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Enhancement of Antimicrobial Activity of Four Classes of Antibiotics Combined with Garlic

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Abstract: The increased resistance to antimicrobial agents among clinical isolates is a serious problem that dramatically raises the cost of health care worldwide. Seeking alternative approaches to enhance the susceptibility of these microorganisms to killing is a major concern to researchers. One such approach is the combination of some adjuncts with antibiotics. Garlic (*Allium sativum*) is an herbal product traditionally used for many health-related purposes, including protection against microbial infections. This work investigates the effect of combining garlic, in sub-inhibitory concentrations, with four different antibiotics against sixteen selected multidrug resistant Gram-negative clinical isolates belonging to *Pseudomonas* and *Acinetobacter* genera. A combination of 5 and 10 mg mL^{-1} of garlic (for *Acinetobacter* and *Pseudomonas*, respectively) with levofloxacin, gentamicin, azithromycin and doxycycline resulted in a decrease in the antibiotics' Minimum Inhibitory Concentrations (MICs) against the isolates in the range of 4-≥32, 4-≥2048, 2-≥2048 and 2-≥128 fold, respectively. The kinetics of killing of the garlic-gentamicin combination were subsequently followed in four Pseudomonads for 24 h and a significant effect ranging between 2 and 5 log reduction in bacterial count, compared to the control, was obtained. The results show a great potential for the use of garlic as an adjunct to antibiotics for the treatment of infections caused by resistant Gram-negative strains and warrant further investigation.

Key words: Garlic, antibiotics, adjunct, antimicrobial resistance, killing kinetics

INTRODUCTION

The extensive production and widespread use of antibiotics worldwide in clinical and veterinary medicine, agriculture, aquaculture, horticulture as well as other human activities has lead to the evolution of antibiotic resistance among human and animal pathogens (Aminov, 2009). Dissemination of antibiotic resistance results in thousands of deaths each year and imposes a considerable economic and social burden on health care systems (De Kraker *et al*., 2011).

Pseudomonas aeruginosa is a Gram-negative bacterium omnipresent in the environment, in addition to being an important human pathogen. It is a major causative agent of infections in immunocompromised patients, such as those suffering from burn wounds or receiving cancer chemotherapy. *P. aeruginosa* also infects the lungs of cystic fibrosis patients leading to high mortality and morbidity (Rao *et al*., 2011). The reason for such high cost of *Pseudomonas* infections lies in its intrinsic resistance to a broad spectrum of antibiotics and its arsenal of virulence factors (Hancock and Speert, 2000). *Acinetobacter* is yet another Gram-negative bacterium found in soil and fresh water, besides, it has become a serious culprit in causing an array of nosocomial infections (Bergogne-Berezin and Towner, 1996). Such infections are especially challenging because of the organism's high potential to rapidly develop antibiotic resistance, with the looming possibility of multidrug resistance within a few decades (Perez *et al*., 2007).

Garlic (*Allium sativum*) is an herbal product that has been known since ancient times and is used in the folklore medicine to guard against a number of infections because of its antibacterial, antifungal and antiviral properties (Block, 1985; Weber *et al*., 1992). The active ingredient (allicin or diallyl thiosulphinate, Fig. 1a) is produced when a garlic clove is crushed or damaged and alliin (Fig. 1b), (a precursor compound representing about 0.24% (w/w) of each garlic clove, comes in contact with the enzyme alliinase (originally present in a separate vesicle) (Block *et al*., 1984).

In this study we hypothesize that the use of garlic as an adjunct in antibiotic regimens will improve the

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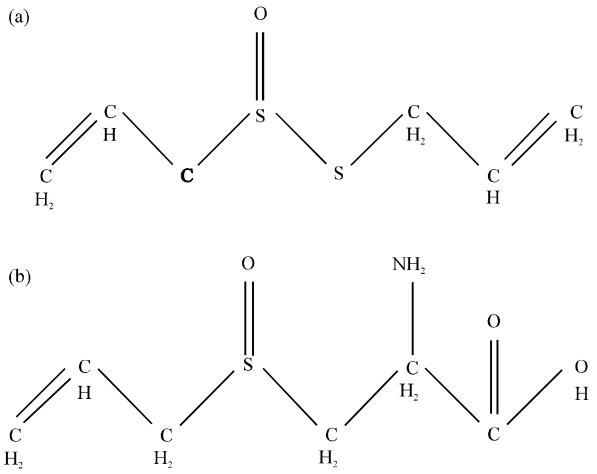

Fig. 1(a-b): The chemical structure of (a) allicin (b) alliin

antimicrobial performance to overcome the problem of increasing antibiotic resistance among clinical isolates. Another objective of the current work is to follow the killing kinetics of the garlic-antibiotic combination, relative to either component alone.

MATERIALS AND METHODS

Test organisms: Sixteen Gram-negative microorganisms were used in this study. They were collected in a clinical microbiology laboratory affiliated with El-Meery tertiary teaching hospital in Alexandria, Egypt. The microorganisms were eight *Acinetobacter baumannii* (designated "A" isolates) and eight *P. aeruginosa* (designated "P" isolates) obtained from various clinical specimens.

Antibiotic powders: Levofloxacin (Amoun Pharmaceutical Company, Egypt), Doxycycline (The Nile Co. for Pharmaceuticals and Chemical Industries, Egypt) and Gentamicin (Schering Plough Corporation, Egypt) were purchased from the corresponding pharmaceutical companies. Azithromycin was obtained as a gift from Amriya Pharmaceutical Co. (Egypt).

Bacterial identification: The microorganisms were identified by means of conventional methods and included morphological, cultural properties and biochemical characteristics that were estimated by API system (BioMérieux, France). For *A. baumannii* isolates, growth at 44°C was used to confirm identity. The identified strains were stored at –70°C in nutrient broth (Oxoid, England) containing 20% glycerol until needed for further tests.

Antimicrobial susceptibility testing: The *in vitro* antimicrobial activity of the four antibiotics, alone and combined with garlic, against the tested isolates was determined by the broth microdilution method in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2006). Microtiter plates, containing two-fold serial dilutions of each antibiotic in Luria-Bertani (LB) medium (Bioshop, Canada) in addition to 5 or 10 mg mL^{-1} of garlic (for *Acinetobacter* and *Pseudomonas*, respectively), were inoculated with each organism to yield the appropriate density (5×10^5 CFU mL^{-1}) in a final volume of 180 μL per well. The plates were then incubated for 24 h at 37°C. To determine the MIC of the antibiotics alone, the same procedure was used except that garlic was replaced with distilled water. The MIC was defined as the lowest concentration of the antibiotic completely inhibiting the growth of the organism as detected by the unaided eye. Susceptibility rates were determined following CLSI breakpoints.

Biocidal activity of garlic-gentamicin combination against selected strains: *In vitro* bactericidal activity of garlic-gentamicin combination against three *Acinetobacter* and four *Pseudomonas* isolates was evaluated using time-kill assays according to CLSI M26-A protocol with some modifications to suit the test conditions. Probe tubes contained garlic (5 or 10 mg mL^{-1} for *Acinetobacter* and *Pseudomonas*, respectively), gentamicin (4 μg mL^{-1} for all isolates, except for A_{13}, A_{16} and P_{25} 2 μg mL^{-1}, corresponding to the next to last inhibitory concentration as determined from antimicrobial susceptibility testing) or garlic-gentamicin combination (at the previous concentrations). Tubes were inoculated with the test microorganisms (10^6 CFU mL^{-1}) and incubated aerobically in a shaking water bath at 37°C for 24 h. Aliquots were removed from each tube and serial dilutions were plated in duplicates onto LB plates after 0, 2.5, 5 and 24 h of incubation. Colony counts were determined after 24 h of incubation at 37°C. Bactericidal activity was defined as a 3 $\log_{10}$ reduction in the bacterial count compared with the initial inoculum at zero time. In each case, a control lacking gentamicin and garlic was included in the procedure.

RESULTS

Susceptibility testing: *In vitro* antimicrobial activity of levofloxacin, gentamicin, azithromycin and doxycycline, alone and in combination with a sub-inhibitory concentration of garlic, was determined by broth microdilution technique against sixteen clinical isolates belonging to the *Pseudomonads* and *Acinetobacter*

Table 1: Distribution of fold decrease in the Minimum Inhibitory Concentration (MIC) of levofloxacin, gentamicin, azithromycin and doxycycline, against the sixteen tested isolates, as a result of combining with garlic

Organism (No. of isolates tested)	Antimicrobial agent	Fold decrease in MIC of antibiotics after combining with garlic (≥)											
		No effect	2	4	8	16	32	64	128	256	512	1024	2048
Acinetobacter baumannii (8)	Levofloxacin	1	0	3	1	0	3	0	0	0	0	0	0
	Gentamicin	2	0	0	0	0	0	0	1	2	0	0	3
	Azithromycin	0	0	3	1	1	1	0	0	0	0	0	2
	Doxycycline	0	1	2	2	1	0	1	1	0	0	0	0
Pseudomonas aeruginosa (8)	Levofloxacin	4	0	0	1	0	1	2	0	0	0	0	0
	Gentamicin	3	0	1	0	0	0	0	3	0	1	0	0
	Azithromycin	3	1	1	0	3	0	0	0	0	0	0	0
	Doxycycline	1	1	5	1	0	0	0	0	0	0	0	0

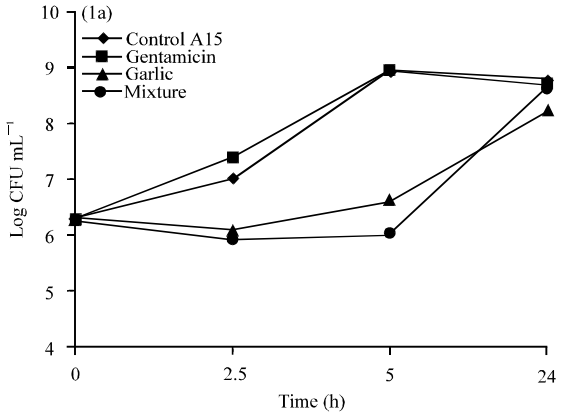

Fig. 2a: Time-kill curve showing the bactericidal activity of garlic-gentamicin combination against A_{15}

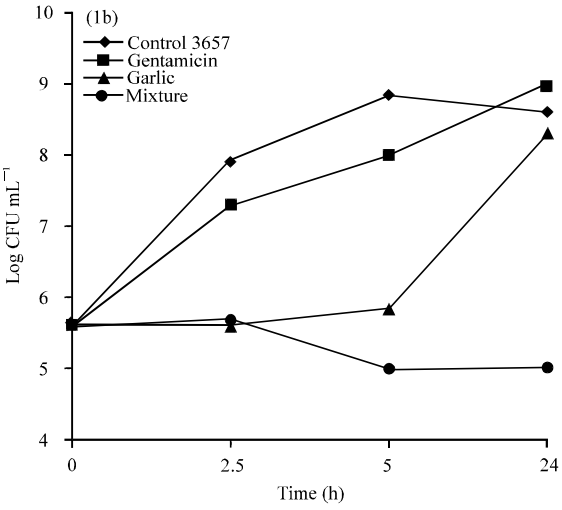

Fig. 2b: Time-kill curve showing the bactericidal activity of garlic-gentamicin combination against P_{3657}

species and the findings are summarized in Table 1. Garlic was initially tested alone for its antimicrobial activity against a large number of clinical *Pseudomonas* and *Acinetobacter* isolates. As expected, garlic demonstrated great potency against the majority of the tested isolates and its inhibitory concentration for each isolate was recorded (data not shown). As for the combination, generally, more promising results were obtained with *Acinetobacter* than with *Pseudomonas* isolates. 65.7% of the combinations tested against *Pseudomonas* species demonstrated variable enhancement in the activity of the antibiotics with the isolates P_{25} and P_{3657} being the most inhibited in response to gentamicin-garlic, followed by levofloxacin-garlic combination. On the other hand, a higher percentage, amounting to 90.6%, of garlic-antibiotic combinations showed even greater inhibitory effect on *Acinetobacter* species, compared to the effect of antibiotics alone. Two isolates, A_{17} and A_{18}, were particularly highly affected by all four antibiotic-garlic combinations.

The two antibiotics whose antimicrobial activities were most highly influenced by the combination with garlic were azithromycin and gentamicin, showing shifts in MIC values ranging between≥2 and≥2048 fold against the tested clinical isolates. This was best evidenced in the finding that 37.5 and 25% of the *Acinetobacter* isolates (A_{17}, A_{18}, A_{22} and A_{17}, A_{18}) displayed≥2048 fold increase in their susceptibility to gentamicin and azithromycin, respectively, after combining with garlic. Doxycycline and levofloxacin came next with MIC shifts ranging between≥2 and≥128 fold, with the highest shifts observed against A_{17} in case of doxycycline and P_{414} and P_{3657} in case of levofloxacin.

Time-kill studies: The *in vitro* bactericidal activity of the garlic combination with gentamicin was then tested using the time-kill assay against seven clinical isolates displaying MIC decrease due to the combination, the effect can be seen in the representative examples shown in Fig. 2a and b. The effect was bacteriostatic at the best with the *Acinetobacter* showing regrowth after 24 h to counteract the inhibitory effect seen at shorter times of exposure to garlic or the combination. Garlic enhanced the antibacterial activity of gentamicin against *Pseudomonas*, an effect that was more pronounced at 24 h with 3.6, 4.2, 4.77 and 2.1 log reduction in viable count of P_{3657}, P_{414}, P_{410}

and P_{25} isolates, respectively, compared to the control at the same time interval (data not shown). At shorter exposure times (2.5 h) and to a lesser extent (5 h), the effect of garlic on the activity of gentamicin was generally less pronounced.

DISCUSSION

The development of bacterial resistance to antibiotics is a severe problem that highlights the importance of developing new strategies to limit the different mechanisms of resistance (Ciofu *et al*., 1994). Garlic (*Allium sativum*) an essential food ingredient, has established strong antibacterial, antifungal and antiviral actions. Allicin (diallyl thiosulphinate) is the main constituent of garlic showing antimicrobial activity and it is generated by the enzyme alliinase when garlic is crushed (Weber *et al*., 1992; Ankri and Mirelman, 1999). Garlic extract has demonstrated a great antibacterial activity for controlling methicillin-resistant *Staphylococcus aureus* and other pathogens (Cutler and Wilson, 2004). Garlic extract also affected *Escherichia*, *Salmonella*, *Staphylococcus*, *Streptococcus*, *Klebsiella*, *Proteus*, *Clostridium*, *Mycobacterium* and *Helicobacter* species as previously demonstrated (Cellini *et al*., 1996).

The present study investigates the potential of using garlic as an adjunct to antibiotic therapy, in an attempt to overcome the increased tolerance among infectious microorganisms towards these antibiotics. The tested antibiotics belong to four different classes with mechanisms of action inhibiting protein synthesis or affecting nucleic acids.

The antimicrobial activity, in terms of MIC, of the four studied antibiotics was tested alone and in the presence of sub-inhibitory concentration of garlic, using broth microdilution method as it was more convenient than the agar diffusion technique in this particular situation. When combined with antibiotics, garlic was found to enhance the antibacterial activity of the tested antibiotics to variable degrees against *Acinetobacter* and *Pseudomonas* species. A previous report was in accordance showing a synergistic antibacterial effect when garlic extract and tobramycin were combined (Shuford *et al*., 2005). In another study, allicin was found to enhance the antibacterial activity of cefazolin, oxicillin and cefoperazone at sub-inhibitory concentrations (Cai *et al*., 2007).

A possible explanation could be blocking of quorum sensing and communication systems of the microorganism as a result of garlic treatment rendering *P. aeruginosa* sensitive to the action of antibiotics and suggesting a means to reduce the virulence and control *P. aeruginosa* infections (Bjarnsholt *et al*., 2005). Moreover, garlic appears to alter the structure and integrity of the outer surface of microbial cells as well as decrease their total lipid content (Iwalokun *et al*., 2004) which allows better access and subsequent inhibition of the tested antibiotics to their respective targets either protein or nucleic acid. Another probable mechanism for the enhancement of antimicrobial activity of antibiotics when combined with garlic could be the well-established garlic's antibiotic function, which is likely to inhibit bacterial attachment. Evidence for this last mechanism was provided by a previous study that showed that sub-MICs of allicin might play a role in the prevention of adherence of *Staphylococcus epidermidis* to microtiter plates (Perez-Giraldo *et al*., 2003). Similarly, Shuford *et al*. (2005) demonstrated that fresh garlic extract inhibited growth of *Candida albicans* in its planktonic, adherent and sessile phases. In addition, the administration of garlic is expected not only to reduce the persistence of biofilms, a structure that plays a major role in increasing antimicrobial resistance, but also to inhibit the expression of bacterial virulence determinants that actively degrade components of the defense system (Kharazmi *et al*., 1986). Nevertheless, in the current work, some tested combinations were ineffective. This agrees with the findings of Jonkers *et al*. (1999) who found no effect of garlic on amoxicillin or clarithromycin against *Helicobacter pylori* suggesting the changeable effect of garlic on the antibacterial activity of antibiotics depending on the strain tested.

In time-kill assays conducted in the current study, the potential bactericidal effect of garlic-gentamicin combination was followed against selected clinical isolates for 24 h. Garlic was found to enhance the activity of gentamicin against *P. aeruginosa* particularly at 24 h, yet the effect was not bactericidal as the combination usually showed a count reduction of less than 3 logs, compared to the count at zero time. This comes in contrast with the findings of Shuford *et al*. (2005) who found that the superior activity of garlic occurred at 1 versus 48 h of treatment and this probably relates to the half-life of fresh garlic extract at 37°C and would be an important consideration in the development of in vivo uses. However, data obtained by Shuford and colleagues (Shuford *et al*., 2005) somewhat agree with our results for the *Acinetobacter* and may imply that a genus related factor might be involved. Another possible explanation for the little or no effect observed with the *Acinetobacter* could be the development of biofilm. These findings are consistent with other works that found that the *in vitro* activity decreases as the biofilm phenotype develops (Bjarnsholt *et al*., 2005).

The results at hand show great promise and merit thorough investigation to further document and determine the exact mechanism of action of the different components

of garlic extract in a synergistic combination with antibiotics. This is the aim of our next research project since the current work is one in a series conducted to elucidate the combined effect of garlic and antibiotics in overcoming antibiotic resistance among bacterial cells.

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