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The Combined Effects of Shallot Extract by the Vacuum Rotary Evaporator Technique with Common Antibiotics against Multidrug-Resistant Bacteria

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ABSTRACT

Introduction: *Allium hirtifolium* (Persian shallot) belongs to the Alliaceae family. Recently, the ethanolic extract of Persian shallot has significant activity against some important clinical pathogens. The present study aimed to evaluate the *in-vitro* antibacterial potency of the ethanolic extracts of Persian shallot (Iranian Moosir) combined with common antibiotics against five clinically important antibiotic-resistant pathogens.

Methods: Antibacterial activities were determined using the disc-diffusion method. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were also evaluated using the broth microdilution method for the extract and selected antibiotics. In addition, the checkerboard technique was applied to evaluate the combined effects of the extract and antibiotics.

Results: The MICs of the extract and antibiotics were within the ranges of 4-16 mg/ml and 4-128 µg/ml, respectively. The MBCs of the extract and antibiotics were within the ranges of 8-16 mg/ml and 8-128 µg/ml, respectively. The results of the checkerboard technique showed that amikacin and trimethoprim/sulfamethoxazole had synergistic effects, while levofloxacin, imipenem, and vancomycin exerted antagonistic effects on the isolates in combination with the extract.

Conclusion: The *in-vitro* application of Persian shallot extract combined with amikacin and trimethoprim/sulfamethoxazole is recommended to effectively inhibit the growth of five clinically significant antibiotic-resistant pathogens.

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Introduction

Bacterial antibiotic resistance is a major public health concern across the world, which is mainly caused by the decreased efficacy of antibiotics due to the worldwide spread of resistant bacterial genes. The discovery of the first antibiotic was a remarkable breakthrough the treatment of infections. However, cases of antibiotic resistance were reported soon afterwards, and a dramatic rise has been observed in the rate of antibiotic-resistant infections over the past years (1-3). Therefore, further investigations are essential as to discover new antimicrobial compounds; for instance, the combination of herbal extracts and antibiotics is speculated to overcome this crisis (4, 5).

Numerous medicinal plants are known in the Iranian traditional medicine (6). The therapeutic potential of traditional medicine is a major area of interest in the field of infectious disease control (5). *A. hirtifolium* (Persian shallot) belongs to the Alliaceae family and is native to the west of Iran, especially the Zagros Mountains (7, 8).

Similar to the other members of the Alliaceae family, Persian shallot has significant antifungal, antibacterial, antiprotozoal, antiviral, and anti-inflammatory properties (7-10). Recent investigations have been focused on the

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antimicrobial properties of Persian shallot (Iranian Moosir), and they have mostly evaluated the antimicrobial properties of this compound (11-13). Until recently, no reliable evidence was presented regarding the antibacterial effects of Persian shallot in combination with antibiotics. The present study aimed to assess the antibacterial effects of Persian shallot ethanolic extract on some resistant bacteria and investigate the antibacterial effects of Persian shallot in combination with antibiotics on some resistant bacteria.

Materials and Methods

Bacterial Strains

Five antibiotic-resistant bacteria were isolated from urine cultures and selected for the examination of the antibacterial effects of the extract. The selected isolates included *Pseudomonas aeruginosa*, vancomycin-resistant *Enterococcus* (VRE), *Klebsiella pneumoniae, Acinetobacter baumannii*, and *Staphylococcus aureus.* The cultured organisms on blood agar plates were obtained from the microbiology laboratory of Qaem Hospital in Mashhad, Iran and confirmed by microbiological tests (Table 1).

Table 1. Identification tests for clinical obtained organisms

Organisms / Tests	*E. Faecium*	*S. aureus*	*K. pneumonia*	*P. aeruginosa*	*A. baumannii*
Gram Staining	(+)	(+)	(-)	(-)	(-)
Motility	.	(-)	.	(+)	(-)
Indole	.	.	(-)	(-)	(-)
H_2S	.	.	(-)	(-)	(-)
Catalase	(-)	(+)	(+)	(+)	(+)
Oxidase	(-)	(-)	(-)	(+)	(-)
Citrate	.	.	(+)	.	(+)
Methyl red	.	.	(-)	.	.
Voges-Proskauer	.	.	(+)	.	.
Lysine decarboxylase	.	.	(+)	.	.
TSI	.	.	.	K/N	K/N
Urease	.	.	.	(+)	(-)
Mannitol	(+)	(+)	.	.	.
Coagulase	.	(+)	.	.	.
DNase	.	(+)	.	.	.
Novobiocin	.	Sensitive	.	.	.
Growth in 6.5% NaCl	(+)	(+)	.	.	.
bile esculin	(+)	.	.	.	.
PYR	(+)	.	.	.	.
Vancomycin	Resistant	.	.	.	.
Growth at 45℃	(+)	.	.	.	(+)
Bacitracin	Resistant	.	.	.	.
Optochin	Resistant	.	.	.	.

K/N: No Sugars Fermented; (+): Positive result; (-): Negative result

Antimicrobial Screening Tests

In total, 14 commonly used antibiotic discs were purchased from Padtan Teb Co., Iran, and the antimicrobial susceptibility of the strains was examined using the disc-diffusion method in accordance with the guidelines of the Clinical Laboratory Standards Institute (CLSI 2018) (14).

Preparation of the Plant Material for Extraction

Fresh Persian shallot was prepared commercially and originated from the farms in

Mashhad. The plant sample was confirmed by the Research Center for Plant Sciences at Ferdowsi University of Mashhad, Iran (E1040-FUMH). The bulbs were separated, washed with tap water, and sliced to small pieces. At the ratio of 1:10 w/v, the Persian shallot pieces were combined with 50% hydroalcoholic solvent (96% ethanol, Merck, Darmstadt, Germany) and placed on a shaker at 200 grams and room temperature for 24 hours. The solution was filtered using Whatman No.1 filter paper, concentrated in a vacuum rotary evaporator at the temperature of 45°C in 220 grams, and placed in an oven at the temperature of 45°C until drying.

Checkerboard Assay of the Extract and Antibiotics

Five antibiotics were obtained from Sigma-Aldrich (www.sigmaaldrich.com). The antibiotic selection for the broth microdilution synergy test was based on formulary, site of action, and CLSI guidelines.

The checkerboard assay has been described in the study by Mandal et al. (15). Bacterial suspensions equivalent to 0.5 McFarland standard ($1.5x10^8$CFU/ml) were inoculated into plates by streaking in the duplicates before incubation at the temperature of 37°C for 24 hours. Based on the minimum inhibitory concentration (MIC) results, the fractional inhibitory concentration (FIC) was calculated from the lowest concentration of the antibiotic and extract combination, thereby allowing no observable growth of the examined isolates on the plates (16). In addition, each factor was determined for their FIC value using the following standard formula:

$$FIC\ extract = \frac{MIC\ of\ extract\ in\ combination\ with\ antibiotic}{MIC\ of\ extract\ alone}$$

$$FIC\ antibiotic = \frac{MIC\ of\ antibiotic\ in\ combination\ with\ extract}{MIC\ of\ antibiotics\ alone}$$

The correlations between the extract and selected antibiotics were measured using the following formula:

FIC index = ΣFICi = FIC (antibiotic) + FIC (extract)

Based on the checkerboard technique, FICi was defined as FICi≤0.5 as synergy and antagonism by FICi>4.0. In addition, FICi within the range of 0.5-1.0 was explained as additivity, while the range of 1-4 denoted indifference (17).

Results

Antibiotic Susceptibility Assay

The results of the antibiotic susceptibility test revealed that the isolates were resistant to most of the antibiotics (Table 2).

Table 2. Antibiotic susceptibility by agar disk diffusion technique

Bacterial Isolates	AM	GM	AN	CP	LEV	CAZ	IMI	SXT	V	E	NOR	D	TE	CC
P. aeruginosa	-	7mm (R)	12mm (R)	11mm (R)	6mm (R)	10mm (R)	13mm (R)	-	-	-	6mm (R)	-	-	-
VRE	8mm (R)	-	-	6mm (R)	8mm (R)	-	-	-	9mm (R)	-	-	18mm (S)	20mm (S)	-
A. baumannii	-	13mm (I)	17mm (S)	12mm (R)	14mm (I)	9mm (R)	10mm (R)	6mm (R)	-	-	-	6mm (R)	-	-
S. aureus	-	7mm (R)	-	-	10mm (R)	-	-	6mm (R)	-	8mm (R)	-	-	10mm (R)	6mm (R)
K. pneumoniae	8mm (R)	6mm (R)	8mm (R)	10mm (R)	11mm (R)	9mm (R)	6mm (R)	6mm (R)	-	-	-	-	-	-

R: Resistant; S: Sensitive; I: Intermediate; AM:Ampicillin; GM:Gentamicin; AN:Amikacin; CP:Ciprofloxacin; LEV:Levofloxacin; CAZ:Ceftazidime; IMI:Imipenem; SXT: Trimethoprim/sulfamethoxazole; V:Vancomycin; E:Erythromycin; TE:Tetracycline; NOR:Norfloxacin; VRE: Vancomycin-Resistant *Enterococcus*; D: Doxycycline

Determination of the minimum inhibitory concentrations (MIC)

In general, the sensitivity of the isolates from the most resistant to the most sensitive strains was in the following sequence: *K. pneumoniae*>*S. aureus*>*A. baumannii*>VRE>*P. aeruginosa*. Table 3 shows the MICs of the Persian shallot extract and antibiotics using the broth microdilution method.

Determination of the Minimum Bactericidal Concentration (MBC)

Table 4 shows the minimum bactericidal concentrations (MBCs) of the Persian shallot extract and antibiotics. Accordingly, the results

regarding the pattern of the MBCs were similar to those of the MICs.

The Checkerboard Assay Results

Table 5 shows the results of the checkerboard assay. Accordingly, the Persian shallot extract had synergistic effects against all the isolates in combination with Ampicillin (AM) and Sulfamethoxazole/Trimethoprim (SXT), while antagonistic effects were observed with V, IMI, and LEV.

Table 3. MIC of Persian shallot extract and different antibiotics

Bacterial isolates	AN (µg/ml)	LEV (µg/ml)	IMI (µg/ml)	SXT (µg/ml)	V (µg/ml)	MIC Extract (mg/ml)
P. aeruginosa	128(R)	16(R)	16(R)	-	-	4
VRE	-	128(R)	-	-	>128(R)	4
A. baumannii	16(S)	4(I)	32(R)	128(R)	-	4
S. aureus	-	16(R)	-	128(R)	8(I)	8
K. pneumoniae	128(R)	8(R)	128(R)	128(R)	-	16

R: Resistant; S: Sensitive; I: Indifference; AN:Amikacin; LEV:Levofloxacin; IMI:Imipenem; V:Vancomycin; SXT: Trimethoprim/sulfamethoxazole VRE: Vancomycin-Resistant *Enterococcus*

Table 4. MBC of Persian shallot extract and different antibiotics

Bacterial isolates	AN (µg/ml)	LEV (µg/ml)	IMI (µg/ml)	SXT (µg/ml)	V (µg/ml)	MIC Extract (mg/ml)
P. aeruginosa	128	32	32	-	-	8
VRE	-	128	-	-	128	8
A. baumannii	16	8	64	128	-	16
S. aureus	-	32	-	128	16	8
K. pneumoniae	128	16	128	128	-	16

AN:Amikacin; LEV:Levofloxacin; IMI:Imipenem; V:Vancomycin; SXT: Trimethoprim/sulfamethoxazole VRE: Vancomycin-Resistant *Enterococcus*

Table 5. Combination effect of the extract and antibiotics

	Interaction (Extract/Antibiotic)				
Bacterial isolates	**AN**	**LEV**	**IMI**	**SXT**	**V**
P. aeruginosa	S	A	A	-	-
VRE	-	A	-	-	A
A. baumannii	S	A	A	S	-
S. aureus		A	-	S	A
K. pneumoniae	S	A	A	S	-

AN:Amikacin; LEV:Levofloxacin; IMI:Imipenem; V:Vancomycin; SXT: Trimethoprim/sulfamethoxazole VRE: Vancomycin-Resistant *Enterococcus*; S: Synergism; A: Antagonism

Discussion

Multidrug-resistant bacteria is a complicated health concern due to the ability of bacteria to develop resistance to antimicrobial drugs (2, 4). Drug combination therapy could be utilized to increase the spectrum of antimicrobial activity to overcome resistant strains (2). The combination of medicinal plant extracts and synthetic antimicrobial agents is considered to be a definitive treatment for infectious diseases (18). Prior studies have highlighted the importance of the antimicrobial properties of *A. hirtifolium* (10, 12, 19). Therefore, the use of these compounds has attracted the attention of researchers given the fewer side-effects in combination with antibiotics (7, 20).

According to the results of the present study, most of the isolates were resistant to antibiotics at varied levels. Five antibiotics were selected based on various mechanisms of action, including amikacin to inhibit bacterial protein synthesis, imipenem to inhibit bacterial cell wall synthesis, trimethoprim/sulfamethoxazole to inhibit folic acid synthesis, vancomycin to inhibit cell wall synthesis, and levofloxacin to inhibit DNA gyrase and topoisomerase IV (21).

Our findings are consistent with the previous observations demonstrating that both gram-positive and gram-negative isolates are susceptible to herbal extracts. Among the five tested isolates in the current research, the *A. hirtifolium* extract had the most potent antibacterial activity against *P. aeruginosa,* VRE, and *A. baumannii,* while *K. pneumonia*e was the most resistant isolate to the extract. Various concentrations of the MIC and MBC in similar studies could be attributed to factors such as extraction methods, chemical properties of the solvent, plant variety, and diversity of the interactions between isolates and extracts.

Previous studies have reported the chemical composition and bioactive compounds of Allium

plants, especially the volatile organosulfur-containing compounds such as diallyl trisulfide, diallyl thiosulfinate (allicin), diallyl disulfide, and S-allylcysteine. Furthermore, these plants contain polyphenolic and flavonoid compounds (e.g., shallomin), which have antimicrobial properties (11, 12, 24, 25).

The results of the present study demonstrated the synergistic effects of the amikacin (AN) plus extract and SXT plus extract. However, synergism was not observed with the other selected antibiotics plus extract, while antagonism was detected with the V and IMI at the same action sites plus extract. The underlying mechanism of the synergistic and antagonistic effects of Allium plants remains unknown. *A. hirtifolium* has been reported to decrease oxygen uptake, induce oxidative stress, inhibit the synthesis of lipids, proteins, and nucleic acids, damage cell membrane integrity, and reduce the growth of the organism (12). Nevertheless, data is scarce regarding the antagonisms and synergism of extract plus antibiotics, and it remains unclear which factors may influence the mechanism. Antagonism may be associated with the destruction or change in the antibiotic structure by herbal extracts.

Limitations of the Study

One of the limitations of the present study was the source of Moosir, which might have affected the ingredients and could be analyzed via high-performance liquid chromatography.

Conclusion

According to the results, the ethanolic extract of *A. hirtifolium* affected the bioactive antimicrobial source. Therefore, the combinatorial use of *A. hirtifolium* extracts and antibiotics could effectively eradicate the drug-resistant pathogens involved in hospital-acquired infections.

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