

The Antibacterial Effect of Aqueous Extract of Garlic against Resistant Enterococci

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ABSTRACT

Background and Objective: Enterococci are relatively nonvirulent bacteria that rarely cause disease. Antimicrobial treatment of Enterococci is often challenging due to their antibiotic resistance. This study aimed to investigate the antibacterial activity of aqueous extract of garlic against Enterococcal isolates.

Methods: In this descriptive study, 120 Enterococcus isolates including 70 multidrug-resistant isolates were collected from hospitals of Babol, Iran. Isolates' susceptibility to different antibiotics and the antibacterial activity of garlic extract were assessed using methods of minimum inhibitory concentration (MIC) measurement. The experiments were performed according to the Clinical & Laboratory Standards Institute guidelines, using Tryptic soy broth medium and disc diffusion method.

Results: Among the 120 Enterococcal isolates, 95 (79.2%) and 25 isolates (20.8%) were *E. faecalis* and *E. faecium*, respectively. Of the all Enterococcal isolates, the highest resistance was to erythromycin (95.8%), tetracycline (88.3%) and ampicillin (65.8%). While, the minimal level of resistance was to chloramphenicol (6.8%), vancomycin (20%) and ciprofloxacin (25%). Also, 53.3% of Enterococcal isolates showed simultaneous resistance to at least three antibiotics (tetracycline, erythromycin and ampicillin). Such resistance in *E. faecium* isolates was higher compared to *E. faecalis* (68% vs. 55.7%). The range of antibacterial activity of garlic extract against isolated Enterococci was determined by growth inhibition zone of 16.8 ± 1.8 mm and MIC of between 4 to 32 mg/ml.

Conclusion: This study indicates the clear anti-enterococcal effect of aqueous extract of garlic and confirms the use of garlic in treatments by medicinal plants.

Keywords: garlic extract, multi-drug resistance, minimum inhibitory concentration, Enterococcus .

INTRODUCTION

Garlic (*Allium sativum*) is a plant from the Liliaceae family, which is endemic in central Asia and can be found today in all parts of the world. From centuries ago, different species of this plant were used as a spice and food additive in cooking, and also applied as a drug in herbal medicine for treatment of various types of diseases (1). Garlic has antibiotic, anti-cancer, antioxidant, anti-inflammatory, hypoglycemic and cardioprotective properties (2). The antibacterial effects of garlic on different bacteria types has been reported (3,6). Enterococci are commensal gut bacteria and as opportunistic pathogens, are capable of causing urogenital tract infections, endocarditis, meningitis, intra-abdominal abscesses, bacteremia, neonatal sepsis and nosocomial infections (5). Almost 20 species are in the genus *Enterococcus*, two of which (*E.faecalis* and *E.feacium*) are responsible for almost 90% of all Enterococcal infections in humans (5). The pathogenicity of Enterococci is affected by resistance to various antibiotics, rather than its virulence factors. Selective pressure caused by antibiotic overuse during the past 50 years on one hand, and the capacity of Enterococci to acquire and disseminate antibiotic resistance determinants on the other, are some of the risk factors that highlight the need for continuous monitoring of antibiotic resistance in these bacteria. Multiple antibiotic resistance development increase healthcare costs and leads to relapse of previously controlled diseases. This in turn increases the incidents of opportunistic and chronic infections in the world (6). Patients often prefer medicinal plants due to their lower costs and fewer unwanted side effects compared to chemical drugs (7). Given the above facts, antibacterial effects of garlic and abundance of its cultivation and its either raw or processed consumption in Iran, this study aimed to investigate the antibacterial properties of garlic aqueous extract against various multidrug-resistant (MDR) Enterococci in city of Babol. Studying the range of antibacterial properties of garlic against the isolated strains may confirm the potential role of this plant in treatment of microbial infections.

MATERIAL AND METHODS

The Bakri and Douglas methods were used in this descriptive study to prepare the

aqueous extract of garlic. First, 80 grams of garlic were peeled after weighing and washing, then crushed and thoroughly mixed and homogenized by an electric mill in 100 ml of sterile distilled-water. The mixture was centrifuged for 20 min at 6000 rpm. The resulting supernatant was passed through the Whatman (Grade 1) filter and then became sterile by passing through a 0.45 μ filter (Millipore). The final concentration of the garlic extract was estimated as 512 mg/ml, after subtracting the weight of the insolubles from the weight of raw garlic cloves (8). The Enterococcal isolates used in this study were obtained from patients with bacteremia, pneumonia and urinary tract infections. Isolates' identity was confirmed by colony morphology and Gram staining, while their biochemical properties were assessed using esculin hydrolysis in the presence of bile, growth in the presence of NaCl 5.6% and Pyrrolidonyl Arylamidase test. Carbohydrate fermentation tests (arabinose, mannitol, sorbitol, sorbose and lactose) were used to determine the species (9). Antibiotic susceptibility of Enterococcus isolates was assessed by disk diffusion test using erythromycin (μ g 15), tetracycline (μ g 30), ampicillin (μ g 10), gentamicin (μ g 10), ciprofloxacin (μ g 5), vancomycin (μ g 30) and chloramphenicol (μ g 15) (MAST Co., UK). High level of gentamicin resistance test was done using 120mg gentamicin disks. In order to assess the Enterococcus strains' susceptibility to garlic extract, a suspension of Enterococcal isolates with turbidity equivalent to 0.5 McFarland units was prepared in Mueller Hinton Broth. Then, 0.1 ml of the suspension was cultured on Mueller Hinton agar medium and sterile blank disk were soaked in the garlic extract by Whatman filter paper No.1 (Padtan Teb Co.) with a diameter of 5 mm. After 5 minutes of drying at 60 °C, the disks were placed on the plate's surface. Blank discs soaked in distilled water were used as negative controls. After 24 hours of incubation at 37 °C, the diameter of inhibition zone around the discs were measured in millimeters. The minimal inhibitory concentration (MIC) was determined by microdilution in wells using Tryptic soy broth. A suspension was prepared from the 24-hour culture of bacteria with final concentration of 1.5×10^6 CFU/ml, which

was later diluted 1/100 by Mueller Hinton broth. Stock vancomycin antibiotics with concentration of 10 mg/ml was obtained using pure vancomycin powder (SERVA Co.) and antibiotic dilutions of 1536, 768, 384, 192, 96, 48, 24, 12, 6, 3, 1.5 and 0.75 µg/ml were prepared. In this method, bacterial suspension with 1/100 dilution along with the prepared concentrations of antibiotics was added to the microplates. After incubation at 37 °C for 24 hours, MIC of strains was determined by considering the lowest concentration of antibiotics at which no growth was observed. *E. faecalis* ATCC 29212 (susceptible) and *E. faecium* BM 4147 (resistant) strains were used as positive and negative controls, respectively. Obtained results were interpreted according to guidelines of the National Committee for Clinical Laboratory Standards (10). Data were analyzed using SPSS-14 software.

RESULTS

In this study, 120 clinical isolates of Enterococci were investigated including 47 urine samples (39.1%), 34 blood samples (28.3%), 21 skin and soft tissue samples (17.5%) and 15 samples from the respiratory tract (18%). Of the 120 Enterococcal isolates, 24 isolates (20%) were vancomycin-resistant (through microdilution) (MIC > 8µg) and grew well around the vancomycin disc. Of the 95 *E. faecalis* isolates, five isolates were vancomycin-resistant (5.2% of this specie and 4.2% of all isolates), and out of 25 *E. faecium* strains, 19 were resistant to this antibiotics (76 % of all isolates of this species and 15.8% of

all isolates) (Table 1). The results of the antibiogram test and MIC determination are presented in Table 2. The investigated Enterococci showed the highest level of resistance against erythromycin (95.8%), tetracycline (88.3%) and ampicillin (65.8%), while showing minimal resistance against chloramphenicol (6.8%), vancomycin (20%) and ciprofloxacin (25%). Also, 53.3% of all the Enterococcal isolates showed simultaneous resistance against at least three antibiotics (tetracycline, erythromycin and ampicillin). This type of resistance was observed more in the *E. faecalis* (68%) isolates compared with the *E. faecium* isolates (55.7%). The MIC of garlic's aqueous extract for 95 *E. faecium* isolates were as follows: 74 resistant samples (78% , MIC ≥ 8), 6 semi-susceptible samples (6.3% , MIC 4-8 µg/ml) and 15 susceptible samples (15.8% , MIC ≤ 4µg/ml). The MIC amounts for the 25 *E. faecium* isolates included: 21 resistant samples (84% , MIC ≥ 8), 1 semi-susceptible sample (4% , MIC 4-8 µg/ml) and 2 susceptible samples (8% , MIC ≤ 4µg/ml). The results of disk diffusion and agar dilution were completely concordant which indicates higher resistance of *E. faecium* in comparison to *E. faecalis*. Garlic Aqueous extract's average diameter of growth inhibition zone in the studied Enterococci was measured as 16.8 ± 1.8 mm. The results of this study indicated reduced growth of MDR bacterial colonies in the presence of garlic's aqueous extract after incubation at 37 °C for 24 hours. The diameter of growth inhibition zone for any MDR specie was reported as 17.3 ± 0.4 mm.

Table 1- Distribution of Enterococcus strains against Vancomycin in terms of species

Enterococcus strains	VSE* Number	VRE** Number	Total Number (%)
<i>E. faecalis</i>	90	5	95 (79)
<i>E. faecium</i>	6 (5)	19	25 (21)
Total	96	24	120(100)

Table 2- antibiotic susceptibility testing of Enterococcal isolates

Antibiotic	Sensitive (percent)	Semi-Sen (percent)	Resistance (percent)	Disc Difussion	MIC (µg/ml)
Erythromycin	0.8	3.4	95.8	15	0.01-128>
Tetracycline	8.3	3.4	88.3	30	0.08-128>
Ampicillin	6.6	27.6	65.8	10	0.08-128>
Gentamicin	45.9	20.8	33.3	10	0.08-1024
Ciprofloxacin	40	35	25	5	0.04-32
Ciprofloxacin	80	0	20	30	0.02-256>
Chloramphenicol	86.6	6.7	6.7	15	0.02-256>

DISCUSSION

Enterococci are linked to one of the most important types of intestinal infections. Increased bacterial resistance against antibiotics highlighted the need for developing antibacterials to enhance infection control. Use of medicinal plants is one of the most commonly used methods of treatment and symptom control since ancient times. In this study, a total of 120 Enterococcal isolates were investigated. Various studies in Iran suggests that this bacterium is endemic in hospitals and has an important role in hospital infections (11,12). In a study in Babol, the frequency of isolation for *E. faecalis* and *E. faecium* were reported as 63% and 33%, respectively (13). Meanwhile, Hayes et al. reported 53.2 % and 31.4% isolation for these species (14). In this study, 115 Enterococcal isolates (98.8% of all isolates) were erythromycin-resistant. This level of resistance was in agreement with findings of Farokh and Oskoyi (15), but significantly higher than the level reported by Dadfarma et al. in Hamedan (68%)(13). In a similar study in India, 85% of the Enterococcal isolates were erythromycin-resistant (16) and in a study in Italy except the *E. faecalis* isolates, 87% of *E. faecium* isolates were also resistant to this antibiotic (17). Erythromycin is considered as an alternative for penicillin in allergic patients and is used in infection prophylaxis such as subacute bacterial endocarditis. The level of tetracycline resistance in this study was 88.3%, while Oskoyi and Farokh reported 21% resistance for this antibiotic in Tehran (15). This increased resistance may be associated with ease of access and consumption of this antibiotic in the country. In this regard, Sing's investigation in India showed 40% resistance to this antibiotic in Enterococci (18). In another study in India, the resistance to aminoglycosides has been reported as 37% (19). Among the 120 Enterococcal isolates in this study, 30 isolates were ciprofloxacin-resistant (25%), while the level of resistance to this antibiotic was higher in *E. faecium* compared to *E. faecalis* (80% vs. 10.5%). Moreover, 20.9% of all Enterococci were resistant to vancomycin (MIC \geq 8 μ g/ml). There are very limited studies on vancomycin resistance in Enterococci strains with often divergent results. While Asgarian et al.

reported the prevalence of vancomycin resistant Enterococci in hospitalized patients in city of Shiraz as 14% (20), a study in India in 2003 reported only 1% vancomycin resistance in these bacteria (16). Nevertheless, recent studies have reported 80.2% resistance to this antibiotic in India (18). In the present study, the level of resistance to other antibiotics was also higher among vancomycin resistant isolates. The study of AslaniMehri et al. investigated the MIC of 165 isolates using the agar dilution method and their results showed that 7 isolates (4.2%) were susceptible to erythromycin (MIC \leq 0/5 μ g/ml), 11 isolates (6/6%) were intermediate (MIC 1-4 μ g/ml) and 147 isolates (89%) were resistant to erythromycin (MIC \geq 8 μ g/ml). Furthermore, the results of the disk diffusion and agar dilution methods in the mentioned study were completely concordant. Among 23 *E. faecium* isolates, 21 (91.3%) were resistant to erythromycin which indicates higher antibiotic resistance in this specie compared to *E. faecalis*. Of 165 enterococci isolates, 18 (10.9 %) had MIC of $<$ 128 μ g/ml and 147 isolates (89%) had MIC of $>$ 128 μ g/ml. MIC₅₀ and MIC₉₀ values of *E. faecalis* and *E. faecium* were higher than 128 μ g/ml (20). In a study in Shiraz, all vancomycin-resistant enterococci isolates were also resistant to ampicillin, penicillin and gentamicin (21). According to the results of the present study, the average diameter of inhibition zone of garlic's aqueous extract in the tested Enterococci was 16.8 ± 1.8 mm. Lwalokun et al. reported the average diameter of the inhibition zone of this extract in Gram-positive bacteria, between 20.2 to 21.8 mm (2). This difference may be due to diversity of tested garlic extract concentrations and the method of antibacterial properties assessment, since the present study used the disk diffusion method, while the other study had used the well diffusion method. According to the same study, the diameter of growth inhibition zone increased after 24 hours, thus the antibacterial effects of garlic's aqueous extract were reported as dose- and time-dependent (2). On the other hand, longer periods may reduce the concentration of garlic's active constituents. In this regard, Lemar et al. reported that fresh garlic extract has higher effects on morphology and growth inhibition of *Candida*

compared to its old extract (22). Ruddock et al. study compared the antibacterial activity of natural garlic health products and fresh garlic extract against *Neisseria gonorrhoeae*, *Staphylococcus aureus* and *Enterococcus faecalis* using the microdilution method. Garlic extract of health products had 47% inhibitory effect on *N. gonorrhoeae*, and only 16% antibacterial effect against *S. aureus* and *E. faecalis*. In general, products containing garlic compounds have higher antimicrobial activity in comparison with fresh garlic extract (23). The results of the present study confirm the importance of genetic resistance patterns of Enterococci and continuous monitoring of this resistance in the region. Moreover, the results indicate the effect of garlic extract on elimination of Enterococcus strains and antibiotic resistant strains in particular. However, further studies are recommended on other Enterococci strains before its clinical application in pre-clinical

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CONCLUSION

The present study indicates the clear anti-enterococcal effect of aqueous extract of garlic and confirms the use of garlic in treatments by medicinal plants.

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CONFLICT OF INTEREST

There are no conflicts of interest.

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