Evaluation the synergistic interactions between Ascorbic acid and antibiotics against *Pseudomonas aeruginosa* isolated from different clinical infections

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ABSTRACT

The present study was carried out to shed light upon some materials that worked in increasing antibiotic activity against Pseudomonas aeruginosa. Two hundred and twelve samples including wound swab (70), ear swab (53), urine samples (41), burn swab (33), respiratory tract swab (15) were collected from different hospitals and private clinics in Wasit province during the period from the mid of October 2022 to the end-April 2023. Antibiotic susceptibility test was conducted for 54 bacterial isolates of Pseudomonas aeruginosa, tested by disc diffusion method using (12) antibiotics and the results showed a different percentage of resistance to each antibiotic as (Gentamycin, amikacin, ampicillin, bacitracin, Ciprofloxacin, Norfloxacin, chloramphenicol, erythromycin, tetracycline, streptomycin, tobramycin, Trimethoprim sulfamethoxazole). The results revealed that Ciprofloxacin was the most effective antibiotic against bacterial isolates followed by amikacin and then by Norfloxacin, and the isolates are completely resistant to both erythromycin and tetracycline. Twelve isolates were selected to detect the effect of ascorbic acid when was combined with antibiotics and tested by using disk diffusion assay. Various concentrations of the ascorbic acid were used, starting from (1 to 22.2 mg). The results showed that there is a synergistic interaction between Ascorbic acid and most of the antibiotics, Also, the synergistic effect increases with increasing concentration of the Ascorbic acid. The antibiotic chloramphenicol had the greatest effect, as the area of inhibition increased in 11 out of 12 isolates. Also, the tests showed that ascorbic acid had an antagonistic effect on some antibiotics such as norfloxacin and tobramycin, where the inhibition area decreased in 9 and 8 isolates, respectively

Keywords:

Introduction

Pseudomonas aeruginosa expresses a high level of resistance. In addition to its virulence factors which involved the resistance mechanisms [1], Ascorbic acid has antibacterial, antiviral activity and is essential to stimulate the immune function (activate phagocytic leukocytes) [2],Species because it is widespread in the environment and causes a wide variety of infections in humans, animals, and plants. Also, it is more important pathogens that are considered as a causal factor of nosocomial infections [3]. Also, it causes several diseases in the human body, like wound and burns infections, eye infection urinary tract infection, otitis media, bacteremia, bones and joints infections [4]. P. aeruginosa can secrete many toxic proteins that are believed to act as virulence factors and these proteins can cause Extension tissue damage and bloodstream invasion [5]. Antibiotic resistance has been found in Pseudomonas aeruginosa, including aminoglycosides, quinolones, and -lactams [6]. In general, P. aeruginosa's principal methods for resisting antibiotic attacks may be divided into

intrinsic, acquired, and adaptive resistance. Multidrug Resistance; Pseudomonas aeruginosa is naturally resistant to several antibiotic agents by mutational changes or acquisition of genetic materials. The appearance of MDR strains happens as a result of anti-microbial therapy's selective pressure, besides acquired multi-drug resistance to many antibiotics leading to elevated morbidity and mortality [7], Due to loss of membrane permeability, efflux pumping of antimicrobial agents, and acquired susceptibility by the development of resistance genes,. The rise of multi-resistant P. aeruginosa poses a public health risk by limiting effective antibiotic treatment with commercially available medicines. Antibiotic-resistant bacteria are on the rise across the world, posing a serious threat of treatment failure [8].

2. Materials and Methods

2.1 Materials Cultures media were Blood agar base (Mast diagnostic, England), Cetrimide agar (Micromedia, USA), MacConkey agar (Oxoid, England), Muller-Hinton agar (Rashmi, India), Nutrient broth (Oxoid (England). Ascorbic acid (Merck and Co., Inc., West Point, PA, USA) solution in distilled sterile water was adjusted to pH 7.0 with 10 N NaOH (SIGMA, St. Louis, MO, USA) and added to the media at the required concentration.

2.2 Methods

2.2.1 Samples collection. In this study, a total of (212) collection of specimens from (wound swab, ear swab, burn swab, otitis, and respiratory tract infection) of patients were hospitalized at Al-Zahraa Teaching Hospital. The specimens collected during the period from October 2022 to the end-April 2023.

Bacterial identification

The collected sample (212) was cultured on MacConkey agar, blood agar, Cetrimide agar, and incubated at 37°C for 24 hours. Then, isolated bacteria were identified according to colonial morphologies, shape, size, color, and pigment productio

Antibacterial agents

Twelve different forms of antibiotics were used, described in Table (2.1).

Table (1): List of antibacterial agents used in sensitivity testing.

Antibacterial agents	codes	concentration Mg/ disk	Antibacterial agents	codes	concentration Mg/ disk
Gentamycin	GM	10	Chloramphenicole	C	30
Amikacin	AK	30	Erythromycin	E	15
Ampicillin	amp	10	Tetracycline	TE	30
Bacitracin	BA	10	Streptomycin	S	10
Ciprofloxacin	CIP	5	Tobramycin	TOB	30
Norfloxacin	NOR	10	Trimethoprim-sulfamethoxzole	SXT	5

Antibacterial susceptibility test

This test was done according to the procedure defined by Murray, et al., (2007); twelve different forms of antibiotics were used, described in Table (1) above,Antimicrobial Susceptibility P. aeruginosa isolates was determined by the disk diffusion test (DDT) according to [9],twelve different forms of antibiotics were used, described in Table (1) above,Sensitive and resistant isolates were detected depending on the recommendations made by [10].

Determining Effect of Ascorbic acid in Combination with Antibiotics

Different molarities of ascorbic acid solution were prepared to start from (22.2 mg), and the

lowest molarities of ascorbic acid that causes inhibition against bacteria were determined by using a diffusion assay. The solution of Ascorbic acid was prepared and the desired concentration of antibiotics was added. The paper disk was socked in the final solution, and disk diffusion assays were used to determine the inhibition zone of antibiotic disks against bacteria according to the Clinical And Laboratory Standards Institute [10].

Statistical Analysis.

The Chi-square test was used to statistically analyze all of the data using the system SPSS IBM version 20 program. Statistical significance was defined as a P-value of less than 0.001 [11].

Results and Discussion

Prevalence of *Pseudomonas aeruginosa* according to the clinical samples.

The Prevalence of *Pseudomonas aeruginosa* according to the clinical samples was illustrated in Table (3-2). The current study shows the highest percentage of *Pseudomonas aeruginosa* infections was observed in wound infection 23(32.85%), this bacterium can be considered the major agents of nosocomial infections in wound followed by an ear infection, and the frequency of *Pseudomonas aeruginosa* was 13(24.52%), then UTI 11(26.82%)While the lower incidence was 2 (13.3%)in Respiratory tract.

Table(1)Prevalence of *Pseudomonas aeruginosa*isolates according to the clinical samples.

Clinical Samples	Total	Positive samples	Negative samples
Wound	70	23 (32.85)	47 (67.14)
Ear	53	13 (24.52)	40 (75.47)
Uti	41	11 (26.82)	30 (73.17)
Burn	33	5 (15.15)	28 (84.84)
Respiratory tract	15	2 (13.33)	13 (86.66)
Total	**212**	**54 (25.47)**	**158(74.53)**
X^2			5.09(NS)
P value			**0.472***

*** No significant difference (P>0.05)**

The present results percentages of the isolated *Pseudomonas aeruginosa* are incompatible with Al-Saeedi,(2018) who report the wounds obtained 24(33.8%), followed by ear and UTI19(26.8%) and 12(16.9%) respectively. Also agree with study in Iraq by (Ahmad,2017) show similarity with this study that revealed the percentage of *Pseudomonas aeruginosa* isolated from the wound, UTI and ear were 32%, 27%, and 17% respectively except burn was(24%).

Severe burns are very devastating forms of trauma that require immediate and specialized medical care. The immunosuppression state, triggered by the burn trauma, and the wound local microenvironment are favorable elements for microbial colonization and proliferation (Wu,2018). Among the burn wound pathogens, the Gram-negative bacterium *P. aeruginosa* presents the highest incidence and becomes, generally, predominant in developed infections (Fournier,2015).

P. aeruginosa is the third most prevalent pathogen linked to catheter-associated UTIs in hospitals (Jarvis and Martone, 2009). *P. aeruginosa* virulence is multifaceted and has been related to cell-associated components such as alginate and lipopolysaccharide (LPS), Exoenzymes, or secretory virulence factors such as protease, elastase, phospholipase, pyocyanin, exotoxin A, exoenzyme S, hemolysins (rhamnolipids), and siderophores, as well as flagellum, pilus, and non-pilus adhesins (Veesenmeyer *et al*.,2009). These variables have been linked to the pathogenesis of P. aeruginosa-induced illnesses such as respiratory tract infections, burn and wound infections, UTIs and Otitis (Lysczak *et al*.,2002).

Antibiotics susceptibility testing for Pseudomonas aeruginosa

Antibiotics are being tested resistance in microorganisms is critical for classifying their actions based on the types of antibiotics used, as well as their medicinal use and efficacy in disease care. Furthermore, it will provide a visual representation of the subsequent transmission of genetic elements responsible for resistance across species and, as a result, the identification of resistance spread [15].The requisite test for screening purposes should be capable of detecting a large number of isolates. The disk diffusion method (used in this study) is quick and simple, but it has a lower degree of accuracy since the zone of inhibition is influenced by the medium structure and interaction of certain ions with antibiotics distributed through the medium [16].Thirty-seven isolates were subjected to susceptibility testing according to the CLSI , 2020 [10] guidelines using different antibiotics namely (Gentamycin, amikacin, ampicillin, bacitracin, Ciprofloxacin, Norfloxacin, chloramphenicol, erythromycin, tetracycline, streptomycin, tobramycin, Trimethoprim-sulfamethoxazole) the test show at the figure (2,3).

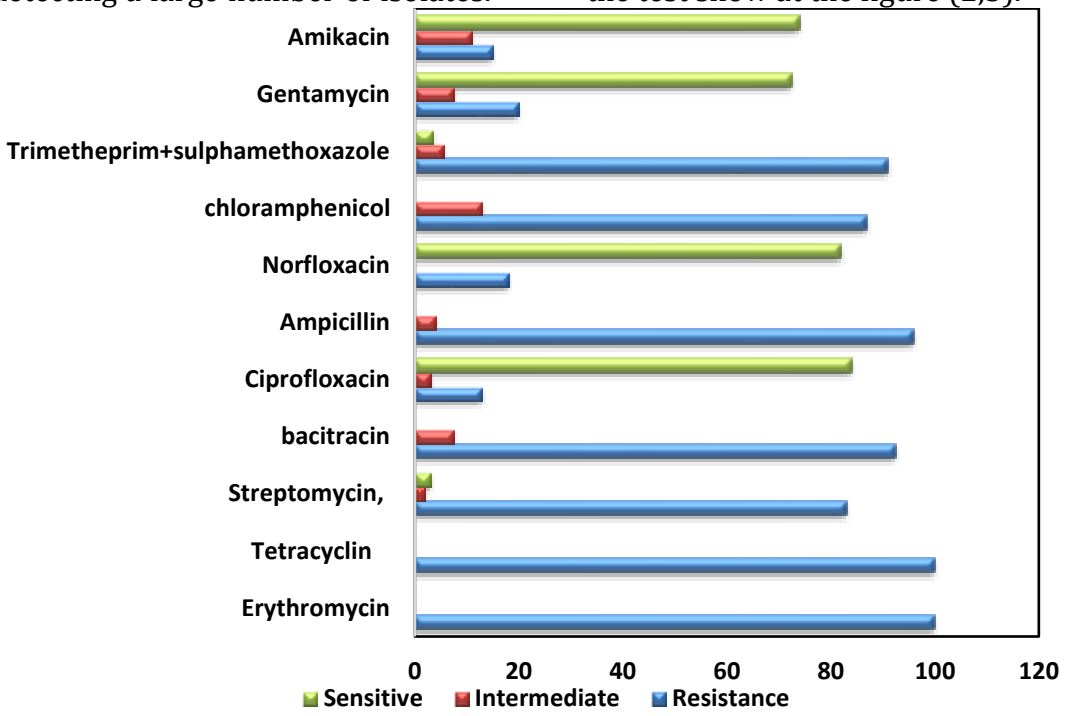

Figure (2): The percentage of antimicrobial resistance profiles of *pseudomonas aeruginosa*

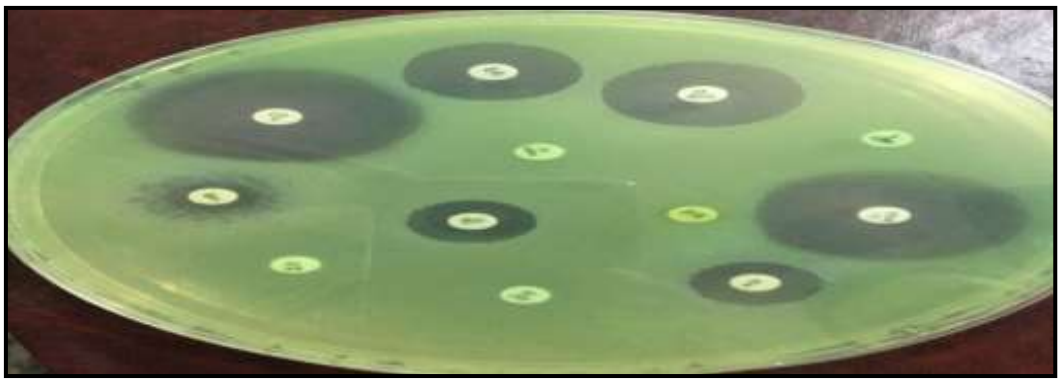

Figure (3): Sensitivity test by using discs diffusion assay.

The unselective use of antibiotics is potentially leading to a higher incidence of infections with resistant microorganisms such as P. aeruginosa. Regarding, the antimicrobial sensitivity agar it was noticed that the isolates were lack of susceptibility to many tested antimicrobial agents. The primary mechanism of the microorganism's resistance relies on its ability to shut out various agents rather than the production of antibiotic inactivating enzymes. Consequently, most antibiotics are of limited value in the treatment of P. aeruginosa infection [17]. The results indicated that the isolates are completely resistant to both erythromycin and tetracycline (100%). These findings agreed with [18,19] were reported the P. aeruginosa is completely resistant to erythromycin and tetracycline, Resistance to erythromycin may be due to the common use of this antibiotic which leads to increase microbial resistance to this antibiotic, while resistance to Tetracycline may be due to the resistance gene carried by plasmid [20]. In this study, isolates showed high resistance to ampicillin (96.29%). This result agrees with the results obtained by [21] who found that Ps. aeruginosa was completely resistant to ampicillin (100%), while [33] revealed that Ps. aeruginosa was resistant to ampicillin at 89.9%. Regarding streptomycin, all bacterial isolates showed high resistance percentage to it (83.33%). This result disagreed with those of [19] who reported high resistance to Ps. aeruginosa(90%). Resistance to gentamycin recorded a low resistance percentage. The Percentage of resistance was (20.37%). [22], reported gentamycin sensitivity for Ps. Aeruginosa 40%, 30% respectively. Ciprofloxacin on the other hand had shown a good effect on the bacterial isolates. Most isolates were found to be sensitive; for example, ciprofloxacin sensitivity was 87% for Ps. aeruginosa, This result was almost comparable to the results reported by [23] who found that ciprofloxacin sensitivity of Ps. aeruginosa was (86%). Sensitivity of bacteria to ciprofloxacin (quinolones) because quinolones act principally by inhibiting bacterial DNA Gyrase, so preventing supercoiling of the DNA, a process that is necessary to compacting chromosome into the bacterial cell. Results showed high resistance to erythromycin with (100%). While [24] mentioned that Ps. aeruginosa resistance to erythromycin was (69.5%). Resistance to Trimethoprim-sulphamethoxazole "SXT" were (90.74%). [19] found the same result as the SXT resistance to Ps. Aeruginosa (100%). The Percentage of resistance to Tobramycin was (24%). [25] reported different findings, the resistance percentage was (80.7%) in Baghdad and disagreed with [23] in Iraq, who recorded percentage resistance with (57.2%). Amikacin percentage resistance in this study was (14.81%). This result disagrees with the study by [26] result was (34.9%).

Enhancement of antibiotics activities

Effect of ascorbic acid in combination with antibiotics

The susceptibility testing of *P. aeruginosa* against antibiotics alone and their combination with ascorbic acid was checked by the disc diffusion method. Combination discs of antibiotic and ascorbic acid in different concentrations (1,6.4.13.8.22.2 mg) were prepared and their efficacy against *P. aeruginosa* was checked by measuring their zone of inhibition around disks, in addition to using several methods of adding acid to the disks of antibiotics, such as the pouring method and the method of pipette addition Synergistic effect were produced when antibiotic and ascorbic acid use together. It was observed that the activity of antibiotics was greatly enhanced and the resistance of bacteria was reversed with ascorbic acid. Total 12 samples of *P. aeruginosa* were checked for their susceptibility against antibiotics and antibiotic+ Ascorbic acid combination.The results showed that ascorbic acid increases the zone diameters of Ampicillin, Tetracycline, and Bacitracin 83.33 % (10 of 12) of the isolates, while Amikacin and Ciprofloxacin appeared a positive effect on half of the samples and a negative effect on the other half. The synergic effect also recorded with using Gentamicin and Erythromycin appeared in 7 out of 12 (58.33%) of the samples. Regarding chloramphenicol, it had a significant positive activity on 11/12 (91.66%) of the samples. Furthermore, Norfloxacin had inhibitory activity on 9/12 (75%) of isolates,

unlike streptomycin and Trimethoprim-sulfamethoxazole, it had a synergistic effect on the same number of isolates. , while for tobramycin, it had an interaction effect, as the inhibition zone was reduced in 8 out of 12 (66.66%) of the bacterial isolates at different concentrations Figure (3-4,5,6)

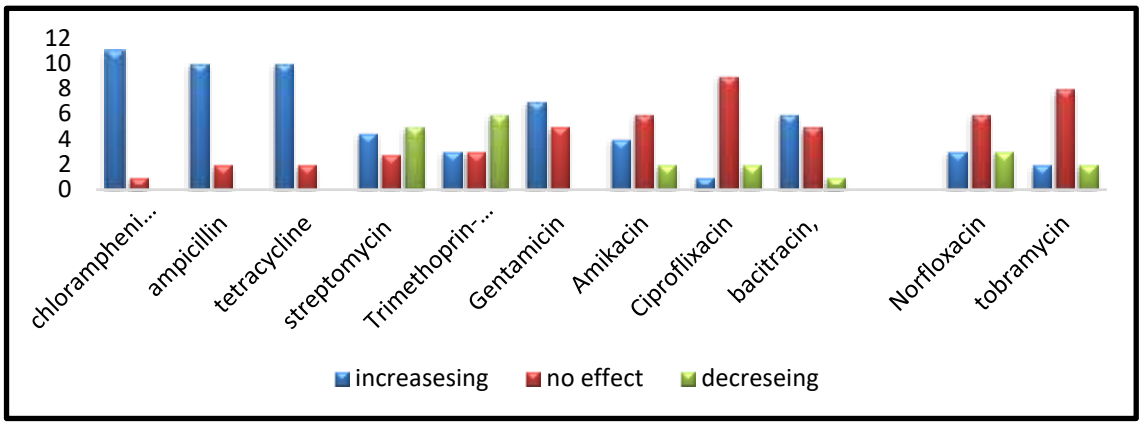

Figure (4): Effect of Antibiotic ascorbic acid combination on Zone of inhibition diameters.

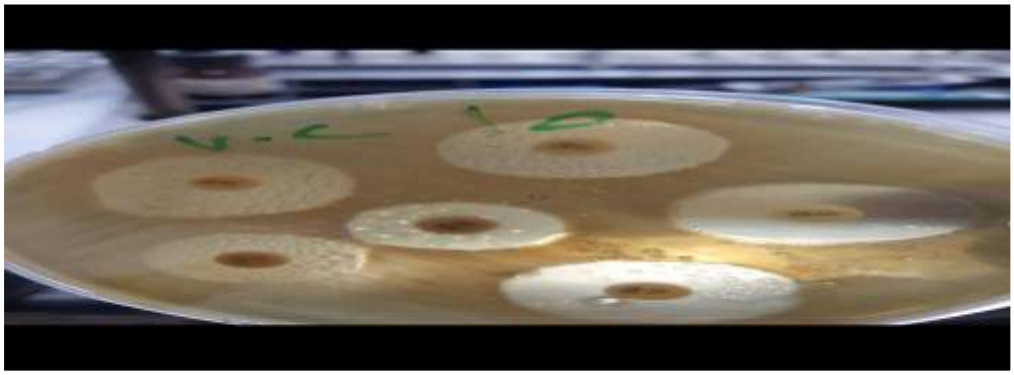

Figure (5): The effect of antibiotic and ascorbic acid(22.2 mg).

In this study, Ascorbic acid has been found to enhance the diameter zone of chloramphenicol in 11 out of 12 samples that turn into sensitive against *P. aeruginosa,* followed by ampicillin, bacterin, and tetracycline with ten samples. While, streptomycin and Trimethoprim-sulfamethoxazole, had a synergistic effect on 9 isolates. Also, the increases of zone diameters appeared with using Gentamicin and Erythromycin in 7 out of 12 samples and in 6 samples by using Amikacin and Ciprofloxacin. In comparison to Norfloxacin alone and tobramycin alone, the average zone of inhibition diameters was reduced in the Norfloxacin (9/12) and tobramycin (8/12) ascorbic acid combination. Antibiotics combined with ascorbic acid have been shown to have a synergistic effect in vitro, increasing the efficacy of treatment against resistant *P. aeruginosa* bacteria. The present results are compatible with a study done by[27] Karim, (2007), Synergistic effect between aminoglycoside and ascorbic acid was shown against *Pseudomonas aeruginosa* bacteria. Also, Erythromycin and SXT showed a synergistic effect with ascorbic acid toward *Pseudomonas aeruginosa* isolates. This study also agrees with [28], In vitro tests were conducted against 12 multi-resistant *Pseudomonas aeruginosa* isolates using a combination of ascorbic acid (AA) and 12 antibiotics. AA chloramphenicol, streptomycin, and tetracycline all showed synergic action. Any antibiotic was shown to be ineffective, with the exception of Amikacin, which was shown to be antagonistic. the results from the current study were revealed that ascorbic acid somehow increases the antibiotic sensitivity and the increase in the zone of inhibition was observed with chloramphenicol, ampicillin, bacterin, tetracycline, streptomycin, Trimethoprim-sulfamethoxazole, Gentamicin, Erythromycin, and Ciprofloxacin [29]. According to studies, ascorbic acid suppresses *P. aeruginosa* growth by interfering with sugar absorption via membrane components such as the phosphotransferase system; this system is sensitive to acerbate and is reversibly inhibited by ascorbic acid.

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 (H2O2) produced by the auto-oxidation of ascorbic acid, which causes antibiotics to have a higher potency [30]. It's important to note that ascorbic acid is required in human tissues, and the RDA for adult nonsmoking men and women is "120" mg per day [31]. According to findings from the heart and reproductive research, regular dosages of ascorbic acid (100 or 500 mg/day) can lower the risk of heart attack and boost the pregnancy rate [32].Ascorbic acid is also commonly employed as a food additive and preservative, as well as a significant antioxidant in the pharmaceutical and cosmetic sectors and Increased ascorbic acid levels in the blood may limit the efficiency of antibiotic therapy with chloramphenicol, hence increased AA intake is of particular concern, for example, but it might be useful in combination with other antibiotics like tetracycline. Because ascorbic acid levels are likewise elevated on human tears when vitamin C -1 g/day is supplemented, it might interact with both systemic and on-topic antibiotics, such as those used to treat eye infections [30].

Conclusions

We can conclude that, The sensitivity assay showed a high synergistic activity of ascorbic acid when mixed with antibiotics to increase the potency of the antibiotics against *Pseudomonas* bacteria, especially the antibiotic chloramphenicol. Ascorbic acid has an antagonistic effect for some antibiotics such as norfloxacin and tobramycin, as the area of inhibition for these antibiotics decreased when the vitamin was present.

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