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Antibacterial Activity of Ascorbic Acid (Vitamin C) Against Urinary Quinolones Resistant Coliform Species

Mohammed T. Mahmood [1], ***Basima A. Abdullah*** [2]
[1]Department of Basic Sci. / College of Nursing / University of Mosul, Iraq
[2]Department of Biology / College of Science / University of Mosul, Iraq

ABSTRACT

One hundred and ten clinical samples were collected from both gender who visited or admitted to AL- Salam General Teaching Hospital from patients suffering from urinary tract infections (UTIs). All samples were cultured on selective media as MacConkey and blood agar aerobically.

Coliform isolates are identified to species level depending on biochemical and physiological tests and using Rapid ™ ONE panel kit to be conformed to the diagnosis. The susceptibility of coliforms species to a variety of antibiotics has been investigated. The results showed the majority of them were resistant to most antibiotics. Coliforms species isolated from UTIs indicated high level of resistance to the fluoroquinolone agents, resistance was observed most often to nalidixic acid followed by norfloxacin and ciprofloxacin. The minimum inhibitory concentration (MIC) values of ciprofloxacin against coliforms species ranged from 4-128 µg / ml. Ascorbic acid (Vitamin C) showed excellent antibacterial activity against urinary quinolones resistant species alone or in combination with antibiotics. The MIC of ascorbic acid was (5mg / ml) against all tested species. The sub-MIC of ascorbic acid (1mg / ml) showed synergistic effect with ciprofloxacin against urinary ciprofloxacin-resistant coliform spp..

INTRODUCTION

Enterobacteriaceae family may account for approximately (80%) of clinically significant isolates of gram-negative bacilli and they may account for (50%) of all clinically significant isolates in clinical microbiology laboratories . Coliforms species may account for approximately (10%)of the family (Willey *et al*., 2011 and Lyonga *et al*., 2014). Coliforms refer to those members of the family that ferment lactose such as Escherichia , Klebsiella , and Enterobacter species (Melkamsew *et al*., 2012 and Akhter *et al*., 2014). Coliforms species are associated with many types of infections including urinary tract and diabetic wound infections (AL-Kalifawi , 2014 and Rocha *et al*., 2015).

Quinolones are widely used to treat clinical infections in both in and out patients ; therefore a survey of quinolone resistance would be especially useful. Their use now accounts for about (11%) of overall prescriptions of antimicrobials in human medicine and one of them , ciprofloxacin , is the most used antibiotic in the world (Lyonga *et al*., 2014 and Tarchouna *et al*., 2015).

Quinolone resistance was for a long time considered to be entirely mediated by mutations in chromosomal genes encoding quinolone targets (that is , DNA gyrase and topoisomerase

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IV) and/or in regulatory genes of outer-membrane proteins or efflux pumps . Plasmid carrying *qnr* genes have been found to transmit quinolone resistance . These genes encode pentapeptide repeat proteins that block the action of ciprofloxacin on bacterial DNA gyrase and topoisomerase IV (Rushdy *et al*., 2013 and Al - Marjani *et al*., 2015).

Ascorbic acid (AS) , also known as AscH2 , ascorbate , or Vitamine C is a water-soluble ketolactone with two ionizable hydroxyl groups. AS is a natural antioxidant agent that is synthesized by plants and most animals except humans , primates and guinea pigs (Abbas, 2012 and Isela *et al* ., 2013). High doses of AS in combination with antibiotics were shown to inhibit the growth of *Helicobacter pylorii in vitro* as well as *in vivo* (Zhang *et al*., 1997 and Tabak *et al* ., 2003). Also , combination of AS with antibiotics had synergistic properties against *Pseudomonas aeruginosa* (Cursino *et al* ., 2005) . Moreover , AS is used as adjuvant in the treatment of cancer (Isela *et al* ., 2013) . Also , Ochoa-Brust *et al* ., (2007) , investigated the efficacy of AS for it's effect on UTIs in pregnant women who are divided into two groups , One group received AS while the other group was used as a control , the results of this study revealed that the AS group had significantly lower incidence of UTIs than the control group .

Several reports have stated that the antibacterial effect of fluroquinolones were enhanced by vitamin C (Carisson *et al* ., 2005) . AS provided a degree of protection against adhesion by some uropathogenes and colonization of biomaterials utilized within the urinary tract (Habash *et al* .,1999). Furthermore , combination of AS with levofloxacin were shown to inhibit the bacterial adherence and urethral catheter biofilm formation (El- Gebaly *et al* ., 2012) . The aim of the present study is to Evaluate the biological activity of ascorbic acid in ciprofloxacin resistant coliforms .

MATERIAL AND METHOD

Isolation and identification of coliforms bacteria

A total of 110 urine samples were collected from patients of both gender who visited or admitted to AL- Salam General Teaching Hospital in Mosul city of Ninavah province / Iraq. Samples were transported quickly by sterile transport media and sterile cotton swabs to the laboratory for culturing on MacConky agar and Blood agar . The isolates were identified to species level depended on morphological, biochemical and physiological tests and confirmed by RapIDTM ONE system (Remel \ USA).

Detection of quinolone resistance isolates

The bacterial isolates were tested for their susceptibility to ciprofloxacin 10 µg / disk , norfloxacin 10 µg / disk , and nalidixic acid 30 µg / disk by using standard disk diffusion method (Bauer et al.,1966). All isolates which were resistant to ciprofloxacin are suspected to harbor *qnr* genes (Pakzad et al ., 2011) .

DNA extraction

Genomic DNA was extracted from all ciprofloxacin resistant isolates using Wizard Genomic DNA purification kit supplemented by (Promega \ USA) according to manufacture instructions. The purity and concentration of genomic DNA were measured using Biodrop spectrophotometer . The method described by Sambrook and Ruseel (2001), was used for prepare horizontal agarose gel electrophoresis for genomic DNA and PCR product .

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Agarose at concentration of 0.7gm / 100ml was prepared for genomic DNA , and 1.2gm / 100 ml for PCR product.

Primers used

Primers sequences were taken from previous articles(Table1). All primers were synthesized by Alpha DNA company , Canada.

Table (1):Primer used for the amplification of *qnr* genes .

Genes	Oligonucleotides (5`→ 3`)	Product size	Reference
qnrA	**F** ATTTCTCACGCCAGGATTTG **R** GATCGGCAAAGGTTAGGTCA	516 bp	Pakzad *et al* ., (2011)
qnrB	**F** GATCGTGAAAGCCAGAAAGG **R** ACGATGCCTGGTAGTTGTCC	469 bp	
qnrS	**F** ACGACATTCGTCAACT GCAA **R** TAAATTGGCACCCTGTAGGC	417 bp	

All PCR reactions were performed in 25µl volumes in Eppendorf tube, PCR conditions program depending on Pakzad et al ., (2011) , with some modification was used for detection of fluoroquinolones resistance genes as shown in (Table 2) .

Table (2). Program conditions for amplification of *qnr* genes .

Stage	Temperature °C	Time (min.)	Cycle number
Initial denaturation	94	3	1
Denaturation	94	1	32
Annealing	53	1	
Extension	72	1	
Final extension	72	7	1
Hold	4	3	1

Antibacterial Activity of Ascorbic Acid

Preparation of Stock Solution

Stock solution of AS was prepared by weighting 1 gram of drug powder and dissolving in 1 ml of sterilized D.W to obtain 1000 mg / ml concentration (El- Gebaly *et al* ., 2012) . Final concentration of 40 , 80 , and 160 mg / ml were obtained through serial dilutions to explore the antibacterial activity against quinolines resistance isolates . AS solution was

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covered of light with aluminum foil . All solutions were prepared in the moment of their use (Isela *et al* ., 2013) .

Evaluation of Antibacterial Activity of AS Against Quinolones Resistant Isolates

The antibacterial effect of AS on quinolones resistant isolates was determined using the agar disk diffusion method (Bauer *et al*.,1966) , all the quinolones resistant isolates were examined for their susceptibility to AS alone or in combination with the same antibiotic disks . The pipette delivery method was used for impregnating AS to empty sterilized disks and quinolones disks , the sterile disks and antibiotic disks were placed in petri dishes (150 × 20 mm) approximately 5 mm apart , and impregnated with 20µl of AS concentrations by using digital micropipette , disks were placed on the agar surface . The plates were left for 30 min at room temperature to allow the diffusion of AS , then they were incubated at 37°C for 18 hours . After the incubation time , the diameter of inhibition zones were measured in mm (AL - Barrak and Mohammed , 2011 and AL - Heety , 2013) .

MIC of Ascorbic Acid in the Presence or Absence of Ciprofloxacin

The MIC of the fresh AS solutions alone or in combination with ciprofloxacin against all ciprofloxacin resistance isolates was determined using the broth dilution method (Andrews , 2001 and Winn *et al* ., 2006) . Ascorbic acid was used to final concentrations of 1, 5 , 10 , 15 , and 20 mg / ml and mixed with bacterial suspension (Isela *et al* ., 2013) . Ascorbic acid at different concentrations was added to the tubes containing increasing concentrations (8 , 16 , 32 , 64 , and 128 µg / ml) of ciprofloxacin . Tubes containing an identical amount of broth , but free of ciprofloxacin and ascorbic acid , and tubes separately containing the antibiotic or As were included in each assay as a growth control . After 18 – 24 hours of incubation at 37°C , the lowest concentration of AS separately or in combination with ciprofloxacin , which prevented the development of turbidity , was regarded as the MIC (Cursino *et al* ., 2005).

Statistical analysis

All statistical analysis was conducted using the Statistical Package for Social Sciences (S.P.S.S.) version 19 from IBM Company, USA. The x2 test was used for statistical comparison of groups , values < 0.05 were regarded as significant .

Results

Identification of coliforms bacteria was first made by the bacteriological methods, biochemical tests and RapID™ ONE panel kit was used for accurate identification of isolates at species level (Figure 1). The result showed that the isolated bacteria belonged to five genera ; the number and percentage of coliforms isolated from UTIs are listed in (Table 3).

The total number of coliform bacteria was (55) isolates. Among the isolates , *Escherichia coli* was the predominant isolates 28 (50.9%) from clinical samples, followed by *Klebsiella pneumoniae* which isolated from 17 (30.9%) samples .

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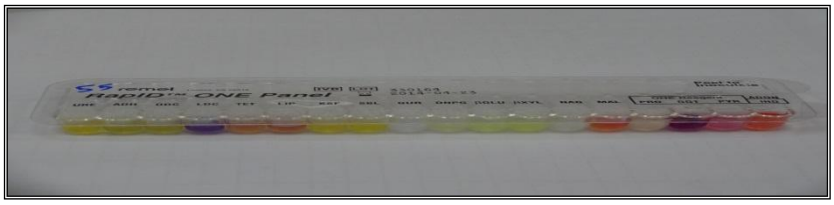

Figure (1): Identification of *K. pneumoniae* by RapID™ ONE panel kit

Table (3): Number and percentage of coliforms spp. isolated from UTIs

Coliforms	**Type of infection**
	UTIs No. (%)
E. coli	28 (50.91)
K. pneumonia	17 (30.91)
Ent. cloacae	5 (9.1)
Ent. aerogenes	2 (3.635)
Serratia marcescens	2 (3.635)
Serr. Fonticola	1 (1.81)
Total	55 (100)

The susceptibility of (55) coliforms spp. against quinolone agents were studied to evaluate the pattern of their resistance using disk diffusion method . Out of (55) bacterial isolates , 48 (87.27%) isolates were resistant to nalidixic acid , whereas 38 (69.1%), and 37 (67.27%)isolates were resistant to norfloxacin and ciprofloxacin respectively(Table 4) . This finding was relatively close to that of Ogbolu *et al* .,(2012) who found that the resistance rate of nalidixic acid among gram-negative bacilli was(98.3%) , while that for ciprofloxacin was (76.5%) . Many reports have indicated that the widespread use of fluoroquinolons is contributing to the increasing percentages of fluoroquinolone insusceptible bacterial strains , including coliforms (Namboodiri *et al*., 2011 and Majumdar *et al*., 2012).

Table (4): Fluoroquinolones resistance among coliforms spp. isolated from UTIs

Type of infections	**Bacterial isolates**	**Antibiotics**		
		CIP No (%)	NOR No. (%)	NA No.(%)
UTIs	*E. coli* (n=28)	19 (67.9)	21 (75)	24 (85.7)
	K. pneumoniae (n = 17)	14 (82.4)	14 (82.4)	16 (94.1)
	Ent. cloacae (n = 2)	0 (0)	0 (0)	1 (50)
	Ent. aerogenes (n = 5)	3 (60)	3 (60)	4 (80)
	Serr. marcescens (n = 2)	1 (50)	1 (50)	2 (100)
	Serr. fonticola (n = 1)	0 (0)	0 (0)	1 (100)
Total	N = 55	37	38 (69.1%)	48 (87.27%)

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		(67.27%)		

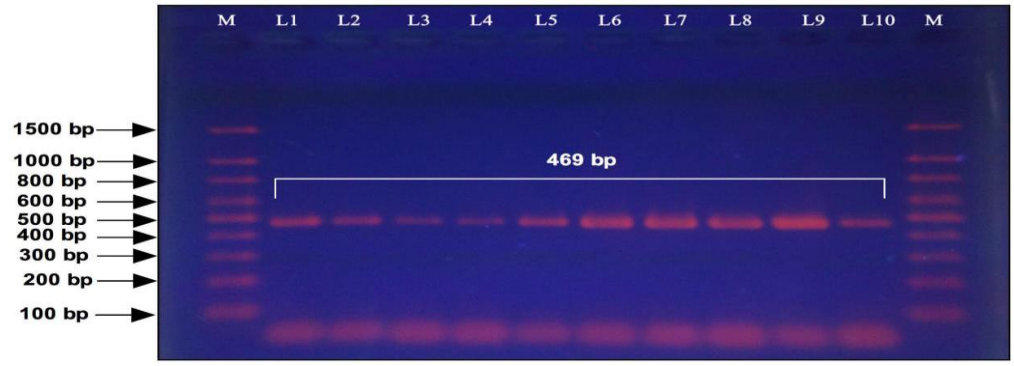
Molecular detection of *qnrA* , *qnrB* , and *qnrS* genes by PCR

Molecular detection of *qnrA* , *qnrB* , and *qnrS* genes by PCRPolymerase Chain Reaction (PCR) technique has been used to amplify *qnr* genes, which may be responsible for flouroquinolones resistance . The PCR analysis showed that among the (37) ciprofloxacin resistant isolates , the *qnr* genes screened in the present study was present in a total of 14/37 (37.83%) isolates as shown in (Table 5). The *qnrB* gene (469bp) was detected in 10(52.6%) *E. coli* , in 3(21.4%) *K. pneumoniae* , and in 1(33.3%) *Ent. aerogenes* isolates as shown in(Figures 2). None of the isolates had *qnrA* and *qnrS* type genes in our study.

Table (5) : Number and percentage of *qnr* genes in ciprofloxacin resistant isolates of coliforms

Ciprofloxacin resistant coliforms	*qnr* gene types		
	qnrA **N. (%)**	***qnrB*** **N. (%)**	***qnrS*** **N. (%)**
E. coli (n = 19)	0(0)	10 (52.6)	0(0)
K. pneumoniae (n = 14)	0(0)	3 (21.4)	0(0)
Ent. aerogenes (n = 3)	0(0)	1 (33.3)	0(0)
Serr. marcescens (n = 1)	0(0)	0(0)	0(0)
Total (n = 37)	0(0)	14 (37.83)	0(0)

In China , Wang *et al*.,(2008) found that the prevalence rates of *qnr* genes among ciprofloxacin-resistant isolates of *E. coli* and *K. pneumoniae* were (7.5%) and (11.9%) respectively , these rates are lower than our results . Differences in distribution of the *qnr* genes may be attributed to difference in geographical area , or may be due to difference in selection criteria (EL-Mahdy , 2015).



Figure(2): Electrophoresis of PCR amplification of *qnrB* gene *E. coli* with expected product length 469bp in (1.2%) agarose gel. M lane, left and right (100bp ladder). Lane 1 – 10 poitive *qnrB* gene.

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Antibacterial Activity of Ascorbic Acid Against Quinolones Resistant Isolates

The method used for the antibacterial activity was agar disk diffusion method , and all the quinolones resistant isolates were examined for their susceptibility to ascorbic acid alone or in combination with the same antibiotic disks . The present study showed that ascorbic acid had an antibacterial activity against urinary resistant isolates . Table (6). revealed that zones of inhibition was increased with the increase of ascorbic acid concentration added to each disk . When ascorbic acid was added at different concentrations to each antibiotic disks , the zones of inhibition of the combination were higher than those of ascorbic acid alone for all tested coliforms as in Figure (3).

Shishodia *et al*., (2013) had reported that high intake of ascorbic acid can be helpful for infection affecting the urinary tract . A high intake of ascorbic acid tends to increase the acidity of urine which is not well tolerated by the bacteria responsible for UTIs . The antibacterial effect of ascorbic acid is explained on the basis of its low molecular weight and hydrophilic nature that can readily cross the bacterial cell membrane through specific transporters on the bacterial cell membrane which enable it to reveal the antioxidant effect as antimicrobial compound (Hussein and Ali , 2013). The enhancement of antibiotic activity or the reversal of antibiotic resistance by non-conventional antibiotics affords the classification of these compounds as modifiers of antibiotic activity (Cursino *et al* ., 2005) . So , according to the result of the present study , we suggest that ascorbic acid could be quinolones modifier . In contrast , Goswami *et al*., (2006) have reported that application of exogenous ascorbic acid to *E. coli* resulted in reduced antibacterial activity of ciprofloxacin which was the result of scavenging superoxide anions and hydrogen peroxide species . Moreover , Masadeh *et al* ., (2012) had reported that vitamins C and E have antagonistic properties when they are used concurrently with ciprofloxacin . Also , another study conducted by Alba *et al*., (2008) who revealed that ascorbic acid did not modify susceptibility patterns of norfloxacin against uropathogenic bacteria .

Table (6): Antibacterial activity of ascorbic acid alone and in combination with quinolones using disk diffusion method

Ciprofloxacin resistant coliforms	AS concen. (mg/ml)	Zones of inhibition (mm)				P values
		AS alone	Cip.10 + AS	Nor.10 + AS	Nal.30 + AS	
E. coli	80	15	20	21	20	1.000
	40	13	17	19	18	
	20	10	14	15	15	
K. pneumoniae	80	14	18	19	21	1.000
	40	12	16	16	18	
	20	10	14	15	16	
E. aerogenes	80	17	20	21	19	1.000
	40	13	18	18	17	
	20	11	15	15	14	

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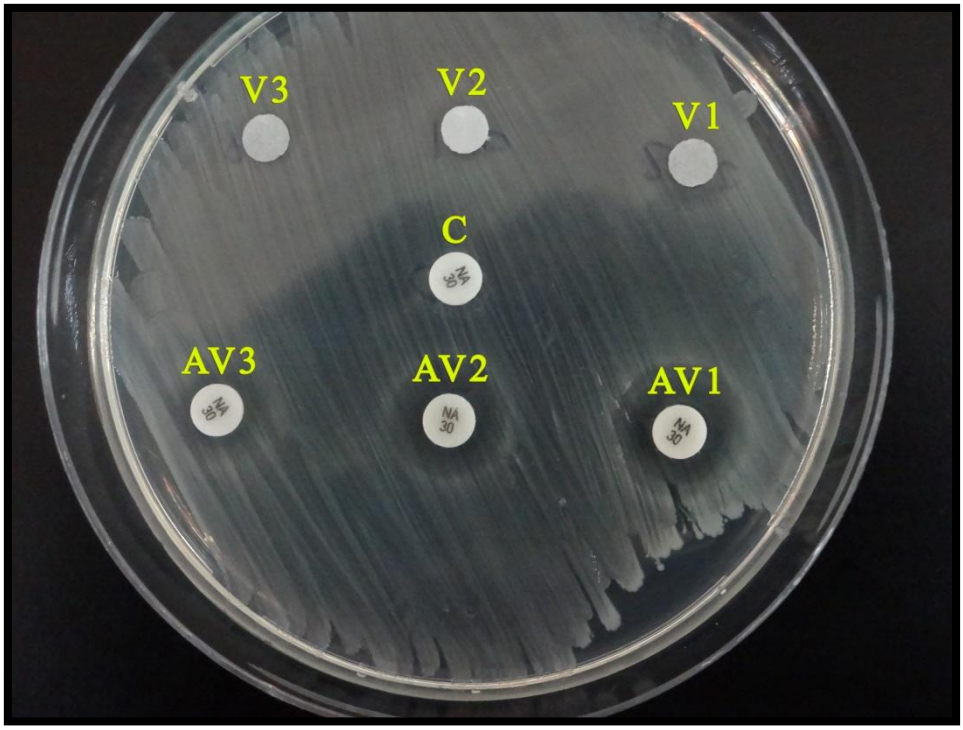
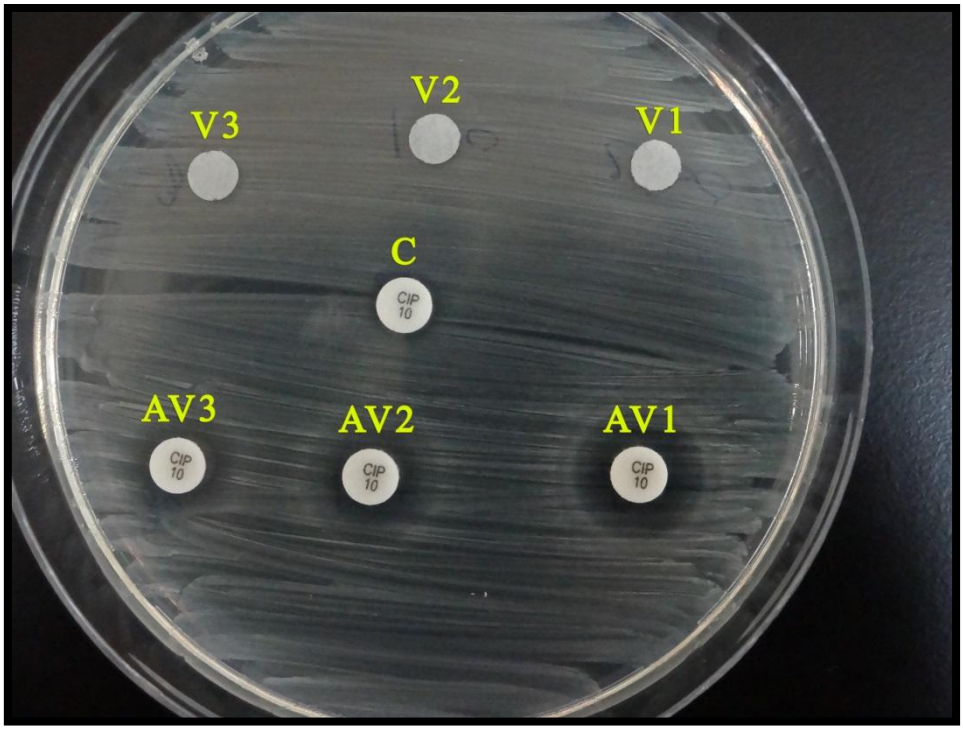
However , ascorbic acid was reported as efflux pump inhibitor in hemolytic *E. coli* . As a result , it can enhance the activity of ciprofloxacin against *E. coli* strains (Serry *et al* ., 2008) .



(A): Ascorbic acid alone and in combination with naldixic acid against *E.coli.*



(B): Ascorbic acid alone and in combination with ciprofloxacin against *Ent. aerogenes*.

Figure (3): Antibacterial activity of ascorbic acid alone and in combination with quinolone disks (A + B). V= Ascorbic acid V1 - 80mg , V2 - 40 mg , V3 - 20mg , AV= Antibiotic + ascorbic acid , C= Antibiotic alone .

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Minimal Inhibitory Concentration (MIC) of Ascorbic Acid Alone or in Combination with Ciprofloxacin

The MIC of ascorbic acid in Table (7) showed that ascorbic acid give similar MIC value (5 mg/ml) for *E. coli* , *K. pneumoniae* , and *Ent. aerogenes* . The lower MIC of ascorbic acid was found in this study than those reported by Isela *et al*., (2013) might be due to the differences in bacterial isolates and sites of samples collection .

The possible interaction between ascorbic acid and ciprofloxacin was evaluated in the current study . The synergistic effect of sub-MIC of ascorbic acid (1mg / ml) with ciprofloxacin at different concentrations against urinary ciprofloxacin- resistant isolates are shown in Table (4.13). The sub-MIC of ascorbic acid showed synergy with ciprofloxacin in *E. coli* and *K. pneumoniae* isolates . This is indicated by significantly lower MIC values for the combination of sub-MIC of ascorbic acid and ciprofloxacin as compared to MIC values of ciprofloxacin alone . The enhancement of the inhibitory effect of ascorbic acid + ciprofloxacin combinations could be explained by the fact that reducing pH of the medium by ascorbic acid addition could increase the activity of ciprofloxacin according to what have been reported by El - Gebaly *et al*., (2012) . While ascorbic acid did not modify the MIC patterns of ciprofloxacin against *Ent. aerogenes* . Results of this study could be of clinical significance due to the common use of vitamin supplementation , especially , ascorbic acid (vitamin C) with antibiotics .

Table (7): MIC values of ascorbic acid alone and in combination with ciprofloxacin against coliform species

Ciprofloxacin resistant isolates	MIC values		
	Ciprofloxacin (µg / ml)	Ascorbic acid (mg / ml)	Ciprofloxacin + Ascorbic acid (µg / ml)
E. coli	128	5	64
K. pneumoniae	128	5	64
Ent. aerogenes	8	5	8

We can conclude from the results that combination of ciprofloxacin with ascorbic acid (Vitamin C) tends to enhance the activity of fluoroquinolones.

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