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Research Paper

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

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ABSTRACT

Introduction: *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*.

Materials & Methods: 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 methicillin-susceptible *S. aureus* (MRSA and MSSA). The effect of *S. striata* extract on the persister cells and the Caco-2 cell line (cytotoxic effect) was evaluated.

Results: 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 *S. striata* extract had the lowest and the highest effects on the elimination of MRSA and MSSA persister cells, respectively. Moreover, the 400 mg/mL concentration of *S. striata* extract had the highest effect on inhibiting the growth of Caco-2 cells.

Conclusion: As an alternative and novel therapeutic option to traditional antibiotics, the *S. striata* extract may be useful in combating *S. aureus* persister cells as well as the cancer cells. Due to the difficulties associated with the eradication of persister cells, which may lead to untreatable persistent infections and pose a serious challenge to medicine, this finding is encouraging; however, more studies are required to confirm and generalize the results.

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1. Introduction

Today, infectious diseases are a leading cause of human death worldwide, and among these, *Staphylococcus aureus* is one of the major pathogens causing different infectious diseases such as skin and soft tissue infections, osteomyelitis, endocarditis, and bacteremia. Unfortunately, in recent decades, *S. aureus* has become more resistant to many different antibiotics, including methicillin (methicillin-resistant *S. aureus*; MRSA) and vancomycin (vancomycin-resistant *S. aureus*; VRSA) [1]. Importantly, these resistances help *S. aureus* to cause dangerous infections and a high rate of morbidity and mortality [2].

The large majority of the sensitive bacterial population is eliminated when it is exposed to bactericidal antibiotics, but individual bacteria, called persister cells, survive antibiotic treatment. In 1944, Joseph Bigger first described persister cells in a population of staphylococci exposed to penicillin. Persister cells are capable of occuring 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 biological processes; among these, persister cell formation is probably the most important. Persister cells are phenotypic variants, and they are completely different from resistant bacteria [4]. Unfortunately, persister cell formation plays an important role in chronic and recurrent infections caused by *S. aureus* [5].

For a long time, people living in the west of Iran have used many different medicinal plants to treat many illnesses. One of the important medicinal plants is *Scrophularia striata* Boiss. Over time, people have found that this medicinal plant has a powerful effect on many microbial infections, so they have used it for the treatment of many infectious diseases. Fortunately, many studies have shown its remarkable effects on the elimination of different microbial infections [6-9].

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

In the present study, the effect of *S. striata* extract on the elimination of persister cells derived from *S. aureus* was investigated.

The Caco-2 cell line is a human epithelial cell line that is widely used as a model of the intestinal epithelial barrier. This cell line is derived from 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 effects of the *S. striata* extract on cancer 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 follows: *S. aureus* ATCC 43300 as a standard strain of MRSA and *S. aureus* ATCC 25923 as a standard strain of MSSA. Conventional culture media such as Blood agar, Luria-Bertani (LB) agar, and LB broth were used for culturing the bacterial strains. Identification and confirmation methods were applied to identify and verify *S. aureus* (MRSA and MSSA) according to the Clinical & Laboratory Standards Institute (CLSI) 2022 guidelines [11].

2.2. Preparation of *S. striata* extract

The *S. striata* was collected from the natural habitat of the plant in the west of Iran and identified and approved by the Herbarium Department of the Medicinal Plants Research Center, University of Tehran, Tehran, Iran, with the voucher number 7183-TEH. The plants were dried and the aqueous extract was prepared using the percolation method [7]. Then, 0.1 g, 0.25 g, 0.4 g, 1 g, and 2 g of *S. striata* powders were individually dissolved in 1 mL of 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 generate persister cells from MRSA and MSSA strains, we first prepared the exponential-phase cells of MRSA and MSSA as follows: the LB broth overnight cultures of bacteria were diluted in fresh medium and then incubated for 2 h. Afterward, the cultures were incubated until they reached an OD600 of 0.5 (exponential phase). Subsequently, the cultures were challenged with rifampin (640 µg/mL; 40× MIC) and ampicillin (100 µg/mL) at 37 ˚C in order to form persister cells [12, 13].

2.4. Determination of minimal inhibitory concentration

The minimal inhibitory concentration (MIC) of rifampin for MRSA and MSSA strains was determined by the macrodilution broth method [11].

In addition, the MIC of *S. striata* extract was evaluated for persister cells of MRSA and MSSA. For this purpose, different concentrations of *S. striata* extract was evaluated. Afterward, dilutions of 25 mg/mL, 50 mg/mL, 100 mg/mL, 200 mg/mL, and 400 mg/mL of *S. striata* extract were prepared. Then, the MIC of *S. striata* extract for the persister cells of MRSA and MSSA was determined by the macrodilution broth 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 cytotoxic effect of the *S. striata* extract. First, the Caco-2 cells were cultured in RPMI 1640 medium at 37 in a humidified atmosphere with 5% CO_2 for 24 h. The next day, the cells were seeded into 96-well plates (at a density of 3×10^4 cells/100 µL/well) at 37 overnight. Afterward, the cells were challenged with 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 *S. striata* extract. After 24 h of incubation at 37 in a humidified atmosphere with 5% CO_2, 10 µL of prepared MTT solution was added to each well and incubated for 3–4 h at 37 °C. Then 100 µL of solubilization solution was added to each well and the absorbance was measured using a microplate reader at 570 nm. Finally, the cell viability percentage was calculated using the Equation 1 [15, 16]:

1. *Cell viability (%) =×100*

3. Results

3.1. Identification and verification of bacterial strains

The bacterial strains ATCC 43300 (MRSA) and ATCC 25923 (MSSA) were confirmed using the standard identification methods described in the CLSI 2022 guidelines. Specifically, the MRSA strain was resistant to cefoxitin (30 µg disk), while the MSSA strain was susceptible.

3.2. Persister cell formation from MRSA and MSSA

As described in the materials and methods section, 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 cells, while under the same conditions, persister cells from MSSA could not be formed. In contrast, in the presence of 100 µg/mL ampicillin, only MSSA could form the persister cells.

3.3. Minimal inhibitory concentration determination

The MIC of rifampin for MRSA and MSSA strains was 8 µg/mL and 16 µg/mL, respectively. Moreover, MRSA showed no growth at 16 µg/mL and MSSA showed no growth at 32 µg/mL of rifampin.

As described in the Materials and Methods section, the persister cells derived from MRSA and MSSA were challenged with different concentrations of *S. striata* extract. The 25 mg/mL, 50 mg/mL, and 100 mg/mL concentrations had no role in eliminating the persister cells of MRSA and MSSA. The 200 mg/mL concentration of *S. striata* extract affected the growth of MRSA and MSSA persister cells. However, the 400 mg/mL concentration had the highest effect, as at this concentration, the persister cells of MRSA and MSSA showed no growth (Figure 1).

3.4. Viability of Caco-2 cell line following treatment by *S. striata* 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 *S. striata* extract on the growth of Caco-2 cells was evaluated by the MTT assay. The Caco-2 cell line showed the highest viability at the 5 mg/mL concentration. However, more than 90% of Caco-2 cells were killed and did not grow at the 400 mg/mL concentration (Figure 2).

4. Discussion

The bacterial infections continue to pose a significant threat to human healthcare, making them a prominent concern in medicine. Various bacterial species are capable of causing a variety of infections, and among them, one of the most important bacterial species is *S. 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

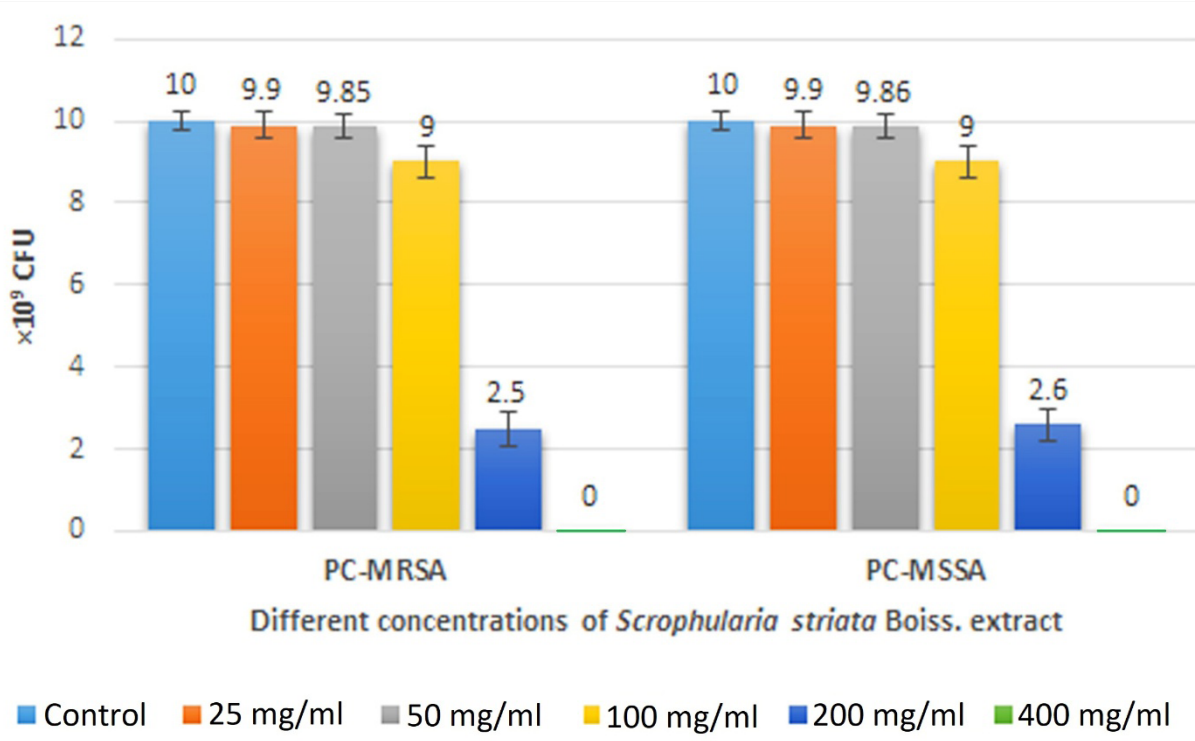

Figure 1. The effect of different concentrations of *S. striata* extract on persister cells derived from MRSA and MSSA

Abbreviations: MRSA: Methicillin-resistant *S. aureus*; MSSA: Methicillin-susceptible *S. aureus*; PC-MRSA: Persister cells derived from MRSA; PC-MSSA: Persister cells derived from MSSA; CFU: Colony-forming unit.

Note: As shown, the 25 mg/mL, 50 mg/mL, and 100 mg/mL concentrations of *S. striata* extract had no significant role in eliminating MRSA and MSSA persister cells. However, the 200 mg/mL concentration affected the elimination of MRSA and MSSA persister cells. Moreover, the 400 mg/mL concentration had the highest effect, and the persister cells of MRSA and MSSA showed no growth at this concentration. 10×10^9 CFU was considered to represent the complete growth of MRSA and MSSA after 18-24 h of incubation at 37 ˚C in LB broth media.

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 antibiotics is a major problem in the treatment of *S. aureus* infections. Methicillin resistance is a critical form of resistance that holds significant importance in medicine. The treatment of MRSA infections is much more difficult than that of infections caused by methicillin-susceptible strains, thereby representing a significant global healthcare concern [18].

The ability to form persister cells constitutes a significant challenge in combating the infections caused by *S. aureus*. Regrettably, these persistent cells are the main cause of recurrent and chronic infections, and their elimination represents a formidable challenge. *S. aureus* persister cells are resistant to conventional doses of antibiotics, allowing them to cause persistent infections that prove to be challenging to eradicate [3, 5].

Currently, the preferred approach to address bacterial infections, particularly the persistent and resistant infections, is the use of 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 treatment and eradication of various infections. The utilization of medicinal plants for the treatment of infections presents numerous benefits compared with antibiotics, including decreasing adverse effects and reducing treatment costs [19].

S. striata is a medicinal plant that has been successfully utilized by the inhabitants of western Iran for a prolonged period of time as a remedy to treat numerous microbial infections. Numerous investigators have reported the antimicrobial properties of this medicinal plant [6-9].

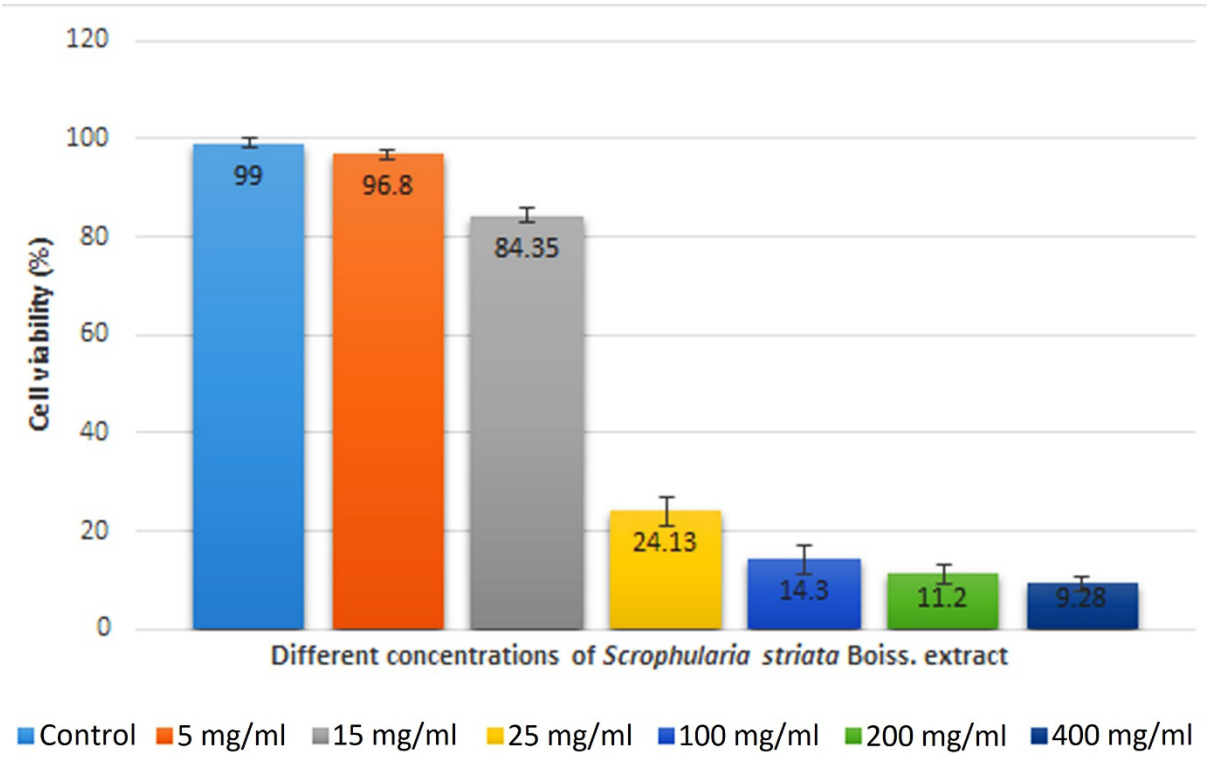

Figure 2. The effect of different concentrations of *S. striata* extract on the growth of Caco-2 cells

Note: The 5 mg/mL and 400 mg/mL concentrations had the lowest and the highest inhibitory effects on the growth of Caco-2 cells, respectively.

Notably, the current study showed a positive correlation between the concentration of *S. striata* extract and its efficacy in inducing bacterial cell death. The 25 mg/mL concentration of the extract had the lowest effect, the 200 mg/mL concentration had a moderate effect, and the 400 mg/mL concentration had the greatest effect in the inhibition of the growth of MRSA and MSSA persister cells. Remarkably, at the highest concentration no persister cells of MRSA and MSSA were able to grow. The present study, for the first time, showed that the extract obtained from *S. striata* 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. (2018) discovered that a novel large molecule polycationic glycopolymer, poly (acetyl, arginyl) glucosamine (PAAG), had a significant effect on the 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 successfully used *S. striata* extract to eliminate the persister cells derived from *S. aureus* strains. Importantly, this medicinal plant demonstrated the potential to effectively eradicate *S. aureus* persister cells, while these cells were shown to form following exposure to 40× MIC rifampin and 100 µg/mL ampicillin.

Canas-Duarte et al. (2014) used LB broth with ampicillin (100 µg/mL) for the analysis of antibiotic-based persister isolation protocols [13]. Alternatively, Sahukhal et al. (2017) used 40× MIC rifampin to form persister cells [12]. Some studies have indicated that different antibiotics vary in their ability to induce persister cell formation and do not induce persister cells equally [21, 22]. In the present study, ampicillin (100 µg/mL) and 40× MIC rifampin were used to form persister cells, and the results showed that MRSA could only form persister cells in the presence of 40× MIC rifampin, whereas MSSA formed persister cells only 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. (2018) showed that the 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. (2020) [24]. Moreover, Zein et al. (2020) used bioac-

tive compounds in the aqueous extract of *E. camaldulensis* leaves to show their cytotoxic effect on Caco-2 cells [25]. In the present study, the results of the MTT assay showed that *S. striata* extract could inhibit the growth of Caco-2 cells. Specifically, the growth of Caco-2 cells was significantly inhibited (more than 90%) at the 400 mg/mL concentration of *S. striata* extract. Considering that the Caco-2 cells are cancer cells, it is plausible that this extract may eventually be utilized as a potential anticancer therapy. Nevertheless, its potential toxicity to normal cells should be investigated as well.

5. Conclusion

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

Compliance with ethical guidelines

This study was approved by the Research Ethics Committee of Shahed University, Tehran, Iran (Code: IR.SHAHED.REC.1400.037).

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Authors' contributions

Conceptualization and study design: Mohammad Hossein Ahmadi and Parviz Owlia; Experiments: Shahriar Bakhti; Data analysis and interpretation: Shahriar Bakhti and Parviz Owlia; Writing the original draft: Mohammad Hossein Ahmadi; Review and editing: Mohammad Hossein Ahmadi; Final approval: All authors.

Conflict of interest

The authors declared no conflict of interest.

Data availability

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

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