

## In Vitro Antibacterial Properties of Aqueous Garlic Extract (AEG) Against Multidrug-Resistant Enterococci

Mohammad Bokaeian,<sup>\*1</sup> Zakaria Bameri

1. Department of Laboratory Sciences, Faculty of Paramedical Sciences, Research Center for Infectious Diseases and Tropical Medicine, Zahedan University of Medical Sciences, Zahedan, Iran
2. Research Center for Infectious Diseases and Tropical Medicine, Zahedan University of Medical Sciences, Zahedan, Iran

Article information	Abstract
<p>Article history: Received: 31 Jan 2012 Accepted: 19 Apr 2012 Available online: 29 Oct 2012 ZJRMS 2013; 15(6): 43-49</p> <p>Keywords: Garlic MIC MDR Enterococcus</p> <p>*Corresponding author at: Department of Laboratory Sciences, Faculty of Paramedical Sciences, Research Center for Infectious Diseases and Tropical Medicine, Zahedan University of Medical Sciences, Zahedan , Iran E-mail: bokaeian.m@gmail.com</p>	<p><b>Background:</b> As relatively avirulent enteric bacteria, enterococci usually cause infections in immune-compromised patients. The antimicrobial treatment, however, is quite challenging, since enterococci are intrinsically resistant to many antibiotics. Objective of the present study was to examine the antibacterial activity of aqueous garlic extract on isolates of enterococci.</p> <p><b>Materials and Methods:</b> In this descriptive research, a total of 120 enterococcus isolates including 70 multidrug-resistant isolates causing different infections were collected from three hospitals in Zahedan. The susceptibility of isolates to different antibiotics was measured by agar diffusion test and antibacterial activity of garlic extract was measured using disc-diffusion and microbroth dilution methods.</p> <p><b>Results:</b> Among 120 enterococcus samples, 95 (79.2%) and 25 (20.8%) isolates were <i>E. faecalis</i> and <i>E. faecium</i> respectively. The highest resistance was observed in erythromycin (95.8%) and the lowest resistance (6.7%) in chloramphenicol, while 88.3% and 65.8% of the isolates were resistant to tetracycline and ampicillin respectively. Moreover, 58% of the isolates were Multi-Drug Resistant (MDR) and showed resistance to at least three antibiotics. Antibacterial activity of AGE was characterized by inhibition zones of 16.8±1.8 mm and Minimum Inhibitory Concentration (MIC) ranged from 4 to 32 mg/ml.</p> <p><b>Conclusion:</b> The present study suggests that AGE has a significant anti-enterococcal effect and therefore, supports the use of garlic as an herbal remedy in Zahedan.</p> <p>Copyright © 2013 Zahedan University of Medical Sciences. All rights reserved.</p>

### Introduction

Allium sativum, commonly known as garlic, is a plant belonging to the family of Liliaceae, which is native to central Asia and nowadays can be found throughout the world. Over the last centuries, various species of garlic have been used as spice or condiment for flavoring food. In herbal medicine, garlic has been prescribed for treating different kinds of diseases [1]. This plant is thought to be regulating the blood sugar and protecting the cardiovascular system. It also bears antibacterial, anticarcinogen, antioxidant and anti-inflammatory properties [2]. In addition, antibacterial effects of garlic on various types of bacteria have been reported in some studies [3, 4].

Enterococci are among the normal flora of human digestive tract. As opportunistic pathogens, they are capable of causing different types of infections in genitourinary tract, endocarditis, meningitis, intra-abdominal abscess, wound infections, bacteremia, neonatal sepsis and nosocomial infections [5]. There are nearly 20 enterococcus species, two of which, *E. faecalis* and *E. faecium*, are considered to be the cause of about 90% of enterococcal infections in human [5]. The virulence of enterococci originates more from their

resistance to various antibiotics rather than virulence factors. The selective pressure resulted from overuse of antibiotics over the last 50 years on the one hand, and the high capacity of enterococci to receive and spread the contributing factors of antibiotic resistance on the other hand, have dangerously led to a situation in which continuous monitoring of antibiotic resistance is regarded as crucial. Aside from increasing medical care and treatment costs, development of multi-drug resistance has brought about re-emergence of diseases that were under control in the past. This issue in turn has resulted in escalation of opportunistic and chronic infections all around the world [6].

On top of their healing benefits, herbs and medicinal plants are comparatively cheaper than chemical drugs and much easier to use. Hence, they are better accepted and taken by patients, in general [7]. Considering the facts mentioned above as well as the antibacterial effects of garlic, which is widely grown and consumed either raw or processed in Iran, conducting a supplementary study on this subject could provide an opportunity to utilize the final results more extensively and systematically. Furthermore, the variety of compounds in garlic as well as

the genetic diversity of bacteria under study, have led to different results reported about evaluation of antibacterial properties of garlic [2]. Therefore, it is essential to locally and regionally examine the antibacterial effects of garlic. With a research carried out on the scope of antibacterial properties of garlic against newly-emerged isolates of bacteria in Zahedan, the helpful medical usage of this plant would be scientifically verified for treating microbial infections in the whole country. The present work deals with antibacterial properties of Aqueous Garlic Extract (AGE) against multidrug resistant (MDR) enterococci from Zahedan.

## Materials and Methods

In this descriptive research, garlic extract was prepared using Bakri and Douglas method, thus 80 gram of garlic was weighed and rinsed, then the protective layer of cloves were peeled out. Using an electric grinder, garlic was crushed and completely blended and homogenized with 100 ml of sterile distilled water. The mixture was centrifuged at 6000 rpm for 20 minutes. The surface liquid was passed through Whatman no. 1 filter and sterilized through 0.45 micron millipore filter. By subtracting the weight of insoluble materials from that of the intact cloves, the final concentration of garlic in the extract was measured to be 512 mg/ml [8].

The enterococcal isolates studied in this research were collected from patients suffering from burns, bacteremia, and pneumonia and urinary tract infections. Presumptive identification of enterococci was based on their colony morphology, gram staining, 6.5% NaCl tolerance, bile esculin hydrolysis and pyrrolidonylarylamidase (PYR) activity [9].

Susceptibility testing was performed by disc-diffusion method and the following antimicrobial discs and concentrations were used: erythromycin (15 µg), tetracycline (30 µg), ampicillin (10 µg), gentamicin (10 µg), ciprofloxacin (5 µg), vancomycin (30 µg), and chloramphenicol (15 µg) [Oxoid]. All strains were tested for high levels of gentamicin resistance (HLGR) using 120-microgram gentamicin discs. Furthermore, *E. faecalis* ATCC 51299 (vancomycin sensitive) and WHO3 (vancomycin resistant phenotype with MIC=512 µg/ml) were used as controls. For interpretation of the results, CLSI guidelines were followed [10]. Minimum inhibitory concentrations (MICs) were determined by microbroth dilution method using tryptic soy broth and *E. faecalis* ATCC 29212 (MIC=4 µg/ml) and *E. faecium* 4147

(MIC=256 µg/ml) strains were used as negative and positive controls respectively.

In order to examine the susceptibility of enterococcal strains to garlic extract, a suspension was made from each studied enterococcal isolates with a McFarland standard turbidity of 0.5 in Mueller-Hinton broth, 0.1 ml of which was cultivated on Mueller-Hinton agar. Next, blank sterile discs (Whatman No. 1 filter paper, 5 mm in diameter supplied by Padtan Teb Co. Iran) were soaked in garlic extract, dried at 60°C for 5 minutes, and were placed on the surface of the plate. A blank disc dipped in distilled water was used as negative control. After 24 hours of incubation at 37°C, the inhibition zone around the disc was measured in millimeters. Broth dilution was employed to determine the MIC.

Data analysis was done using SPSS-14 software by One-way ANOVA with the significance level  $p < 0.05$ .

## Results

In the present work, a total of 120 isolated enterococci were studied using different samples collected from different body parts with the following percentages: 39.1% urine (47), 28.3% blood (34), 17.5% skin and soft tissues (21), and 15% respiratory system (18) (Fig 1).

In this population, 79.2% of the isolates (95 samples) were characterized as *E. faecalis* and the remaining 20.8% (25) fell under the category of *E. faecium* (Fig 2).

As it can be seen, 20% of the total enterococcal samples (24 isolates) showed resistance to vancomycin (MIC>8µg/ml) and grew well around the vancomycin disc. Five out of the total number of *E. faecalis* isolates (95) were resistant to vancomycin (i.e. 5.2% of all the isolates from this species and 4.2% of the total isolates). Moreover, 19 out of the total number of *E. faecium* isolates (25) were resistant to vancomycin (i.e. 76% of all the isolates from this species and 15.8% of the total isolates) (Table 1).

The results obtained from antibiogram and the MIC determination can be seen in table 2 and figure 3. The enterococci under study showed the highest resistance to such antibiotics as erythromycin (95.8%), tetracycline (88.3%), and ampicillin (65.8%). They showed the lowest resistance to chloramphenicol (6.8%), vancomycin (20%), and ciprofloxacin (25%). In addition, 58.3% of the total isolates showed resistance to at least three antibiotics. The average diameter of growth inhibition zone for the AGE was 16.8±1.8 mm. The minimum inhibitory concentration of garlic extract varied from 4 to 32 mg/ml.

**Table 1.** Frequency distribution of enterococcal isolates resistant to vancomycin in terms of two species

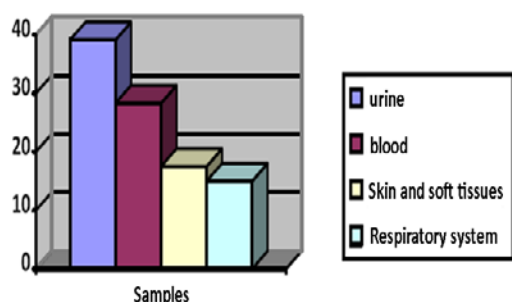
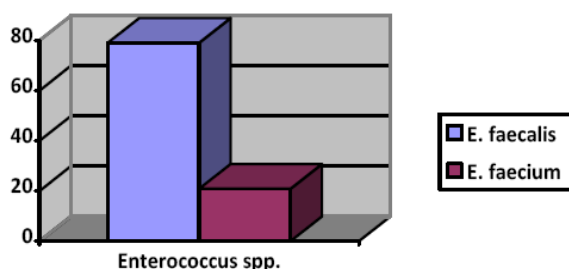
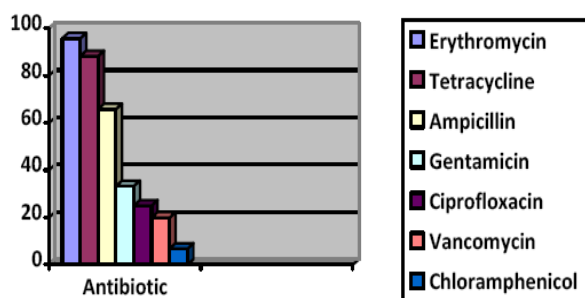
Enterococcal species	Number of VSE <sup>a</sup> (%)	Number of VRE <sup>b</sup> (%)	Total number (%)
<i>E. faecalis</i>	90 (75)	5 (4.2)	95 (79.2)
<i>E. faecium</i>	6 (5)	19 (15.8)	25 (20.8)
Total	96 (80)	24 (20)	120 (100)

VSE<sup>a</sup>: vancomycin-susceptible

VRE<sup>b</sup>: vancomycin-resistant

**Table 2.** Susceptibility of enterococcal isolates to the examined antibiotics

Antibiotics	Disc potency	Number of Resistant isolates (%)	Number of Moderate isolates (%)	Number of Sensitive isolates (%)	MIC ( $\mu\text{g/ml}$ )
Erythromycin	15	115 (95.8)	4 (3.4)	1 (0.8)	<0.101-128
Tetracycline	30	106 (88.3)	4 (3.4)	10 (8.3)	<0.08-128
Ampicillin	10	79 (65.8)	33 (27.6)	8 (6.6)	<0.08-128
Gentamicin	10	40 (33.3)	25 (20.8)	55 (45.9)	<0.08-1024
Ciprofloxacin	5	30 (25)	42 (35)	48 (40)	0.06-16
Vancomycin	30	24 (20)	0 (0)	96 (80)	<0.02-256
Chloramphenicol	15	8 (6.7)	8 (6.7)	104 (86.6)	<0.02-256

**Figure 1.** Frequency distribution of enterococcal isolates in terms of samples from different body parts**Figure 2.** Frequency distribution of the isolates in terms of two enterococci species**Figure 3.** Frequency distribution of enterococci resistant to the examined antibiotics

## Discussion

In the present study, 120 enterococcal isolates were collected in total, including 47 samples from urine, 34 from blood, 21 from skin and soft tissues, and 18 from respiratory system. 95 isolates (79.2%) belonged to *E. faecalis* species, while the remaining 25 isolates (20.8%)

belonged to *E. faecium*. Various studies conducted in Iran suggest that such bacteria are endemic in hospitals and play an important role in nosocomial infections [11, 21]. In the research done regarding Hamedan, it was reported that *E. faecalis* and *E. faecium* covered 63% and 33% of the collected samples respectively [13]. In their study, Hayes and colleagues reported a proportional distribution of 53.2% and 31.4% for the two species respectively [14].

In the present study, however, 115 of the enterococcal samples (95.8% of the total isolates) showed resistance to erythromycin, which is comparable to the results obtained by Oskoui and Farrokh [15] while it shows a considerable difference compared to the study done by Dadfarma and colleagues from Hamedan (68%) [13].

A similar study carried out in India showed that 85% of enterococcal isolates were resistant to erythromycin [16]. In another study regarding Italy, all the *E. faecalis* isolates and 87% of the *E. faecium* isolates were reported to be resistant to this antibiotic [17]. Erythromycin is known as an alternative to penicillin prescribed for patients who are allergic to the latter antibiotic. Furthermore, it can be used in the prophylaxis measures taken to prevent such infections as subacute bacterial endocarditis. Unnecessary overdose or inappropriate intake of erythromycin and other macrolides for treating bacterial infections in Iran and other regions can significantly aggravate the resistance level in this category of antibiotics.

The level of resistance to tetracycline was measured to be 88.3% in this research. According to the study done by Oskoui and Farrokh from Tehran, the level of resistance to this antibiotic was 81.2% [15] which indicates a 40% increase compared with the previous study conducted in the same city [11]. The boosted resistance can be associated with the fact that tetracycline is an easy-to-access antibiotic in Iran and is frequently prescribed as a cheap medication in hospitals and the society. Along the same lines, the research done by Singh in India suggests a 40% resistance of enterococci to such antibiotic [18].

Based on the results obtained from the present study, 65.8% of the isolates showed resistance to ampicillin, which was more intensely in *E. faecium* compared to *E. faecalis* (70% versus 63.1% resistance). In the study done regarding Hamedan, however, resistance to ampicillin was reported 59.6% and 39% for *E. faecium* and *E. faecalis* respectively [13]. In another study concerning India, 66% of the enterococcal isolates were resistant to ampicillin [16].

Moreover, 17 isolates (14.1% of the total) exhibited a High-Level Gentamicin Resistance (HLGR), while 23

isolates (19.1 of the total) showed a medium-level resistance to this antibiotic. As for the Hamedan study, HLGR was reported to be 43.7% in enterococcal isolates [13]. According to Oskoui and Farrokh, the overall gentamicin resistance was 78.1% [15]. The research conducted in 2003 concerning India, 26% of the enterococcal isolates showed a high level of resistance to aminoglycosides [16] which was previously reported to be 37% by another research in the same country [19].

Enterococci with  $MIC \geq 500 \mu\text{g/ml}$  and highly resistant to gentamicin have become a major problem to antibiotic therapy. The combination of penicillin and gentamicin has so far been applied as a treatment strategy for enterococcal infections. With the emergence of HLGR strains, however, such strategy has faced a serious challenge. HLGR is generally triggered by acquiring genes encoding Aminoglycoside-Modifying Enzymes (AMEs) including acetyl-transferase and phospho-transferase. These genes are transferred by transposons and plasmids, causing resistance to clinically-used aminoglycosides except for streptomycin. In the present study, multi-drug resistance in HLGR isolates was observed to be more prevalent than in other isolates, which is consistent with the similar results reported by Dadfarma and colleagues [13].

Among the total 120 enterococci isolates examined, 30 of them (25%) were resistant to ciprofloxacin, effect of which was observed more intensely on *E. faecium* compared to *E. faecalis* (80% versus 10.5%). According to Dadfarma and colleagues, slightly over half of the enterococci were ciprofloxacin-resistant which made no large difference between the two species in this respect [13]. In the research done by Johnson and colleagues, the level of ciprofloxacin-resistance in *E. faecium* and *E. faecalis* were reported to be 28% and 5% respectively [20]. On the other hand, all the enterococci in Oskoui and Farrokh research showed resistance to ciprofloxacin [15]. Furthermore in their study, Mathur and colleagues from India concluded that 88% of the enterococcal isolates were ciprofloxacin-resistant and 20.9% of the isolates (25 of the total 120) showed resistance to vancomycin ( $MIC > 8 \mu\text{g/ml}$ ) and grew well around the disc [16]. Studies conducted regarding the frequency distribution of resistant enterococci strains in Iran has been limited, often producing different results. For instance in the study done by Dadfarma and colleagues, none of the *E. faecalis* isolates showed resistance to vancomycin and only 23.4% of the *E. faecium* isolates were sensitive to this antibiotic [13]. In contrast, 14% of the patients in Shiraz hospitals were affected by vancomycin-resistant enterococci [21] and it was reported to be 51.6% in a study concerning Tehran [15]. According to the similar study done in India in 2003, only 1% of the enterococcal isolates were vancomycin-resistant [16] while in a more recent study, the level of resistance was 80.2% [18]. As for South Korea, it has increased from 2.6% to 16% between 1997 and 2006 years [22].

Vancomycin resistance originated from plasmids was first observed in European regions in 1980's and later in

the United States. Since then, vancomycin-resistant enterococci (VRE) have dramatically increased throughout these countries, and today, such bacteria are regarded as one of the main factors contributing to nosocomial infections [7].

In the present research, however, the level of resistance to vancomycin was unbalanced in terms of species, so that only 5 out of the total 95 *E. faecalis* isolates (5.2%) showed resistance to this antibiotic, while 19 out of the total 25 *E. faecium* isolates (76%) were reported to be vancomycin-resistant. In a two-year study concerning South Korea, 90.7% of the vancomycin resistant isolates belonged to *E. faecium* [23] and according to the study done in India, only 1% of the *E. faecalis* isolates were observed to be vancomycin-resistant [16].

The antibiotic resistance of enterococci to *glycopeptide* antibiotics such as vancomycin and teicoplanin is significant, because they are normally prescribed as a drug of choice and ultimate resort for treating nosocomial infections caused by MDR gram-positive bacteria [15].

In the present study, the level of resistance to other antibiotics was higher in vancomycin-resistant isolates, particularly *E. faecium*. In the study regarding Shiraz, however, all species of vancomycin resistant enterococci showed resistance to such antibiotics as ampicillin, penicillin and gentamicin [21].

Among the total 120 enterococcal isolates examined, 8 samples (6.7%) were chloramphenicol-resistant. In the study concerning Hamedan, resistance to this antibiotic in *E. faecium* and *E. faecalis* were reported to be 49% and 41% respectively [13]. In the research done by Johnston and colleagues, the level of resistance to chloramphenicol was reported to be 5% [17]. Additionally, the resistance level was observed to be 36% in Busani and colleagues' research [17].

Among the total 120 enterococcal isolates examined, 8 samples (58.3%) simultaneously exhibited resistance to at least three antibiotics. Such resistance behavior was seen more intensely in *E. faecium* compared to *E. faecalis* isolates (68% versus 55.7%), which is consistent with the results of Hamedan research [13]. The level of MDR have been reported differently in several other studies. For instance, in the study conducted by Hayes and colleagues, such resistance was reported to be 52.7% [14] while according to Singh from India, 99.5% of the enterococci examined showed multi-drug resistance [18].

The outbreak and development of MDR enterococci have become a global problem. Therefore, it is extremely crucial to be aware of the characteristics of such resistance in order to adopt a suitable treatment strategy. The increasing growth of MDR isolates implies that more attention should be paid on discovering the useful antimicrobial agents which function more effectively and bring about less toxicity. A thorough research carried out on the antibacterial properties and components of plants can be a fundamental step to get ahead.

In present study, the average diameter of growth inhibition zone for the AGE was  $16.8 \pm 1.8$  mm. According to Iwalokun and colleagues, the average diameter of

inhibition zone for the AGE in gram-positive bacteria varied from 20.2 to 21.8 mm [2]. The difference could be due to the concentration of garlic extract and the way its antibacterial property had been measured. We employed the disc-diffusion method here, while those researchers had employed the agar well diffusion method. Based on the report given by Iwalokun, the diameter of growth inhibition zone had extended over 24 hours. As a result, antibacterial effects of AGE were associated with two variables of time and dose [2]. The concentration observed in effective compounds of garlic can be reduced over a long period of time. Regarding such fact, Lemar and colleagues reported that fresh garlic extract is more effective on morphology and growth inhibition of *Candida* compared to old garlic extract [24]. The dose dependency of garlic extract has also been reported in several other studies [25]. Considering the aggregated resistance of bacteria to many commonly-used antibiotics, great efforts have been made to obtain more detailed information about plant compounds and their function in treating microbial infections.

The MIC in the present study varied from 4 to 32 mg/ml, which is almost comparable to the results obtained by Iwalokun and Sivam [2, 3]. In contrast, the level of MIC was reported to be much higher by Ross and colleagues [26]. As mentioned earlier, the effective compounds of garlic vary from one method of preparation to another. Based on the research done by Aliporyegane and colleagues, the MIC level in the extract of garlic powder for salmonella and shigella was 12.5 mg/ml, while the MIC level in the extract of garlic tablets was reported to be 40 mg/ml [27]. In the study done by Hosseini-Jazani, the MIC level in garlic extract was observed to be much lower [4]. In the latter study, the effects of chloroform garlic extract on acinetobacter strains were examined. Therefore, the gap between MIC levels of garlic extract can reflect the difference in genus and species of bacteria as well as the difference in extraction methods and the outcome compounds.

The intensity and range of antibacterial effects of garlic depends to a great extent on the medium in which it grows. In the study done by Kazemi and colleagues, it was concluded that the anti-enterococcal effects of garlic extract in the southern areas of Iran (including Jiroft) was more intense than the garlic grown in North of the country (Babol) [25]. The difference in the examined strains can also affect the obtained results, which explains why researchers dealing with the effects of garlic extract on various bacteria have reported discrepant results [27]. There are several factors contributing to such discrepancies, including the variety in compounds, their different amounts, and also their synergistic effect on sulfhydryl groups existing in the growth media [2].

It has been reported that the amount of allicin and diallyl sulfide in AGE varies from one extraction method to another [2, 28]. According to the reports provided by Prakash and colleagues, allicin has significant antibacterial effects as one of the components of garlic in VRE strains. Furthermore, the MIC of allicin was

recorded to be 75 µg/ml after 4 hours, and 150 µg/ml after 24 hours [7]. These findings not only demonstrate the anti-enterococcal properties of garlic against VRE strains, but it also implies that such effects depend on time.

The antibacterial function of garlic has been mainly associated with the existing allicin in garlic [29]. The antibacterial mechanism of allicin is fairly unknown. A few studies, however, suggest that the main antimicrobial effect of allicin could be due to its chemical reaction with thiol groups of various enzymes [29].

In the present study, AGE reacted effectively against all the enterococcal samples including MDR isolates, and its MIC varied from 4 to 32 mg/ml. According to the report given by Kazemi and colleagues, AGE reacted effectively against all the *E. faecalis* strains [25]. Moreover, Fani and colleagues observed that AGE has antibacterial effects on multidrug-resistant strains of *S. mutans* isolates collected from decayed teeth [30]. In the latter study, it was reported that AGE has antibacterial effects on all the isolates of *S. mutans* (both MDR and non-MDR), the MIC level of which ranged from 4 to 32 µg/ml, and it was recommended by the researchers to be used in mouthwash and toothpaste products [30].

We found that even the MDR enterococci could be susceptible to garlic extract. Based on a few reports, garlic extract and its containing allicin showed bacteriostatic effects on vancomycin-resistant enterococci. Furthermore, garlic extract and vancomycin together have synergistic effect on such enterococci [29]. According to Hosseini Jazani, it was also reported that MDR strains of *Acinetobacter* with high-level antibiotic resistance are susceptible to low concentrations of garlic [4]. SH groups in allicin are probably able to form disulfide bonds with SH groups in bacterial enzymes and then take control of TN/546 transposon (which encodes vancomycin resistance) by connecting to their containing enzymes. It consequently intensifies the susceptibility of VRE strains to vancomycin [29]. The function of forming disulfide bonds is confirmed, because the synergistic effect could be controlled by adding cysteine (which interferes with allicin through SH groups) and mercaptoethanol (which breaks down disulfide bonds) [29].

The positive effect of garlic extract on antibiotic-resistant enterococci demonstrates the difference between antibacterial function of AGE and antibacterial function of the examined antibiotics. Therefore, the problem of antibiotic-resistance behavior and multi-drug resistance to antibiotics might be solved by using the effectively purified compounds of garlic. Iwalokun and colleagues reached the same findings, since they similarly observed and concluded that AGE leaves effects on resistant strains of such bacteria as *S. pneumoniae*, *P. aeruginosa*, *E. coli* and shigella [2]. In addition, they reported that garlic extract influences the pathogenesis of bacteria by controlling their toxic production [2].

Generally, the conclusions drawn by the present research, on the one hand, propose that more attention should be paid on the patterns of genetic-resistance in

enterococci and continuous monitoring of such resistance in Zahedan. On the other hand, it indicates that garlic extract is effective on destroying enterococci, particularly MDR strains. Finally, we still need to launch more research projects examining other species of enterococci before we actually begin to clinically use garlic extract for treating enterococcal infections.

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### Authors' Contributions

All authors had equal role in design, work, statistical analysis and manuscript writing.

### Conflict of Interest

The authors declare no conflict of interest.

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