Effect of *Allium sativum* Extract on Erythromycin and Methicillin Resistant Bacteria Isolated from Hospital Operating Room

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ABSTRACT

***Background*:** This study aimed to evaluate antibacterial effect of *Allium sativum*, garlic extract on erythromycin and methicillin resistant bacteria isolated from an operating room in a teaching hospital in Tehran, I.R. Iran.

***Methods*:** The antibacterial effect of garlic extract was investigated on 70 bacterial strains. The selected isolates were resistant to erythromycin and or methicillin, which were isolated from an operating room. Antibiotic sensitivity was done using an agar well diffusion procedure and either a micro dilution method. Each of the bacterial strains were exposed to concentrations of 4, 8, 12, 16, 20 and 24 µg/ml of garlic extract, separately. The growth rate of the strains was determined by measurement of the inhibition zone diameter, colony count and either by measurement of the optical density.

***Results*:** The results showed that 70 (100%) of the strains in agar well diffusion method were sensitive to 4- 12 µg/ml of garlic extract with MIC 8 µg/ml. While, the results of micro dilution method showed that 40 out of 70 strains were MIC ≥ 12 µg/ml for GE.

***Conclusion*:** The results of this study indicated that the MIC and minimum bactericidal concentration of garlic extract were 8 µg/ml and 16 µg/ml, respectively. These finding indicated that garlic extract inhibit the growth of erythromycin and methicillin resistant bacterial strains.

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Introduction

Emerging of bacterial resistance is a major global problem for health authorities. Thus, finding new antibacterial agents is of a high significance (1, 2). *Allium sativum* (Garlic), as an antibacterial agent against "superbug" bacteria such as vancomycin-resistant *enterococci* (VRE) has been extensively studied (3). The antibacterial effect of crude and aqueous garlic extract (AGE) against *Pseudomonas aeruginosa* has been investigate and the results revealed that the MIC of imipenem was far less in comparison with the MIC of AGE (4). Also, an investigation has shown that the AGE has antibacterial effects against *Staphylococcus aureus* in hamburgers (5). An *in vitro* study of anti-mycobacterial as well as anti-bacterial activity of garlic extract has been assessed using microtiter plate method which showed anti- mycobacterial activity as compared to standard drugs. In addition, garlic oil has demonstrated significant antibacterial activity, particularly against methicillin resistant *S. aureus* isolates (6). *In vitro* antibacterial assay of essential oils (EOs) obtained from fresh bulbs of garlic have shown a considerable antimicrobial activity against *S. aureus*, *P. aeruginosa* and *E. coli* with inhibition zones of 14.8, 21.1, and 11.0 mm, respectively (7).

An investigator assessed the antibacterial effects of different concentrations of garlic extract against human dental plaque microbiota. In this study, four different concentrations of garlic extract, 5%, 10%, 20%, and 100%, were used against *S. mutans*, *S. sanguis*, *S. salivarius*, *P. aeruginosa* and *Lactobacillus* sp. And it was shown that the inhibition zones of different concentrations were not significantly different from one another and concluded the usefulness of using 5% garlic extract (8). Another study has assessed the inhibitory effect of garlic on *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* and the time-killed curve of these bacteria were determined and showed that the garlic extract may have therapeutic effects on periodontitis (9).

Although, many studies show that the isolated bacteria from the operating rooms are resistant to different chemicals disinfectants, no reports have been published about the use of AGE on such resistant bacteria. Also, a proper control of the antibiotic resistant bacteria is highly in demand.

Previously, we showed that more than 50 different erythromycin and methicillin resistant bacterial species were isolated and characterized from the operating room of a teaching hospital in Tehran, I.R. Iran (10). The aim of this study was to assay the antibacterial effects of garlic aqueous extract on resistant bacteria isolated from the operating room.

Material and method

Seven months after harvested Garlic bulbs from region of Ramhormoz Ahvaz, IR Iran (Southern of Iran) were used. The 70 bacterial isolates were obtained from the air in an operating room in a hospital in Tehran and stored at -20 °C. Cloxacillin (500 mg) (Pharmacy Co., Tabriz, Iran) and antibiotic disks (Patan Teb Co, Iran) were used.

Bacterial culture

Bacterial suspensions were prepared (0.5 McFarland) and 100 µl of each bacterial vial was added to 5 ml of sterilized Brain Heart Infusion broth and incubated at 37 °C for 18 hours. After adjusting the cell concentration to OD=7 (1.5×10^8 CFU/ml), a 50 µl of this suspension was added to each well (11).

Garlic extract preparation

Garlic bulbs were extracted according to the Bakri and Douglas method with minor modifications (12). Garlic bulbs (100 grams) were peeled, cleaned, washed and dried. Then they were blended and after which 200 ml of sterile water was added. Then, for 30 minutes at a frequency of 1-minute rotation and 2-minute break was applied until a dough-like mixture obtained. Then, the

dough was placed on a 15 cm Whatman filter paper to be filtered. Finally, 110 ml of garlic extract was obtained which was sterilized via a bacteriological Millipore filter 0.45 micrometers and kept in refrigerator until use.

Determination of the Garlic extract concentration

The concentration of active ingredients in garlic aqueous solution was ascertained. A 1000 µl of distilled water was weighted and the average of the three measurements was calculated in triplicate. The average was around 1.33 g/ml. The weight difference of distilled water with a weight of garlic extract represents the weight of active ingredient in the garlic extract. It was determined that one ml of garlic extract contained 0.28 grams or 280 micrograms of active ingredients as allicin (280µg/ml).

Antibacterial assessment of garlic extract

Agar well diffusion method

In order to evaluate the effects of garlic extract on the isolated bacteria, the agar well diffusion method was used (13). A 100 µl of the cell suspension of each bacterial strains was spread on BHI agar. Then, five wells (5 mm) were punched in a line in the inoculated BHI agar plates and marked. Each well was then filled with 50 µl of the defined dilutions of the garlic extract containing 4, 8, 12, 16, and 20 µg active gradients, respectively. The plates incubated for 24 hours at 37 °C. The diameter of inhibition zone was then measured and recorded.

Broth Micro Dilution Method

Assessment of antibacterial effects of garlic extract was carried out as follows; A 24 wells micro plate was used for the analysis of four strains of bacteria. Six different concentrations of garlic extract were prepared (4, 8, 12, 16, 20, and 24 µg/well). Another parallel plate was used as positive and negative controls (11, 14). The garlic extract solution containing 50-100 µl was added to each row wells of the plate (form 1 to 6 wells). Then, in each of the wells 800 µl of sterile Mueller Hinton broth was added. In addition, to each wells a 100 µl of each bacterial suspension was added. The plates were then incubated for 24 hours at 37 °C. After that, the plate's turbidity was measured by ELISA reader (Biotek Co.) at the wavelength 450 nm. In order to determine the bactericidal or bacteriostatic effect of different concentrations of each garlic extract, a swab was taken and transferred radially in the Mueller-Hinton agar surface and were incubated for 24 hours at 37 °C. After incubation period, the bacterial growth of each well was determined. The results were analyzed statistically, using SPSS software version 19.

Result

Measurement of the active ingredients of the garlic extract concentration revealed that from a mixture of 100 grams of garlic bulbs with 200 ml distilled water has yielded 110 ml of a liquid with 0.28 grams per ml of active ingredients.

Antibiotic susceptibility testing results by disk diffusion methods indicated that 26 (37.14%) out of 70 (100%) strains of *S. aureus* were resistant to 5 µg methicillin disk (figure 1). In addition, the results of cloxacillin agar well diffusion method showed that another 11 (15.71%) methicillin resistant strains resist against 1-5 µg of cloxacillin. This comparative method had a partial correlation with 5 µg methicillin and 1 µg cloxacillin. This finding revealed that cloxacillin might be better than methicillin for detecting resistant strains of *S. aureus* accurately. The MIC of cloxacillin was determined as ≥ 5µg.

The results showed that 26 (37.14%) out of 70 isolates, were resistant to methicillin (5 µg) and erythromycin (15 µg) (Figure 1). Furthermore, 50% of the strains were resistant to erythromycin and 53% to methicillin alone. Amongst the isolates, 35% of the bacterial isolates obtained from air in the operating room were resistant to penicillin (10 µg) and 11% of strains showed

tolerance and 89% of them were sensitive to vancomycin (30 µg).

The results of quantification of garlic extract indicated one ml of garlic extract contained 0.28 grams or 280 micrograms active substance ingredients, which have assumed as allicin content (280µg/ml).

Antibacterial effects of garlic extract by two methods (agar well diffusion method, Broth Micro Dilution Method) revealed that in a period of less than 6 hours all bacteria were sensitive to garlic extract. The results of time killed curves caused by different concentrations of garlic extract were performed for each of the strains, separately (Fig. 3). The MIC and MBC for methicillin-sensitive *S. aureus* (MSSA) were 4 µg/ml and 8 µg/ml, respectively (Fig. 2). The MIC and MBC for methicillin-resistant *S. aureus* (MRSA) were 12 µg/ml and 20 µg/ml, respectively (Fig. 2).

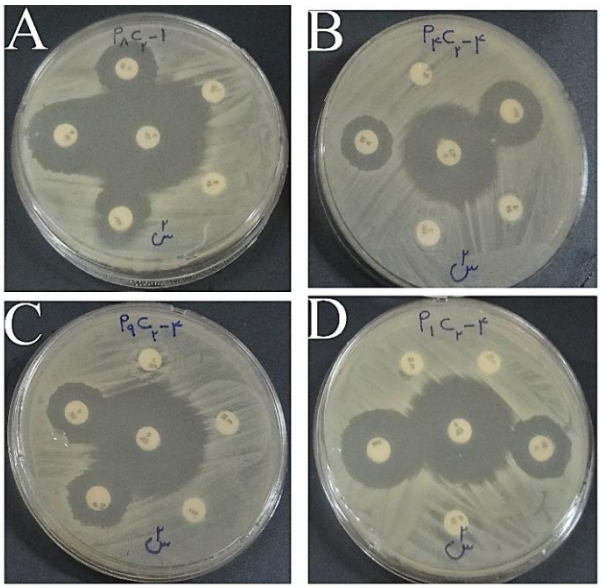

Fig 1. As a simplified, kier– Bay disk diffusion aga for four isolate have shown. All isolated bacteria wer sensitive to Ciprofloxacin, Vancomycin an trimethoprim-sulfamethoxazole (SXT); while, the were resistant to Methicillin (ME) and Erythromyci (E). It obviously revealed that, bacterial strain in plate A, B and D not only resistant to ME and E but also wer resist to penicillin (10 µg). While the bacteria in plat C was sensitive to penicillin (10 µg).

Data analysis by non-parametric Mann-Whitney test showed a significant effect of antibacterial activity of garlic extract. Comparing the pooled results of different concentrations of garlic extract on the survival of 40 species bacterial that were resistant to methicillin or erythromycin is shown in figure 3.

The results indicated that 4 µg/ml of garlic extract within 6 hours, with 95% confidence interval, eliminate 67.5% of the tested bacteria. However, 32.5% of tested bacteria were not killed when treated against 4 µg/ml. Similarly, concentration of 8 µg/ml of can eliminate 77.5% of bacteria. The major results indicated that minimum of 16 µg/ml of garlic extract garlic extract could kill 100% of the tested bacterial.

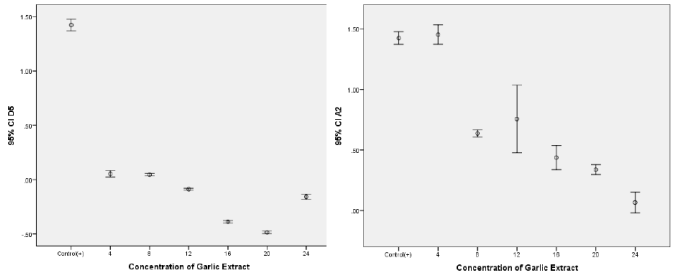

Fig 2. The six hour of time killing curves caused by different concentration of GE have shown. In part, A: the effect of different concentrations of garlic extract on *Staphylococcus aureus* methicillin sensitive and on the part B the effects of the same garlic extract concentrations on MRSA survival is shown. The 4µg/ml of allicin could inhibit the growth of bacteria in plat A, and the MIC of this organism is estimated to be ≥ 8/ml µg. while the MIC for resistant bacteria on plat B was ≥ 16 µg/ml. the calculated MBC for sensitive bacteria were ≥ 12 µg/ml and for resistant bacteria ≥ 24 µg/ml was obtained respectively.

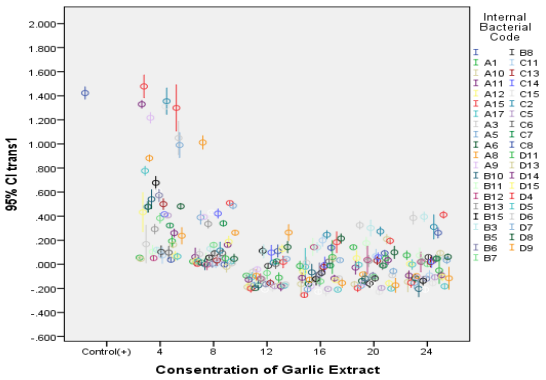

Fig 3. Comparison of the cumulative effects of different concentration of garlic extract on 40 antibiotic resistance bacteria has shown. On the right side of the figure presented the internal code of tested bacterial isolates.

Allium sativum as an edible plant with a history of human use of over 7,000 years (15) exhibits antibacterial, antifungal, antiviral, and antiprotozoal effects which therefore can act as an natural antibiotic (16, 17), However, few studies have attempted to determine the weight of garlic extract concentration. Based on American National Library of Medicine stated, from 1963 up to know more than 1600 research paper on garlic have been published ranging from clinical value of garlic in metabolic syndrome (18) to antimicrobial effects (19).

Various garlic extract preparation methods have shown to exhibit a wide spectrum of antibacterial activity against clinical isolates of Gram-negative, Gram-positive and acid-fast bacteria (20, 21).

Recently it has been shown that garlic extract could prevent the formation of *staphylococcus* enterotoxins A, B, and C1 and thermo nuclease (22). Cavallito and Bailey have demonstrated that the antibacterial action of garlic is mainly due to allicin (23). The sensitivity of various clinical bacterial isolates to pure of allicin have also been reported (24). These finding represent a broad spectrum antibacterial effect of allicin. Also, in some cases the 50% lethal dose concentration has been shown to be lower than that which is required for many new antibiotics (24, 25). Interestingly, various bacterial strains resistant to antibiotics such as MRSA as well as other multidrug-resistant enterotoxicogenic strains of *E. coli*, *enterococcus*, *S. dysenteriae*, *S. flexneni*, and *S. sonnei* found to be sensitive to allicin (26, 27).

In this study, we found that low concentrations as low as 4 to 20 µg/ml of GE display a powerful antibacterial property against multidrug resistance (MDR) bacteria such as MRSA, MRSE, *P. aeruginosa* than previously reports. Others have shown that 40% and 70% concentrations of garlic extract after 30 and 60 seconds could completely inhibit and they concluded that garlic extract might be useful as an alternative antibacterial and new treatment modality with fewer side effects (28). Another study has investigated the garlic extract antibacterial effects on MDR and non-MDR of *S. mutans* and showed that the MIC ranging from 4 to 32 µg/ml could prevent dental caries (29). The garlic extract MIC concentration was nearly similar to our study with the exception that we used MDR bacteria.

In addition, antibacterial effect of garlic extract on *S. aureus* strains in hamburger have been reported (30). Similarly, antibacterial effects of garlic extract (5%, 10%, 20%, and 100%) against *S. mutans*, *S. sanguis*, *S. salivarius*, *P. aeruginosa*, and *lactobacillus* spp was shown. Compared with 0.2% concentration of chlorhexidine gluconate, it was shown that the inhibition zones of the different concentrations of garlic extract were not significantly different from those of *S. mutans*, *S. sanguis*, *S. salivarius*, *P. aeruginosa* and *lactobacillus* spp. Additionally, the inhibition zones of 5%, 10% and 20% concentrations were not significantly different from one another (31). In such cases, the researcher can never be expressed the quantitatively of the used garlic extract concentrations. In another study results showed that the ethanolic extracts of garlic of 67µg/ml against *P. aeruginosa* (32) were as high as three times of our study. It may be possible that garlic extract partially neutralized by alcohol. However, various aspects of antimicrobial activity of garlic

has been reported and even a new peptide has been purified and characterized from garlic which shows antimicrobial properties (33). Thus, the active ingredient of garlic is allicin and this compound has been artificially synthesized.

In a study, 30 strains of MRSA were assayed and the results have indicated that 88% of bacterial tested had MIC 16 µg/ml of allicin, and all strains were inhibited at 32 µg/ml. Furthermore, about 88% of the isolates have shown MBCs 128 µg/ml, and all killed by 256 µg/ml. About 82% of these strains, showed intermediate or full resistance to mupirocin (34). While, in our study all strain (100%) were killed at 20 µg/ml (as MBC cut of point) and 10% of the strains with MIC 8 µg/ml survived. Our results exhibited that *Allium sativum*'s MIC values ranging from 8 to 24 µg/ml was capable of 10^5 CFU/ml reduction in bacterial populations. The tremendous effects of garlic extract may be related to the origin or geographical location of investigated.

According to the results of this research, the prepared garlic extract may be an appropriate candidate for application as a disinfectant in hospital environment. The importance of this study is the fact that all of the investigated bacteria have been isolated from an operating room and interestingly, more than 50% of them were resistant to several antibiotics and 26% were resistant to methicillin and erythromycin. Based on our results, the main cause of antibiotic resistance of bacteria in the air of hospital's operating rooms may be unknown, but it is important to note that all bacteria were isolated after washing and disinfecting the operating room.

Conclusion

The results of this study revealed that the garlic extract is a powerful antibacterial agent against drug resistant bacteria which were isolated from an operating room. A significant advantage of the garlic extract in this study was the broad-spectrum activity. The MIC for resistant bacteria was ≥ 16 µg/ml and the MBC was ≥24 µg/ml. We, therefore, recommend the possibility of adding an aromatic inert material to garlic extract may be useful to be used as a disinfectant agent for operating rooms.

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Authors' Contribution:

Ramezan Ali Ataee and Ali Mehrabi Tavana have developed the original idea, Hassan Araqizade help for operating room sampling, Ramezan Ali Ataee, Rezvan Yoose, and Mohammad Hosein Ataee have designed the experimental protocols, abstracted the data, and prepared the manuscript.

Conflict of interest

The authors declare that they have no conflicts of interest.

Financial disclosure

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