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RESEARCH ARTICLE

Modulation of antibiotic activity against *Pseudomonas aeruginosa* by N-acetylcysteine, ambroxol and ascorbic acid

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ABSTRACT:
The possible synergy between antibiotics and each of N-acetylcysteine (NAC), ambroxol and ascorbic acid against five clinical isolates of *Pseudomonas aeruginosa* was evaluated. Synergy was found with 50% of isolates. NAC showed higher synergy than ambroxol and ascorbic acid. The synergy rates were 80%, 55% and 15% for NAC, ambroxol and ascorbic acid, respectively. Combinations of NAC with each of cefepime, ceftazidime, cefoperazone and meropenem and those of tetracycline with each of NAC and ambroxol showed the highest synergy. NAC showed synergy with all combinations except with levofloxacin with which indifference was found. The synergy rates were higher with β-lactam antibiotics. Antagonism was observed with gentamicin. Ambroxol showed stronger synergy with tetracycline, chloramphenicol and cefepime than with ceftazidime, meropenem, levofloxacin and cefoperazone. Indifference was found with gentamicin, levofloxacin, cefoperazone, ceftazidime, chloramphenicol, cefepime and meropenem. On the other hand ascorbic acid showed weak synergistic activity. Ascorbic acid could only potentiate chloramphenicol, meropenem, cefepime and cefoperazone. Indifference was found with levofloxacin, cefepime, ceftazidime, gentamicin, tetracycline, chloramphenicol and meropenem. These results suggest the use of combinations of NAC, ambroxol and ascorbic acid with antibiotics to combat the antibiotic resistance of *Pseudomonas aeruginosa*.

KEYWORDS: *Pseudomonas aeruginosa*, NAC, ambroxol, ascorbic acid, antibiotics, synergy

INTRODUCTION:
Pseudomonas aeruginosa is very common in nosocomial infections. It is the causative agent of about 10-15% of these infections.[1] It exhibits high resistance to different groups of antibiotics by intrinsic or acquired mechanisms. This remarkable resistance makes the treatment of *P. aeruginosa* infections very difficult.[2,3] It is of great value to investigate new drugs to overcome such resistance either by use of these agents individually or in combination with antibiotics.

N-acetylcysteine (NAC) is a sulfhydryl group-containing antioxidant and a mucolytic agent that is used in therapy of bronchitis.[4,5] It dissolves mucus by disrupting disulphide bonds in mucus. NAC has antimicrobial activity. This activity is due to competitive inhibition of cysteine utilization in bacteria and reaction of its thiol group with bacterial cell proteins.[6]

The antibacterial activity of NAC was reported by several investigators; thus Parry and Neu[7] found that NAC can be inhibitory to both gram-positive and gram-negative bacteria and *P. aeruginosa* was more sensitive to NAC. Moreover, they found that NAC combined with carbenicillin or ticarcillin increased their activity against *P. aeruginosa*. Roberts and Cole[8] reported the bactericidal activity of NAC against *P. aeruginosa* and its potentiating effect on carbenicillin.

Ascorbic acid is a sugar acid with antioxidant properties. Owing to its antioxidant activity, ascorbic acid is a common preservative and an important ingredient in pharmaceutical and cosmetic industries.[9-11] Ascorbic acid was found to augment the bactericidal activity of erythromycin, sulphamethoxazole-trimethoprim but not tetracycline against *P. aeruginosa*.[12,13] In addition, ascorbic acid can act as a β-lactamase inhibitor in *P. aeruginosa*.[14] Furthermore, ascorbic acid was reported as efflux pump inhibitor in hemolytic *E. coli*. As a result, it can enhance the activity of different classes of antimicrobials against *E. coli*.[15]

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Table 1. MIC and MBC in mg/ml of NAC and ascorbic acid against *P. aeruginsa*.

Isolates	NAC		Ascorbic acid		Ambroxol	
	MIC	MBC	MIC	MBC	MIC	MBC
P1	2	8	4	4	3.75	7.5
P2	2	4	4	4	3.75	3.75
P3	2	4	4	4	3.75	3.75
P4	2	2	4	4	3.75	7.5
P5	2	4	4	4	3.75	7.5

Ambroxol is an expectorant useful in the treatment of bronchial asthma and chronic bronchitis.[16] Moreover, it exhibits antioxidant and anti-inflammatory properties.[17]

This study was performed to investigate the antimicrobial action of NAC, ascorbic acid and ambroxol against *P. aeruginosa* and their augmenting effects when combined with antibiotics.

MATERIALS AND METHODS:

Bacterial strains

Five clinical isolates of *Pseudomonas aeruginosa* obtained from intensive care unit patients with lower respiratory tract infections in Zagazig University Hospitals by endotracheal aspiration were used in this study.

Determination of MIC and MBC

The minimum inhibitory concentrations (MICs) of the tested antibiotics; Levofloxacin, gentamicin (EIPICO, 10th of Ramadan City, Egypt), meropenem (Astra Zeneca UK Limited, Cheshire, United Kingdom), cefoperazone (Pfizer, Egypt), ceftazidime (Smith Kline Beecham, Egypt), cefepime (Bristol-Myers Squibb, Egypt), tetracycline and chloramphenicol (CID, Egypt) in addition to NAC, ambroxol and ascorbic acid (Sigma, St. Louis, USA) were determined by the broth microdilution method according to CLSI.[18] Bacterial inocula were prepared and standardized to match a 0.5 McFarland standard. The bacterial suspensions were then diluted with sterile saline to have an approximate cell density of 10^6 CFU/ml. Fifty µl aliquots of the bacterial suspension in Mueller-Hinton broth (Oxoid, Hampshire, England) were added to the wells of a microtiter plate containing 50 µl of twice the two fold serially diluted concentrations of antimicrobial agents. Microtiter plates were sealed in a plastic bag and incubated at 37 ºC for 20 hours. The MIC was the lowest concentration of antimicrobial agent or drug that can completely inhibit visible growth in the wells and the data were interpreted according to CLSI guidelines.[19] For determination of the minimum bactericidal concentration (MBC), 10µl of broth showing no growth was transferred from the microtiter plate wells to plates of Mueller Hinton agar (Oxoid, Hampshire, England). Plates were incubated for 24h at 37°C and MBC was calculated as the lowest concentration that could cause 99.99% reduction in growth as determined by the absence of growth or appearance of less than five colonies.

Synergy testing[20]

To determine the effect of combining NAC, ambroxol and ascorbic acid with antimicrobial agents, the MICs of these antimicrobial agents were determined in the presence of 1/4 MIC of the three drugs. The wells of microtiter plates with 50 µl of four fold the final concentration of each of NAC, ambroxol or ascorbic acid and antibiotics were inoculated with standardized bacterial suspensions to have a final inoculum of $5x10^5$ CFU/ml. The microtiter plates were sealed and incubated at 37 ºC for 20 hours.

The MIC was calculated as the lowest concentration of antimicrobial agent that can completely inhibit visible growth in the wells. Fractional inhibitory concentration (FIC) of antibiotic was determined according to Mackay et al. FIC of drug A= MIC drug A in combination/MIC drug A alone. The result of the combination may be synergistic (FIC $\leq$ 0.5), indifferent (FIC > 0.5 to 4), or antagonistic (FIC > 4).

Table 2. Modification of susceptibility of *P. aeruginosa* to antibiotics by NAC.

Isolates	Cefoperazone		Ceftazidime		Cefepime		Meropenem	
	MIC (µg/ml)	MIC_{NAC} (µg/ml)	MIC (µg/ml)	MIC_{NAC} (µg/ml)	MIC (µg/ml)	MIC_{NAC} (µg/ml)	MIC (µg/ml)	MIC_{NAC} (µg/ml)
P1	512	8	2048	8	128	0.25	4	1
P2	512	4	128	0.5	256	0.25	256	4
P3	1024	16	256	1	512	2	256	4
P4	256	2	2048	8	128	0.25	16	8
P5	256	4	2048	8	128	0.125	4	2

MIC_{NAC}, Minimum inhibitory concentration of antibiotics in the presence of sub-MIC of NAC.

Table 2. Continued

Isolates	Tetracycline		Chloramphenicol		Gentamicin		Levofloxacin	
	MIC (µg/ml)	MIC_{NAC} (µg/ml)	MIC (µg/ml)	MIC_{NAC} (µg/ml)	MIC (µg/ml)	MIC_{NAC} (µg/ml)	MIC (µg/ml)	MIC_{NAC} (µg/ml)
P1	8	4	8	8	1024	4096	128	128
P2	512	64	256	128	2048	128	256	256
P3	256	32	256	128	64	4	256	256
P4	64	8	512	256	2048	1024	128	128
P5	64	4	512	256	1024	8192	128	128

MIC_{NAC}, Minimum inhibitory concentration of antibiotics in the presence of sub-MIC of NAC.

Table 3. Modification of susceptibility of *P. aeruginosa* to antibiotics by ascorbic acid.

Isolates	Cefoperazone		Ceftazidime		Cefepime		Meropenem	
	MIC (µg/ml)	MIC_{ASC} (µg/ml)	MIC (µg/ml)	MIC_{ASC} (µg/ml)	MIC (µg/ml)	MIC_{ASC} (µg/ml)	MIC (µg/ml)	MIC_{ASC} (µg/ml)
P1	512	512	2048	2048	128	128	4	4
P2	512	512	128	128	256	256	256	128
P3	1024	1024	256	256	512	512	256	512
P4	256	128	2048	2048	128	32	16	16
P5	256	256	2048	2048	128	128	4	8

MIC_{ASC}, Minimum inhibitory concentration of antibiotics in the presence of sub-MIC of ascorbic acid.

Table 3. Continued

Isolates	Tetracycline		Chloramphenicol		Gentamicin		Levofloxacin	
	MIC (µg/ml)	MIC_{ASC} (µg/ml)	MIC (µg/ml)	MIC_{ASC} (µg/ml)	MIC_{ASC} (µg/ml)	MIC_{ASC} (µg/ml)	MIC (µg/ml)	MIC_{ASC} (µg/ml)
P1	8	16	8	8	1024	2048	128	128
P2	512	1024	256	256	2048	2048	256	1024
P3	256	256	256	64	64	64	256	512
P4	64	128	512	128	2048	8192	128	256
P5	64	64	512	32	1024	1024	128	256

MIC_{ASC}, Minimum inhibitory concentration of antibiotics in the presence of sub-MIC of ascorbic acid.

Table 4. Modification of susceptibility of *P. aeruginosa* to antibiotics by ambroxol.

Isolates	Cefoperazone		Ceftazidime		Cefepime		Meropenem	
	MIC (µg/ml)	MIC_{Amb} (µg/ml)	MIC (µg/ml)	MIC_{Amb} (µg/ml)	MIC (µg/ml)	MIC_{Amb} (µg/ml)	MIC (µg/ml)	MIC_{Amb} (µg/ml)
P1	512	256	2048	2048	128	64	4	8
P2	512	512	128	32	256	128	256	32
P3	1024	512	256	64	512	512	256	32
P4	256	256	2048	2048	128	64	16	32
P5	256	256	2048	512	128	64	4	4

MIC_{Amb}, Minimum inhibitory concentration of antibiotics in the presence of sub-MIC of ambroxol.

Table 4. Continued

Isolates	Tetracycline		Chloramphenicol		Gentamicin		Levofloxacin	
	MIC (µg/ml)	MIC_{Amb} (µg/ml)	MIC (µg/ml)	MIC_{Amb} (µg/ml)	MIC (µg/ml)	MIC_{Amb} (µg/ml)	MIC (µg/ml)	MIC_{Amb} (µg/ml)
P1	8	4	8	4	1024	1024	128	128
P2	512	256	256	32	2048	2048	256	64
P3	256	128	256	16	64	64	256	64
P4	64	32	512	256	2048	4096	128	128
P5	64	32	512	1024	1024	1024	128	128

MIC_{Amb}, Minimum inhibitory concentration of antibiotics in the presence of sub-MIC of ambroxol.

RESULTS:

N-acetylcysteine, ambroxol and ascorbic acid showed antibacterial activity against *P. aeruginosa* (Table 1). The bactericidal activity of NAC was achieved at values of MIC to 4 MIC. Ascorbic acid showed similar MIC and MBC values, while ambroxol was bactericidal at concentrations of MIC to 2 MIC.

The synergistic effect of sub-MIC of NAC (0.5 mg/ml) with the tested antibiotics against the five clinical *P. aeruginosa* isolates was shown in table 2. Synergy was found with all combinations except with levofloxacin. NAC and levofloxacin combination was indifferent in all isolates. The synergy rate was 100% with β-lactam antibiotics, tetracycline, 80% with chloramphenicol and 60% with gentamicin. Antagonism was observed with gentamicin in 20% of isolates.

Sub-MIC of ambroxol (0.9 mg/ml) showed synergy with tetracycline in all isolates, with chloramphenicol and cefepime in 80% of isolates, with ceftazidime in 60% of isolates and with meropenem, levofloxacin and cefoperazone in 40% of isolates (Table 3). Interaction of ambroxol, on the other hand, was indifferent with gentamicin in all isolates, with levofloxacin, meropenem and cefoperazone in 60% of isolates, with ceftazidime in 40% of isolates and with cefepime and chloramphenicol in 20% of isolates.

Ascorbic acid (1mg/ml) showed weak synergy with antibiotics (Table 4). Synergy was found with chloramphenicol in 60% of isolates, with meropenem, cefepime and cefoperazone in 20% of isolates. Indifference was the result of combining ascorbic acid with ceftazidime, levofloxacin, gentamicin and tetracycline in all isolates, with cefoperazone, cefepime and meropenem in 80% of isolates and with chloramphenicol in 40% of isolates.

DISCUSSION:

Dietary supplements such as ascorbic acid may be prescribed with antibiotics when treating infectious diseases. Furthermore, N-acetylcysteine (NAC) and

ambroxol are used as mucolytics or expectorants in combination with antibiotics for treatment of lower respiratory tract infection [21,16], therefore, it is important to study their antimicrobial activity and their effect on bacterial antibiotic susceptibility.

In this study, direct antimicrobial activities were found for each of NAC, ambroxol and ascorbic acid. NAC could inhibit the growth of *P. aeruginosa* at 2 mg/ml and exerted bactericidal activity at 2-8 mg/ml. These results were lower than that reported by Zhao and Liu[6] who found that the minimum inhibitory concentrations of NAC for 18 out of 20 *P. aeruginosa* isolates studied were 10 to 40 mg/ml and Roberts and Cole[8] who reported that concentrations of 20-50 mg/ml of NAC were bactericidal against *P. aeruginosa*. Whereas, lower results were found with Parry and Neu[7] who found that MICs of NAC against *P. aeruginosa* were 2-20 µg/ml.

The use of NAC, ambroxol and ascorbic acid in combination with antibiotics resulted in synergy in 50% of isolates. Stronger synergy was achieved by NAC. It showed synergy rate of 80%, whereas ambroxol and ascorbic acid augmented the antipseudomonal activity in 55% and 15% of the isolates, respectively. Combinations of NAC with each of cefepime, ceftazidime, cefoperazone and meropenem in addition to those of tetracycline with each of NAC, ambroxol and ascorbic acid showed the highest synergy.

Only levofloxacin interaction with NAC did not show synergism or antagonism. On the contrary, synergy was found with the tested β-lactam antibiotics; cefepime, ceftazidime, cefoperazone and meropenem in addition to tetracycline and chloramphenicol (Figure 1). The combination of NAC with Gentamicin was either synergistic or antagonistic. Combining NAC was more effective with β-lactams and tetracycline. Synergy was stronger with β-lactams than with tetracycline. The susceptibility increased by 256-1024 folds with cefepime, by 256 folds with ceftazidime, by 64-128 folds with cefoperazone and by 2-64 folds with meropenem. Lower synergy was obtained with tetracycline (2-16 folds).

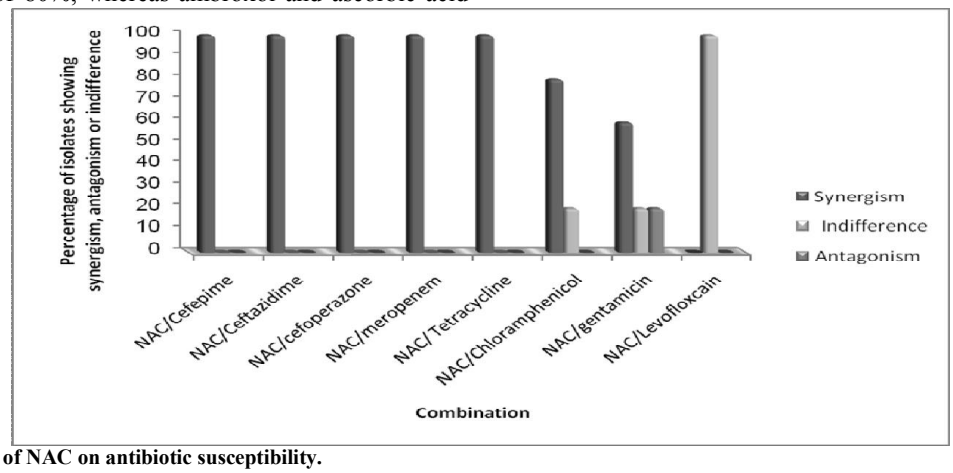

Figure 1. Effect of NAC on antibiotic susceptibility.

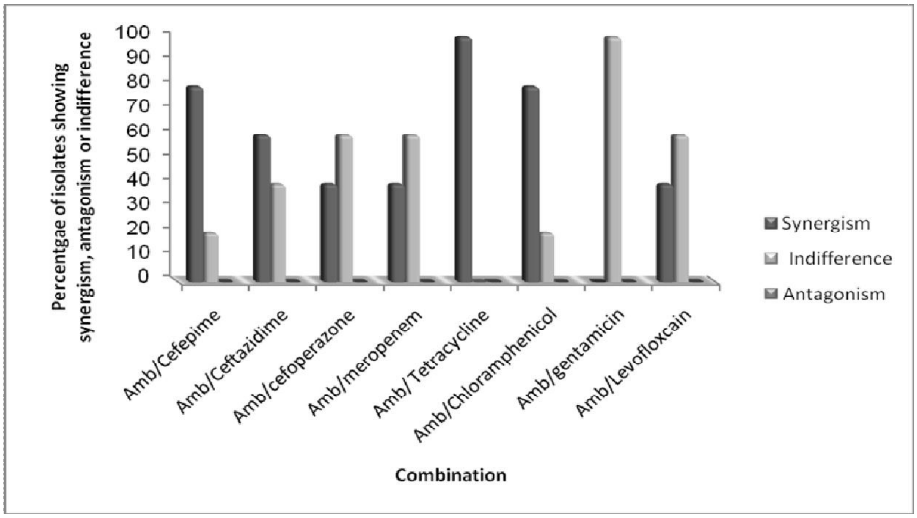

Figure 2. Effect of ambroxol on antibiotic susceptibility.

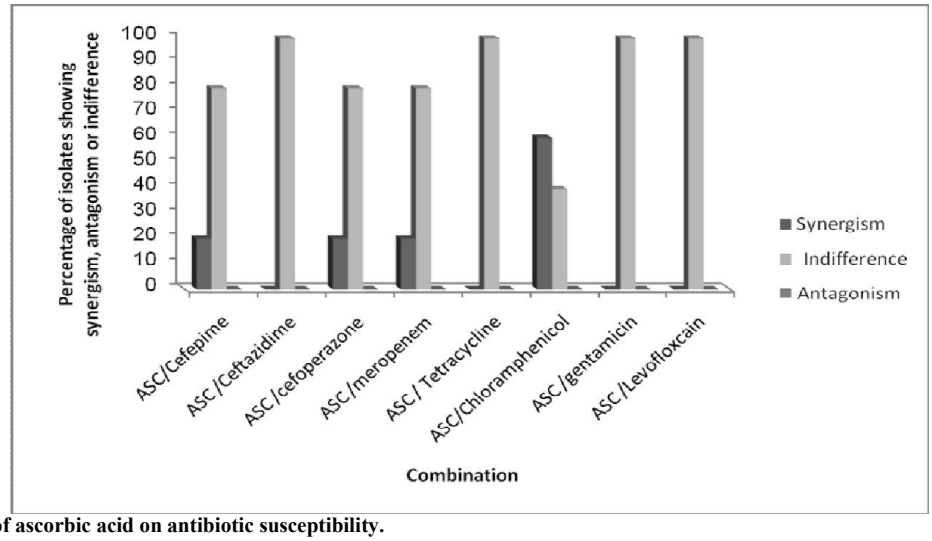

Figure 3. Effect of ascorbic acid on antibiotic susceptibility.

Parry and Neu [7] reported the potentiating effect of NAC on the antipseudomonal activity of β-lactams carbenicillin and ticarcillin and the antagonistic effect with the aminoglycosides gentamicin and tobramycin. Zhao and Liu[6] found that interaction of NAC with ciprofloxacin was synergistic in 10 out of 20 *P. aeruginosa* isolates and no antagonism was observed. Moreover, the augmenting effect of 10 mg/ml NAC on the antipseudomonal activity of carbenicillin was demonstrated by Roberts and Cole[8].
Goswami and Jawali[22] found that the presence of NAC (10 mM) can either reduce the antibacterial activity of aminoglycosides, fluoroquinolones, or enhance the efficacy of ß-lactams against *P. aeruginosa.* The susceptibility decreased moderately to ciprofloxacin and markedly to the aminoglycosides streptomycin, kanamycin, and spectinomycin due to protection exerted by the thiol compound NAC against aminoglycoside and fluroquinolone antibiotics. On the other hand, synergistic effect was found with the ß-lactams penicillin and ampicillin, and no change in MICs was observed with each of chloramphenicol and tetracycline.

In this study, it is noteworthy that NAC combined with cefepime, cefoperazone and ceftazidime could reverse the resistance of the five tested isolates of *P. aeruginosa* and render them sensitive to the tested antibiotics.

Similar results were observed with meropenem; two resistant isolates were rendered susceptible while one resistant isolate showed intermediate susceptibility to meropenem when combined with NAC. Moreover, NAC was used at concentration of 0.5 mg/ml; a lower concentration than the commonly used therapeutic concentration for management of severe respiratory disorders which ranges between 4 and 10 mM per day.[23]
No synergistic or antagonistic interaction was demonstrated for ambroxol with gentamicin. (Figure 2). Synergy was found for the combinations of ambroxol with tetracycline, chloramphenicol, cefepime, ceftazidime, meropenem, levofloxacin and cefoperazone. Interaction between ambroxol and tetracycline was more effective compared to other antibiotics.

Gillissen and Nowak[24] reported that ambroxol enhances the penetration of the fluoroquinolone gatifloxacin. Wiemeyer[25] demonstrated the increase in the bronchopulmonary levels of ampicillin, amoxicillin and erythromycin by 234% for ampicillin and 27% for each of amoxicillin and erythromycin when co-administered with ambroxol.

With ascorbic acid, Synergy was found with chloramphenicol which demonstrated higher synergy than that found with meropenem, cefepime and cefoperazone (Figure 3). Ceftazidime, gentamicin, levofloxacin and tetracycline displayed no synergism or antagonism with ascorbic acid. These results were different from those reported by Crusino et al.[26] who studied the interaction of ascorbic acid (1mg/ml) with kanamycin, streptomycin, ampicillin, tobramycin, tetracycline and chloramphenicol against 12 multidrug resistant *P. aeruginosa.* No synergism or antagonism was observed with the β-lactam antibiotic ampicillin. Aminoglycosides were variably affected by ascorbic acid; tobramycin was antagonized, while kanamycin and streptomycin were potentiated. Synergy was found with tetracycline and chloramphenicol, while antagonism was achieved with chloramphenicol. Tetracycline was more augmented than other antibiotics. On the other hand, Shoeb et al.[27] found that ascorbic acid (10mg/ml) increased the susceptibility to ampicillin by inhibition of β-lactamase production in *P. aeruginosa.* Amabile-Cuevas et al.[28] reported that ascorbic acid could augment tetracycline against *Staphylococcus aureus*. In his studies, Goswami et al.[29,21] found that ascorbic acid could protect *E. coli* against gentamicin and ciprofloxacin by induction of a protective phenotype for the former and antioxidant mediated reactive oxygen species scavenging

for the latter. On the contrary no effect was found with penicillin, ampicillin, tetracycline or chloramphenicol.
Hancock and Wong[30] found that L-ascorbate could enhance the permeability of *P. aeruginosa* outer membrane, a property that can enhance the diffusion of antibiotics into the cells of *P. aeruginosa.* Moreover, ascorbic acid potentiated chloramphenicol, neomycin and tetracycline against *P. aeruginosa* by affecting the cell surface to enhance the permeability to antibiotics.[13] Furthermore, ascorbic acid was reported as efflux pump inhibitor in hemolytic *E. coli*. As a result, it can enhance the activity of ciprofloxacin, cefoperazone and chloramphenicol against *E. coli*, while it exerted no potentiating activity with tetracycline.[15] In adults, the recommended daily intake of ascorbic acid is 120 mg [31], while in this study, ascorbic acid was used at a much lower concentration;1 mg/ml.

Comparing antibiotics according to the rate of synergy exerted by the tested agents, it was found that chloramphenicol, tetracycline and cefepime were more augmented. Synergy was found with chloramphenicol against 73.3% of isolates and with each of cefepime and tetracycline in 66.7% of isolates. Lower synergy rates were observed with ceftazidime, cefoperazone and meropenem (53.3% of isolates each), whereas, gentamicin and levofloxacin were the least potentiated (20% and 13.3% of isolates, respectively).

In conclusion, this study suggests the use of NAC, ambroxol and ascorbic acid to augment the antibiotic activity against *P. aeruginosa.* The combinations of NAC with ß-lactams are more recommended because of higher synergy than other combinations.

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