In Vitro Antibacterial Properties of Pistachio (Pistacia *Vera L.*) Rosy Hull Phenolic Extracts

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Abstract

Introduction: Plants have a wide range of secondary metabolites with antimicrobial activity such as polyphenols, quinines, flavonoids, and alkaloids, with most of them acting as a plant defense against pathogens. This study aimed to evaluate the in vitro activity of pistachio rosy hull extracts against Gram-positive (Bacillus cereus, Listeria monocytogenes, Staphylococcus aureus, and Streptococcus pyogenes), Gram-negative (Escherichia coli, Klebsiella pneumonia, and Pseudomonas aeruginosa), and clinical isolates including methicillin-resistant S. aureus (MRSA) bacterial strains.

Materials and Methods: The antibacterial activity of extracts was determined using disc diffusion and microdilution broth methods. The synergistic effects and susceptibility pattern of clinical isolates were determined using antibiotic discs.

Results: All extracts of rosy hull showed a significant bacteriostatic and bactericidal effect on *S. aureus*. However, the aqueous extract was the most active one against MRSA strains. The MICs of the aqueous and methanolic hull extracts against *S. aureus* were 0.78 and 1.56 mg/mL, respectively. The MICs of the aqueous and methanolic hull extracts against MRSA strains were 3.12 and 6.25 mg/mL, respectively. Furthermore, in many cases, the extracts enhanced the action of antibiotics against Gram-positive bacteria including *S. aureus* and *Listeria monocytogenes*.

Conclusions: The antibacterial activity of pistachio rosy hull was reported for the first time in this study. The results suggested that pistachio rosy hull is a rich source to be used to combat problems caused by MRSA in hospitals as well as *S. aureus* skin infections.

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1. Introduction

Plants have a wide range of secondary metabolites with antimicrobial activity such as polyphenols, quinines, flavonoids, tannins, coumarins, terpenoids, and alkaloids, with most of them acting as a plant defense against pathogens (1). Plant extracts are also considered as potential antimicrobial agents in food industries due to the adverse effects of synthetic antimicrobials (2). It has been shown that essential oils and their components have antibacterial effects on food-borne pathogens both *in vitro* and in foods especially Gram-positive ones (3).

Pistacia Vera L., which is the only species grown commercially, belongs to the Anacardiaceae family. Iran, the United States of America, and Turkey are the main countries that produce pistachio. Three species of pistachio grow as wild plants in Iran, including *P. vera*, *P. khinjuk*, and *P. atlantica*. Iran produces more than 300,000 tons pistachio per year and is the major pistachio exporter in the world (4, 5). *Pistacia* species were traditionally used for treating a range of health problems in different countries. Parts of the plant such as leaf, resin, fruit, and aerial parts have had many applications in traditional medicine (6). Several studies have reported antioxidant and antimicrobial activities of different parts of the pistachio tree (7-11). Pistachio fruits are covered by the fleshy hull (mesocarp and epicarp). It has been shown that pistachio hull has a high content of phenolic and antioxidant compounds (12, 13). The chemical composition of pistachio hull essential oil has been evaluated during fruit development. The most important compounds at fruit development stages, ripe fruits, leaves, and unripe fruits were alpha-pinene and terpinolene (14, 15).

Currently, antibiotic resistance is a serious health problem caused by frequently using antibiotics against bacteria in humans and animals and also the mobility of populations (16). It seems likely that antibiotic resistance will raise concerns over the treatment of bacterial infections, increase in mortality, and economic problems in the near future (17). In the last couple of decades, methicillin-resistant *Staphylococcus aureus* (MRSA) has caused an increasing number of infections worldwide. Community-associated MRSA (CA-MRSA) has recently changed the epidemiology of MRSA infections compared to hospital-associated MRSA (HA-MRSA) (18). Individuals without any risk factors are exposed to CA-MRSA infections leading to frequent skin and soft-tissue infections (19). Therefore, there is an urgent need to develop novel agents to fight infections caused by MRSA strains. Plant-derived compounds are of considerable interest to

identify natural agents with antibacterial activities. Continued exploration of compounds derived from plants can lead to the development of several kinds of antibacterial compounds including antibiotics, multidrug resistance pump (MDR) inhibitors, and those that target virulence factors (20). The main aim of this research was to evaluate *in vitro* antibacterial effects of pistachio rosy hull extracts on reference Gram-positive and Gram-negative bacteria as well as clinically isolated *S. aureus* strains.

2. Materials and Methods

2.1. Plant materials

Pistachio (*Pistacia vera* L.) rosy hulls were collected from Rafsanjan, Kerman province. The hulls were air-dried at room temperature for 2 weeks, ground into powder, and then stored at –20°C.

2.2. Pistachio rosy hull phenolic extracts

The hull phenolic compounds were extracted using three different solvents (water, methanol, and water/methanol (1:1 v/v)). Briefly, 50 g of powdered sample was macerated with 200 mL of each solvent for 48 h at room temperature under continual shaking. Each extract was filtered by filter paper under vacuum and then dried at 35°C using a rotary evaporator. And finally, crude hull extracts were dissolved in sterile distilled water to prepare a stock solution (400 mg/mL) for further studies on strains.

2.3. Bacterial strains and culture media

We used several reference strains obtained from Persian Type Culture Collection: *Bacillus cereus* PTCC 1154, *Escherichia coli* PTCC 1395, *Klebsiella pneumonia* PTCC 1053, *Listeria monocytogenes* PTCC 1297, *Pseudomonas aeruginosa* PTCC 1707, *Staphylococcus aureus* PTCC 1189, and *Streptococcus pyogenes* PTCC 1447. Moreover, three clinical strains (*S. aureus*) isolated from patients with a skin infection were studied in the clinical laboratory of Rafsanjan University of Medical Sciences. All bacteria were cultured in Mueller-Hinton and BHI (Brain Heart Infusion) broth at 37°C for 24 h and stored at 4°C. Mueller-Hinton broth and agar were used to test the susceptibility of the bacteria to the hull extracts and antibiotics.

2.4. Antibacterial susceptibility tests

Antibacterial activity was evaluated by disc diffusion, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) methods. Initially, a 0.5 McFarland standard was prepared using sterile saline and overnight cultures. Then, Mueller-Hinton agar plates were inoculated with a standard suspension of each strain using sterile cotton swabs. The discs, 6 mm in diameter, were impregnated with 30 µl of different concentrations of crude extracts

(10, 50, and 200 mg/mL) and placed on agar plates. All plates were incubated for 24 h at 37°C to record inhibition zones due to antibacterial activity. The disc diffusion tests were conducted in triplicate. The MIC of each strain was evaluated through the microdilution (tube) broth method according to CLSI methods (2006). Briefly, two-fold dilutions of extracts were prepared in Mueller-Hinton broth to achieve various concentrations, ranging from 200 to 0.39 mg/mL for the hull extracts. The 0.5 McFarland suspension was further diluted in broth to prepare 1 x 10^6 CFU/mL inoculum and then 1 mL of the inoculum was added to each test tube (1 mL). Thus, dilution (1:2) of concentrations and inoculum led to 5 x 10^5 CFU/mL final inoculums in each tube. The test tubes were incubated for 24 h at 37°C. After incubation, the lowest concentration of the extracts without any visible bacterial growth was considered to be MIC compared to the positive control. In addition to the MIC, the MBC of each strain was then evaluated by subculturing 100 µl of the suspension from tubes without visible turbidity onto Mueller-Hinton agar plates. The agar plates were incubated for 24 h at 37°C and the lowest concentration of the extracts without any colony formation was considered to be MBC.

2.5. Antibiotic susceptibility of isolates

The antibiotic susceptibility pattern of three clinical isolates of *S. aureus* was determined by a disc diffusion method using methicillin (5 µg), penicillin (10 U), vancomycin (30 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), and ceftriaxone (30 µg). Inoculum suspensions (0.5 McFarland standards) of isolates were spread on Mueller–Hinton agar plates using sterile cotton swabs and the plates were incubated overnight at 37°C. The susceptibility profiles of *S. aureus* isolates were reported according to the updated CLSI interpretive criteria (2012).

2.6. Synergistic/ antagonistic interactions with antibiotics

Synergistic and antagonistic effects on several standard antibiotics were evaluated by disc diffusion assay. Briefly, the ciprofloxacin (5 µg), chloramphenicol (30 µg), ceftriaxone (30 µg), and cefixime (5 µg) discs were impregnated with 30 µl of the pistachio hull extracts (50 mg/mL). The disc diffusion tests were conducted in triplicate. The inhibition zones were recorded to determine whether the extracts had any synergistic effect on antibiotics.

3. Results

3. 1. Evaluation of antibacterial activity of pistachio rosy hull extracts

The results reported in Table 1 show the activity of different hull extracts against bacterial strains determined by the disc diffusion test. The most sensitive strain to the three types of extracts was *S. aureus* that produced inhibition zones to all concentrations whereas *K. pneumonia* did not produce any inhibition zones around the discs. Furthermore, the extracts were effective against other bacterial strains, Gram-positive and Gram-negative, at high concentrations (50 and 200 mg/mL). The MIC and MBC results of extracts are shown in Table 2. *S. aureus* had the lowest MIC and MBC of aqueous (MIC, 0.78 mg/mL), methanolic and hydroalcoholic (MIC, 1.56 mg/mL) extracts, while *K. pneumonia* had the highest MIC value (MIC, 100 mg/mL) compared to other bacteria. In contrast to other bacterial strains, all extracts exhibited very poor bactericidal activity (MBC> 200 mg/mL) against *B. cereus*. According to the findings presented in Table 1 and 2, the antibacterial activity of the aqueous extract of the rosy hull was more effective than that of the methanolic and hydroalcoholic extracts against *S. aureus*, *B. cereus*, *E. coli*, *L. monocytogenes*, and *S. pyogenes*.

3. 2. Effect of pistachio rosy hull extracts on *S. aureus* isolates

Table 3 presents the determination of antibiotic sensitivity of *S. aureus* isolates using disc diffusion method. The results showed that all clinical isolates were resistant to penicillin and two isolates were identified as MRSA (methicillin-resistant *S. aureus*). Additionally, *S. aureus* (isolate 1) was found to be resistant to chloramphenicol and ceftriaxone (Table 3). Although all of the hull extracts exhibited considerable antibacterial effects, aqueous extract was highly active against *S. aureus* clinical isolates (Table 1). The MIC value of aqueous extract was 3.12 (mg/mL) for clinical isolates (Table 2).

3.3. The synergy of hull extracts with antibiotics against bacterial strains

Finally, the combined effects of aqueous and methanolic hull extracts with several antibiotics were examined to find out whether there was any synergy against the tested bacterial strains (Table 4). Both extracts produced synergistic effects on chloramphenicol against almost all strains. The synergistic effect of the aqueous extract was also observed with ceftriaxone and cefixime. Interestingly, most of the antibiotics in combination with extracts showed synergistic activity against *S. aureus*.

Table 1- Antibacterial activity of pistachio rosy hull extracts against standard strains and clinical isolates. The data are shown as mean ± SEM of three experiments

Solvent	**Methanol/Water**			**Methanol**			**Water**		
Concentration (mg/mL)	**10**	**50**	**200**	**10**	**50**	**200**	**10**	**50**	**200**
Bacterial strains	**Inhibition zones (mm)**								
***B. cereus* PTCC 1154**	-	8.00 ± 0.00	13.67 ± 0.33	-	-	10.00 ± 0.58	-	-	11.33 ± 0.67
***E. coli* PTCC 1395**	-	13.33 ± 0.67	21.00 ± 0.58	-	-	12.33 ± 0.67	-	-	13.67 ± 0.67
***K. pneumonia* PTCC 1053**	-	-	-	-	-	-	-	-	-
***L. monocytogenes* PTCC 1297**	-	10.33 ± 0.33	18.67 ± 0.33	-	-	8.67 ± 0.33	-	-	11.00 ± 0.58
***P. aeruginosa* PTCC 1707**	-	-	12.33 ± 0.33	-	7.33 ± 0.33	11.33 ± 0.88	-	-	11.67 ± 0.67
***S. aureus* PTCC 1189**	19.33 ± 0.67	28.00 ± 0.58	34.67 ± 0.67	16.00 ± 0.58	23.00 ± 0.58	27.33 ± 0.67	16.67 ± 0.67	23.00 ± 0.58	30.00 ± 1.15
***S. pyogenes* PTCC 1447**	-	11.67 ± 0.67	18.67 ± 0.67	-	-	12.67 ± 0.33	-	-	15.67 ± 0.88
***S. aureus* (isolate 1)**	7.00 ± 0.00	14.33 ± 0.88	22.67 ± 0.88	-	10.33 ± 0.33	17.33 ± 0.67	-	9.00 ± 0.00	18.00 ± 0.58
***S. aureus* (isolate 2)**	8.00 ± 0.00	16.33 ± 0.33	22.33 ± 1.33	-	11.33 ± 0.33	16.00 ± 1.00	-	10.00 ± 0.00	15.00 ± 1.00
***S. aureus* (isolate 3)**	7.67 ± 0.33	14.00 ± 0.00	21.33 ± 0.88	-	10.33 ± 0.33	14.33 ± 0.33	-	8.67 ± 0.33	14.67 ± 1.20

Table 2- MIC and MBC of pistachio rosy hull extracts (mg/mL) against bacterial strains and clinical isolates

	Methanol/Water		Methanol		Water	
	MBC	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**
Bacterial strains						
***B. cereus* PTCC 1154**	6.25	> 200	12.5	> 200	12.5	> 200
***E. coli* PTCC 1395**	6.25	12.5	12.5	50	12.5	50
***K. pneumonia* PTCC 1053**	100	200	100	200	100	200
***L. monocytogenes* PTCC 1297**	3.12	6.25	25	50	12.5	25
***P. aeruginosa* PTCC 1707**	25	50	12.5	25	12.5	25
***S. aureus* PTCC 1189**	0.78	1.56	1.56	3.12	1.56	3.12
***S. aureus* (isolate 1)**	3.12	6.25	6.25	12.5	6.25	12.5
***S. aureus* (isolate 2)**	3.12	12.5	6.25	12.5	6.25	12.5
***S. aureus* (isolate 3)**	3.12	6.25	6.25	12.5	6.25	12.5

Table 3- Antibiotic susceptibility of *S. aureus* clinical isolates. P, Penicillin; C, Chloramphenicol; CP, Ciprofloxacin; VA, Vancomycin; CRO, Ceftriaxone; ME, Methicillin; S, Susceptible; I, Intermediate; R, Resistant

Antibiotics	**P**	**C**	**CP**	**VA**	**CRO**	**ME**
Isolates						
***S. aureus* (isolate 1)**	R	R	S	S	R	R
***S. aureus* (isolate 2)**	R	S	S	S	I	R
***S. aureus* (isolate 3)**	R	S	S	S	S	I

Table 4- Effects of aqueous and methanolic rosy hull extracts of pistachio in combination with antibiotics against bacterial strains. C, Chloramphenicol; CP, Ciprofloxacin; CRO, Ceftriaxone; CFM, Cefixime. Data are shown as mean ± SEM of three experiments

	Antibiotics				**Antibiotics+ aqueous extract**				**Antibiotics+ methanolic extract**			
	C	CP	CRO	CFM	C	CP	CRO	CFM	C	CP	CRO	CFM
Bacterial strains	**Inhibition zones (mm)**											
***B. cereus* PTCC 1154**	25.33 ± 0.33	25.33 ± 0.67	8.33 ± 0.33	-	26.67 ± 0.33	23.00 ± 0.58	8.67 ± 0.33	-	28.67 ± 0.67	25.33 ± 0.33	8.00 ± 0.00	-
***E. coli* PTCC 1395**	25.67 ± 0.67	40.33 ± 0.33	35.33 ± 0.33	27.00 ± 0.58	26.33 ± 1.20	38.67 ± 0.67	36.33 ± 1.20	30.00 ± 1.00	30.00 ± 0.58	36.33 ± 0.67	37.00 ± 0.00	30.67 ± 1.33
***K. pneumonia* PTCC 1053**	25.33 ± 0.88	35.33 ± 0.33	30.33 ± 0.33	31.33 ± 0.33	28.67 ± 0.67	35.33 ± 0.88	33.00 ± 1.15	34.33 ± 0.88	25.67 ± 0.67	29.67 ± 1.20	27.67 ± 0.88	29.00 ± 1.15
***L. monocytogenes* PTCC 1297**	24.67 ± 0.67	23.67 ± 0.33	14.00 ± 0.00	-	25.00 ± 0.58	21.67 ± 0.88	15.33 ± 0.67	10.33 ± 0.33	27.67 ± 0.88	22.67 ± 0.33	15.67 ± 0.33	9.00 ± 0.00
***P. aeruginosa* PTCC 1707**	13.33 ± 0.33	37.00 ± 0.58	20.33 ± 0.67	-	16.67 ± 0.33	37.33 ± 0.88	22.33 ± 0.67	-	11.33 ± 0.33	36.00 ± 0.58	20.33 ± 0.33	-
***S. aureus* PTCC 1189**	25.00 ± 0.00	28.33 ± 0.33	22.00 ± 0.58	7.67 ± 0.33	25.33 ± 0.88	30.33 ± 0.33	21.33 ± 0.88	15.33 ± 0.67	26.67 ± 0.67	28.67 ± 0.88	22.33 ± 0.88	10.00 ± 0.58

4. Discussion

Although many antibiotics are currently available worldwide, most of the bacterial pathogens exhibit resistance to different kinds of antibiotic drugs. Therefore, researchers try to develop novel antimicrobials and vaccines against infections caused by bacteria such as MRSA.

There are a limited number of studies on antimicrobial effects of hull extracts of different plants (21). For instance, it has been shown that green hull extracts of *Juglans regia* have antibacterial activity against several bacterial pathogens. In this study, the ethanolic green hull extract of *J. regia* had the best activity compared with other extracts (22). Another report investigated antibacterial activity of aqueous hull extracts of some legumes used in India (mung bean, Bengal gram, and pigeon pea). The hull extracts had high phenolic and flavonoid content. The results showed that the hull extracts were effective against Gram-positive bacteria (*B. cereus* and *S. aureus*) whereas they had no effect on Gram-negative bacteria (*E. coli* and *P. fluorescens*) (23).

Despite a small number of reports on pistachio hull activities, its antioxidant and antimicrobial properties have been demonstrated. Rajaei et al. (2010) found that water was the best solvent for the extraction of phenolic compounds of pistachio green hull. They also revealed that Gram-positive bacteria (*B. cereus* and *S. aureus*) were susceptible to pistachio green hull extracts, but there were no inhibition zones against Gram-negative bacteria (13). In another study, water was identified as a solvent to extract the highest phenolic compounds of pistachio hull (12). A recent study by Bisignano et al. investigated the antimicrobial activities of polyphenols from raw shelled and roasted salted pistachios. Interestingly, polyphenolic extracts showed bactericidal effects against Gram-positive bacteria, particularly *S. aureus and L. monocytogenes* (24). However, these extracts were inactive against other microorganisms including Gram-negative bacteria, fungi, and yeasts. The data generally indicated that Gram-positive bacterial strains are more sensitive to polyphenols than Gram-negative bacterial strains.

The present study provided a comprehensive evaluation of the pistachio rosy hull extracts properties against reference and clinical bacterial strains (MRSA). Following the studies described above, our results indicated that the aqueous extract suppresses bacterial pathogens growth more effectively due to higher bioactive compounds. Our findings demonstrated that pistachio rosy hull extracts had a maximum inhibitory effect on the growth of *S. aureus* as a very important pathogenic microorganism resistant to several available antibiotics. It is noteworthy that high concentrations (> 50 mg/mL) of the aqueous extract at showed more antibacterial potency than the antibiotic discs tested against *S. aureus*.

Similar observations were made against clinically isolated strains, and therefore the hull extract prepared by water had a major impact on MRSA strains.

Fortunately, unlike most medicinal plants that grow in a particular area, pistachio trees are often found in Asia, Europe, and North America. Rosy hull is a by-product of pistachio processing and may lead to pollution of the environment. It is estimated that an enormous amount of the pistachio hull is produced annually during the harvest season. There is only one recently published article on bioactivities of pistachio rosy hull extract that revealed its antioxidant and antimutagenic properties (25). Thus, further studies are needed to find practical applications of pistachio hull in medicine and agriculture. Further pharmacological and microbiological studies are required to elucidate the impact of pistachio hull on different microorganisms. Besides, *in vivo* investigations and some human trials are necessary for assessing antimicrobial potentials of plant-derived phenolic compounds (26).

Collectively, the pistachio rosy hull can be considered as a novel source of anti-Staphylococcal agents. Due to its strong action against *S. aureus* and MRSA strains as well as the enhancement of anti-Staphylococcal activity of antibiotics as shown in the present study, pistachio rosy hull can be used to develop formulations against antibiotic-resistant *S. aureus* strains, especially in health care facilities. This study also points to the potential activity of the pistachio rosy hull compounds against food-borne and oral pathogens.

Conflict of Interest

The authors declared no conflict of interest.

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