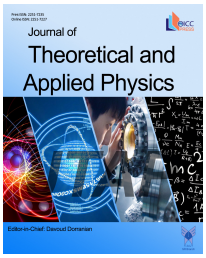

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Photodynamic activation using herbal extract and low-power laser against gram-positive (*Streptococcus mutans*) and gram-negative (*Klebsiella pneumoniae*) bacteria: threats and resistance mechanisms

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Original Research

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Abstract:

This paper investigates the efficacy of photodynamic activation (PDA) using herbal extract in combination with low-power laser treatment against both gram-positive (*Streptococcus mutans*) and gram-negative (*Klebsiella pneumoniae*) bacteria. The study explores the threats posed by these bacteria and highlights their resistance mechanisms. Various experiments, including colony formation assays, minimal inhibitory concentration tests, and bacterial viability assays, were conducted to assess the antibacterial effects of PDA in the presence and absence of laser irradiation. Additionally, spectroscopic analysis of the herbal extract was performed to elucidate their composition and potential synergistic interactions with PDA.

Keywords: Photodynamic activation; Herbal extract; Low-power laser; Antibacterial effects; Resistance mechanisms

1. Introduction

Bacterial infections continue to pose significant challenges in healthcare, contributing to morbidity, mortality, and healthcare costs worldwide. Among the diverse array of bacterial pathogens, gram-positive and gram-negative bacteria represent major categories with distinct structural and functional characteristics, yet both are implicated in a wide range of infectious diseases. Gram-positive bacteria, such as *Streptococcus mutans* is primarily known for their role in dental caries (tooth decay), but it can also contribute to more serious conditions like bacteremia and endocarditis. On the other hand, gram-negative bacteria, exemplified by *Klebsiella pneumoniae*, are notorious for their ability to cause severe healthcare-associated infections, including urinary tract infections, pneumonia, and bloodstream infections, often in immunocompromised individuals. The clinical management of bacterial infections is further complicated by the emergence and spread of antibiotic-resistant strains, which undermine the efficacy of conventional antimicrobial therapies. In this context, there is an urgent need for the development of alternative strategies to combat bacterial pathogens and mitigate the threat of antibiotic resistance [1–3]. One promising approach is photodynamic activation (PDA), an antimicrobial modality that harnesses the combined action of photosensitizing agents and light irradiation to selectively target and destroy microbial cells. PDA offers several advantages over traditional antibiotics, including broad-spectrum activity, reduced likelihood of inducing resistance, and potential synergistic interactions with other antimicrobial agents. Herbal extract has garnered attention as potential photosensitizing agents for PDA due to their rich repertoire of bioactive compounds with antimicrobial properties [4–9]. These natural products of-

fer a renewable and sustainable source of antimicrobial agents and have been traditionally used in folk medicine for treating various infectious diseases [10–12]. Additionally, low-power laser irradiation serves as a non-invasive and precise means of delivering light energy to activate photosensitizers, thereby enhancing the antimicrobial effects of PDA [13–21]. This study seeks to investigate the efficacy of PDA using herbal extract in combination with low-power laser treatment against gram-positive (*S. mutans*) and gram-negative (*K. pneumoniae*) bacteria [22–24]. By elucidating the mechanisms underlying bacterial susceptibility to PDA and exploring potential synergistic interactions between herbal extract and light irradiation, this research aims to contribute to the development of novel therapeutic strategies for combating bacterial infections. Moreover, understanding the resistance mechanisms employed by these bacteria in response to PDA could inform the design of strategies to overcome or mitigate resistance development, thereby improving patient outcomes and public health. In light of the growing threat posed by antibiotic-resistant bacteria and the limitations of current antimicrobial therapies, exploring innovative approaches such as PDA with herbal extract and low-power laser irradiation holds promise for addressing the global challenge of bacterial infections and antibiotic resistance.

2. Materials and methods

2.1 Selection and preparation of herbal extract

The herbal extract was meticulously chosen based on its reported antimicrobial activities against gram-positive and gram-negative bacteria [25–28]. The plant material used in this study was sourced from mecca and the exact part of the plant used for extraction was the flowers. These parts were chosen due to their high absorption peak for photosensitizing agents. This extract (*H. sabdariffa*) was procured from reputable suppliers and subjected to rigorous quality control measures to ensure purity and consistency. Extraction procedures were optimized to maximize the yield of bioactive compounds while minimizing potential degradation. The extraction technique, Soxhlet extraction was employed based on the solubility and stability of target compounds in an ethanolic solvent and rotary and freeze dryer were used as shown in Figure 1 [29–34]. Previous studies have examined the conservation status of *Hibiscus sabdariffa*, the plant species used in this study. Global organizations such as the International Union for Conservation of Nature (IUCN) and the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) have evaluated this plant to ensure it is not listed as endangered or threatened with extinction. The selection of *H. sabdariffa* and its potential impact on human health were carefully considered. The plant material used in our study was responsibly collected, taxonomically identified, and confirmed by the herbarium of the Islamic Azad University, Central Tehran Branch of Iran. *Hibiscus sabdariffa* is deemed safe for human consumption.

2.2 Cultivation of bacterial strains

Pure cultures of *Streptococcus mutans* ATCC 35668 and *Klebsiella pneumoniae* ATCC 13883 strains were obtained from authenticated culture collections with known antibiotic susceptibility profiles. Bacterial strains were cultured on appropriate agar media, such as Mueller-Hinton agar medium for *K. pneumoniae* and Blood Agar medium for *S. mutans*, and incubated under standardized conditions to ensure optimal growth. Subculturing was performed to maintain bacterial viability and purity throughout the experimental procedures. The well diffiusion of *H. sabdariffa* was performed on two bacteria according to Clinical and Laboratory Standards Institute (CLSI) guidelines [35].

2.3 Photodynamic activation experiments

PDA experiments were conducted using a meticulously designed protocol to evaluate the antimicrobial efficacy of herbal extract in combination with low-power laser irradiation against *S. mutans* and *K. pneumoniae*. Bacterial suspensions in appropriate growth media were treated with varying concentrations of herbal extract for predetermined incubation periods to allow for adequate interaction between the photosensitizing agents and bacterial cells. Minimum inhibitory concentration (MIC) tests were conducted in mi-

Figure 1. The steps of providing the ethanolic *H. sabdariffa* extract.

croplates using the standard broth microdilution method as recommended by the Clinical and Laboratory Standards Institute (CLSI) [36]. The tests were performed twice: once in the absence of laser irradiation and once in its presence. During the laser treatment phase, each well in the microplate was exposed to low-power laser irradiation at specific wavelengths and doses using a calibrated laser system. Control groups, including untreated bacterial suspensions and suspensions treated with herbal extract or laser irradiation alone, were included for comparison.

2.4 Low-power laser irradiation setup

A low-power laser system equipped with a suitable light source and optical components was utilized for PDA experiments [37]. The laser parameters, including 532 nm wavelength, 4.16 nm bandwidth of laser, 20 mW power output, 6, 12, 18, and 20 min irradiation time, and 1 mm^2 spot size, 25 mW/cm^2 light intensity, and energy for each times periods, 9, 18, 27, and 30 J/cm^2 were meticulously optimized to ensure precise and reproducible delivery of light energy to the bacterial suspensions. Laser safety precautions were strictly adhered to during experimental procedures to minimize the risk of exposure to laser radiation as shown in Fig. 2.

2.5 Colony formation assays

After PDA treatment, to assess the colony-forming units (CFUs), 100 μL from the control well (containing bacteria without the extract) and from the two wells preceding the MBC well and the MBC well itself (both containing bacteria and the herbal sample) were serially diluted in sterile normal saline across 9 consecutive tubes (0.1 mL of the well content and 0.9 mL of the diluent). Subsequently, 100 μL from tubes 7, 8, and 9 were transferred to Muller-Hinton agar plates and incubated overnight for bacterial growth. After 18–24 h, the number of viable colonies was enumerated, and the percentage reduction in colony-forming units (CFU) compared to untreated controls was calculated to evaluate the antimicrobial efficacy of PDA [38]. Each experiment was conducted three times to ensure accuracy.

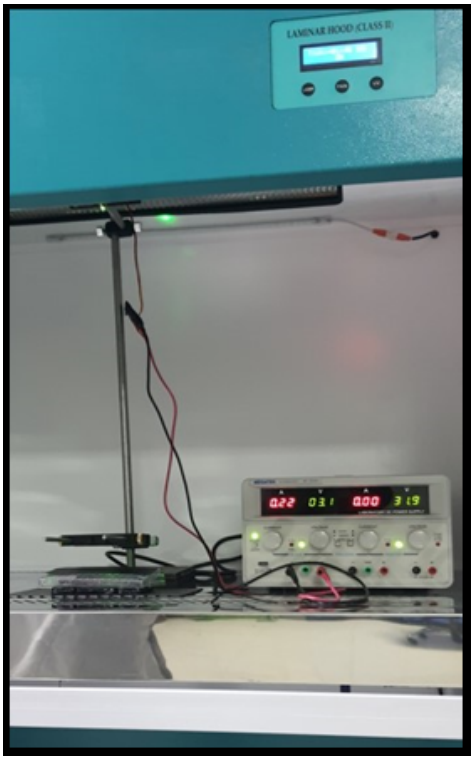

Figure 2. The setup of green low- level laser.

2.6 Minimal inhibitory concentration (MIC) tests

The MIC of herbal extract against *S. mutans* and *K. pneumoniae* strains was determined using broth microdilution assays following established guidelines. Serial dilutions of herbal extract were prepared in appropriate growth media, and standardized bacterial inoculum was added to each well of microtiter plates. The plates were incubated under optimal conditions for bacterial growth, and the lowest concentration of herbal extract that inhibited visible bacterial growth was recorded as the MIC.

2.7 Spectroscopic analysis of herbal extract

The chemical composition of the herbal extract was analyzed using spectroscopic technique to elucidate the presence of bioactive compounds responsible for their antimicrobial activity (UV-vis spectroscopy) [39]. This provided valuable insights into the composition and potential synergistic interactions of herbal extract with PDA.

2.8 Statistical analysis

Statistical analysis was performed using appropriate software to evaluate the significance of differences between treatment groups. Parametric and non-parametric tests, such as t-tests, were employed based on the distribution and nature of the data. Data were expressed as mean $\pm$ standard deviation (SD), and p-values less than 0.05 were considered statistically significant. The robustness and reproducibility of experimental results were ensured through repeated experiments and adequate sample size determination.

3. Results

3.1 Antibacterial effects of photodynamic activation

The antibacterial effects of photodynamic activation (PDA) using herbal extract (*H. sabdariffa* extract) in combination with low-power laser irradiation were evaluated against both gram-positive (*S. mutans*) and gram-negative (*K. pneumoniae*) bacteria. Colony formation assays revealed a significant reduction in bacterial viability following PDA treatment compared to untreated controls as shown in Fig. 3. Specifically, treatment with herbal extract and low-power laser irradiation resulted in a dose-dependent decrease in the number of viable bacterial colonies for both *S. mutans* and *K. pneumoniae* strains. Moreover, the combination of herbal extract and laser irradiation exhibited enhanced antibacterial efficacy compared to treatment with herbal extract or laser irradiation alone, indicating synergistic interactions between the photosensitizing agents and light energy.

The photodynamic activation (PDA) of the plant extract exhibited significant antibacterial efficacy through the generation of reactive oxygen species (ROS). Upon exposure

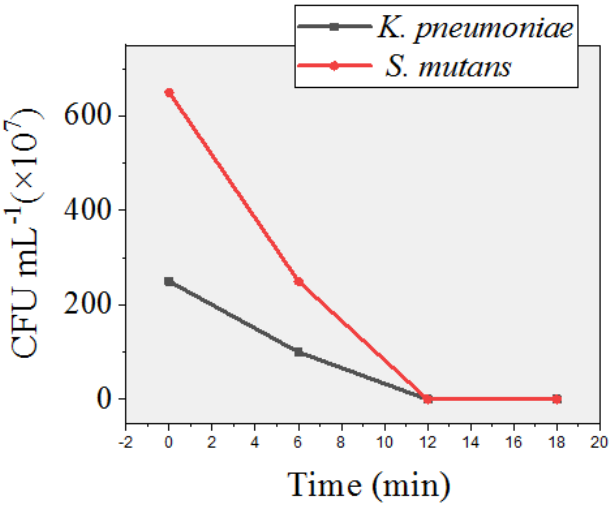

Figure 3. The result of CFUs/mL of *S. mutans* and *K. pneumoniae* employing *H. sabdariffa* ethanolic extract separately in the absence and in the presence of laser irradiation at 3 times, 6, 12, and 18 min.

to light, the photosensitizing compounds in the extract absorbed the light energy and transitioned to an excited state. This excited state facilitated the transfer of energy to molecular oxygen, producing singlet oxygen ($^1O_2$) and other ROS such as superoxide anions (O_2^-) and hydroxyl radicals ($OH^\cdot$). These ROS are highly reactive and can cause oxidative damage to vital cellular components of bacteria, including lipids, proteins, and nucleic acids. The biophysical phenomena underlying this process include, "Absorption of Photons" the photosensitizers absorb photons and become excited. "Energy Transfer" excited photosensitizers transfer energy to molecular oxygen, generating ROS. "Oxidative Damage" ROS interact with bacterial cells, causing damage to membranes, enzymes, and DNA, leading to cell death. Experimental results showed a substantial reduction in bacterial colonies after PDA treatment, confirming the approach's effectiveness. The reduction in bacterial load was statistically significant ($p < 0.05$) compared to control groups, demonstrating the potent antibacterial action facilitated by the photodynamic mechanism.

3.2 Dose-dependent effects of herbal extract

The dose-response relationship between herbal extract concentrations and bacterial viability was meticulously characterized. Gradual increases in the concentration of herbal extract resulted in a proportional decrease in bacterial viability, as evidenced by the reduction in colony-forming units (CFU) on agar plates in Figure 3. Notably, higher concentrations of herbal extract exhibited greater antimicrobial efficacy against both *S. mutans* and *K. pneumoniae* strains, reaching a plateau at maximal inhibitory concentrations.

3.3 Enhanced bacterial killing with low-power laser irradiation

Low-power laser irradiation significantly potentiated the antibacterial effects of PDA, leading to enhanced bacterial killing compared to PDA treatment alone. Bacterial suspensions treated with herbal extract and low-power laser irradiation demonstrated a substantial reduction in viable bacterial colonies compared to those treated with herbal extract without laser irradiation. The synergistic action of herbal extract and laser irradiation resulted in more pronounced microbial growth inhibition and increased bactericidal activity against both *S. mutans* and *K. pneumoniae* strains as shown in Fig. 4.

3.4 Minimal inhibitory concentration (MIC) values

Minimal inhibitory concentration (MIC) tests were performed to determine the potency of herbal extract against *S. mutans* and *K. pneumoniae* strains. The MIC values, defined as the lowest concentration of herbal extract that inhibited visible bacterial growth, were meticulously determined using broth microdilution assays. The herbal extract exhibited varying MIC values against different bacterial strains, with lower MIC values indicative of greater antimicrobial potency. The MIC values obtained for *S. mutans* and *K. pneumoniae* strains highlighted the efficacy of PDI in inhibiting bacterial growth at concentrations below those causing cytotoxic effects in Fig. 4.

3.5 Spectroscopic characterization of herbal extract

Spectroscopic analysis of herbal extract provided valuable insights into its chemical composition and potential synergistic interactions with PDI. UV-vis spectroscopy revealed characteristic absorption peaks corresponding to specific bioactive compounds present in herbal extract. The absorption peak in Figure 5 has shown, that the presence of bioactive ingredients and the red color of the extract tell us, the presence of anthocyanin pigments too. We have the fingerprints of phenolic and non-phenolic compounds (bioactive ingredients and pigments) from recent studies, like Flavonoids (in the range of $240-285$ nm and others in the range of $300-400$ nm) [40–42] Tannins (280 nm) [43], Anthocyanin and Anthocynadin (predominantly one between 270–290 nm and the other in the visible spectrum at 500–550 nm) [42, 44]. Also, the hole of inhibitions of *H. sabdariffa* extract for two bacteria were good as seen in

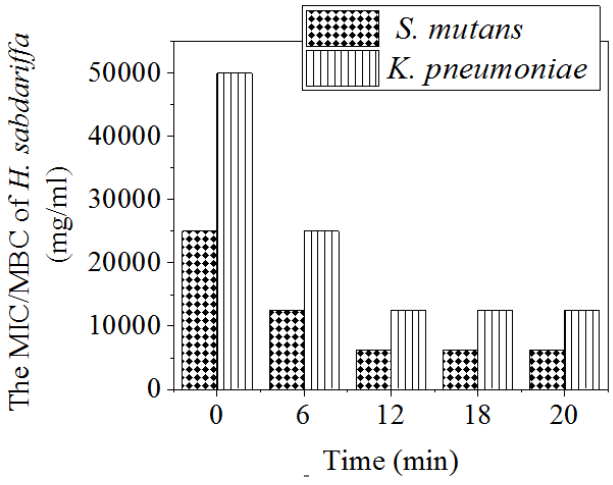

Figure 4. The MIC/MBC values of *H. sabdariffa* against on *S. mutans* and *K. pneumoniae*.

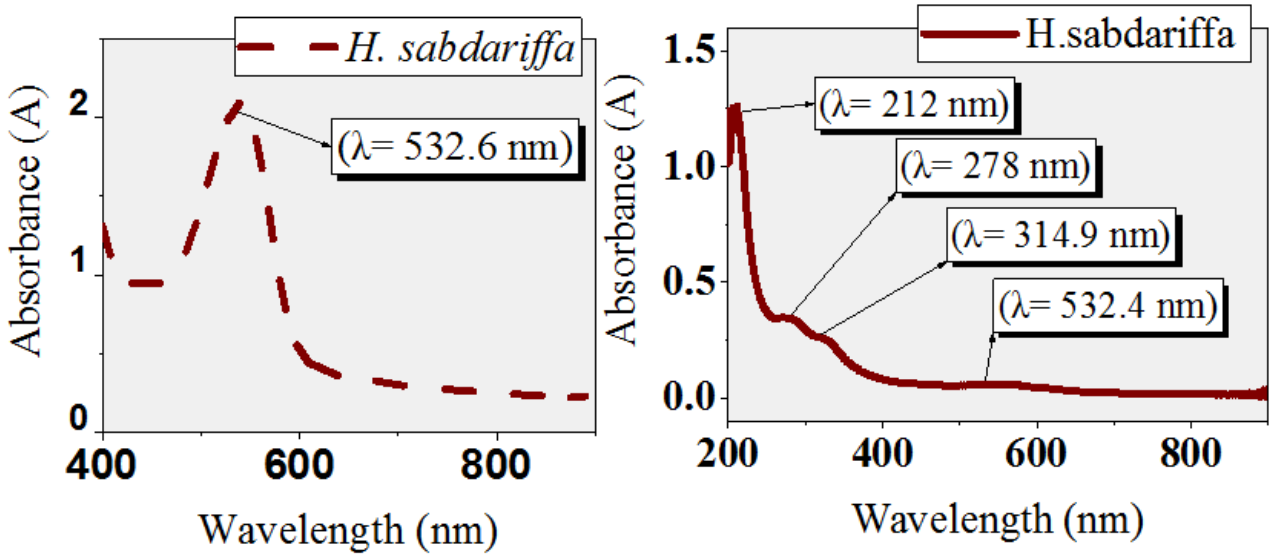

Figure 5. The absorption peaks of ethanolic extract of *H. sabdariffa* in UV-VIS spectroscopy.

Fig. 6.

3.6 Statistical analysis

Statistical analysis of experimental data was conducted to assess the significance of differences between treatment groups. The t-test was employed to compare mean or median values between groups. The results were expressed as mean ± standard deviation (SD), with p-values less than 0.05 considered statistically significant. The robustness and reproducibility of experimental findings were ensured through rigorous experimental design, adequate sample size determination, and repeated measurements. Numerical results of the MIC/MBC test in the absence and the presence of laser irradiation against bacteria are shown in Table 1.

4. Conclusion

In conclusion, the findings of this study underscore the potential of photodynamic activation (PDA) using herbal extract and low-power laser irradiation as a promising therapeutic approach for combating bacterial infections caused by both gram-positive (*S. mutans*) and gram-negative (*K. pneumoniae*) bacteria. Through comprehensive investigations of the antibacterial effects of PDA and elucidation of the underlying mechanisms, this research has contributed valuable insights into the development of innovative strategies to address the global challenge of antibiotic resistance and improve patient outcomes in healthcare settings [45]. The results demonstrate the robust antimicrobial efficacy of PDA against both *S. mutans* and *K. pneumoniae*, as evidenced by the significant reduction in bacterial viability following treatment. Importantly, the synergistic action of herbal extract and low-power laser irradiation potentiates the bactericidal effects of PDA, leading to enhanced microbial killing compared to treatment with herbal extract or laser irradiation alone. This synergistic interaction highlights the importance of combining photosensitizing agents with light energy to maximize the therapeutic efficacy of PDA and overcome bacterial resistance mechanisms. Furthermore, the dose-dependent effects of herbal extract and the determination of minimal inhibitory concentration (MIC) values provide valuable insights into the optimal dosing regimen for PDA and the potency of herbal extract (*H. sabdariffa*) against bacterial strains. These findings pave the way for the rational design of PDA protocols tailored to specific bacterial infections, optimizing treatment outcomes and minimizing potential adverse effects. The spectroscopic characterization of

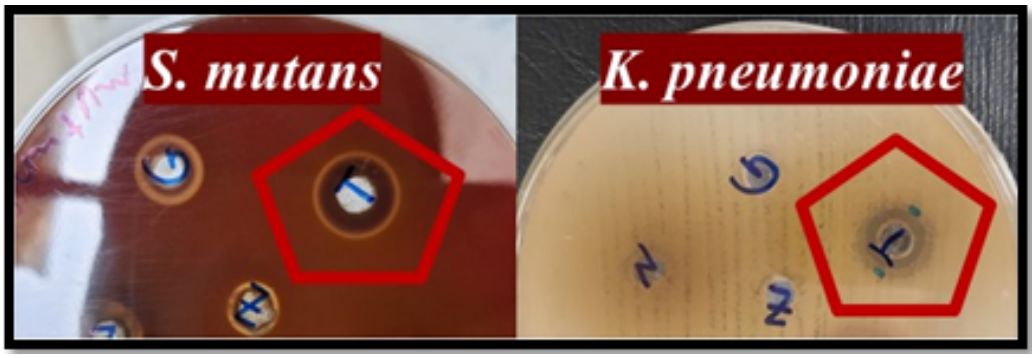

Figure 6. The hole of inhibition of *H.sabdariffa* extract on *S. mutans* and *K. pneumoniae* bacteria.

Table 1. The numerical results of the MIC/MBC test of *H. sabdariffa* in the absence and the presence of laser irradiation against *S. mutans* and *K. pneumoniae*.

	In the absence of laser irradiation	Sample *H. sabdariffa*	
	(min)	*S. mutans* bacteria	*K. pneumoniae* bacteria
The MIC/MBC (mg/mL)	t = 0	50000	25000
	t = 6	25000	12500
	t = 12	12500	6250
	t = 18	12500	6250
	t = 20	12500	6250
	Standard deviation	0.001	0.001
	P values	0.037	0.037

herbal extract elucidates their chemical composition and potential synergistic interactions with PDA, offering mechanistic insights into the antimicrobial mechanisms underlying PDA. This comprehensive understanding of the chemical properties of herbal extract and their interactions with light energy facilitates the development of novel photosensitizing agents with enhanced antimicrobial activity and improved therapeutic efficacy. In light of the growing threat posed by antibiotic-resistant bacteria and the limitations of current antimicrobial therapies, the findings of this study have significant implications for the development of alternative treatment modalities for bacterial infections. By harnessing the power of PDA and herbal extract, healthcare providers can explore innovative approaches to combat antibiotic resistance and improve patient care outcomes. Further research is warranted to optimize PDA protocols, elucidate additional synergistic interactions between photosensitizing agents and light sources, and evaluate the clinical applicability of this approach in diverse healthcare settings. Overall, this study underscores the potential of PDA as a promising adjunctive therapy for the treatment of bacterial infections and highlights the importance of multidisciplinary approaches in addressing the global challenge of antibiotic resistance. By embracing innovative strategies such as PDA, we can pave the way toward a future where effective antimicrobial treatments are accessible to all and where bacterial infections are effectively controlled, thereby improving public health and saving lives.

Ethical Approval
This manuscript does not report on or involve the use of any animal or human data or tissue. So the ethical approval is not applicable.

Authors Contributions
All the authors have participated sufficiently in the intellectual content, conception and design of this work or the analysis and interpretation of the data (when applicable), as well as the writing of 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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