's capacity to ferment lactose and result in colonies with a pink tint this agree with (Hameed et al., 2022). A typical mauve color colony was produced by the chrome agar positive isolate. This medium was thought to be a sensitive and quick way to diagnose *E. coli O157*. This finding was consistent with those from (Lateef et al., 2018).

Sensitivity testing

10 antibacterial agents and ascorbic acid were tested in agar diffusion assay. They produced different degrees of zones of inhibition against *E. coli O157*. The sizes of inhibition zones (measured by length of diameter) were different according to each antibacterial agent and concentration of each. Chloramphenicol, ciprofloxacin, rifampicin, norfloxacin, gentamycin, kanamycin, netillin, amikacin, erythromycin, cefixime and ascorbic acid were the antibiotic that used. The size of inhibition zone was (31.45mm, 29.16mm, 20.16mm, 27.56mm, 20.63mm, 18.23mm, 22.63mm, 18.88mm, resistance, resistance and 18.0mm) respectively Figure 1. Alttai et al. (2023) find out that the highest sensitivity of *E. coli* bacteria to the antibiotic ciprofloxin, trimethoprim, and gentamicin was 96, 94, and 86%, respectively. In comparison, the highest percentage of resistance of *E. coli* to the antibiotics cephalothin, tetracycline, erythromycin, and amoxicillin was 100, 64, 64, and 62%, respectively.

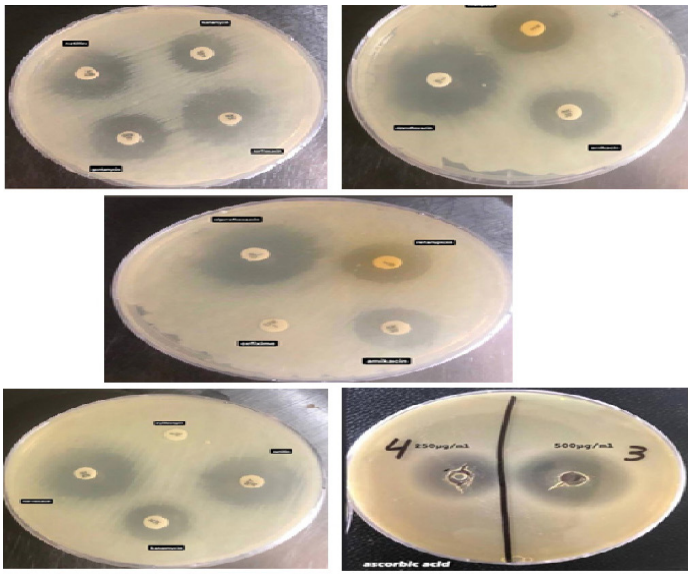

Figure 1: Sensitivity of *E. coli O157* to different antibiotic and ascorbic acid.

Pharmacodynamics analysis ciprofloxacin, erythromycin and ascorbic acid against *E. coli O157*

Minimum inhibitory concentration

The MIC values of ciprofloxacin, erythromycin and ascorbic acid estimated by micro-dilution assay were 2, 256µg/ml and 32 mg/ml, respectively Figure 2.

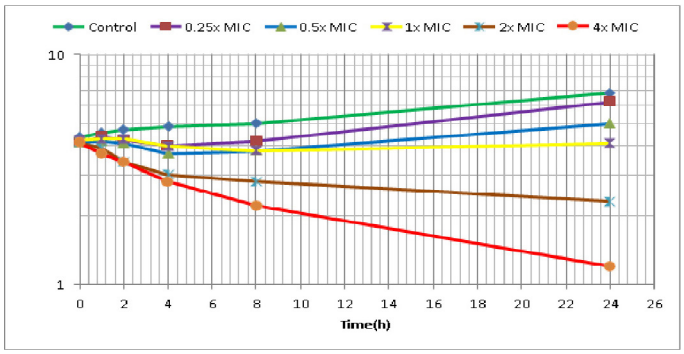

Figure 2: Time kill curve kinetics of ciprofloxacin against *E. coli O157*.

Due to uropathogens rising resistance to commonly used antibiotics such as erythromycin and cefixime, urinary tract infections caused by these pathogens are now much more difficult to treat. All of the UPEC isolates analyzed for this investigation showed multi-drug resistance.

The high prevalence rate of multi-drug resistant (MDR) isolates and their resistances to some regularly used antibiotics shown in our study are linked to the intensive and excessive use of these antibiotics in the treatment of urinary tract infections (UTIs) (Laxminarayan et al., 2013; Paphitou, 2013). The results of (Mohammed and Ibrahim,

2022) showed that the *E. coli O157* isolate was found to be multidrug-resistant (MDR).

Time-kill curve kinetics

Based on the highest MIC recorded from the microdilution MIC assay, which was 2, 256 µg/ml of ciprofloxacin and erythromycin, respectively, for the *E. coli O157*, time-kill curve kinetic results were calculated. The in vitro concentrations involved in the study were 0.25x, 0.5x, 1x, 2x, and 4x MICs.4 x (MICs) and 2x MIC concentrations of ciprofloxacin and erythromycin showed a drop in growth curve at 24th hr.While 1x MIC, 0.5x MIC and 0.25x MIC failed to achieve a significant bacteriostatic or bactericidal effect Figures 2, 3. The killing time's area under curve (AUC) was calculated and compared to the growth rate of the control inoculum; the difference between the area under the curve values for the various treatments served as the endpoint, and the highest bactericidal effect was indicated by the area under the curve with the lowest values (Table 1). In both antibiotics the AUC results showed that both 2x (MICs), and 4x (MICs) achieved the highest significant bacteriostatic effect in comparison to other concentrations. While The 1x MIC, 0.5x MIC, 0.25x MIC and control groups that failed to achieve a significant bacteriostatic or bactericidal effect.

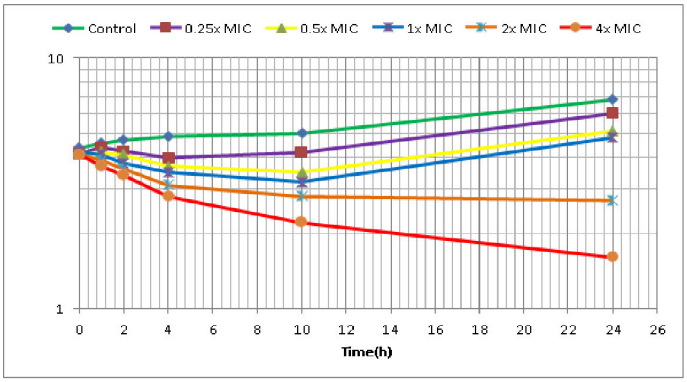

Figure 3: Time kill curve kinetics of erythromycin against *E. coli O157*.

The change in the logarithmic number of bacterial colonies over a predetermined chronological pattern is what determines the Time Killing Curve Kinetic, a comprehensive and unified method to evaluate the bacteriostatic and bactericidal effects of antibiotics (Mouton et al., 2005). The bacteriostatic effect of Ciprofloxacin is expected since the 4x MIC and 2 x MIC of ciprofloxacin has located within the determined value of *E. coli* sensitivity toward ciprofloxacin which was 2µg/ml. This bacteriostatic effect resulted from it interfere with the transient nature of the topoisomerase-induced double-strand breaks by stabilizing DNA/topoisomerase/drug ternary complex that traps the enzyme-bound double-strand breaks, thereby blocking bacterial growth (Heisig, 2009; Aldred et al., 2014).

On the other hand, a bacteriostatic effect of erythromycin is expected since the 4x MIC and 2 x MIC of ciprofloxacin has located within the determined value of *E. coli* sensitivity toward ciprofloxacin which was 256µg/ml. This bacteriostatic effect resulted By attaching to the 50S subunit of the bacterial ribosome, where it binds to the 23S ribosomal RNA molecule, it prevents the production of peptide chains, hence limiting protein synthesis (Tenson et al., 2003: Liang and Han, 2013). While 1xMIC, 0.25xMIC, and 0.5xMIC concentrations of both antibiotic failed to provide a discernible bacteriostatic or bactericidal impact, this failure may be related to an antibiotic's inability to sufficiently eradicate bacteria with high virulence (Kusumoto et al., 2017).

Pharmacodynamics analysis of double Minimum inhibitory concentration (MICS)

The results showed that the MICs of ciprofloxacin/ascorbic acid (CIP/Ascorbic acid) and erythromycin/ ascorbic acid (ERY/Ascorbic acid) against *E. coli O157:H7* isolate were 0.5/8 and 128/16, respectively. The checkerboard assays showed, the minimum inhibitory concentration (MICs) of these antibiotics were decreased significantly in antibiotics combinations (double) and exhibited a synergistic effect (FIC index < 1) against the selected isolate.

A fractional inhibitory concentration value of (cip 0.5/8 µg/ml ascorbic) was (0.75) less than 1.0 indicates synergistic effect of interaction while the FIC value of combination (ery 128/16µg/ml ascorbic) (1.06) more than 1.0 indicates antagonistic effect.

Table 1: Area under the time-kill curve of ciprofloxacin, erythromycin and combination against *E. coli* O157 (h*log CFU/ml).

Antibiotics	Control	0.25 × MIC	0.5× MIC	1 × MIC	2 × MIC	4× MIC
Ciprofloxacin	495.00±1.15aA	476.66±2.40bcB	430.00±1.15bC	374.66±1.45bD	312.00±1.15bE	224.00±1.15bF
Erythromycin	497.33±1.20Aa	488.66±2.02aB	447.33±1.45aC	385.00±1.15aD	325.00±1.15aE	245.33±0.88aF
Cip/Ascorbic	495.00±1.15Aa	475.33±2.02cB	409.66±1.45dC	348.66±3.52dD	214.00±2.08dE	124.33±1.76dF
Ery/Ascorbic	497.00±1.15aA	480.33±1.45bB	424.66±1.45cC	362.33±1.45cD	241.00±2.08cE	172.33±1.45cF
LSD	4.71					

Means with a different small letter in the same column are significantly different (P<0.05). Means with a different capital letter in the same row are significantly different (P<0.05).

The synergistic effect of ascorbic acid with antibiotics may be due to ascorbic acid's effect on some metabolic activity associated with protein synthesis inside bacterial cells, making the organisms more permeable to antibiotics through its effect on the cell membrane, allowing antibiotics to penetrate the cell more easily and effectively, or it could be due to the effect of (H_2O_2) produced by the auto-oxidation of ascorbic acid, which causes antibiotics to have a higher potency (Kramarenko et al., 2006). Other research has shown that certain antibiotics increase the production of reactive oxygen species (free radicals) in various bacterial species (Becerra and Albesa, 2002; Albesa et al., 2004) suggesting that ascorbic acid may serve as a free radical scavenger. According to obtained results we choose (cip/ascorbic) combination.

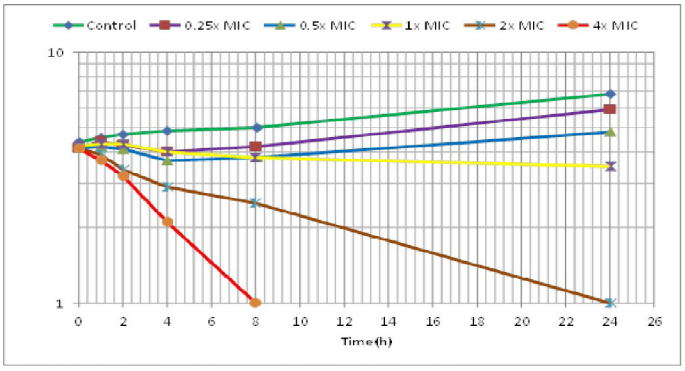

Figure 4: Time kill curve kinetics of CIP/ASCORBIC against *E. coli O157*.

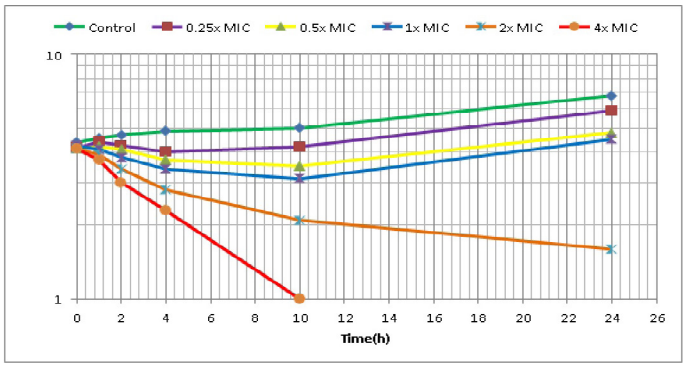

Figure 5: Time kill curve kinetics of ERY/ASCORBIC against *E. coli O157*.

Time-kill curve kinetics

Results of time-kill curve kinetics of double combination were based on the highest MIC recorded from the microdilution MIC assay against *E. coli O157*, the in-vitro concentrations of combination (CIP/Ascorbic) involved in the study were 0.25x MIC (0.5 µg/ml/4mg/ml), 0.5x MIC (1 µg/ml/8mg/ml), 1x MIC (2 µg/ml/16mg/ml), 2x MICs (4 µg/ml/32mg/ml), and 4x MICs (8 µg/ml /64mg/ml). On other hand, in-vitro concentrations combination (ERY/Ascorbic) involved in the study were 0.25x MIC (64 µg/ml/4mg/ml), 0.5x MIC (128 µg/ml/8mg/ml), 1x MIC (256 µg/ml /16mg/ml), 2x MICs (512 µg/ml /32mg/ml), and 4x MICs (1024 µg/ml /64mg/ml).The results showed, that 4 x (MICs) concentrations of CIP/Ascorbic achieved the bactericidal effect by Reducing ≥ 3 log10 of the total number of CFU/ml of *E. coli O157* at the 8th hr compared with 4x (MICs) of ERY/Ascorbic which reducing ≥ 3 log10 at the 10th hr. While 2x (MICs) concentrations of CIP/Ascorbic showed a distinguished bactericidal effect by reducing ≥ 3 log10 at the 24th hr compared with 2 x (MICs) concentrations of ERY/Ascorbic which showed a drop in growth curve at 24th hour. In addition to, 1 x MIC of CIP/Ascorbic showed a small drop in growth curve at 24th hours, in comparison to 1 x MIC of ERY/Ascorbic and 0.25x MIC and 0.5 x MIC of CIP/Ascorbic and ERY/Ascorbic that failed to achieve a significant bacteriostatic or bactericidal effect Figures 4, 5. The area under the time of killing curve was calculated and compared to the growth rate of the control inoculum; the difference in area under the curve values between the various treatments was set as the endpoint, and the lowest area under the curve corresponds to the greatest bactericidal effect, as shown in the Table 1. According to the obtained curves Figures 4 and 5 and the calculated areas under each one of them (Table 1), The results of AUC showed that (2x MICs and 4x MICs) CIP/Ascorbic and (4x MICs) of ERY/Ascorbic combination achieved the highest significant bactericidal effect (P≥ 0.05). While, 1x MIC concentration of CIP/Ascorbic achieved significant purely bacteriostatic effect (P≥0.05) against *E. coli* throughout 24 hrs compared to 1x MIC of ERY/Ascorbic and 0.5x MIC and 0.25x MIC and control of both combination which failed to achieve a significant bacteriostatic or bactericidal effect. The time-kill curve results of CIP/Ascorbic and ERY/Ascorbic combination were established on tracking the antibacterial effect of estimated MIC 2 µg/ml/16mg/ml and 256 µg/ml/16mg/ml respectively for *E. coli* in addition to down-scaled and up scaled derived MIC concentrations (0.25x MIC, 0.5x MIC, 1x MICs, 2x MICs, and 4x MICs) throughout 24 hrs. A such bacteriostatic or bactericidal effect of CIP/Ascorbic combination resulted from it is synergistic activities of the combination; the mechanism of ascorbic acid which effect on the cell membrane, ascorbic acid effects some metabolic activities connected to protein synthesis inside bacterial cells, making the organisms more permeable to antibiotics and improving the efficiency with which antibiotics can enter the cell. As a result, this will increase the amount of ciprofloxacin that enters the cell wall and exerts its bactericidal activity against *E. coli* by inhibiting DNA gyrase and bacterial topoisomerase IV, which kills the bacteria and delays the development of resistance (Appelbaum and Hunter, 2000; Dijkmans et al., 2017). More ever, combination of ERY/Ascorbic showed a bacteriocidal effect only in 4 x MIC at 24th hrs and bacteriostatic effect in 2 x MIC at 24th hrs this in agreement with (Rawal, 1974, 1978) that showed the bactericidal

effect of erythromycin against P. aeruginosa were found to be improved by ascorbic acid. While at low concentration there was no bacteriostatic nor bacteriocidal effect this is in agreement with (Wang et al., 1992) who showed that the effect of ascorbic acid on erythromycin is unknown. The pH range between 5.5 and 8.0 is ideal for erythromycin's antibacterial action at acid pHs, erythromycin loses its effectiveness. The observed effects of ascorbic acid on erythromycin could not have been produced by a pH factor because the pHs of culture medium were maintained neutral during incubation periods for the effective ascorbic acid concentration test. The current study's findings indicated that CIP/Ascorbic combinations are more likely than ERY/Ascorbic combinations to be synergistically effective in vitro bactericidal against multi-drug resistant *E. coli O157*. In regard to this, it has been suggested that CIP/Ascorbic combinations, even at low doses, could be employed to treat infections brought on by multidrug resistant (MDR) *E. coli O157*.

CONCLOUSION AND RECOMMENDATIONS

In vitro study with ciprofloxacin, and erythromycin against isolated *E. coli* showed that this microorganism is highly sensitive to ciprofloxacin and resistance to erythromycin. *In-vitro* study exposure of *E. coli* to 2, 4 MIC of ciprofloxacin success to kill bacteria after 24 and 8 hours, respectively in comparison with erythromycin only 4 MIC kill bacteria in 24 hrs. The effectiveness of ciprofloxacin increased in combination with ascorbic acid (highest synergism) compared to individual antibiotic acting alone.

ACKNOWLEDGMENT

I would like to thank the staff of the Physiology and Pharmacology Department, College of Vet. Med. Baghdad University for their flexibility, professional ideas, and advice throughout my study.

NOVELITY STATEMENT

The novelty of the study is focused on pharmacologically effect of Ascorbic acid with Antibiotics Against *E.coli O157* lead to synergistic effect against bacterial diseases *in vivo* and in *vitro*.

AUTHOR'S CONTRIBUTION

The authors each contributed equally.

Conflict of interest

The authors have declared no conflict of interest.

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