# Elimination of Persister Cells Originating from *Staphylococcus aureus* by *Scrophularia striata* Boiss. Extract

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## Abstract

*Staphylococcus aureus* is a significant pathogen responsible for various infections. It has shown increasing resistance to multiple antibiotics, with persister cell formation playing a crucial role in recurrent infections. *Scrophularia striata* Boiss., a medicinal plant known for its antimicrobial properties, is assessed in this study for its potential to eliminate persister cells derived from *S. aureus*. The identification and confirmation of bacterial strains as well as the minimum inhibitory concentration (MIC) tests were performed according to CLSI, 2022. Rifampin (640 µg/ml; 40×MIC) and ampicillin (100 µg/ml) were used to trigger persister cell formation from methicillin-resistant and -susceptible *Staphylococcus aureus* (MRSA and MSSA). The effect of *Scrophularia striata* Boiss. extract on the persister cells and Caco-2 cell line (cytotoxicity effect) were evaluated. The MIC of rifampin for MRSA and MSSA was 8

µg/ml and 16 µg/ml, respectively. The 25 mg/ml and 400 mg/ml concentrations of *Scrophularia striata* Boiss. extract had the lowest and the highest effect in deletion of MRSA and MSSA persister cells, respectively. Moreover, the 400 mg/ml of *Scrophularia striata* Boiss. extract had the highest effect in inhibiting the growth of Caco-2 cells. As an alternative and novel therapeutic option, instead of traditional antibiotics, the *Scrophularia striata* Boiss. extract may be useful to fight against the *S. aureus* persister cells as well as the cancer cells. Due to the difficulties in eradication of persister cells which may lead to untreatable persistent infections and become a serious challenge in medicine, this finding is encouraging, however, more related studies are required to confirm and generalize the results.

**Keywords:** *Staphylococcus aureus*; MRSA; MSSA; persister cells; *Scrophularia striata* Boiss.

## 1. Introduction

Today, infectious diseases contribute the main part of human death worldwide and among these, *Staphylococcus aureus* (*S. aureus*) is one of the important bacteria to create different infectious diseases such as skin and soft tissue infections, osteomyelitis, endocarditis, and bacteremia. Unfortunately, since the recent decades *S. aureus* has become more resistant to many different antibiotics for instance methicillin (methicillin-resistant *Staphylococcus aureus*; MRSA) and vancomycin (vancomycin-resistant *Staphylococcus aureus*; VRSA) (1). Importantly, these resistances help the *S. aureus* to cause dangerous infections and a high range of morbidity and mortality (2).

The large majority of sensitive bacterial population are removed when they are affected by bactericidal antibiotics but the individual bacteria, called the persister cells, survive from

antibiotic treatment. For the first time, in 1944, Joseph Bigger showed the persister cells in a population of staphylococci influenced by penicillin. The persister cells are capable to occur in different bacterial populations and cause many problems such as the failure of antimicrobial therapy and recurrent infections (3).

Toxin-Antitoxin (TA) systems are involved in many applications, among these, probably persister cell formation is the most important one. The persister cells are phenotypic variants and they are completely different from resistant bacteria (4). Unfortunately, persister cell formation play an important role in chronic and recurrent infections caused by *S. aureus* (5).

Since a long time ago, people who live in the west of Iran have used many different medicinal plants to cure many illnesses. One of the important medicinal plants is *Scrophularia striata* Boiss. Over the time, people have found that this medicinal plant has a powerful effect on many microbial infections so they have used this magic medicinal plant for treatment of many infectious diseases. Fortunately, many researches have shown its amazing effect on deletion of different microbial infections (6-9).

Due to the problems caused by the bacterial persister cells, (treatment difficulties, chronic and persistent infections), this is necessary to find and apply the novel treatment options.

In the present study, the effect of *Scrophularia striata* Boiss. extract in deletion of persister cells derived from *S. aureus* was investigated.

The Caco-2 cell lines are the human epithelial cell line that widely used as a model of the intestinal epithelial barrier. This cell line is derived from a colon carcinoma and it has been widely used for drug permeability studies (10). In the present study, we also selected the Caco-2 cell line to evaluate the cytotoxicity effect of the *Scrophularia striata* Boiss extract on the cancerous cells.

## 2. Materials and Methods

### 2.1. Identification and validation of bacterial strains

In the current study, two standard strains of *S. aureus* were used as following: *S. aureus* ATCC43300 as a standard strain of MRSA, *S. aureus* ATCC25923 as a standard strain of MSSA. Conventional culture media such as Blood agar, Luria-Bertani (LB) agar and LB broth were used for culture of the bacterial strains. Identification and confirmation methods were applied to specify and verify the *S. aureus* (MRSA and MSSA) according to CLSI 2022 standard guideline (11).

### 2.2. Preparation of *Scrophularia striata* Boiss. extract

The *Scrophularia striata* Boiss. was collected from the natural habitat of the plant in west of Iran and identified and approved by the Herbarium Department of the Medicinal Plants Research Center of Tehran University, Tehran, Iran, with voucher number of 7183-TEH. The Plants were dried out and the aqueous extract was provided using the percolation method (7). Then, 0.1g, 0.25g, 0.4g, 1g, and 2g powders of *Scrophularia striata* Boiss. were individually dissolved in 1ml sterile distilled water and the suspensions were filtered through a 0.22-µm filter and stored at 4°C.

### 2.3. Formation of persister cells from MRSA and MSSA

To make persister cells from MRSA and MSSA strains, we first prepared the exponential phase cells of MRSA and MSSA as following; the LB broth overnight cultures of bacteria were diluted in fresh medium and then incubated for 2hrs. Afterward, the cultures were incubated

until they reached an OD600 of 0.5 for exponential phase. Subsequently, the cultures were challenged with rifampin (640 µg/ml; 40× MIC) and ampicillin (100 µg/ml) further in 37℃ in order to form the persister cells (12, 13).

### 2.4. Determination of minimal inhibitory concentration (MIC)

The MIC of rifampin for MRSA and MSSA strains was determined by macro broth dilution method (11).

In addition, the MIC of *Scrophularia striata* Bois. extract was evaluated for persister cells of MRSA and MSSA. For this purpose, the different concentrations of *Scrophularia striata* Boiss. extract was evaluated. Afterward, 25 mg/ml, 50 mg/ml, 100 mg/ml, 200 mg/ml, and 400 mg/ml of *Scrophularia striata* Bois. extract dilutions were prepared. Then, the MIC of *Scrophularia striata* Boiss. extract for the persister cells of MRSA and MSSA was determined by macro broth dilution method (11). Finally, the loss of viability was determined by counting the Colony Forming Units (CFUs) (14).

### 2.5. Cell culture and the MTT assay

The MTT assay was performed to evaluate the cell toxicity effect of the *Scrophularia striata* Boiss. extract. First, the Caco-2 cells were cultured in RPMI 1640 medium at 37℃ in a humidified atmosphere with 5% CO2 for 24 hours. The next day, the cells were seeded into 96-well plates (at a density of $3\times10^4$ cells/100 µl/well) at 37℃ over night. Afterward, the cells were challenged by different concentrations (400 mg/ml, 200 mg/ml, 100 mg/ml, 50 mg/ml, 25 mg/ml, 15 mg/ml, 10 mg/ml, and 5 mg/ml) of *Scrophularia striata* Boiss. extraction. After

24 hrs growth at 37℃ in a humidified atmosphere with 5% CO2, 10 µl of prepared MTT solution were added to each well and incubated for 3–4 hrs at 37 °C, and then 100 µl of solubilization solution were added to each well and the absorbance of them were measured using a microplate reader at 570 nm. Finally, the cell viability percentage of the MTT assay was calculated through the following equation (15, 16):

$$\text{Cell Viability (\%)} = \left\{\frac{Mean sample\ OD}{Mean\ blank\ OD}\right\} * 100$$

## 3. Results

### 3.1. Identification and verification of bacterial strains

The bacterial strains ATCC43300 as MRSA and ATCC25923 as MSSA standard strains were confirmed using the CLSI 2022 guideline standard identification methods as, the MRSA strain was resistant to cefoxitin (30 µg disk) while the MSSA was susceptible.

### 3.2. Persister cell formation from MRSA and MSSA

As described in methods, the MRSA and MSSA were challenged with rifampin and ampicillin in order to form persister cells. When MRSA was challenged with rifampin (40× MIC), it was able to form persister cell while in the same condition, persister cells of MSSA couldn't be formed. But in the presence of 100 µg/ml ampicillin, only the MSSA could form the persister cells.

### 3.3. MIC determination

The MIC of rifampin for MRSA and MSSA strains was 8 µg/ml and 16 µg/ml, respectively. Moreover, MRSA in 16 µg/ml and MSSA in 32 µg/ml of rifampin didn't have any growth.

As described in methods, the persister cells derived from MRSA and MSSA were challenged with different concentrations of *Scrophularia striata* Boiss. extract. As a result, 25 mg/ml, 50 mg/ml, and 100 mg/ml concentrations had no significant role in deletion of the persister cells of MRSA and MSSA. The 200 mg/ml concentration of *Scrophularia striata* Boiss. extract affected on the growth of MRSA and MSSA persister cells; although, the 400 mg/ml concentration had the highest effect as, in this concentration, the persister cells of MRSA and MSSA didn't have any growth (Figure 1).

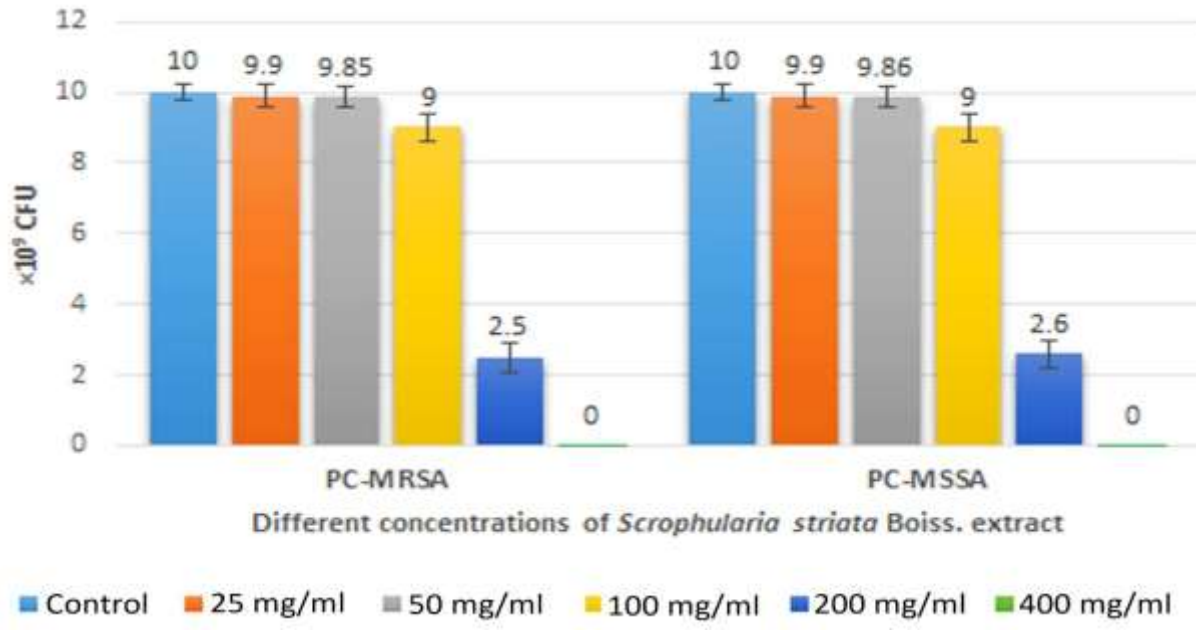


**Figure 1.** The effect of different concentrations of Scrophularia striata Boiss. extract on persister cells derived from MRSA and MSSA.

As shown, the 25 mg/ml, 50 mg/ml, and 100 mg/ml concentrations of Scrophularia striata Boiss. extract had no significant role in deletion of MRSA and MSSA persister cells. But, the effect of 200 mg/ml concentration in deletion of MRSA and MSSA persister cells could not be ignored. Moreover, the 400 mg/ml concentration had the highest effect and the persister cells of MRSA and MSSA didn't show any growth in this concentration. MRSA: methicillin-resistant Staphylococcus aureus; MSSA: methicillin-susceptible Staphylococcus aureus; PC-MRSA: persister cells derived from MRSA; PC-MSSA: persister cells derived from MSSA; CFU: colony forming unit. (Note: 10*109 CFU considered as the complete growth of MRSA and MSSA after 18-24h incubation at 37℃ in LB broth medium).

### 3.4. The viability of Caco-2 cell line influenced by *Scrophularia striata* Boiss. extract

The effect of different concentrations (400 mg/ml, 200 mg/ml, 100 mg/ml, 50 mg/ml, 25 mg/ml, 15 mg/ml, 10 mg/ml, and 5 mg/ml) of *Scrophularia striata* Boiss. extract on the growth of Caco-2 cells was evaluated by MTT assay. The Caco-2 cell line had the highest growth in 5 mg/ml concentration. However, more than 90 percent of Caco-2 cells killed and couldn't grow in the 400 mg/ml concentration (Figure 2).

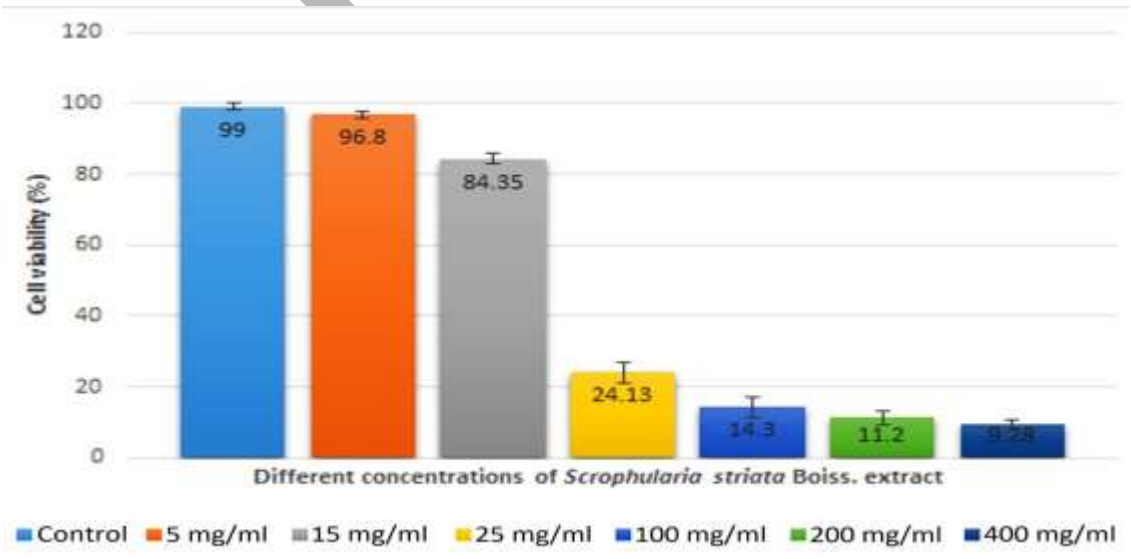

**Figure 2.** The effect of different concentrations of Scrophularia striata Boiss. extract on the growth of Caco-2 cells.

The 5 mg/ml and 400 mg/ml concentrations had the lowest and the highest inhibiting effect on the growth of Caco-2 cells, respectively.

## 4. Discussion

The bacterial infections continue to pose a significant threat to human healthcare, making them a prominent concern within the field. The various strains of bacteria are found to be capable of triggering a variety of infections, and among them, one of the crucial bacterial species is *Staphylococcus aureus*, a Gram-positive bacterium commonly found in the nasal passages and on the skin of humans and animals. It can cause a range of infections from minor skin infections to life-threatening illnesses such as pneumonia, endocarditis, and sepsis. *S. aureus* is known for its ability to develop resistance to antibiotics, making it a significant public health concern (2, 17). Resistance to the antibiotics is the major problem in treatment of the infections caused by *S. aureus*. The resistance to methicillin is a critical form of resistance that holds significant importance in medicine. The treatment of MRSA infections is much more difficult than the sensitive strains, thereby representing a significant global healthcare concern (18).

The ability to form persister cells constitutes a significant challenge in combatting the infections caused by *S. aureus*. Regrettably, these enduring cells are the main cause of recurrent as well as chronic infections and their elimination represents a formidable challenge. The persister cells of *S. aureus* are resistant to conventional doses of antibiotics making them to create persistent infections that prove to be challenging to eradicate (3, 5).

Currently, the preferable approach to address bacterial infections, particularly the persistent and resistant ones, is by using alternative therapeutic options instead of antibiotic treatments. One of the primary approaches employed in healthcare is traditional medicine, particularly the utilization of botanical remedies (19). In many countries such as Iran, the medicinal plants have been employed for the eradication and treatment of various infections. The utilization of medicinal plants for the treatment of infections presents numerous benefits in comparison with antibiotics, for example, decreasing the side effects (adverse effects) and also reducing the treatment costs (19).

*Scrophularia striata* Boiss. is a medicinal plant that has been efficaciously utilized by the inhabitants of western Iran for a prolonged period of time as a remedy cure the numerous microbial infections. Numerous investigators have reported the antimicrobial properties of this magic plant in a favorable manner (6-9).

Notably, the current study showed a positive correlation between the concentration of *Scrophularia striata* Boiss. extract and its efficacy in inducing bacterial cell death. As, the 25 mg/ml concentration of the extract had the lowest, the 200 mg/ml concentration had a moderate, and the 400 mg/ml concentration had the greatest effect in inhibition of MRSA and MSSA persister cells growth and, amazingly, in the last concentration any persister cells of MRSA and MSSA couldn't grow. The present study, for the first time, showed that the extract obtained from *Scrophularia striata* Boiss. had the potential to eradicate both MRSA and MSSA persister cells. While this finding is promising, further investigation is required to fully ascertain the effectiveness of the extract. Narayanaswamy *et al*. discovered that a novel large molecule polycationic glycopolymer, poly (acetyl, arginyl) glucosamine (PAAG) had a significant effect in elimination of *Pseudomonas aeruginosa* persister cells. They also showed that PAAG had greater efficacy against persisters than antibiotics currently being used to treat

persistent chronic infections such as tobramycin, colistin, azithromycin, aztreonam, and clarithromycin, *in vitro* (20). Here we efficiently used *Scrophularia striata* Boiss. extract to eliminate the persister cells derived from *S. aureus* strains. Amazingly, this medicinal plant demonstrated the potential to effectively eradicate the persister cells of *S. aureus* while it was observed that the formation of these cells could occur when exposed to 40× MIC Rifampin and 100 µg/ml ampicillin.

Canas-Duarte *et al*. used LB broth with Ampicillin (100 µg/mL) for the analysis of antibiotic based persisters isolation protocols (13). Alternatively, Sahukhal *et al*. used 40× MIC Rifampin to form persister cell (12). Some studies have indicated that the various antibiotics are different in persister cell formation and the antibiotics do not form persister cells equally (21, 22). In the present study, Ampicillin (100 µg/mL) and 40× MIC Rifampin were used to form the persister cells and the results showed that MRSA could only form persister cell in the presence of 40× MIC Rifampin and the persister cell of MSSA could only form in the presence of Ampicillin (100 µg/mL).

Numerous studies have been conducted to assess the impact of various compounds on the growth inhibition of the Caco-2 cell line. Esghaei *et al*. showed that hydroalcoholic extract of *Camellia sinensis* considerably inhibited the growth of Caco-2 cells (23). Likewise, a cytotoxic effect of *Eucalyptus camaldulensis* essential oil on Caco-2 cells was documented by Taheri *et al*. (24). Moreover, Zein *et al*. used bioactive compounds in the aqueous extract of *Eucalyptus camaldulensis* leaves to show its cytotoxic effect on Caco-2 cells (25). Surprisingly, in the present study, the results of MTT assay showed that *Scrophularia striata* Boiss. extract could inhibit the growth of Caco-2 cells; as, the growth of Caco-2 cells was significantly inhibited (more than 90 percent) in the 400 mg/ml concentration of *Scrophularia striata* Boiss. extract. Considering that the Caco-2 cells are a form of cancer cells, it is plausible that this particular

extract may eventually be utilized as a potential anticancer therapy. Nevertheless, its potential toxicity towards normal cells should be investigated as well.

Finally, the development of persister cells from *S. aureus* represents a significant challenge in the field of medicine, necessitating a prompt and effective medical intervention through exploiting alternative and novel therapeutic options instead of traditional antibiotic treatments. The findings of the present study propose that the *Scrophularia striata* Boiss. extract may be applicable to fight against persister cells derived from *S. aureus* strains, as well as the cancer cells; However, although these findings are encouraging, more related in vitro and in vivo studies are required to confirm this potential strategy against the persister bacteria as well as the cancer cells.

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None to declare.

**Author contributions**

S.B. performed most of the experiments and analysis of the data. M.H.A. conceived the project, designed the experiments, participated in writing, review & editing. P.O. participated in the conceptual design and data analysis. All the authors approved the final version of the manuscript.

**Ethics Approval**

This study was approved by the Research Ethics Committee of Shahed University (Approval ID: IR.SHAHED.REC.1400.037).

## Conflict of Interest

The authors declare that they have no conflict of interest.


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## Data Availability

The data that support the findings of this study are available upon request from the corresponding author.

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