WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 7.523
Volume 7, Issue 09, 1523-1555. Research Article ISSN 2277– 7105

# A STUDY OF THE CURING POTENTIAL OF ASCORBIC ACID ON A SELECTED MULTIDRUG RESISTANT *Escherichia coli* UROPATHOGEN STRAIN

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Article Received on
21 March 2018,

Revised on 10 April 2018,
Accepted on 01 May 2018,

DOI: 10.20959/wjpr20189-12152

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## INTRODUCTION

### Background to the Study

World Health Organization (WHO) has identified multi drug resistant (MDR) bacteria as one of the top three threats to human health (Bassetti *et al*., 2011). Antibiotics are naturally occurring compounds produced by microorganisms. They have the capacity to kill susceptible microorganisms or to inhibit their growth (Todar, 2012). The use of antibiotics has been one of the reasons for the increase in life expectancy in the 20$^{th}$ century (Todar, 2012). Despite this impressive feat, there have been records of disease causing microbes that have become resistant to antibiotics (Livermore, 2004; Pereira *et al*., 2013).

Resistance to antibiotics is of two types and they are intrinsic (natural) and acquired resistance. Intrinsic resistance occurs when microbes naturally do not possess target sites for antibiotics and therefore, the drugs do not affect them or they naturally have low permeability to those agents due to differences in the chemical nature of such drugs and the microbial membrane structures especially for antibiotics that selectively attack cell membrane (Fluit *et al.*, 2001). Others are due to overproduction of the target sites of the antimicrobial agent, expression or suppression of a gene invivo in contrast to the situation invitro, production of antibiotic inactivating enzymes and exotoxin production.

Multidrug resistance develops when a pathogen is resistant or acquires resistance to two or more antibiotics which do not have to be related (WHO, 2012). The increasing number of multiple antibiotic resistant pathogens has become a serious threat to human health (CDC,

2013; WHO, 2014). Moreover, there has been an upsurge in antibiotic resistant strains of clinically important pathogens in recent times and this has led to the emergence of new bacterial strains that are multi resistant (WHO, 2001; Albinu *et al*., 2003; Albinu *et al.*, 2004). The primary causes of antibiotic resistance in bacteria are mobile elements called plasmids, conjugative transposons and integrons (Su *et al*., 2012; Amaral *et al*., 2013).

Curing is the process of removing plasmids from a bacterial cell (Trevors, 1986). The resulting bacterial organism then becomes sensitive to the selective agent. It was initially thought that this phenomenon would proffer solution in controlling the development of antibiotic resistance in formerly antibiotic susceptible bacteria. Novobiocin, ethidium bromide, acriflavine, acridine orange, ascorbic acid and elevated temperatures have been used as curing agents (Ramesh *et al*., 2000). There are a number of reports demonstrating the ability of various chemical and physical agents to increase the rate of loss of plasmid DNA from bacteria (Sonstein and Baldwin, 1972; Stanier, 1984; Kumar and Surendran, 2006).

Ingram *et al*. (1972) found that drug resistance of *P. aeruginosa* could be eliminated by treatment with SDS and Pattnakik *et al.* (1995) reported that acridine orange could not affect *P.aeruginosa* due to impermeability of its cell wall while ethidium bromide and SDS cured antibiotic resistance plasmid at a concentration of 1-2% and 700-3000ug/ml for SDS and ethidium bromide respectively. Al-Amar *et al.* (1999) treated an isolate of *P. aeruginosa* with 1000ug/ml dilution of acridine orange and reported that there were no cured cells as the plasmid profile of cured cells was the same as that of untreated samples with a conclusion that acridine orange had no effect on *Pseudomonas aeruginosa* as a curing agent. It was also reported that ethidium bromide was effective as a curing agent at 600, 700 and 1000ug/ml dilutions for the same isolate of *Pseudomonas aeruginosa.*

It has been reported that phenothiazines have the ability to control overexpression of efflux pump systems and thus are able to remove or reduce antibiotic resistance (Viveiros *et al*., 2010; *Amaral et al*., 2013). Mukherjee *et al*. (2012) reported the use of 1000-3000ug/ml dilutions of a type of phenothiazine and an anti-psychotic drug called thioridaxine to cure a multidrug resistant strain of *Pseudomonas aeruginosa.*

**Justification of the Study**

The non-availability and high cost of new generation antibiotics with limited effective lifespan has resulted in increase in morbidity and mortality (Williams, 2000). Hence, the

proliferation of multidrug resistant pathogens continues within and around us. It is important that their resistance trend be put under check through intensive research and antibiotic surveillance (Akortha and Filgona, 2009).

Besides, it is common these days for physicians to report increasing number of treatable infections that fail to respond to antibiotic treatment. Antibiotic resistant bacteria may keep people sick longer and sometimes unable to recover. The development of microbial resistance to antibiotics has resulted in the search for new antibiotics in order to maintain a pool of effective drugs at all times and unless antibiotic resistance problems are detected and tackled as they emerge and action taken to enforce the necessary and judicious use of antibiotics, the society could be faced with previously treatable diseases such as tuberculosis, pneumonia, gonorrhea, typhoid etc that have become untreatable as in the era before antibiotics were discovered (Todar, 2012).

Multidrug resistance is now common among familiar pathogens such as *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae* to mention but a few. Multidrug resistant (MDR) *E.coli* and other Enterobacteriaceae isolates are characterized by non-susceptibility to at least one agent in three or more antibiotic categories (Magiorakos *et al.*, 2012). *Escherichia coli* has high resistance for older generation of human and veterinary antibiotics including ampicillin, streptomycin, tetracycline as well as increasing resistance to newer antibiotics such as fluoroquinolones and cephalosporins (Tadesse *et al*., 2012). Moreso, as a gram negative organism, *E.coli* is resistant to many antibiotics that are effective against gram positive organisms (Johnson *et al*., 2006). *E.coli* bacteria often carry multidrug resistance plasmids and under stress, readily transfer those plasmids to other species (Salyers *et al* 2004). Extended spectrum beta lactamase producing *E.coli* is highly resistant to an array of antibiotics and infections by these strains of *E.coli* are highly difficult to treat (Paterson and Bonomo, 2005). Virulent strains of *E.coli* can cause infantile gastro-enteritis, pelvic inflammatory disease, neonatal meningitis, 85% community acquired UTIs, 50% hospital acquired UTIs and more than 80% cases of uncomplicated pyelonephritis (Bergeron, 1995; Todar, 2007).

Treatments that increase frequency of elimination of plasmids will certainly enhance sensitivity (effectiveness) of antibiotics in situ. Ramesh *et al*. (2000) and Potrus (2009) have used dilutions of ascorbic acid to induce antibiotic resistance loss in *Pediococcus acidilactivi* CFR K7 and *Serratia marcescens* respectively. Dilutions of ascorbic acid have also been used

to cure antibiotic resistance plasmid genes in *Azotobacter chroococcum* (Garg *et al.*, 2003) and *Staphylococcus aureus* (Amabile-Ceuvas, 1988, 1991). Hence, there is not much documented work on use of dilutions of ascorbic acid (an anti-oxidant and a non-antibiotic agent) to reduce resistance loss in MDR bacterial pathogens and particularly *Escherichia coli* isolated from an infected urinary tract in terms of removal of antibiotic resistance genes (if plasmid mediated).

**Overall Aim of the Study**

The overall aim of the study is to investigate the curing potency of ascorbic acid dilutions on a selected multidrug resistant *Escherichia coli* uropathogen strain.

**The Specific Objectives of the study are:**

i. To determine the antibiotic susceptibility patterns or antibiograms of fifteen (15) *Escherichia coli* strains (coded EC1 – EC15) before exposure to dilutions of ascorbic acid after incubation at 37$^{o}$C for 24hr.
ii. To determine the occurrence and distribution of multidrug resistant *E.coli* uropathogen strains among the isolates.
iii. To determine the dilutions of ascorbic acid that induced ≤50% loss in resistance after treatment on a selected uropathogen strain and incubation at 37$^{o}$C for 6hr,12hr,18hr and 24hr.
iv. To determine the cumulative means of ≤50% loss of resistance as induced by ascorbic acid dilutions on the selected MDR *E.coli* uropathogen after 6hr, 12hr, 18hr and 24hr incubation at 37$^{o}$C.
v. To determine effect of ascorbic acid dilutions treatment on the selected MDR *E.coli* uropathogen strain on the minimum inhibitory concentration (MIC) of ampicillin invitro.

**MATERIALS AND METHODS**

**Study Area**

This study will be carried out in the Medical Microbiology Department of the University of Benin, Benin City, Nigeria. Benin City is the capital of Edo State in Midwestern part of Southern Nigeria. It has a population of 1, 147, 188 (Census, 2006) with a density of 950/km$^2$ (2, 500/sq miles), occupies an area of 1,204 km$^2$ (465sq miles) and is approximately 40 kilometres (25miles) north of Benin River. Benin City is also situated 320kilometres (200miles) by road, east of Lagos.

**Study Design/Sampling**

Pure cultures of *Escherichia coli* are to be randomly obtained from 24hr Cystine Lactose Electrolyte Deficient (CLED) agar and MacConkey agar plates which had been inoculated with freshly voided midstream urine samples obtained from in-patients and out-patients of the University of Benin Teaching Hospital, Edo State, Nigeria diagnosed with symptoms of urinary tract infections (UTIs).

**Power and sample size estimation**

To ensure that power will be high to detect reasonable departures from the statement of research question, a power analysis will be carried out to determine that effect, using the following formula described by Naing *et al.* (2006).

$N = Z^2 P (1-P)/d^2$

Where N = sample size

Z = statistics for a level of 95% confidence interval = 1.96

P = urinary tract infection prevalence rate = 59.2% (2015)

d = precision (allowable error) =5% = 0.05

A power of 0.60 will give an estimated sample size of 250 samples and a mean difference of 0.25 at p=0.05.

**Selection Criteria**

**Inclusion criterion**

Only *Escherichia coli* pure cultures grown on 18-24hr sterile CLED and MacConkey agar plates from midstream urine samples will be used for the study.

**Exclusion criterion**

All pure cultures of *Escherichia coli* grown on other culture media and on CLED or MacConkey agar but not from urine samples will not be recruited for this study.

**Ethical Approval and Informed Consent**

Ethical approval for this study will be sought from the ethical committee of University of Benin Teaching Hospital (UBTH), Benin City, Edo State as well as the informed consent of visiting UTI diagnosed out patients before urine samples would be collected.

**Sampling and Processing**

The status of the *E.coli* isolates (strains) will be re-confirmed by gram reaction, biochemical and sugar fermentation tests by standard methods (Cowan and Steel, 1993; Cheesbrough, 2003; Oyeleke and Manga, 2008). After confirmation, all gram negative, raised, entire, circular, motile, indole positive, methyl red positive, voges praskauer negative, citrate negative, urease negative, lactose and glucose fermenting colonies will be stocked aseptically on sterile Nutrient agar slants and incubated at 37$^{o}$C for 24hr. The resulting axenic cultures will be kept at 4$^{o}$C in the refrigerator for further use after appropriate labeling. All stocked *E.coli* strains will be subjected to antibiotic sensitivity testing to determine their antibiograms as well their multidrug resistance statuses after which they will be treated with dilutions of ascorbic acid.

**Antibiotic Sensitivity Testing**

Each of the fifteen (15) stocked *E.coli* strains will be subcultured on sterile CLED agar medium and incubated at 37$^{o}$C for 24hr. Antibiotic sensitivity testing will then be carried out on the resulting pure culture colonies using the agar disc diffusion method on sterile Mueller Hinton agar (MHA) plates initially described by Bauer *et al.* (1966) and recommended by the Clinical Laboratory Standard Institute (2006). A loopful of each colony of the uropathogen strains will be picked aseptically using a flamed and cooled wire loop and placed in the centre of the sterile MHA plates. This will then be spread all over the plates applying the caution of not touching the edges of the plates. The seeded plates will be allowed to stand for about 2mins to allow the agar surface to dry. A pair of forceps will be flamed and cooled and used to pick an antibiotic multidiscs (Abitek, Liverpool) containing ciprofloxacin (5ug), ampicillin (10ug), gentamycin (10ug), ceftazidime (30ug), cefuroxime (30ug), ofloxacin (5ug), amoxicillin/clauvulanic acid (30ug) and nitrofurantoin (300ug). The discs will be placed at least 22.0mm from each other and 14.0mm from the edge of the plates (Ochei and Kolhatkar, 2008). Antibiotic discs will be selected on the basis of their clinical importance and efficacy on various pathogenic strains of *Escherichia coli*. The seeded plates will be allowed to stand for 10mins before incubation (Mbata, 2007).

At the end of incubation, the diameters of the zones of inhibition from one edge to the opposite will be measured to the nearest millimeter using a transparent ruler (Byron *et al.*, 2003). Isolates will be grouped as resistant or sensitive based on the scheme provided by NCCLS (2000) and CLSI (2009). Isolates that show resistance against three or more

antibiotics will be termed multiple drug resistant strains (Jan *et al*., 2004; Santo *et al.*, 2007) and will be noted.

**Preparation of Ascorbic acid Dilutions**

The dilutions of ascorbic acid (100 – 1000ug/ml) will be prepared according to Ramesh *et al*. (2000) using RV/O. Ten Spartan vitamin C tablets (Kunimed Pharmachem Ltd, Lagos) each having a dosage of 100mg will first be dissolved in 100ml of sterile water to obtain a stock concentration of 10,000ug/ml. To obtain 100ug/ml dilution, 0.5ml of stock will be mixed with 49.5ml sterile water (diluent). The next dilution of 200ug/ml will be prepared by mixing 2ml of stock solution with 98ml of diluent. Other dilutions will be prepared by mixing various volumes of stock with their corresponding volumes of sterile water thus: 1.5ml+48.5ml (300ug/ml), 3ml+72ml (400ug/ml), 4ml+76ml (500ug/ml), 3ml+47ml (600ug/ml), 7ml+93ml (700ug/ml), 6ml+69ml (800ug/ml), 4.5ml+45.5ml (900ug/ml) and 6ml+54ml (1000ug/ml).

**Growing Broth Culture of a Selected MDR *Escherichia coli* uropathogen**

A pure stock culture strain will be selected from among the eleven MDR strains based on their antibiograms from the sensitivity testing earlier done. An inoculum of the selected MDR organism will be aseptically picked from its slant stock culture using flamed and cooled wire loop and inoculated into 10ml sterile Nutrient broth (LabM, UK). The inoculated broth will be incubated at $37^{o}$C for 18hr. The resulting turbid broth culture will then be diluted according to a modified method of Shirtliff *et al*. (2006). Using a sterile pipette, 0.1ml of the overnight broth culture will be mixed with 19.9ml (1:200 dilution) of sterile nutrient broth (LabM, UK). This will be properly mixed and used as working inoculum and should contain $10^5$ to $10^6$ organisms and will be used within 30mins (Ochei and Kolhatkar, 2008).

**Treatment of Selected MDR *E.coli* strain with prepared Ascorbic acid dilution**

The treatment of the selected MDR *Escherichia coli* isolate with the prepared ascorbic acid dilutions will be done according to a modified method previously described by Byron *et al*. (2003). Eleven (11) sterile medium sized test tubes (capped with cotton wool) will be arranged on a test tube rack and labeled with the ascorbic acid dilutions of 100-1000ug/ml and the selected *E.coli* strain. The $11^{th}$ tube will be labeled as organism control. Using a sterile 5ml needle and syringe, 4.5ml of Mueller Hinton broth (Liofilchem Diagnostics, Italy) will be dispensed into each tube including the $11^{th}$ tube. Using a sterile 2ml needle and syringe, 0.5ml of diluted selected *E.coli* strain broth culture will be aseptically dispensed into

each tube. With another sterile 2ml needle and syringe, 0.5ml of each of the prepared ascorbic acid dilutions will be dispensed rinsing the syringe intermittently with sterile water. No ascorbic acid agent will be introduced into the control tube. The content of each tube will be mixed and capped properly. All tubes will then be incubated in a water bath at 37$^{o}$C for 6hr, 12hr, 18hr and 24hr (the same set of tubes will be used).

**Antibiotic Sensitivity Testing of Ascorbic acid treated selected *E.coli* strain**

At the end of 6hr, 12hr, 18hr and 24hr incubation periods, all tubes will be removed from the water bath and arranged on test tube racks. Sterile Mueller Hinton agar (Biotech Lab. USA) plates labeled corresponding to the number of test tubes will be prepared and also arranged. Using sterile 2ml needle and syringe, an aliquot (about 0.04ml) of the mixture content of each will be taken and inoculated on the surface of each plate as labeled with the ascorbic acid dilutions. Inoculation on sterile MH agar plates will be done after 6hr, 12hr, 18hr and 24hr incubation and plates will be labeled as such. A sterile wire loop will be used to spread the inoculum on the surface of the plate aseptically and the plates will be allowed to stand for 10mins. Using a pair of sterile forceps, the same gram negative multidiscs used before treatment will be picked and impregnated on the seeded MH agar plates. Seeded plates will be allowed to stand for 10mins before incubation (Mbata, 2007). Plates will then be incubated at 37$^{o}$C for 24hr.

Zones of inhibition will be measured as previously described for before treatment with ascorbic acid dilutions. Results will be recorded as after ascorbic acid treatment. Differences in zones of inhibition before and after treatment will be calculated in terms of ≤50% resistance reduction as benchmark (Akortha and Filgona, 2008).

**Ascorbic acid Dilutions' Treatment of selected *E.coli* strain and the Effect on MIC of One of the resisted antibiotic drugs (based on antibiogram before treatment)**

Ascorbic acid effect on the minimum inhibitory concentration (MIC) of one of the antibiotic drugs resisted by the selected multidrug resistant *E.coli* uropathogen will be determined. Serial doubling dilutions of the randomly selected antibiotic (using its standard MIC as a basis) will be carried out. The idea is that any ascorbic acid dilution that can reduce the MIC of a particular antibiotic below its known invitro MIC may consequently assist in reducing the resistance (hence, increase the sensitivity) of the selected MDR *E.coli* strain to that particular antibiotic. At the molecular level, this has implication in partial removal of conjugative plasmids that code for enzymes that degrade antibiotics etc. With the standard or

known MIC being in the middle, higher and lower dilutions of the selected antibiotic will be aseptically prepared. For instance, if the selected antibiotic has an MIC of 10ug and the capsule or tablet has a dose of 250mg, hence, 80, 40, 20, 10, 5, 2.5, 1.25, 0.63 and 0.32ug/ml dilutions of the antibiotic will be prepared from 250mg capsule or tablet of the antibiotic. Nine sterile 100ml transparent screw capped bottles will be labeled with the above dilutions. The 250mg capsule will initially be dissolved in 100ml sterile water in a separate sterile 100ml transparent bottle and mixed properly and this will give 2,500ug/ml stock solution concentration. This sterile antibiotic stock solution will then be diluted in turn, into each of the dilutions. Therefore, to prepare 80ug/ml from 2,500ug/ml stock, 0.4ml of stock will be added to 12.1ml of diluent (sterile water). To obtain 40ug/ml ampicillin, 0.2ml stock and 12.3ml diluent. To reduce contamination to the barest minimum, the diluents will first be measured and dispensed appropriately into their labeled bottles and sterilized at 121$^{o}$C for 15mins in an autoclave after which they will be allowed to cool and the various calculated mini-volumes of the stock drug solutions will aseptically be added. The dilutions of ascorbic acid as already prepared earlier will be used.

**Statistical Analysis**

Simple percentages and averages will be used in data analyses. Significant resistance loss will be calculated as equal to or greater than 50% obtained from the percentage differential of antibiotic disc zones of inhibition after and before treatment with the inducing agent.

**RESULTS**

The antibiotic sensitivity patterns of *Escherichia coli* (EC1 – EC15) uropathogens strains (isolated from midstream samples) to ciprofloxacin, ofloxacin (fluoroquinolones), ceftazidime, cefuroxime (cephalosporins), gentamycin (aminoglycoside), ampicillin, amoxicillin/clauvulanic acid (penicillins) and nitrofurantoin (urinary antiseptic) are shown in Table 1. In terms of overall resistance of the strains to each antibiotic, 15(100.0%), 13(86.7%), 9(60.0%), 8(53.3%), 6(40.0%), 5(33.3%), 5(33.3%) and 3(20.0%) of the *E.coli* uropathogen strains were resistant to ampicillin, amoxicillin/clauvulanic acid, ceftazidime, cefuroxime, gentamycin, ciprofloxacin, ofloxacin and nitrofurantoin in that decreasing order. This means the most and least resisted drugs were ampicillin and nitrofurantoin respectively.

Vertically, in decreasing order, zones of inhibition recorded were 17.0±5.3mm, 15.0±5.3mm, 14.5±5.0mm, 10.5±6.0mm, 9.5±4.9mm, 8.7±3.6mm, 1.5±0.1mm and 0.0±0.0mm for nitrofurantoin, ciprofloxacin, ofloxacin, ceftazidime, cefuroxime, gentamycin,

amoxicillin/clauvulanic acid and ampicillin respectively. Hence, nitrofurantoin and ampicillin recorded the highest and lowest mean± standard error zones of inhibition.

Horizontally, Table 1 also showed the mean±standard error zones of inhibition of each *E.coli* strain to all the selected antibiotics. Uropathogen strains EC4, EC8, EC15, EC2, EC6, EC12, EC13, EC9, EC5, EC3 and EC10 recorded mean± standard error zones of inhibition of 0.0±0.0mm, 0.0±0.0mm, 1.0±0.2mm, 6.6±3.1mm, 7.6±3.0mm, 7.9±1.7mm, 8.9±2.2mm, 9.3±1.5mm, 9.8±2.8mm, 12.1±4.0mm and 13.8±4.4mm respectively in that increasing order. Other strains such as EC11, EC14, EC1 and EC7 recorded much higher mean± standard error zones of inhibition of 14.4±6.2mm, 16.8±6.3mm, 16.9±5.5mm and 18.5±6.4mm respectively in that increasing order. Out of the fifteen strains, EC7 strain recorded the highest mean± standard error zones of inhibition.

**Table 1: Antibiotic Susceptibility Patterns of Fifteen (15) *Escherichia coli* isolates bef before exposure to dilutions of ascorbic acid after incubation at 37$^{o}$C for 24hr.**

| | **Selected Antibiotics** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **CAZ** 30ug | **CRX** 30ug | **GEN** 10ug | **CPR** 5ug | **OFL** 5ug | **AUG** 30ug | **NIT** 300ug | **AMP** 10ug | **Mean±SE** |
| EC1 | 22mm | 17mm | 13mm | 30mm | 22mm | 0.0 | 31mm | 0.0 | **16.9±5.5** |
| EC2 | 0.0 | 0.0 | 0.0 | 23mm | 20mm | 0.0 | 10mm | 0.0 | **6.6±3.1** |
| EC3 | 26mm | 25mm | 15mm | 0.0 | 0.0 | 0.0 | 31mm | 0.0 | **12.1±4.0** |
| EC4 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0mm | 0.0 | **0.0±0.0** |
| EC5 | 0.0 | 16mm | 0.0 | 17mm | 13mm | 13mm | 19mm | 0.0 | **9.8±2.8** |
| EC6 | 0.0 | 0.0 | 10mm | 17mm | 18mm | 0.0 | 16mm | 0.0 | **7.6±3.0** |
| EC7 | 30mm | 21mm | 14mm | 24mm | 20mm | 9mm | 30mm | 0.0 | **18.5±6.4** |
| **EC8** | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | **0.0±0.0** |
| EC9 | 0.0 | 0.0 | 8mm | 15mm | 27mm | 0.0 | 24mm | 0.0 | **9.3±1.5** |
| EC10 | 30mm | 27mm | 21mm | 0.0 | 0.0 | 0.0 | 32mm | 0.0 | **13.8±4.4** |
| EC11 | 21mm | 16mm | 14mm | 21mm | 26mm | 0.0 | 17mm | 0.0 | **14.4±6.2** |
| EC12 | 0.0 | 0.0 | 0.0 | 22mm | 25mm | 0.0 | 16mm | 0.0 | **7.9±1.7** |
| EC13 | 0.0 | 0.0 | 20mm | 30mm | 21mm | 0.0 | 0.0 | 0.0 | **8.9±2.2** |
| EC14 | 28mm | 20mm | 16mm | 25mm | 25mm | 0.0 | 20mm | 0.0 | **16.8±6.3** |
| EC15 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 8mm | 0.0 | **1.0±0.2** |
| **Mean±SE** | **10.5±6.0** | **9.5±4.9** | **8.7±3.6** | **15.0±5.3** | **14.5±5.0** | **1.5±0.1** | **17.0±5.3** | **0.0±0.0** | |
| **% Overall Resistance of Strains** | **9(60%)** | **8(53.3%)** | **6(40%)** | **5(33.3%)** | **5(33.3%)** | **13(86.7%)** | **3(20%)** | **15(100%)** | |

CPR = Ciprofloxacin, AMP = Ampicillin, GEN = Gentamycin, CAZ = Ceftazidime, CRX = cefuroxime, OFL = Ofloxacin, AUG = Amoxicillin/Clauvulanic acid, NIT = Nitrofurantoin

**Table 2** shows the occurrence and distribution of multidrug resistant *E.coli* uropathogen isolates after antibiotic sensitivity was carried out. Whereas EC5 strain was resistant to three drugs (namely ceftazidime, a cephalosporin, gentamycin, an aminoglycoside and ampicillin, a

penicillin), EC3, EC6, EC9 and EC10 strains each resisted 4(50.0%) drugs as shown. Uropathogen strains EC2, EC12 and EC13 resisted 5(62.5%) drugs each as shown also. Strain EC15 resisted 7(87.5%) drugs while strains EC4 and EC8 each resisted all 8(100%) drugs tested. In increasing order 1(9.0%) strain, 4(36.4%) strains, 3(27.3%) and 3(27.3%) strains resisted 3drugs, 4drugs, 5drugs and more than 6drugs respectively. On the whole, 11(73.3%) out of the 15(100%) strains were multidrug resistant.

**Table 2: Distribution of Multidrug Resistant *E.coli* Uropathogen Strains Among Total Isolates.**

| Isolates' Strain Codes | No of Antibiotics Resisted | | | | |
|---|---|---|---|---|---|
| | 3drugs | 4drugs | 5drugs | ≤ 6drugs | Drugs Resisted |
| EC2 | - | - | + | - | CAZ, CRX, GEN, AUG, AMP |
| EC3 | - | + | - | - | CPR, OFL, AUG, AMP |
| EC4 | - | - | - | + | CAZ, CRX, CPR, OFL, AUG, AMP, GEN, NIT |
| EC5 | + | - | - | - | CAZ, GEN, AMP |
| EC6 | - | + | - | - | CAZ, CRX, AUG, AMP |
| **EC8** | - | - | - | + | **ALL EIGHT DRUGS** |
| EC9 | - | + | - | - | CAZ, CRX, AUG, AMP |
| EC10 | - | + | - | - | CPR, OFL, AUG, AMP |
| EC12 | - | - | + | - | CAZ, CRX, GEN, AUG, AMP |
| EC13 | - | - | + | - | CAZ, CRX, AUG, AMP, NIT |
| EC15 | - | - | - | + | ALL EIGHT EXCEPT NIT. |
| **TOTAL n=11** | **1(9.0%)** | **4(36.4%)** | **3(27.3%)** | **3(27.3%)** | |

CAZ, CRX (cephalosporins), CPR, OFL (fluoroquinolones), AUG, AMP (penicillins), GEN (aminoglycoside), NIT (urinary antiseptic).

Data of zones inhibition of EC8 strain before and after exposure to the prepared dilutions of ascorbic acid are shown in **Table 3.** Zones of inhibition before and after treatment were mathematically computed to obtain ≤50% loss of resistance according to a scheme provided by Akortha *et al.* (2011). Before treatment with the doses of ascorbic acid, EC8 uropathogen strain was resistant to 8(100%) of antibiotics tested. After treatment and incubation for 6hr, 12hr, 18hr and 24hr, EC8 strain became sensitive to only 6 (75.0%) of the eight tested antibiotics and these were ceftazidime (a cephalosporin), gentamycin (an aminoglycoside), ciprofloxacin, ofloxacin (fluoroquinolones), amoxicillin/clauvulanic acid (branded augmentin) and nitrofurantoin (a urinary antiseptic). Multidrug resistant EC8 strain remained resistant to 2(25.0%) of the antibiotics used and these included cefuroxime and ampicillin.

Treatment with 100ug/ml ascorbic acid dilution recorded more than 50% reduction in EC8 resistance to ceftazidime after 18hr and 24hr incubation each. No significant effect was seen after 6hr incubation and more than 40% resistance reduction was recorded after 12hr incubation. With the same ascorbic acid dilution treatment, EC8 strain recorded less than 20% resistance reduction to nitrofurantoin after 18hr incubation and there was no effect recorded after 6hr incubation. More than 100% resistance reduction to augmentin was recorded by 100ug/ml AA treatment after 18hr and 24hr incubation while 100% resistance reduction was recorded after 6hr and 12hr incubation each (Table 3).

Ascorbic acid treatment dose of 200ug/ml on EC8 strain recorded no significant resistance reduction to both ceftazidime and nitrofurantoin after 6hr to 24hr incubation. The same organism recorded more than 50% and 70% resistance reduction to nitrofurantoin after 18hr and 24hr incubation respectively with 300ug/ml ascorbic acid. The same dilution induced no significant reduction to ceftazidime. The test organism (**EC8**) after 6hr, 12hr, 18hr and 24hr incubation with 400ug/ml ascorbic acid concentration, recorded 52.4%, 61.9%%, 81% and 100% resistance reduction respectively to ceftazidime. Incubation of EC8 strain with 400ug/ml dose yielded 52.9% and 52.9% resistance reduction after 18hr and 24hr respectively to nitrofurantoin.

Similarly, after 18hr and 24hr incubation of EC8 strain treated with 500ug/ml dilution, more than 60% and 70% resistance reduction was recorded to ceftazidime and less than 40% and 50% resistance reduction to nitrofurantoin was noted after 18hr and 24hr incubation periods.

In the case of 600ug/ml dilution, 12hr, 18hr and 24hr incubation period with EC8 strain recorded 52.4%, 71.4% and 90.5% resistance reduction to ceftazidime respectively and 52.9%, 52.9% and 58.8% RR to nitrofurantoin after the same incubation periods respectively. Noteworthy is that the same dilution induced 233%, 300%, 300% and 367% RR to augmentin after 6hr, 12hr, 18hr and 24hr incubation periods respectively and also less than 20%, 30%, 30% and 40 RR to gentamycin after the same incubation periods respectively **(Table 3).**

After 18hr and 24hr incubation of EC8 strain with 700ug/ml dilution, significant resistance reduction (RR) of 71.4% and 81.0% respectively to ceftazidime was recorded. Lower incubation periods yielded less than 5% and 40% reduction in resistance. The same curing agent dilution treatment induced no significant RR to nitrofurantoin after all four incubation periods **(Table 3).** Similar to the effect of 700ug/ml AA dilution, 71.4% and 71.4% RR to ceftazidime was induced by 800ug/ml AA dilution after 18hr and 24hr incubation

respectively. Ascorbic acid dose of 900u/ml after 18hr and 24hr incubation effected 71.4% and 109.5% respectively to ceftazidime with no significant RR to nitrofurantoin after all four incubation periods with the same dilution.

Ascorbic acid dilution of 1000ug/ml treatment of EC8 strain after 6hr, 12hr, 18hr and 24hr incubation periods yielded resistance reduction to six of the antibiotics tested and they included ceftazidme, gentamycin, ciprofloxacin, ofloxacin, augmentin and nitrofurantoin respectively. Eighty one percent, 100%, 100% and 128% RR to ceftazidime were recorded respectively after the four incubation periods. The same dilution yielded 52%, 52%, 79% and 86% RR respectively to ofloxacin after the four incubation periods. The same dilution after the four incubation periods also induced 150%, 150%, 167% and 233% RR respectively to amoxicillin/clauvulanic acid. One thousand microgram per milliliter dilution of AA after 18hr and 24hr incubation periods recorded 64.7%% and 70.6% RR respectively to nitrofurantoin. The same dilution recorded less than 50%, less than 50%, less than 50% and 53% RR to ciprofloxacin after the four incubation periods respectively. Although resistance reduction to gentamycin was induced by 1000ug/ml, none was significant after the same incubation periods.

**Table 3: Dilutions of ascorbic acid that induced ≤50% loss in resistance after treatment on EC8 uropathogen strain and incubation at 37°C for 6hr, 12hr, 18hr and 24hr.**

| Ascorbic Acid Dilutions (ug/ml) | Selected Antibiotics (Zones of Inhibition and % Improvements in Sensitivity) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Treatments & Time | CAZ mm | CRX mm | GEN mm | CPR mm | OFL Mm | AUG Mm | NIT mm | AMP Mm |
| | **Before** | **10.5** | **9.5** | **8.7** | **15.0** | **14.5** | **1.5** | **17.0** | **0.0** |
| **100** | 6hr | 13.0 (23.0%) | 0.0 | 0.0 | 0.0 | 0.0 | 3.0**(100%)** | 10.0 (0.0%) | 0.0 |
| | After 12hr | 15.0 (42.9%) | 0.0 | 0.0 | 0.0 | 0.0 | 3.0**(100%)** | 19.0 (11.8%) | 0.0 |
| | 18hr | 16.0 **(52.4%)** | 0.0 | 0.0 | 0.0 | 0.0 | 4.0**(167%)** | 20.0 (17.7%) | 0.0 |
| | 24hr | 16.0 **(52.4%)** | 0.0 | 0.0 | 0.0 | 0.0 | 4.0**(167%)** | 22.0 (29.4%) | 0.0 |
| **200** | 6hr | 10.0 (0.0%) | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 11.0 (0.0%) | 0.0 |
| | After 12hr | 10.0 (0.0%) | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 11.0 (0.0%) | 0.0 |
| | 18hr | 11.0 (4.8%) | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 11.0 (0.0%) | 0.0 |
| | 24hr | 13.0 (23.8%) | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 11.0 (0.0%) | 0.0 |
| **300** | 6hr | 10.0 (0.0%) | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 19.0 (11.8%) | 0.0 |
| | After 12hr | 11.0 (4.8%) | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 24.0 (41.2%) | 0.0 |
| | 18hr | 12.0 (14.3%) | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 26.0 **(52.9%)** | 0.0 |
| | 24hr | 12.0 (14.3%) | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 30.0 **(76.5%)** | 0.0 |
| **400** | 6hr | 16.0 **(52.4%)** | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 14.0 (0.0%) | 0.0 |
| | After 12hr | 17.0 **(61.9%)** | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 20.0 (17.7%) | 0.0 |
| | 18hr | 19.0 **(81.0%)** | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 26.0 **(52.9%)** | 0.0 |
| | 24hr | 21.0 **(100.0%)** | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 26.0 **(52.9%)** | 0.0 |
| **500** | 6hr | 13.0 (23.8%) | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 9.0 (0.0%) | 0.0 |
| | After 12hr | 15.0 (42.9%) | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 17.0 (0.0%) | 0.0 |
| | 18hr | 17.0 **(61.9%)** | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 23.0 (35.3%) | 0.0 |
| | 24hr | 18.0 **(71.4%)** | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 25.0 (47.1%) | 0.0 |
| **600** | 6hr | 14.0 (33.3%) | 0.0 | 10.0(15% | 0.0 | 0.0 | 5.0(**233** | 22.0 (29.4%) | 0.0 |
| | After 12hr | 16.0 **(52.4%)** | 0.0 | 11.0(26% | 0.0 | 0.0 | 6.0(**300** | 26.0 **(52.9%)** | 0.0 |
| | 18hr | 18.0 **(71.4%)** | 0.0 | 11.0(26% | 0.0 | 0.0 | 6.0(**300** | 26.0 **(52.9%)** | 0.0 |
| | 24hr | 20.0 **(90.5%)** | 0.0 | 12.0(38% | 0.0 | 0.0 | 7.0(**367** | 27.0 **(58.8%)** | 0.0 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **700** | 6hr | 12.0 (14.8%) | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 17.0 (0.0%) | 0.0 |
| | After 12hr | 14.0 (43.3%) | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 18.0 (5.9%) | 0.0 |
| | 18hr | 18.0 **(71.4%)** | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 19.0 (11.8%) | 0.0 |
| | 24hr | 19.0 **(81.0%)** | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 22.0 (29.4%) | 0.0 |
| **800** | 6hr | 11.0 (8.0%) | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 12.0 (0.0%) | 0.0 |
| | After 12hr | 15.0 (48.9%) | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 14.0 (0.0%) | 0.0 |
| | 18hr | 18.0 **(71.4%)** | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 14.0 (0.0%) | 0.0 |
| | 24hr | 18.0 **(71.4%)** | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 18.0 (5.9%) | 0.0 |
| **900** | 6hr | 11.0 (10.0%) | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 14.0 (0.0%) | 0.0 |
| | After 12hr | 12.0 (14.8%) | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 16.0 (0.0%) | 0.0 |
| | 18hr | 18.0 **(71.4%)** | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 17.0 (0.0%) | 0.0 |
| | 24hr | 22.0 **(109.5%)** | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 20.0 (17.7%) | 0.0 |
| **1000** | 6hr | 19.0 (**81.0%)** | 0.0 | 9.0(3.5% | 21.0(40 | 22.0(**52** | 3.0(**150** | 24.0 (41.2%) | 0.0 |
| | After 12hr | 21.0 (**100.0%)** | 0.0 | 10.0(15% | 21.0(40 | 22.0(**52** | 3.0(**150** | 24.0 (41.2%) | 0.0 |
| | 18hr | 21.0 (**100.0%)** | 0.0 | 11.0(26% | 22.0(47 | 26.0(**79** | 4.0(**167** | 28.0 (**64.7%)** | 0.0 |
| | 24hr | 24.0 **(128.6%)** | 0.0 | 11.0(26% | 23.0**(53** | 27.0(**86** | 5.0(**233** | 29.0 (**70.6%)** | 0.0 |

A summary of the means of percentage losses (reduction) of antibiotic resistance after 6hr, 12hr, 18hr and 24hr incubation and ≤50% loss of resistance are shown in **Table 4**. Mean percentage reduction of resistance after 6hr, 12hr, 18hr and 24hr incubation of EC8 strain with ascorbic acid dilutions of 400ug/ml, 500ug/ml, 600ug/ml, 700ug/ml, 800ug/ml, 900ug/ml and 1000ug/ml recorded 73.8%, 50.0%, 61.9%, 52.6%, 50.0%, 51.3% and 102.4% RR to ceftazidime respectively. Only 1000ug/ml dilution recorded ≤50% loss of resistance of 67.6%, 175.3% and 54.5% to ofloxacin, amoxicillin/clauvulanic acid and nitrofurantoin respectively.

Ascorbic acid dilutions of 100ug/ml and 600ug/ml also induced ≤50% loss of resistance of 133.4% and 300.4% respectively to amoxicillin/clauvulanic acid. More than 45% resistance reduction to nitrofurantoin was recorded each for 300ug/ml and 600ug/ml ascorbic acid dilutions **(Table 4)**.

**Table 4: Cumulative means of ≤50% loss of resistance as induced by exposure of EC8 uropathogen strain to ascorbic acid dilutions after 6hr, 12hr, 18hr and 24hr incubation at 37°C.**

| Ascorbic Acid Dilutions ug/ml | Antibiotics That Recorded ≤50% loss of resistance | | | | |
|---|---|---|---|---|---|
| | Treatments & Time | CAZ Mm | OFL mm | AUG mm | NIT mm |
| | **Before** | **10.5** | **14.5** | **1.5** | **17.0** |
| 100 | 6hrs | 23.0% | 0.0% | 100.0% | 0.0% |
| | After 12hrs | 42.9% (42.7%) | 0.0% | 100.0% **(133.4%)** | 11.8% (14.7%) |
| | 18hrs | 52.4% | 0.0% | 166.7% | 17.7% |
| | 24hrs | 52.4% | 0.0% | 166.7% | 29.4% |
| 200 | 6hrs | 0.0% | 0.0% | 0.0% | 0.0% |
| | After 12hrs | 0.0% (7.2%) | 0.0% | 0.0% | 0.0% |
| | 18hrs | 4.8% | 0.0% | 0.0% | 0.0% |
| | 24hrs | 23.8% | 0.0% | 0.0% | 0.0% |
| 300 | 6hrs | 0.0% | 0.0% | 0.0% | 11.8% |
| | After 12hrs | 4.8% (8.4%) | 0.0% | 0.0% | 41.2% (45.6%) |
| | 18hrs | 14.3% | 0.0% | 0.0% | 52.9% |
| | 24hrs | 14.3% | 0.0% | 0.0% | 76.5% |
| 400 | 6hrs | 52.4% | 0.0% | 0.0% | 0.0% |
| | After 12hrs | 61.9% **(73.8%)** | 0.0% | 0.0% | 17.7% (30.9%) |
| | 18hrs | 81.0% | 0.0% | 0.0% | 52.9% |
| | 24hrs | 100.0% | 0.0% | 0.0% | 52.9% |
| 500 | 6hrs | 23.8% | 0.0% | 0.0% | 0.0% |
| | After 12hrs | 42.9% **(50.0%)** | 0.0% | 0.0% | 0.0% (20.6%) |
| | 18hrs | 61.9% | 0.0% | 0.0% | 35.3% |
| | 24hrs | 71.4% | 0.0% | 0.0% | 47.1% |
| 600 | 6hrs | 33.3% | 15.5% | 233.3% | 29.4% |
| | After 12hrs | 52.4% **(61.9%)** | 26.7% (26.8%) | 300.2% **(300.4%)** | 52.9% (48.5%) |
| | 18hrs | 71.4% | 26.7% | 300.2% | 52.9% |
| | 24hrs | 90.5% | 38.3% | 367.7% | 58.8% |
| 700 | 6hrs | 14.8% | 0.0% | 0.0% | 0.0% |
| | After 12hrs | 43.3% **(52.6%)** | 0.0% | 0.0% | 5.9% (11.8%) |
| | 18hrs | 71.4% | 0.0% | 0.0% | 11.8% |
| | 24hrs | 81.0% | 0.0% | 0.0% | 29.4% |
| 800 | 6hrs | 8.0% | 0.0% | 0.0% | 0.0% |
| | After 12hrs | 48.9% **(50.0%)** | 0.0% | 0.0% | 0.0% |
| | 18hrs | 71.4% | 0.0% | 0.0% | 0.0% |
| | 24hrs | 71.4% | 0.0% | 0.0% | 5.9% |
| 900 | 6hrs | 10.0% | 0.0% | 0.0% | 0.0% |
| | After 12hrs | 14.3% **(51.3%)** | 0.0% | 0.0% | 0.0% |
| | 18hrs | 71.4% | 0.0% | 0.0% | 0.0% |
| | 24hrs | 109.5% | 0.0% | 0.0% | 17.7% |
| 1000 | 6hrs | 81.0% | 52.4% | 150.0% | 41.2% |
| | After 12hrs | 100.0% **(102.4%)** | 52.2% **(67.6%)** | 150.0% **(175.3%)** | 41.2% **(54.5%)** |
| | 18hrs | 100.0% | 79.1% | 167.7% | 64.7% |
| | 24hrs | 128.6% | 86.5% | 233.3% | 70.6% |

The data on the effect of ascorbic acid dilutions on the minimum inhibitory concentration (MIC) of ampicillin on the MDR **EC8** strain are shown in **Table 5.** After 18hr incubation at 37$^{o}$C, inoculum control tubes showed turbidity (cloudiness) as expected. Sterile broth control and drug control tubes separately remained clear at the end of incubation as expected. Ascorbic acid dilutions of 200, 400 and 900ug/ml did not affect the minimum inhibitory concentration of ampicillin (a penicillin and one of the two antibiotics that **EC8** strain remained resistant to after treatment) as the MIC remained 10ug. Dilutions of 100, 600, 700 and 800ug/ml reduced ampicillin MIC to 5ug each which is a two-fold reduction. Ampicillin MIC was reduced to 2.5ug (a four-fold reduction) by the effect of 1000 ug/ml dilution only and this was the highest MIC reduction. Ascorbic acid dilutions of 300ug/ml and 500ug/ml increased the MIC of ampicillin by two fold and four fold respectively

**Table 5: Effect of Ascorbic acid dilutions treatment of EC8 strain on the minimum inhibitory concentration (MIC) of Ampicillin Invitro.**

| Ascorbic Acid Dilutions | Post Treatment MIC | Ampicillin Dilutions (µg/ml) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 80 | 40 | 20 | 10 | 5 | 2.5 | 1.25 | 0.63 | 0.32 | sterilebroth control | Inoculum control | Drug control |
| 100µg/ml | **5µg (½-fold)** | _ | _ | _ | _ | _ | + | + | + | + | _ | + | _ |
| 200µg/ml | No Change | _ | _ | _ | _ | + | + | + | + | + | _ | + | _ |
| 300µg/ml | 20µg (2-fold) | _ | _ | - | + | + | + | + | + | + | _ | + | _ |
| 400µg/ml | No Change | _ | _ | _ | _ | + | + | + | + | + | _ | + | _ |
| 500µg/ml | 40µg (4-fold) | _ | - | + | + | + | + | + | + | + | _ | + | _ |
| 600µg/ml | **5µg (½-fold)** | _ | _ | _ | _ | - | + | + | + | + | _ | + | _ |
| 700µg/ml | **5µg (½-fold)** | _ | _ | _ | _ | - | + | + | + | + | _ | + | _ |
| 800µg/ml | **5µg (½-fold)** | _ | _ | _ | _ | - | + | + | + | + | _ | + | _ |
| 900µg/ml | No Change | _ | _ | _ | _ | + | + | + | + | + | _ | + | _ |
| 1000µg/ml | **2.5µg (¼-fold)** | _ | _ | _ | _ | _ | - | + | + | + | _ | + | _ |
| EC8 organism + Amp Dilutions | | - | - | - | - | + | + | + | + | + | + | + | + |

EC8 organism + AA Dilutions + **(100ug/ml)** + **(200ug/ml)** + **(300ug/ml)** + **(400ug/ml)** + **(500ug/ml)** + **(600ug/ml)** + **(700ug/ml)** + **(800ug/ml)** + **(900ug/ml)** + **(1000ug/ml)**

## DISCUSSION

The increasing number of multiple –antibiotic resistant pathogens has become a serious threat to human health (CDC, 2013; RAR, 2014; WHO, 2014). Hence, the emergence and dispersion of antibiotic resistance caused by these multiple antibiotic resistant or MDR pathogens) has reduced the susceptibility of such pathogens to antibiotics in medical treatment (Allen *et al*., 2010). The overall outcome of this is long hospital stay (by patients suffering from diseases caused by these MDR pathogens), increased hospital bills, spread of such pathogens internationally, increased mortality rate among others (Bassetti *et al*., 2011). Non effective antibiotics due to MDR bacteria will affect areas of medicine such as surgery, cancer chemotherapy, transplantation etc. (Spellberg *et al.*, 2007).

The prevalence of resistance to specific antibiotics within a microbial population may be explained by the presence of mobile genetic elements such as integrons, plasmids and transposons found within the population encoding such antibiotic resistance (Tamma *et al*., 2012). Moreso, the existence of plasmid-bearing extended spectrum beta-lactamases (ESBL) and fosfomycin resistance determinants that can spread effectively in Enterobacteriaceae (of which Escherichia coli is a member) have been discovered and are of great clinical concern (Zhao *et al*., 2015).

This study was focused on seeking a scientific pathway to reclaim some commonly used antibiotics which have lost therapeutic value or usefulness owing to development of resistance genes against them by gram negative pathogens such as *Escherichia coli*. Antibiotic sensitivity patterns of fifteen (15) strains (uropathogens) of *Escherichia coli* before ascorbic acid treatment in this study showed that the fifteen uropathogen strains were sensitive to nitrofurantoin except strains EC4, EC8 and EC13. Therefore, 15(100%), 13(86.7%), 9(60.0%), 8(53.3%), 5(33.3%), 5(33.3%) and 3(20.0%) *Escherichia coli* strains were resistant to ampicillin, amoxicillin/clauvulanic acid, ceftazidime, cefuroxime, gentamycin, ciprofloxacin, ofloxacin and nitrofurantoin respectively. This implied that all strains resisted ampicillin (a penicillin antibiotic) while 12(80.0%) strains were sensitive to nitrofurantoin (a urinary antiseptic). Strain EC8 selected and used in this work was resistant to all eight antibiotics used.

In terms of sensitivity, all fifteen strains recorded (in decreasing order) mean±standard zone of inhibition sensitivity of 17.0±5.3mm, 15.0±5.3mm, 14.5±5.0mm, 10.5±6.0mm, 9.5±4.9mm, 8.7±3.6mm, 1.5±0.1mm and 0.0±0.0mm to nitrofurantoin, ciprofloxacin,

ofloxacin, ceftazidime, cefuroxime, gentamycin, amoxicillin/clauvulanic acid and ampicillin respectively. This sensitivity pattern suggests that nitrofurantoin followed by ciprofloxacin, by ofloxacin, ceftazidime and cefuroxime (singly or in synergistic combination of any two different classes) may be drug of choice for treatment of urinary tract infections caused by *E.coli*. While the choice of nitrofurantoin may be cheering to low income patients, the alternative of choosing any of ciprofloxacin, ofloxacin (both fluoroquinolones), ceftazidime or cefuroxime (both cephalosporins) may be a sad one owing to their high cost and sometimes, non-availability. The total resistance recorded by ampicillin was expected owing to its abuse arising from its availability and low cost. Worrisome is the almost total resistance recorded by amoxicillin/clauvulanic acid (branded augmentin) and the reason is because it is popularly or commonly prescribed and used by physicians to treat a good number of human diseases. Some authors have also expressed similar worry over augmentin efficacy (Oluremi *et al.,* 2011). The sensitivity patterns obtained for all fifteen *E.coli* strains used in this study are however, subject to verification and confirmation by other authors.

The multidrug resistant (MDR) statuses of 11(73.3%) out of the fifteen *E.coli* strains showed that 1(9.0%), 4(36.4%), 3(27.3%) and 3(27.3%) strains were resistant to 3drugs, 4drugs, 5drugs and more than 6drugs respectively. *Escherichia coli* strain 8 used in this study was resistant to all the eight antibiotics sampled. Uropathogen EC5 strain resisted 3drugs which belonged to cephalosporin, aminoglycoside and penicillin antibiotic groups. Strain EC3 resisted four drugs which were of fluoroquinolone and penicillin groups. Strain EC13 resisted five drugs belonging to cephalosporin, penicillin and urinary antiseptic groups. The other strains resisted antibiotics of various groups. These strains therefore, qualified as multiple resistant or MDR strains. Isolates are considered MDR if they are resistant to at least three of the antibiotics tested (Santo *et al*., 2007; Jan *et al*., 2004). According to Chethana *et al*. (2013), one of the methods used by various authors to characterize organisms as MDR is based on invitro antimicrobial susceptibility test results when they test resistant to multiple antimicrobial agents, classes or subclasses of antimicrobial agents.

Multidrug resistant organisms are microorganisms that are resistant to one or more therapeutic classes of antimicrobial agents (Adam *et al.*, 2008). The MDR statuses of 11(73.3%) of the *E.coli* strains used in this study is further established by the report of previous authors which stated that multidrug resistant *E.coli* and other enterobacteriaceae isolates are characterized by non-susceptibility to at least one agent in three or more antibiotic

categories (Magiorakos *et al*., 2012). The incidence of 11(73.3%) MDR *E.coli* strains in this study is a high one and this is in line with a report which stated that *E.coli* had been recognized as a contributor to the dissemination of antibiotic resistance genes in natural environments (Henriques *et al.*, 2006; Zhao and Dang, 2012; Alm *et al.*, 2014; Alves *et al*., 2014).

Antibiotic resistant *E.coli* and other enteric bacteria that survived the extensive antibiotic treatments in the gut of humans or animals can enter aquatic systems through discharge from poultry and livestock production and through hospital municipal wastewaters (Pruden *et al*., 2006; Pereira *et al.*, 2013) and therefore, the rivers that are used for recreational activities, irrigation etc can be potent sources through which antibiotic resistant bacteria are disseminated (Su *et al*., 2012; Pereira *et al.*, 2013).

In this study, ascorbic acid dilutions of 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000ug/ml were used to treat and possibly cure an MDR EC8 strain which was resistant to 8(100.0%) of all antibiotics tested with the aim of reducing its resistance significantly or eliminating it completely (via complete or partial plasmid removal). The use of 100 – 1000ug/ml was according to the scheme provided originally by Ramesh *et al.* (2000) and adopted by Potrus (2009). Ascorbic acid or vitamin C is a non-hazardous, non-antibiotic anti-oxidant agent which is cheap, safe to handle and readily available. The loss of 50-100% or ≤50% of resistance after treatment with the above stated dilutions of ascorbic acid was used as the basis of establishing the curing effects of these dilutions. The use of 50% and above loss in resistance as a criterion to determine the extent of plasmid curing was according to the scheme provided by Akortha *et al*. (2011). Stainer *et al.* (1984) reported that the elimination of plasmids by dyes and other natural agents reflects the ability of such an agent to inhibit plasmid replication at a concentration that does not affect the chromosome.

After treatment with the above stated ten (10) invitro ascorbic acid dilutions, *Escherichia coli* strain 8 still remained completely resistant to cefuroxime and ampicillin. Strain 8 *E.coli* however, became sensitive to ceftazidime, gentamycin, ciprofloxacin, ofloxacin, augmentin and nitrofurantoin as a result of the invitro treatment. Hence, there was reduction from 8(100.0%) resistant drugs to 2(25.0%) resistant drugs or from 8(100.0%) resistant drugs to 6(75.0%) sensitive drugs after treatment. Continued resistance to the 2(25.0%) antibiotics listed above (even after treatment) may be due to the fact that resistance genes responsible for resistance to these drugs may be chromosome-mediated or non-conjugative plasmids

(Akortha and Filgona, 2009). After treatment with the doses of ascorbic acid, incubation was carried out for 6hr, 12hr, 18hr and 24hr. This was to avail the dividing cells in EC8 strain pure broth culture the opportunity to respond effectively to the curing effect (if any), of ascorbic acid at the various liquid growth phases of the organism during incubation in Mueller Hinton broth medium.

Growth in liquid medium is synonymous with growth of bacterial pathogens invivo (in the blood circulatory system, tissues, lymphatic system etc) of man and related primates. Incubation in water bath rather than conventional incubator was to ensure direct heat (temperature) contact of diluted broth culture cells and the ascorbic acid dilutions to ensure optimal biochemical and physiological activities of the EC8 strain in the set up. Based on the results therefore, 100ug/ml, 400ug/ml, 600ug/ml and 1000ug/ml dilutions recorded significant resistance loss (and hence, significant sensitivity improvement) of EC8 strain to amoxicillin/clauvulanic acid, ceftazidime, amoxicillin/clauvulanic acid and nitrofurantoin respectively after 6hr incubation (Table 3). It is possible that the lag phase (i.e. phase of physiological and biochemical inactivity) of this strain of *E.coli* is short. This is not in agreement with the report of Thiel (2015) which stated that culture harvest is ideal after 12-16hr of incubation which is the period of transition from logarithmic phase to stationary phase and that it is during the 12-16hr period that bacterial DNA or plasmid is not degraded. The presence of resistance loss after 6hr (or 360mins) incubation however, is in agreement with the report of a previous author which stated that generation time of *E.coli* in the laboratory is between 15-20mins (Todar, 2012).

After 12hr incubation, ≤50% loss of resistance was also recorded by 100ug/ml, 400ug/ml, 600ug/ml and 1000ug/ml ascorbic acid dilutions in that they induced100%, 61.9%, 52.4% and 100% resistance reduction respectively to amoxicillin/clauvulanic acid and ceftazidime. After 18hrs incubation, 100, 400, 500, 600, 700, 800, 900 and 1000ug/ml ascorbic acid dilutions induced resistance reduction of 52.4%, 81%, 61.9%, 71.4%, 71.4%, 71.4%, 71.4% and 100% respectively to ceftazidime. During the same incubation period, 100ug/ml, 600ug/ml and 1000ug/ml dilutions recorded 167%, 300% and 167% RR respectively to amoxicillin/clauvulanic acid. Other significant RR during the 18hr incubation period was recorded by 300ug/ml and 400ug/ml dilutions which induced 52.9% and 52.9% resistance losses respectively to nitrofurantoin and lastly, 1000ug/ml dilution induced 79%, 167% and 64.7% RR to ofloxacin, amoxicillin/clauvulanic acid and nitrofurantoin respectively during

the same incubation period. It is clear therefore that 8(75.0%) of the dilutions tested induced significant RR after 18hr incubation and this may suggest that 18hr incubation is optimal for invivo expression of the physiological and biochemical activities of *Escherichia coli.*

Similarly, after 24hr incubation, 100, 400, 500, 600, 700, 800, 900 and 1000ug/ml dilutions yielded RR of 52.4%, 100%, 71.4%, 90.5%, 81%, 71.4%, 109.5% and 128.6% respectively to ceftazidime. Also, after 24hrs incubation, only 100, 600 and 1000ug/ml recorded 167%, 367% and 233% RR respectively to amoxicillin/clauvulanic acid. During the same incubation period, 300, 400, 600 and 1000ug/ml dilutions induced 76.5%, 52.9%, 58.8% and 70.6% RR to nitrofurantoin. The treatment outcomes under the same incubation period as recorded by 600ug/ml and 1000ug/ml with respect to amoxicillin/clauvulanic acid were 367% and 233% resistance reduction respectively. A resistance reduction of 53% and 86% was recorded by 1000ug/ml dilution with respect to ofloxacin and ciprofloxacin (both fluroquinolones). Clearly, optimal Mueller Hinton broth culture activity of EC8 uropathogen was attained after 18hr and 24hr incubation. This may be because there were more actively dividing cells in the logarithmic phase of the Mueller Hinton broth culture. Hence, this finding tends to lend credence to the choice of 18-24hr incubation period for both agar and broth cultures after appropriate inoculation. Besides, this finding is in line with the report of a previous author (Thiel, 2015).

This longer incubation period may be suggestive of the fact that the cells of EC8 strain are still dividing even up to this period and the fact that dilutions that induced ≤50% loss of resistance losses were achieved more and most after 18hr and 24hr incubation respectively may indicate that the highest biochemical and physiological activities of EC8 uropathogen strain will be attained after 18-24hr incubation at 37$^{o}$C - the conventionally prescribed temperature for human pathogens such as *E.coli* urinary tract pathogens. After 24hr incubation, 100, 400, 500, 600, 700, 800, 900 and 1000ug/ml dilutions recorded ≤50% loss of resistance to ceftazidime and 100, 300, 600 and 1000ug/ml recorded ≤50% loss of resistance to nitrofurantoin (Table 3). This may be because these dilutions have the capacity to remove resistance genes carried on the inherent plasmids present in the organism.

The means of all resistance losses after 6hr, 12hr, 18hr and 24hr periods of incubation for all dilutions showed that only ascorbic acid dilutions of 400, 500, 600, 700, 800, 900 and 1000ug/ml recorded ≤50% resistance losses of 63.1%, 73.8%, 50.0%, 61.9%, 52.6%, 50.0%, 51.3% and 102.4% respectively to ceftazidime. Only 1000ug/ml dilution recorded ≤50% loss

of resistance in EC8 strain to both ceftazidime and nitrofurantoin. Resistance losses of ≤50% of 67.6% and 175.3% to ofloxacin and amoxicillin/clauvulanic acid respectively were also induced by 1000ug/ml AA dilution (**Table 4)**. This particular finding in this study singles out 1000ug/ml and to a lesser extent, 600ug/ml for industrial and pharmaceutical trial/application in terms of drug modification with regard to ceftazidime, ofloxacin, amoxicillin/clauvulanic acid and nitrofurantoin. The implication of this is that 1000ug/ml dilution and to a lesser extent, 600ug/ml ascorbic acid dilutions may possess the capacity to eliminate resistance genes (if plasmid mediated) in MDR EC8 and indeed other MDR *E.coli* strains and therefore, could be administered in the therapeutic control of various infections caused by such bacteria.

According to Ramesh *et al.* (2000), the toxic effect of ascorbate (derivative of ascorbic acid) on cells and bacterial cells was dose dependent. Ascorbic acid causes conformational damage to unprotected cells (Ramesh *et al*., 2000). L- ascorbic acid inhibits a wide range of biological functions and modifies the properties of DNA, generation of hydrogen peroxide and hydroxyl radicals by auto-oxidation and lipid peroxidation of membrane components (Morgan *et al*., 1976; Shamberger, 1984; Sugiyama *et al*., 1991; Halliwell and Aruoma, 1991). The reactive oxygen species such as $OH^-$ and $H_2O_2$ are involved in DNA damage by Fenton – type reaction which has been shown to occur in bacterial cells (Imlay and Linn, 1989).

In this study, higher doses (dilutions) of ascorbic acid (i.e. 400, 500, 600, 700, 800, 900 and 1000ug/ml especially 1000ug/ml) recorded significant resistance losses and perhaps, increased curing or plasmid removal. Lower doses did not induce or yielded insignificant losses. This finding is supported by the report of some authors which stated that the effect of ascorbate in terms of antibiotic resistance genes curing is dose dependent. Since L-ascorbic acid has the capacity to modify the properties of DNA (Morgan *et al*., 1976), it can be reasoned that it will also have effect on extra-chromosomal DNA (which is plasmid).

That EC8 uropathogen significantly became sensitive to ceftazidime, ofloxacin, amoxicillin/clauvulanic acid and nitrofurantoin after 1000ug/ml ascorbic acid treatment **(Table 4)** may open up a new era of therapy. The simultaneous application of this dose of ascorbic acid dilution and the implicated drugs (antibiotics) to patients however, may not only act as an additional antibacterial agent but may help to eliminate the drug resistance genes (if plasmid mediated) from the infectious bacterial cells (Spendler *et al.*, 2006). Hence, patients suffering from urinary tract infections caused by MDR *E.coli* may be administered ascorbate at standard human doses (using 1000ug/ml in particular or 600ug/ml as a basis)

along with ceftazidime, ofloxacin, amoxicillin/clauvulanic acid and nitrofurantoin or any of their combinations (combination therapy). Besides, *Escherichia coli* is catalase negative and catalase is a protective enzyme that which reduces the effects of reactive oxygen species (i.e $OH^-$ and $H_2O_2$) mediated damage to the bacterial DNA (Hardy, 1987) and this is advantageous for curing by ascorbic acid in EC8 uropathogen.

Resistance loss effect of the ten ascorbic acid dilutions was also tested on the minimum inhibitory concentration (MICs) of ampicillin (a penicillin drug). The idea is that a fast and accurate determination of MIC can indicate optimal effective treatment of patients while at the same time, avoiding over prescription. This will save money for healthcare providers as well as reduce development of resistance (NCCLS, 2000; McGowan and Wise, 2001). Findings in this study showed that MIC of ampicillin (which is 10ug based on long standing research), was reduced to 5ug (a two-fold reduction), 5ug (a two-fold reduction), 5ug (a two-fold reduction), 5ug (a two-fold reduction) and 2.5ug (a four-fold reduction) by the synergistic action of ascorbic acid dilutions of 100ug/ml, 600ug/ml, 700ug/ml, 800ug/ml and 1000ug/ml respectively.

According to Dimitru *et al*. (2006), there is a significant correlation between MIC values and the inhibition zone diameters obtained by an antibiotic disc containing a drug in microgram. Dilutions of ascorbic acid reduced the MIC of ampicillin by two, two, two, two and four folds. The lower the MIC and the larger the zone of inhibition, the more susceptible the microorganism is to the antimicrobial agent (in this regard, ampicillin) and conversely, the higher the MIC and smaller the zone of inhibition, the more resistant the microorganism (Dimitru *et al.,* 2006). Again, it is noteworthy the four-fold reduction of the MIC of ampicillin induced by 1000ug/ml ascorbic acid dilution which was the highest reduction. This was followed by two-fold reduction recorded each by 100ug/ml, 600ug/ml, 700ug/ml, 800ug/ml and 1000ug/ml. It should be noted that whereas the first control which contained only EC8 organism and ampicillin dilutions did not show any MIC change, the second control which contained only EC8 organism and ascorbic acid dilutions showed turbidity for all the dilutions. This suggested to some extent, that ampicillin MIC reductions as stated in this study could be solely due to the synergistic action of ascorbic acid and the drug dilutions. Cursino *et al.* (2005) has reported the synergistic interaction of ascorbic acid with antibiotics against multi drug resistant bacteria like *Pseudomonas aeruginosa*.

Tunicamycin at a concentration of 0.08ug/ml and in synergy with beta-lactam antibiotics has been reported to reduce the MIC of oxacillin against methicillin resistant Staphylococcus aureus from 50ug/ml to 0.4ug/ml, a 125-fold reduction (Campbell *et al*., 2011). Farha *et al.* (2012) reported the use of the anti-platelet drug-ticlopidine in synergy with cefuroxime to lower the MIC of MRSA by up to 64-fold. The therapeutic importance (in terms of application) therefore of findings in this regard is that when doses of one of these ascorbic acid dilutions or a combination of any two are incorporated into the manufacture of ampicillin or any other related antibiotic and administered to a patient diagnosed to be suffering from a disease caused by an MDR *E.coli* organism, a better result in terms of therapeutic outcome (cure of the disease) may be achieved. These results are supported by reports of previous authors who carried out related studies (Kohler, 2010; Crowle *et al*., 1992; Shiram *et al*., 2008).

## CONCLUSION

The results of antibiotic sensitivity testing showed that to treat any urinary tract infection caused by an MDR *E.coli* strain, any of nitrofurantoin, ciprofloxacin, ofloxacin, ceftazidime or cefuroxime or a synergistic combination of any two may be effective. Resistance losses (or reduction) of ≤50% were achieved most between 18-24hr incubation suggesting that optimal biochemical and physiological activities may be attained by EC8 strain and indeed other MDR *E.coli* uropathogens during this incubation period at 37$^{o}$C for *E.coli* uropathogens. Ascorbic acid dilutions of 100ug/ml, 400ug/ml, 500ug/ml, 600ug/ml, 700ug/ml, 800ug/ml, 900ug/ml and 1000ug/ml recorded ≤50% mean loss of resistance in EC8 strain to ceftazidime. Noteworthy was 1000ug/ml dilution which induced ≤50% mean loss of resistance to ceftazidime, ofloxacin, amoxicillin/clauvulanic acid and nitrofurantoin. Ascorbic acid dilutions of 100ug/ml, 600ug/ml, 700ug/ml, 800ug/ml and 1000ug/ml as treated with EC8 strain reduced ampicillin MIC to 5ug (two-fold reduction), 5ug (two-fold reduction), 5ug (two-fold reduction), 5ug (two-fold reduction) and 2.5ug (four-fold reduction) respectively. Uropathogen EC8 strain treated with 1000ug/ml reduced ampicillin MIC to 2.5ug (four-fold reduction) which was the highest reduction. The administration therefore of ascorbic acid at standard human doses using 1000ug/ml in particular or even higher dose as a basis along with ceftazidime, ofloxacin, amoxicillin/clauvulanic acid and nitrofurantoin or any of their appropriate combinations may assist in eliminating inherent resistance genes (present on plasmids or chromosomes) in MDR *E.coli*. It is hoped that this

will bring about faster treatment outcomes and perhaps, help reclaim some hitherto first line antibiotics which have long lost their therapeutic usefulness.

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