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Research Article

Chemical Composition and Antibacterial and Anti-biofilm Activity of Acetone Extract of *Pistacia atlantica* Leaf, Fruit, and Gall

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Abstract

Background: Bacterial infectious diseases caused by antibiotic resistance and biofilm formation agents are one of the most important challenges researchers and doctors face. Therefore, many studies have been done on medicinal plants to find phytochemical antibacterial and anti-biofilm agents.

Objectives: In this study, the antibacterial and anti-biofilm activities of the acetone and aqueous extract of *Pistacia atlantica* leaf, fruit, and gall were evaluated against some bacteria.

Methods: The leaves, fruits, and galls of *P. atlantica* were collected from the forests of Lorestan province, Iran. Antibacterial effects of extracts were studied by well diffusion method against *Staphylococcus aureus*, *Bacillus cereus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Escherichia coli*. Microdilution method was used to evaluate the minimum inhibitory concentration and minimum bactericidal concentration of extracts. Anti-biofilm activity of acetone and aqueous extracts in sub-lethal concentration was investigated by crystal violet-stained microtiter method.

Results: Acetone extract of *P. atlantica* had significant antibacterial effects against tested bacteria except for *E. coli*. There was a significant relationship between antibacterial activity and extract concentration ($P<0.05$). The minimum inhibitory concentration of acetone and aqueous extract of leaf, fruit, and galls ranged from 3-12.5 mg/mL. The acetone extract of *P. atlantica* galls had a high inhibitory effect on *S. aureus* and *P. aeruginosa* biofilm formation at a concentration of 12.5 and 25.0 mg/mL, respectively.

Conclusions: The gall extracts of *P. atlantica* have a significant inhibitory effect against bacteria, which is probably related to certain active compounds. These extracts inhibited biofilm formation of *S. aureus* and *P. aeruginosa*.

Keywords: Plant extract, *Pistacia atlantica*, Antibacterial agent, Anti-biofilm

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Background

The use of antibiotics to prevent and control infection is very common, but infection and the prescription of antibiotics have led to the spread of resistant strains. It is estimated that more than 80% of human infections are related to bacterial biofilm formation. Bacterial biofilms are involved in the development of chronic human infections, dental plaque, and catheter infections. Currently, the use of herbal substances for treating diseases and microbial infections has been considered (1).

The Zagros mountain ranges are one of the main areas of wildlife growth. *Pistacia atlantica*, known as Bennet in Iran, is a tree with 2 to 7 meters' height that grows in the cold and humid weather of the Zagros (2, 3). The leaf of the *P. atlantica* is used to treat hypertension, cough, sore throat, eczema, and stomachache. *P. atlantica* leaves inhibit colon cancer cells, in vitro (4, 5). The nut of *P. atlantica* contains fatty acids including oleic acid, linoleic acid, alpha linoleic acid and palmitic acid (3, 6). Phenolic compounds have been identified in *P. atlantica* fruits. The galls are induced by the aphid *Slavum wertheimae* on branches of *P. atlantica* trees (7). Galls are abnormal growths of some parts of plants that are caused by insects. Galls are very diverse in morphologically and biochemically (8, 9). The molecular mechanism of gall formation is still unknown. Ecological studies and phylogenetic analyses have shown that insects control the behavior of galls (10).

It was demonstrated that the essential oil of *P. atlantica* subsp. *Kurdica* had broader antibacterial effects against the Gram-positive bacteria than Gram-negative bacteria and this essential oil was suggested to be used as a preservative in food industry to increase safety and reduce food pathogen risks (4). Kordbacheh et alshowed that active components of *P. atlantica* have high anti-Quorum sensing (QS) activities and may potentially treat chronic infections caused by *Pseudomonas aeruginosa* (11). The extracts of *P. nigra* exhibited antimicrobial activity and antibiofilm effects greater than 70% (12).

Objectives

In this study, the antibacterial and anti-biofilm activities of the acetone and aqueous extract of leaf, fruit, and gall of *Pistacia atlantica* were evaluated against pathogenic bacteria.

Methods

Plant Materials

Leaves, fruits, and galls of *P. atlantica* were collected from forests of Lorestan province, Iran (Oshtaran Kuh-Path Gahar Lake: 49°14'08"E, 33°209'28"N). Samples were approved by experts of the Agricultural and Natural Resources Research Center. The specimens were dried in shade, prepared in powder form and kept in dark glass at 4°C. The dried specimen powder was extracted by Soxhlet procedure for 5-6 hours. The different concentration of extracts was prepared by serial dilution method (final concentrations from 3 to 100 mg/mL).

Microbial Strains

Antibacterial activity of the extracts was evaluated against *Staphylococcus aureus* (ATCC: 6538), *Bacillus cereus* (ATCC: 11778), *Enterococcus faecalis* (ATCC: 29212), *P. aeruginosa* (ATCC: 9027) and *E. coli* (ATCC: 25922). These strains were obtained from the microbial collections of the Iranian Research Organization for Science and Technology (IROST). All bacterial strains were reconstituted according to standard methods. The bacterial suspensions were adjusted to 0.5 McFarland standards.

Screening for Antibacterial Activity

Well diffusion method was used to evaluate the antimicrobial effect of the extracts. Final concentrations of each extract from 3 to 100 mg/mL were added to the wells. Sterile distilled water served as negative control and Imipenem (10 µg/mL) was used as a positive control. After 24 hours of incubation at 37°C, the diameter of the growth inhibition zone around each well was measured (13). The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were determined by the microdilution method. 100 µL of different concentrations of extracts were added in to each well of a 96-well sterile plate, separately. 100 µL of bacterial inoculum (1.5×10^7 cfu/mL) were added to the wells. The plates were incubated at 37°C for 24 hours. After incubation, the optical density (OD) of each well was measured at a wavelength of 630 nm by ELISA reader. After incubation, the lowest concentration with no visible growth was defined as the MIC. The MBC was determined by plating 10µl of well samples on nutrient agar. The lowest concentration with no visible growth on plates was the MBC (14-16).

Screening for Anti-biofilm Activity

The acetone and aqueous extracts of *P. atlantica* were tested for their potential to prevent biofilm formation of *S. aureus* and *P. aeruginosa* by the microtiter plate method (17). The extracts were added to the Brain Heart Infusion broth including 1% glucose (3.0, 6.25, and 12.5, mg/mL). In each well of a sterile 96-well microtiter plate, 150 µL of each concentration was added. All bacterial cultures were adjusted to 10^8 CFU/mL by absorbance (0.08-0.1 at 630 nm) in a spectrophotometer and 150 mL of this bacterial suspension was added to each well (18).

After 24 hours of incubation, the plates were washed and the contents of the wells were evacuated. The biofilm created by cells attached to wells were fixed in the plate wells by adding 150 µL of ethanol for 15 minutes. TThen, the ethanol was removed, and 200 µL of a 2% violet crystal solution (for 20 min) were add . After washing and drying the wells, 200 µL of 33% (v/v) glacial acetic acid (Merck, Germany) were added per well. 300 µL of brain heart infusion (BHI) medium containing 1% glucose was used as negative control and 150 µL of BHI medium containing 1% glucose with 150 mL of bacterial suspension was considered as a positive control. The optical density was measured at 495 nm by an ELISA reader (Statfax-2100, USA). The biofilm formation reduction (%) was calculated by the following formula (19, 20):

$$\left\{\frac{(\text{OD of negative controls}-\text{OD of negative controls})-(\text{OD of Test well}-\text{OD of negative controls})}{\text{OD of negative controls}-\text{OD of negative controls}}\right\}\times 100$$

Gas Chromatography/Mass Spectrometry (GC/MS) Analysis

The ingredient of *P. atlantica* gall were analyzed by GC/MS. The GC/MS analysis was done with an Agilent 5975 GC-MSD system equipped with HP-5MS capillary column (30 m × 0.25 mm, 0.25 µm film thickness). Helium was used as the carrier gas, at a flow rate of 1.2 mL/min. The oven temperature was maintained at 50°C for 3 minutes and programmed to 200°C at a rate of 5°C/min, and kept constant at 290°C for 3 minutes, at the split mode. The injector temperature was set at 280°C. Identification of the components of the extract was accomplished based on comparison of retention times indices using a MS library.

Statistical Tests

Each experiment was repeated three times. Statistical analysis was performed using SPSS software, version 17.0. The differences between the control and treated groups were analyzed by Kruskal–Wallis test. $P\leq 0.05$ was considered to be statistically significant.

Results

The antibacterial activity of the acetone and aqueous extracts of *P. atlantica* at different concentrations (ranging from 3 to 100 mg/mL) against selected bacteria are shown in Tables 1 and 2. The antibacterial activity was observed

in acetone gall extract. Statistical analysis of the data using the Kruskal–Wallis test indicated that the difference between these concentrations was significant ($P<0.05$).

The MIC and MBC values of different extracts of *P. atlantica* are presented in Table 3 . The MIC and MBC values of acetone extracts were lower than aqueous

Table 1. The Antibacterial Activity of *Pistacia atlantica* Acetone Extract at Different Concentrations

Acetone extract	Concentration (mg/mL)	Mean ± SD Inhibition Zone					*P* value
		B. cereus	*S. aureus*	*E. faecalis*	*E. coli*	*P. aeruginosa*	
Fruit	100	16.0±1.0	19.0±2.7	19.0±1.0	9.7±0.6	12.7±0.6	0.023
	50	15.0±1.0	17.3±2.5	16.7±1.5	7.7±0.6	10.3±0.6	0.025
	25	12.0±1.0	16.0±3.0	15.3±0.6	-	-	0.019
	12.5	11.0±1.0	13.0±2.7	13.7±0.6	-	-	0.024
	6.25	9.0±1.0	8.7±1.2	11.3±0.6	-	-	0.021
	3	7.3±1.2	-	-	-	-	0.023
Leaf	100	22.7±2.1	21.7±1.5	17.3±0.6	-	15.0±0.0	0.011
	50	19.7±2.1	20.0±1.0	15.3±0.6	-	11.7±0.6	0.010
	25	13.0±1.7	17.3±1.5	13.3±1.5	-	9.7±0.6	0.012
	12.5	11.0±1.0	11.3±1.2	10.0±1.0	-	-	0.020
	6.25	9.3±0.6	-	8.7±1.0	-	-	0.014
	3	-	-	-	-	-	1.000
Gall	100	24.3±0.6	24.3±1.5	20.7±1.5	12.7±0.6	21.0±0.0	0.036
	50	21.7±2.9	22.3±2.5	18.7±1.5	9.7±1.5	19.0±1.0	0.040
	25	16.3±2.3	21.3±3.2	16.3±0.6	-	17.0±2.0	0.022
	12.5	15.0±1.0	18.3±1.5	15.3±0.6	-	15.3±2.1	0.017
	6.25	12.7±3.1	14.7±0.6	12.7±1.2	-	11.3±1.5	0.014
	3	10.0±0.0	9.0±1.0	9.3±1.2	-	8.7±1.2	0.010
Imipenem		29.0±0.0	35.0±0.0	18.7±0.5	25.0±0.0	-	0.008

Table 2. The Antibacterial Activity of *Pistacia atlantica* Aqueous Extract at Different Concentrations

Acetone extract	Concentration (mg/mL)	Mean ± SD of Inhibition Zone					*P* Value
		B. cereus	*S. aureus*	*E. faecalis*	*E. coli*	*P. aeruginosa*	
Fruit	100	17.3±2.1	17.3±0.6	11.7±0.6	-	10.7±1.2	0.023
	50	15.0±2.0	15.0±1.0	9.3±1.2	-	-	0.025
	25	10.3±2.5	10.3±0.6	8.0±0.0	-	-	0.019
	12.5	-	-	-	-	-	0.024
	6.25	-	-	-	-	-	0.021
	3	-	-	-	-	-	0.023
Leaf	100	16.7±0.6	15.0±0.0	18.0±0.6	10.3±0.6	13.7±1.2	0.013
	50	15.0±0.0	12.0±3.5	14.0±1.0	8.0±0.0	10.3±0.6	0.015
	25	12.0±0.0	9.3±1.2	12.0±1.7	-	7.7±1.5	0.013
	12.5	9.0±1.0	-	9.3±1.2	-	-	0.406
	6.25	-	-	-	-	-	1.000
	3	-	-	-	-	-	1.000
Gall	100	18.0±0.0	18.7±1.2	15.7±2.1	9.3±1.2	15.3±2.1	0.020
	50	16.7±0.6	16.7±0.6	11.7±1.5	-	12.0±1.7	0.013
	25	13.7±2.1	14.7±1.5	8.7±1.2	-	8.7±1.2	0.013
	12.5	10.0±0.0	12.0±2.7	-	-	-	0.010
	6.25	-	8.7±1.2	-	-	-	0.008
	3	-	-	-	-	-	1.000
Imipenem		29.0±0.0	35.0±0.0	18.7±0.5	25.0±0.0	-	0.008

extracts for all tested bacteria. The MIC value of acetone extracts ranged from 3 to 100 mg/mL (Table 3).

The reduction of biofilm formation by *S. aureus* and *P. aeruginosa* is shown in Figure 1. Evaluation of the anti-biofilm activity of acetone extract of *P. atlantica* in concentrations lower than the MIC value showed that leaf and fruit extracts inhibited the production of *S. aureus* biofilm completely at a concentration of 6.25 mg/mL.

The anti-biofilm activity of the extracts decreased by reducing the concentration of acetone and aqueous extracts to 1.5 mg/mL (Figure 1). Also, acetone and aqueous leaf extract at the concentration of 6 mg/ml inhibited biofilm formation of *P. aeruginosa* completely (Figure 1).

Phytochemical analysis results of different part of *P. atlantica* are summarized in Table 4. These results show the presence of the different functional organic materials such as pinene.

Discussion

Herbal extracts have been used for the treatment of diseases since ancient times. The antibacterial effects of some herbs used in traditional medicine have been proven. In this study, the acetone and aqueous extracts of the fruit, leaf, and gall of *P. atlantica* showed a relatively high antibacterial effect against tested bacteria, especially against gram-positive strains.

The diameter of inhibition zone was significantly correlated with the concentration of the acetone and aqueous extracts ($P<0.05$). The acetone and aqueous extracts of *P. atlantica* have a certain antibacterial effect, which is increased by increasing the concentration of extracts. *B. cereus* and *S. aureus* were more sensitive to the aqueous and acetone extracts of the fruit. *P. aeruginosa* was more sensitive to the aqueous and acetone extracts of gall. According to the results of Kruskal-Wallis test, there was a significant difference between the diameter of inhibition zones of all tested bacteria at concentrations of 6.25 to 100 mg/mL in acetone and aqueous extracts. Also, there was a significant difference between the diameter of the inhibition zone aqueous and acetone extract of gall at concentrations of 1 to 100 mg/mL in tested bacteria. On the other hand, the diameter of the inhibition zones caused by the antibacterial activity of the acetone and

Table 3. MIC and MBC of different extracts of *Pistacia atlantica* (mg/mL)

Extract			*B. cereus*	*S. aureus*	*E. faecalis*	*E. coli*	*P. aeruginosa*
Fruit	Aqueous	MIC/MBC	25.0/50.0	25.0/50.0	50.0/100.0	-	100.0/200.0
	Acetone	MIC/MBC	6.25/12.5	6.25/12.5	6.25/12.5	100.0/200.0	50.0/100.0
Leaf	Aqueous	MIC/MBC	12.5/25.0	12.5/25.0	12.5/25.0	50.0/100.0	50.0/100.0
	Acetone	MIC/MBC	12.5/25.0	12.5/25.0	12.5/25.0	-	25.0/50.0
Gall	Aqueous	MIC/MBC	12.5/25.0	6.25/12.5	25.0/50.0	100.0/200.0	25.0/50.0
	Acetone	MIC/MBC	3.0/6.25	50.0/100.0	3.0/6.25	50.0/100.0	3.0/6.25

Table 4. Chemical Composition Assessed by GC-MS of *Pistacia atlantica* Galls

Compounds	Percentage of Probable Presence in Extract	Peak Area (%)	Retention Time (min)
α-Pinene	96	32.57	8.493
β-Pinene	97	11.62	9.944
Delta-3-carene	97	6.37	11.453
l-Limonene	98	6.27	12.505
Verbenene	90	5.64	9.101
Camphene	98	3.86	8.931
Oleic acid	97	3.10	35.811
β-Myrcene	90	2.72	10.601
Campholenealdehyde	87	2.58	16.667
Benzene,1-methyl-3-(1-methylethyl)	97	2.15	12.320
trans-Pinocarveol	86	1.96	17.174
2,4-Pentadienamide	50	1.82	17.841
Palmitic acid	99	1.48	33.664
Fencholenic aldehyde	97	1.24	15.333
Cyclotrisiloxane	91	1.16	5.328
Verbenone	99	1.23	19.287

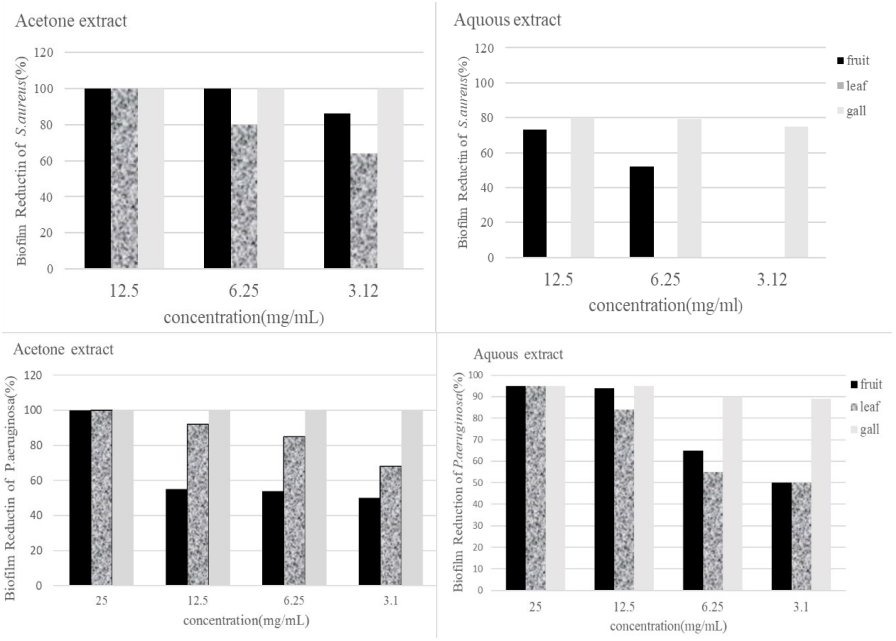

Figure. 1. Reduction of Biofilm Formation by *S. aureus* and *P. aeruginosa* Treated With Sub-lethal Concentration of Acetone and Aqueous Extracts of *P. atlantica* .

aqueous extracts of the leaf, fruit, and gall of *P. atlantica* was different according to the bacterial species tested.

Edrah and colleagues showed the ethanol extract of the *P. atlantica* leaf has antibacterial activity against *S. aureus*, but *S. epidermidis* and *E. coli* were resistant (21). So far, no study has been published on the antibacterial effects of acetone and aqueous extracts of *P. atlantica* against bacteria. Mortazavi and colleagues evaluated the antibacterial activity of *Pistacia khinjuk* fruit against *S. aureus* and *E. coli* and they found that the sensitivity of the ethanol extract of the *P. khinjuk* fruit against *E. coli* is higher than *S. aureus*. The MIC values were reported in the range of 10-20 mg/mL (3). The results of these studies differ in the present study. The acetone and aqueous extracts of *P. atlantica* fruit were effective against *S. aureus* and had no appropriate activity against *E. coli*.

One of the interesting points in this study was the antibacterial activity of acetone and aqueous extracts of *P. atlantica* against *P. aeruginosa*, while the bacterium was resistant to Imipenem. The inhibition zones of acetone and aqueous gall extracts of *P. atlantica* were 21.0 and 15.3 mm, respectively, at a concentration of 100 mg/ml against *P. aeruginosa*. Antibacterial activity of acetone and aqueous gall extracts of *P. atlantica* was higher than the antibacterial activity of Imipenem against *E. faecalis*. The inhibition zones of acetone and aqueous gall extracts of *P. atlantica* at a concentration of 100 mg/mL against *E. faecalis* was 20.7 and 15.7 mm, respectively. Jalayer Naderi and colleagues reported that *Streptococcus mutans* and *P. aeruginosa* were not sensitive to *Pistacia lentiscus,* but *S. aureus* and *Streptococcus sanguinis* were sensitive at concentrations of 50, 100, and 1000 mg/mL of methanol extracts. The MIC of *P. lentiscus* for *S. sanguinis* was 1000 mg/ml (22).

A few studies are available on the anti-biofilm effects of leaves, fruits, and galls of *P. atlantica.* Hosseini and colleagues reported that the extracts of *P. atlantica* resin decreased the *S. mutans* biofilms. They showed β-pinene (70 %), α-copaene (76%) and α-terpinolene (86%) were found to be the major components *P. atlantica* resin. Also the extracts of resin has antibacterial activity against *S. mutans* (4). In this study, extracts of *P. atlantica* similarly reduced the biofilm production of *S. aureus* and *P. aeruginosa* in the range of 3.25 to 25 mg/mL.

Omidi and Sharifi reported that the methanol extracts of *Quercus brantii*, *Pistacia atlantica* and *Elaeagnus angustifolia* inhibited 60, 57 and 72% biofilms formation of *P. aeruginosa*, respectively (23). According to these findings, acetone and aqueous extracts of *P. atlantica* have higher ability to inhibit biofilms than methanol extracts.

Conclusions

Fruit and gall extracts of *P. atlantica* have a significant preventive effect against bacteria, which is probably related to certain active compounds. These extracts inhibited biofilm formation of *S. aureus* and *P. aeruginosa*. Therefore, clinical and pharmacological studies on these extracts are recommended.

Authors' Contribution

MMS and MT designed the experiments; GDG performed the experiments; MMS analyzed the results and wrote the manuscript.

Conflict of Interests
The authors declare that they have no competing interests.

Ethical Approval
Not applicable.

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