



## ***In vitro* anti-biofilm activity of *Quercus brantii* subsp. *persica* on human pathogenic bacteria**

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### **Abstract**

**Background and objectives:** *Quercus brantii* subsp. *persica* is used in folk medicine to treat infections in Iran. There is not available report on the anti-biofilm activity of *Quercus brantii* subsp. *persica*. The aim of the present study was to investigate the effects of *Quercus brantii* subsp. *persica* against bacterial biofilms. **Methods:** Eighty biofilm producing strains of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli* and *Pseudomonas aeruginosa* were collected. *Quercus brantii* subsp. *persica* fruits aqueous extraction (QBAE) was prepared through maceration method. Chemical analysis to distinguish the main components of the QBAE was carried out using thin-layer chromatography. The antibacterial effects of QBAE on bacterial isolates were determined by the Kirby-Bauer and broth microdilution methods. The antibiofilm effects of QBAE on bacterial isolates were determined using a microtiter assay. **Results:** The *Quercus brantii* subsp. *persica* exhibited bacterial growth inhibition and bactericidal activity on the majority of the strains at concentrations between 0.2 and 1.2 mg/mL. The average of biofilm formation inhibition by *Quercus brantii* subsp. *persica* at a minimum inhibitory concentration MIC<sub>50</sub> in *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus epidermidis* and *Staphylococcus aureus* strains were 35%, 45%, 57% and 61%, respectively. coumarins, phenols, terpenes and steroids were found in the QBAE by TLC. **Conclusion:** The results showed that *Quercus brantii* subsp. *persica* aqueous extraction was effective against the tested microorganisms and showed anti-biofilm activity which can be a basis for future studies to investigate for new anti-biofilm drugs.

**Keywords:** anti-biofilm, bacteria, *Quercus brantii* subsp. *persica*

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### **Introduction**

Biofilm is a population of bacteria that grows on living and non-living surfaces [1]. Due to the presence of biofilms in various infections, the study of biofilm is important [2-3].

Antibiotics usually are not effective on biofilm

infections and when antibiotic treatment is stopped, recurrent infections often appear [2]. For these reasons, researchers are looking for ways to manage and prevent biofilm formation.

Today, medicinal plants are not only used as

traditional managements but also regarded as official drugs that were confirmed in pharmacopeias. The Fagaceae family includes nine genera around the world of which three grow in Iran: *Fagus* spp., *Quercus* spp., and *Castanea* spp. Oak (*Quercus*) has many species growing in Iran and *Quercus brantii* subsp. *persica* (or *lindii*) is native to temperate regions. It is a tall tree with a height of twenty meters with oval leaves. *Quercus brantii* subsp. *persica* is used in folk medicine to treat acute diarrhea, burns, oral and skin infections in Iran [4-6]. It has been previously reported that the ethanol extracts of *Quercus brantii* subsp. *persica* fruits possess antibacterial activity [4]. Up to date there are no available reports about the anti-biofilm activity of *Quercus brantii* subsp. *persica*.

The most serious problem of biofilms in clinical practice is antibacterial resistance, and problems in the treatment of infectious diseases caused by biofilm necessitate the search for natural antibacterial agents. This study investigated the effects of *Quercus brantii* subsp. *persica* extracts against bacterial biofilms *in vitro*.

## Experimental

### Biofilm producing bacteria

Eighty biofilm producing strains of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli* and *Pseudomonas aeruginosa* were collected from the Microbiology Laboratory of Tabriz University of Medical Sciences, between 2014 and 2015. Four strains of bacteria from the Persian Type

Culture Collection (PTCC) were also tested as control strain including *E.coli* (PTCC 1112), *P. aeruginosa* (PTCC 2381), *S. epidermidis* (PTCC 1436), and *S.aureus* (PTCC 1311). In the present study, microtiter assay was used for the detection of biofilm-forming isolates [3,7].

### Chemicals and reagents

Dichloromethane, sulfuric acid, ferric chloride, methanol, aluminum chloride, potassium hydroxide, anisaldehyde, iodine and ninhydrin were obtained from Sigma Chemicals, Germany. Muller-Hinton Agar, Muller-Hinton broth, paper disks and crystal violet were

supplied by Merck, Germany. Deionized water was used through the reactions.

### Preparation of *Quercus brantii* subsp. *persica* aqueous extraction (QBAE)

The *Q. brantii* subsp. *persica* selection was based on availability and ethnobotanical use categories relating to infection in Iran [4,6]. *Quercus brantii* subsp. *persica* was identified at the laboratory of the Faculty of Natural Sciences, Tabriz, Iran. The fresh fruit of *Q. brantii* subsp. *persica*, was collected from Kermanshah province, Iran, and were washed several times with water to remove the dust particles. Then dried in a drying oven at 40-45 °C. The dried *Q. brantii* fruit was ground to a fine powder and its mixture was prepared at a concentration of 1 g per 10 mL of distilled water and kept in the dark at room temperature for 24 h. The aqueous solution was filtered through a 0.45 µm pore membrane and stored at -20 °C. Extracts were tested for microbial contamination at every step of processing, by culturing on Muller-Hinton Agar plates.

### TLC analysis

Chemical analysis of the QBAE was carried out using thin-layer chromatography (TLC).

TLC was performed on silica plates 60 F254, 10-20 cm (Merck, Germany) using methanol: dichloromethane (2:8) and observed under ultraviolet light (254 and 366nm). Alkaloids compounds were distinguished with Dragendorff's reagent, ferric chloride 2% and aluminum chloride 1% was used for phenols; potassium hydroxide 5% for coumarins; ninhydrin for aminoacids, and iodine vapor as a general reagent [8].

### Antibacterial effects against the planktonic form

Antibacterial effects of QBAE were performed by the Kirby-Bauer method against planktonic form of strains in Muller-Hinton Agar (Merck, Germany). Paper disks were prepared with QBAE concentration of 0.08, 0.4, 2, and 10 mg/mL. The disks were allowed to dry at room temperature. The bacteria were inoculated on the petri dishes. The blank paper disk (without QBAE) was used as the negative control and imipenem disk was used as the positive control.

The petri dishes were evaluated after 24 h incubation at 37 °C and the inhibition diameter (millimeter) of growth around the disks were measured.

The MIC was obtained by the broth microdilution method. Serial dilutions of QBAE were prepared. Then, 10<sup>5</sup> CFU/mL studied strains (*S. aureus*, *S. epidermidis*, *E. coli* and *P. aeruginosa*) and Muller-Hinton broth (Merck, Germany) was added and incubated at 37°C. Positive control wells consisting 200 µL Mueller-Hinton broth with a bacterial suspension without QBAE and negative control wells consisting of 200 µL of Mueller-Hinton broth were used. Subsequent experiments were conducted to determine the MBC (Minimum Bactericidal Concentration), MBC of QBAE by sub-culturing samples, from the tubes with concentrations beyond the MIC on new petri agar.

#### Biofilm inhibition activity

The microtiter assay was carried out to study the biofilm formation inhibition activity. Here 10<sup>5</sup> CFU/mL bacteria and QBAE in the microplates were incubated at 37 °C. After 48 h, the microplate wells were washed with 200 µL phosphate buffer saline two times. Then, 200 µL of 0.5% crystal violet was added to the wells and incubation continued for 15 min; the wells were washed with water and allowed to dry at room temperature. Extra color attached to the surface was removed by ethanol 95%. Finally, the optical density of stained biofilms was read by ELISA auto reader at 570 nm [7]. The positive and negative controls were used for evaluation of biofilm inhibition activity.

#### Data analysis

In the present study, the entire tests were repeated three times. The results were analyzed by ANOVA and Fisher exact tests in the SPSS software version 19. In this study,  $p \leq 0.05$  was regarded as statistically significant.

#### Results and Discussion

The bacteria were collected from different clinical samples and were screened for capability of biofilm formation. Eighty biofilm-producing isolates were selected.

The results of agar disk diffusion method didn't show significant differences and have been presented in figure 1. According to the results, the maximum diameter of growth inhibition was observed in *S. aureus* followed by *S. epidermidis*, *E. coli* and *P. aeruginosa*. The results of MIC and MBC are presented in figure 2. There were significant differences among the different doses of QBAE and antibacterial effects ( $p < 0.05$ ). The results of MIC showed that the amount of QBAE for growth inhibition in *P. aeruginosa* and *E. coli* were greater than *S. aureus* and *S. epidermidis*. The bactericidal activity of QBAE was exhibited on the majority of the strains at concentrations between 0.4 and 1.2 mg/mL.

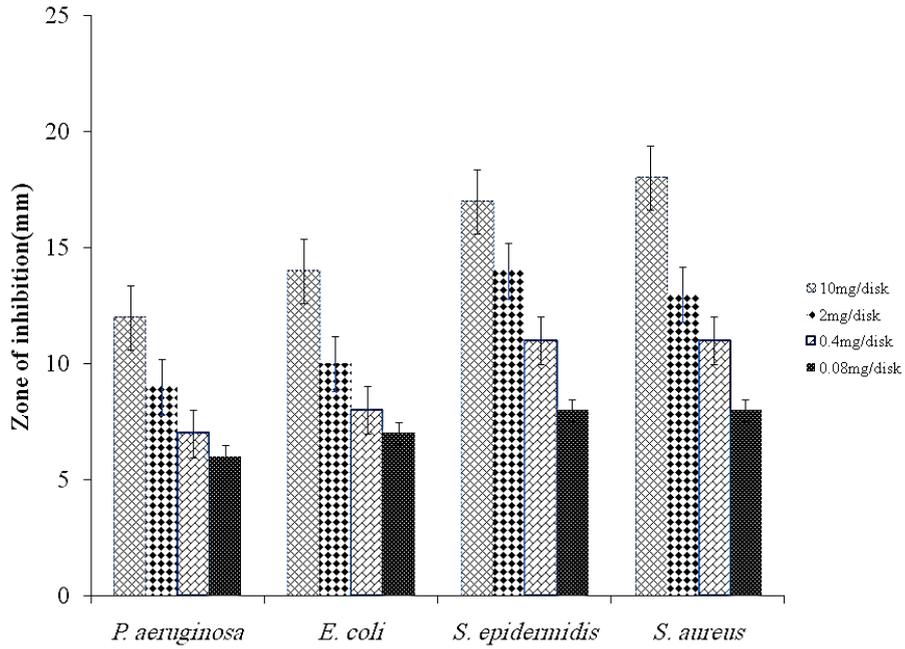
A qualitative chemical screening was also performed with QBAE. The TLC examination revealed the presence of coumarins and phenols in the QBAE.

The mean of biofilm inhibition by QBAE has been shown in table 1 and figure 3.

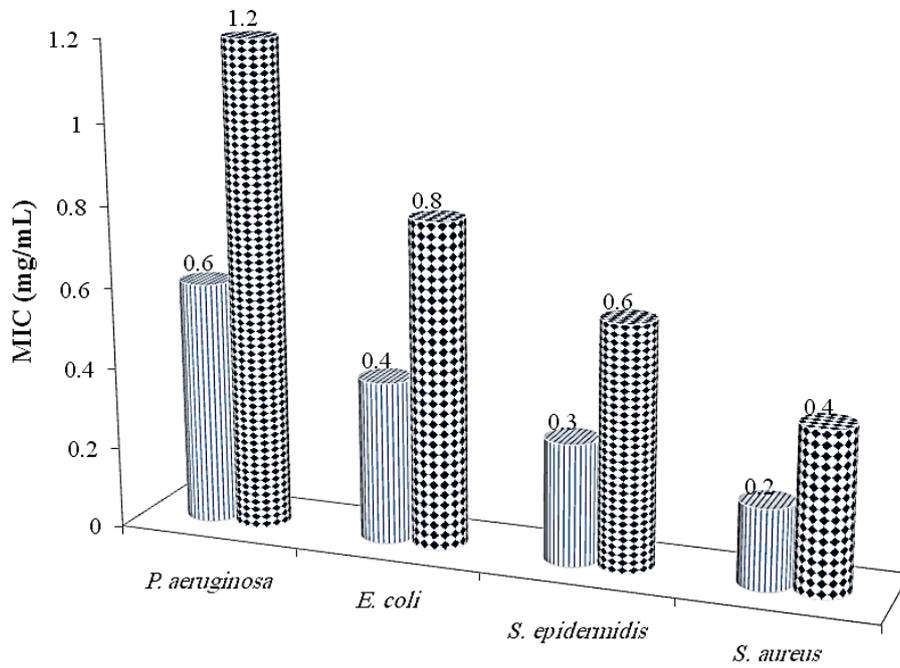
QBAE was found to be more effective against *S. aureus* biofilms than *S. epidermidis*, *E. coli*, and *P. aeruginosa* isolates. There were significant differences among the different doses of QBAE and QBAE anti-biofilm activity ( $p < 0.01$ ).

**Table 1.** The frequency of biofilm inhibition by different concentrations of *Quercus brantii* subsp. *persica* aqueous extract

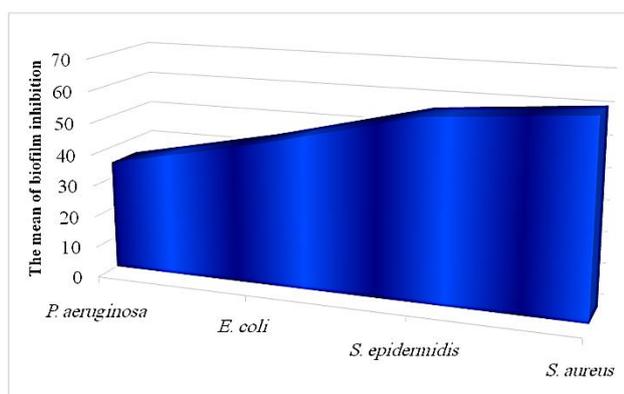
Bacteria	Concentration (mg/mL)	% Biofilm inhibition
<i>S. aureus</i>	0.10	61
	0.05	50
	0.025	27
	0.0125	13
<i>S. epidermidis</i>	0.15	57
	0.075	40
	0.0375	18
	0.01875	9
<i>E. coli</i>	0.20	45
	0.10	18
	0.05	9
	0.025	4
<i>P. aeruginosa</i>	0.30	35
	0.15	23
	0.075	11
	0.0375	3



**Figure 1.** Inhibition zone diameter (mm) produced by the *Quercus brantii* subsp. *persica* aqueous extract on bacteria by disk diffusion method at different concentrations ( $p>0.05$ )



**Figure 2.** MICs and MBCs of *Quercus brantii* subsp. *persica* aqueous extract against bacteria



**Figure 3.** The mean percentage of biofilm inhibition by MIC<sub>50</sub> concentrations of *Quercus brantii* subsp. *persica* aqueous extract (*P. aeruginosa* 0.3 mg/mL, *E. coli*, 0.20 mg/mL, *S. aureus* 0.10 mg/mL, *S. epidermidis* 0.15 mg/mL)

In the present study, the antimicrobial and anti-biofilm effects of QBAE were assessed against four medically important bacteria, including *S. epidermidis*, *S. aureus*, *P. aeruginosa* and *E. coli*. The results of the current study showed that QBAE were effective against all tested isolates. Comparison of growth inhibition diameter indicated that the most sensitive bacteria to QBAE was *S. aureus*. When the concentration of the QBAE increased, the zone of growth inhibition in turn increased, which indicates that inhibition of bacterial growth was associated with QBAE concentration. In all cases, bacteriostatic and bactericidal effects were not detected by the same concentrations; the MBC was more than double the

MIC. According to the MIC results, the lowest MIC was observed for Gram-positive bacteria (*S. aureus* and *S. epidermidis*) and the highest MIC was for Gram-negative bacteria (*E. coli* and *P. aeruginosa*). Similar to our study, Shan *et al.*, [9] reported that Gram-positive bacteria were generally more sensitive to the tested extracts compared to Gram-negative and low permeability of Gram-negative cell wall may be the cause of this issue. According to the low MIC and MBC of the QBAE against bacteria isolates, QBAE may be an appropriate and alternative antibacterial agent against bacterial infections. Microorganisms associated with biofilms behave

differently in growth rates, the ability of resistance to antimicrobial agents and immune system compared to planktonic cells [10-12].

Since the importance and the involvement of biofilms in various infections are increasing, biofilm treatment is considered as one of the main problems of human infections; however, for centuries, plants have been used as treatments for diseases. *Quercus* spp. grow in abundance in the north and west of Iran. In the present study, the aqueous fraction of *Quercus brantii* subsp. *persica* could inhibit the biofilm formation. Biofilm inhibitory concentration values for QBAE ranged between 0.0125 and 0.3 mg/mL. When the concentration of the QBAE increased, the anti-biofilm activity increased too. The obtained results confirmed the antibiofilm potential of the QBAE. Anti-biofilm activities of the extract varied related to the tested organism. The data showed that biofilm of Gram-positive bacteria (*S. aureus* and *S. epidermidis*) were more sensitive than Gram-negative bacteria (*P. aeruginosa*) to *Quercus brantii*. In this study, *S. aureus* biofilms were the most sensitive to QBAE, while *P. aeruginosa* was the most resistant. A probable reason for these observations may be the significant differences in the cell wall of bacteria. Gram-negative bacteria have an extra layer named outer membrane and an exclusive periplasmic space which is not found in Gram-positive bacteria.

The results of TLC showed that the dominant chemical compounds of QBAE were phenols and coumarins. It seems that the reason for the anti-biofilm activity of *Quercus brantii* subsp. *persica* is probably due to the presence of these compounds. Phenols have received some attention newly concerning their antimicrobial effect on bacteria biofilms [13-14]. Sampaio *et al.* indicated that phenol-rich extracts of most *Caesalpinia ferrea* had antibiofilm activity [15]. This finding is similar to our results, which inhibited the establishment of a biofilm by human pathogen bacteria. *Quercus brantii* subsp. *persica* is native to Iran. To date, this is the first report on the anti-biofilm activity of *Quercus brantii* subsp. *persica*. We found only a few studies linking biofilm inhibition with coumarins [16,17].

Additionally, furocoumarins, which are available in *Quercus brantii*, inhibit the growth of bacteria by interacting with the bacterial DNA [4,5]. To date, this is the first report on the anti-biofilm activity of *Quercus brantii* subsp. *persica*. Recently, investigators have conducted numerous studies on the antibacterial activity of *Quercus brantii* subsp. *persica* [4,5,18]. One aspect of this study that should be considered in future research is the possible toxicity of *Quercus brantii*. The selective toxicity of an antibacterial agent on microorganisms is essential and would influence the utility of the *Quercus brantii* subsp. *persica* extract as a medicinal compound. If an antibacterial agent is toxic for human cells it can be useful as an antiseptic or disinfectant. The nature of used solvent affects the secondary metabolite composition of a plant extract. However, antimicrobial effects depend on the nature of the analysts to be separated and techniques used. Here solely water soluble compounds were studied, but *Quercus brantii* subsp. *persica* has many water insoluble compounds. It is suggested that the water insoluble combinations should be studied in the future.

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#### Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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