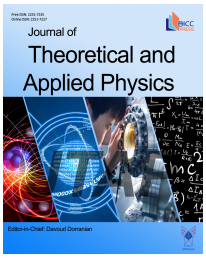

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Optimizing the photodynamic inactivation of *Staphylococcus aureus* using barberry plant extract and low power laser

Maliheh Ranjbaran[1,*], Asra Pourabdellah[2], Jamshid Sabaghzadeh[1], Zahra Aghaebrahimi[1]

[1]*Department of Physics, Central Tehran Branch, Islamic Azad University, Tehran, Iran.*
[2]*Department of Biology, Central Tehran Branch, Islamic Azad University, Tehran, Iran.*

[*]**Corresponding author:** ma.ranjbaran@iau.ac.ir

Original Research

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Abstract:

The rise of drug-resistant infections poses a significant threat to global health, necessitating the exploration of alternative treatment approaches. *Staphylococcus aureus* is a prominent pathogen responsible for a range of infections, including nosocomial infections. Photodynamic inactivation (PDI) has emerged as a promising technique for controlling drug-resistant microorganisms. This study investigates the use of photosensitizers derived from Iranian seedless barberry (Zereshk) in PDI to effectively target and eliminate drug-resistant *S. aureus*. Barberry is known for its rich alkaloid content, particularly berberine, which exhibits antibacterial and anti-inflammatory properties. The results provide insights into the inhibitory effects of barberry extract on the growth of *S. aureus*, as well as the synergistic effect of combining the extract with laser radiation. The findings highlight the potential of barberry-derived photosensitizers as a promising approach for combating drug-resistant *S. aureus* infections, offering a new avenue for effective treatment strategies.

Keywords: *Staphylococcus aureus*; Barberry extract; Low level laser; Medicinal plants; Synergistic effect

1. Introduction

The rise of drug-resistant infections has become a pressing global health concern, posing significant challenges to the effectiveness of traditional antibiotic treatments. In recent years, hospitalized patients have experienced fatal outcomes due to infections caused by drug-resistant strains, resulting in a substantial increase in healthcare expenses across communities [1]. Moreover, these infections have the ability to spread between different diseases, compounding the urgency of finding effective solutions. Scientists and the scientific community have recognized the gravity of the situation, with predictions indicating that if the current trend continues, all antibiotics may become ineffective within a few decades, reverting humanity to a pre-penicillin era. Published reports on antibiotic drug resistance provide evidence of an ongoing progression towards this alarming outcome [2].

Among the pathogens that have developed resistance to antibiotics, *Staphylococcus aureus* (*S. aureus*) stands out as one of the most significant. *S. aureus*, is commonly found as part of the normal flora on the skin or in the nose [3]. *S. aureus* is responsible for a range of infections, from common skin conditions such as boils and abscesses to severe, life-threatening diseases like pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome, and septicemia. It is also one of the leading causes of nosocomial infections, particularly post-surgical wound infections [4–6]. The emergence and spread of drug-resistant strains, including have rendered previously effective treatments ineffective and significantly increased the complexity and cost of healthcare. The urgent need to address drug-resistant infections has spurred researchers to explore alternative treatment approaches that offer greater efficacy, faster results, non-invasiveness, low-toxicity, and resistance-free

outcomes [7].

Photodynamic inactivation (PDI) has emerged as a promising technique for controlling and eliminating drug-resistant microorganisms [8]. This approach utilizes a photosensitizer (PS), which has low side effects in the absence of light but becomes activated when exposed to light at its absorption peak. There are two proposed mechanisms for the antibacterial effects of PDI. The first suggests that the excitation of certain chromophores in photoacceptor molecules affects the redox states and electron flow in the respiratory chain, leading to accelerated ATP synthesis. The second mechanism involves the production of toxic molecules, bioactive species as singlet oxygen, through the interaction between the PS and oxygen. This process involves the excitation of the PS to an unstable and short-lived excited singlet state, followed by intersystem crossing to the triplet state. In this triplet state, the PS can react with molecular oxygen to produce reactive oxygen species (ROS), including singlet oxygen. These oxidizing species can cause damage to lipids, proteins, and nucleic acids in bacterial cells, leading to their destruction [9–11]. By employing photodynamic inactivation, the reliance on systemic antibiotics, especially for skin infections, can be significantly reduced, mitigating the development and spread of antibiotic resistance.

To enhance the effectiveness of photodynamic inactivation on antibiotic-resistant bacteria, researchers have explored various approaches, focusing on optimizing laser light parameters and selecting suitable photosensitizers [12–17]. In this context, the utilization of photosensitizers derived from medicinal plants extracts such as *Hibiscus sabdariffa*, *Opuntia ficus-indica* [18], *Lumnitzera racemosa*, *Albizia procera* and *Cananga odorata* [19], *Calendulae officinalis floridis*, *Chamomillae recutitae floridis*, *Achillea millefolii herbae*, *Hypericum perforatum*, *Eucalyptus viminalis folia* [20] for their potential as source of photosensitizers in photodynamic therapy has gained attention. Medicinal plants are known for their diverse compounds that exhibit significant biological activities. By extraction of bio active compounds from plant sources, researchers can explore their potential application in various industries, including healthcare [12, 17].

Among the medicinal plants under investigation, Iranian seedless barberry or Zereshk (*Berberis*) has shown promise due to its rich alkaloid content, particularly berberine. Berberine, a naturally occurring compound found in various plant species, possesses numerous therapeutic benefits, including inhibiting bacterial growth, reducing inflammation, and displaying antioxidant properties [21–23]. In this study we have used barberry-derived photosensitizers in photodynamic inactivation to present an exciting avenue for effectively targeting and eliminating drug-resistant *S. aureus*. The extraction was performed by Soxhlet extraction method, and the absorption peaks of the extract were determined using a spectrophotometer. The extract was then tested for its antimicrobial activity through disk diffusion and minimum inhibitory concentration (MIC) and MBC tests. The results of colony counting will contribute to the understanding of the potential therapeutic applications of combining barberry extract and low level laser irradiation in combating bacterial infections, providing valuable insights for the development of new antimicrobial agents.

2. Materials and methods

2.1 Preparation of the plant and its extraction

The selected plant for this study was barberry (Zereshk), which was obtained from a store and stored in a sterile refrigerator at an appropriate temperature until the extraction process. The plant, authenticated and taxonomically confirmed by the herbarium of Islamic Azad University, Central Tehran Branch of Iran. Also, The selection of plant and its potential effects on human health was thoughtfully evaluated, taking into account its assessment by prominent global bodies like the International Union for Conservation of Nature (IUCN) and the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). This scrutiny aimed to confirm that the plant is not listed as endangered or at risk of extinction.

The extraction was conducted using a Soxhlet and rotary apparatus. Initially, 100 grams of the plant material were accurately weighed and placed in the Soxhlet apparatus. A suitable solvent, such as 70% ethanol, was used to initiate the extraction process. The extraction process for obtaining the barberry extract took approximately one and a half hours to complete.

After the extraction process using the Soxhlet apparatus, the barberry extracts underwent a 10-minute rotary evaporation step to remove any excess ethanol, which was used as the solvent during extraction. After aiding in the drying process, the extracts were spread onto sanitized glass dishes and left to dry in a ventilated hood. This method aimed to retain the active components within the extracts. Once fully dried, the extracts were gently removed from the glass dishes and transferred into small, pre-sterilized microtubes (as shown in Fig. 1).

2.2 Determining the absorption peak of the extract

The obtained extract was subjected to analysis using a spectrophotometer to determine its absorption peaks. The spectrophotometer was set to a wavelength range of 300 to 750 nanometers. Subsequently, the extract's light absorption was measured, and the resulting absorption peaks were recorded for further analysis.

2.3 Preparation of *S. aureus* bacteria

Staphylococcus aureus ATCC 33591 bacteria were provided from the microbial bank of the Central Tehran Branch, Islamic azad University. The bacteria were specifically selected and prepared for microbiological experiments.

2.4 Disk diffusion test

To assess the antimicrobial activity of the extracts, a disk diffusion test was performed. Initially, a turbidity equivalent to a 0.5 McFarland standard was prepared from the *S. aureus* bacteria. Using a sterile swab, the bacteria were streaked onto a Mueller-Hinton agar plate, ensuring even distribution. Wells were then created on the plate.

In these wells, the desired extracts were diluted with 3.0 grams and 1 mL of water using a micropipette. Then, 50 µL

Figure 1. Preparation of the plant and its extraction, (a) Soxhlet apparatus, (b) rotary apparatus, (c) drying glass dishes and pre-sterilized microtubes.

of the diluted extracts were added to each well.
The agar plate was properly labeled and then incubated at a temperature of 37 °C for a period of 18 to 24 hours. During this incubation period, the extracts would diffuse into the surrounding agar and potentially inhibit the growth of the *S. aureus* bacteria.

2.5 Minimum Inhibitory Concentration (MIC) test

The minimum inhibitory concentration (MIC) test is conducted to determine the lowest concentration of the extract that significantly inhibits the growth of a microorganism after a specified incubation period (typically 16 to 20 hours, depending on the bacterial species). The steps involved in the MIC test are as follows: 1. A pure culture of the microorganism is prepared in a suitable culture media, such as Mueller-Hinton agar. The culture is standardized to approximately one million cells per milliliter using standard microbiological techniques. 2. The antimicrobial agents are serially diluted, usually in a 1:1 ratio. In the next step, equal volumes of the diluted antimicrobial agents and the inoculum are added to each well of the liquid culture media, resulting in a final microbial concentration of approximately 500,000 cells per milliliter. 3. The inoculated antimicrobial agents are typically incubated for 18 hours at an appropriate temperature. Consistency in the incubation period is crucial for reproducibility. 4. The results are observed and reported as turbidity or microbial growth at the bottom of the wells. The last well in the series that does not show visible growth corresponds to the minimum inhibitory concentration (MIC) of the antimicrobial agent.
To determine the MIC of the bacteria, the broth microdilution method was used. Turbidity equivalent to a 0.5 McFarland standard was prepared from *S. aureus*. Then, 95 µL of the culture medium was added to each well of a 96-well plate, followed by the addition of 100 µL of the barberry extract. Finally, 5 µL of the prepared bacterial turbidity was added to each well. The plate was then incubated at 37 degrees Celsius for 24 hours, and the results were observed. The MIC test has several strengths. It is a widely accepted method for determining the susceptibility of microorganisms to antimicrobial agents. The test is relatively simple to perform and requires minimal equipment and resources. The results obtained from the MIC test are quantitative, providing a specific concentration value at which the antimicrobial agent inhibits bacterial growth. This information is valuable for guiding appropriate dosing strategies in clinical settings.
However, there are also limitations to the MIC test. The prolonged incubation period can sometimes lead to higher MIC values, as bacteria may continue to grow before being inhibited by the antimicrobial agent. Additionally, even slight variations in test parameters, such as inoculum size or incubation time, can have a significant impact on the MIC results, making it important to maintain consistency in the testing conditions.
• **Colony count**:
Following the determination of the MIC results, the number of bacterial colonies at the MIC concentration is counted to assess the antimicrobial agent's effectiveness in inhibiting bacterial growth. Three microtubes were then prepared for the bacterium, with each containing specific amounts of culture medium. Barberry extract was added to one microtube, and subsequent transfers and incubation steps were carried out 18 to 24 hours for colony count analysis. This helps in evaluating the effectiveness of the antimicrobial agent in inhibiting bacterial growth.
• **Dilution**:
A sample from microtube containing *S. aureus* bacteria at 0.5 McFarland turbidity was streaked onto a series of tubes filled with autoclaved distilled water. This process was repeated for all tubes, with subsequent dilutions made. Three petri dishes were then prepared for each sample, with aliquots from specific tubes poured onto the dishes. The Muller-Hinton agar medium was spread on the petri dishes using the pour plate method and incubated for 18 to 24 hours. After incubation, colony counts were conducted on each plate, and the *S. aureus* bacteria were then prepared for laser irradiation.
• **Laser irradiation**:
An extract solution with the minimum inhibitory concentration on the bacterium was prepared and irradiated with a 532 nm, 20 mW diode laser for durations of 5, 10, and 15 minutes. To ensure a reliable and consistent evaluation of the laser's effects, the laser power was adjusted based on the area it covered. This normalization process resulted in a laser intensity of 25 mW/cm^2, which represents the power delivered per unit area. The extract solution was then diluted and distributed into various tubes for testing, including microtubes and test tubes. Bacterial cultures were added to the tubes, which were then subjected to laser irradiation for different durations and placed in the incubator for colony count analysis after 18 to 24 hours.

3. Results and discussion

The disk diffusion test revealed a 22 mm halo surrounding the *S. aureus* bacteria treated with barberry extract, indicating a strong antibacterial effect (as shown in Fig. 2 (b)). The larger the halo, the stronger the ability of the extract to kill and inhibit the growth of the bacteria. This result indicates that barberry extract exhibits a high level of antibacterial potency specifically against *S. aureus*. Generally, the effect of barberry extract on gram-positive bacteria is greater than on gram-negative bacteria. This dissimilarity can be attributed to the structural differences in their cell walls.

Gram-positive bacteria have a simpler cell wall structure with a thick peptidoglycan layer, which is more permeable to the extract molecules. This allows the extract to penetrate the cell wall more easily and reach the target sites within the bacterial cell. On the other hand, gram-negative bacteria have a more complex cell wall structure with an outer membrane containing lipopolysaccharides that act as a barrier. This outer membrane limits the penetration of extract molecules, making it more challenging for the extract to reach the target sites within the bacterial cell [24].

Also, the results of our study demonstrate that the barberry extract shows a notable inhibitory effect on the growth of *S. aureus* bacteria, as evidenced by the MIC (minimum inhibitory concentration) value of 9375 mg/L. This indicates that at a concentration of 9375 mg/L, the barberry extract is able to prevent the growth and reproduction of the bacteria. Furthermore, the MBC (minimum bactericidal concentration) value of 18750 mg/L indicates that at a higher concentration, the barberry extract is capable of not only inhibiting the growth but also killing the *S. aureus* bacteria. This suggests that the extract possesses bactericidal properties against the bacteria, effectively eliminating them at a concentration of 18750 mg/L (as shown in Fig. 2 (a)).

Fig. 2 (c) shows the results of colony counting of subjected barberry extract and *S. aureus* to laser radiation at three different time intervals. The barberry extract alone showed minimal bactericidal activity against *S. aureus* bacteria and possesses some inherent antibacterial properties. Nevertheless, when the barberry extract was exposed to laser radiation at various time points, there was a noticeable reduction in the number of bacterial colonies. This suggests that the laser radiation enhances the antibacterial effect of the barberry extract. Furthermore, after 15 minutes of laser irradiation, no bacterial colonies were observed on the growth plate. This indicates a significant reduction in bacterial growth and suggests that the combined effect of the barberry extract and laser radiation is highly effective in inhibiting the growth and potentially killing *S. aureus* bacteria.

Fig. 3 provides a comparative analysis of the number of bacterial colonies counts in the medium containing the culture medium, bacteria, and the extract, with and without laser irradiation. The number of bacterial colonies was carefully assessed and recorded under different laser irradiation time intervals. These results represent the impact of laser radiation on the growth of *S. aureus* bacteria when combined with barberry extract. The results in this figure will likely show a gradual reduction in bacterial colonies with increasing laser irradiation time intervals. After 15 minutes of laser exposure, there will be a significant decrease in the number of bacterial colonies, potentially resulting in complete inactivation. In comparison, without the extract, the colony count was higher at the same dilution factors. This indicates that the combination of barberry extract and laser radiation has a synergistic effect, leading to a more pronounced inhibition of bacterial growth.

To ensure the validity of the experimental results, a control group was included where the laser was applied solely to the culture medium and bacteria without the presence of the

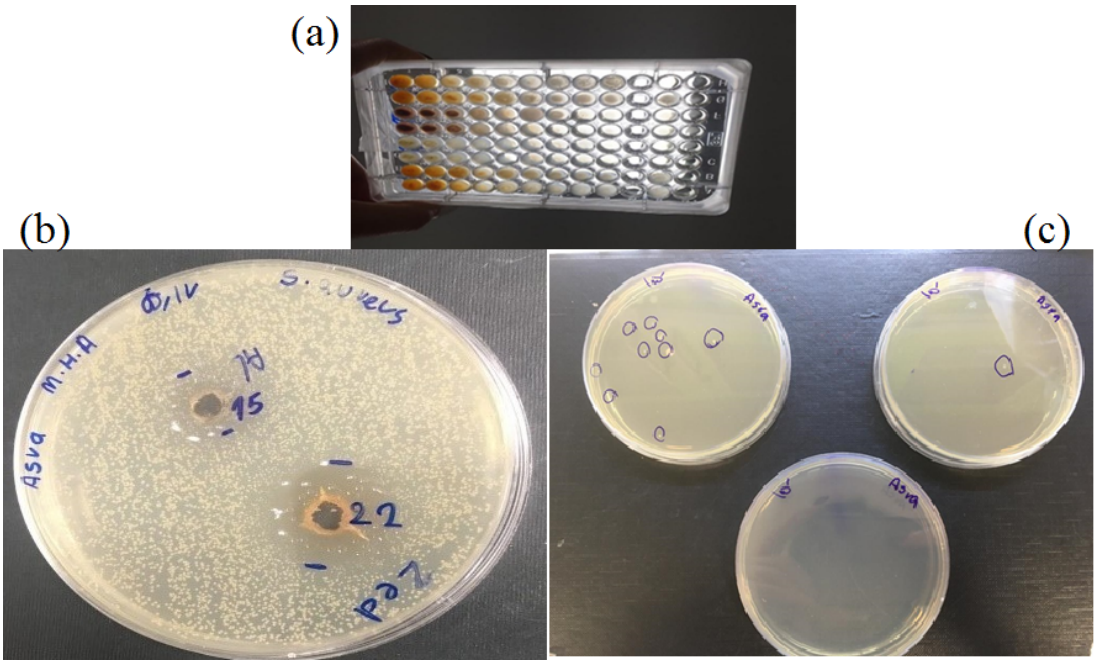

Figure 2. (a) MBC and MIC results, (b) disk diffusion test, (c) results of colony counting.

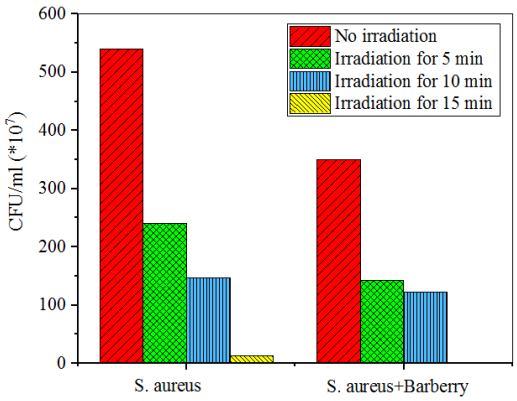

Figure 3. *S. aureus* bacteria colony counting in the presence irradiation and barberry extract.

extract (as shown in Fig. 3). The purpose of this control group was to demonstrate the specific antibacterial properties of both the extract and the laser. The results obtained from this control group align with expectations and provide further confidence in the experimental findings. The control group, consisting of only the culture medium, exhibited an uncountable number of bacterial colonies. This serves as a baseline measurement, confirming the presence of bacterial growth in the absence of any antibacterial interventions.

Our findings support previous research demonstrating the potent antibacterial efficacy of combining low-level laser irradiation with *Hibiscus sabdariffa* and *Opuntia ficus-indica* as photosensitizers against *S. aureus* ATCC 33591 [18]. Furthermore, our research aligns with the potential of other commercially available extracts like *Calendulae officinalis floridis*, *Chamomillae recutitae floridis*, *Achillea millefolii herbae*, *Hypericum perforatum*, and *Eucalyptus viminalis folia* as photosensitizers to inactivate *S. aureus* bacteria [20]. Moreover, our antibacterial testing methods (disk diffusion, MIC, and colony count) have been validated by preceding research using Giemsa stain for photoinactivation of *S. aureus* [25].

4. Conclusion

This study demonstrates the potential of using photosensitizers derived from Iranian seedless barberry (Zereshk) in photodynamic inactivation (PDI) to effectively target and eliminate drug-resistant *Staphylococcus aureus* (*S. aureus*) infections. The results of this study highlight the inhibitory effects of barberry extract on the growth of *S. aureus*, as evidenced by the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values.

The extract demonstrates its ability to prevent the growth and reproduction of the bacteria, indicating its potential as an effective treatment option. Furthermore, the combination of barberry extract and low level laser radiation in PDI shows a synergistic effect, leading to a significant reduction in bacterial colonies. The enhanced antibacterial effect observed after laser irradiation suggests that the extract and laser combination can effectively inhibit the growth and potentially kill drug-resistant *S. aureus*. These findings contribute to the growing body of research on alternative treatment approaches for drug-resistant infections. The use of barberry-derived photosensitizers in PDI offers low-side effect and resistance-free treatment option, addressing the urgent need to combat drug-resistant *S. aureus* infections.

Authors contributions

Maliheh Ranjbaran conducted the experiments, analyzed the data, and wrote the manuscript. A. Pourabdellah performed the experiments, data analysis, and contributed to the conceptualization of the study. Jamshid Sabaghzadeh supervised and directed the research. Zahra Aghaebrahimi contributed to performing the experiments and data analysis. All authors discussed the results and commented on the manuscript.

Availability of data and materials

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

Conflict of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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