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ANTIMICROBIAL ACTIVITY OF THE COMBINED APPLICATION OF PHOTOSENSITIZERS AND RED-SPECTRUM LED RADIATION

Introduction. The irrational and excessive use of antibiotics in clinical practice, as well as the widespread application of antimicrobial agents beyond the medical field, contributes to the accelerated spread of antimicrobial resistance. This, in turn, necessitates the search for effective non-pharmacological approaches to combating infectious agents.

The aim of this study was to investigate the combined effects of photosensitizers (PS) – 0.1% aqueous solutions of azure, methylene blue, methylene green, and brilliant green – and red-spectrum light-emitting diode (LED) irradiation on the growth of opportunistic microorganisms.

Materials and Methods. To determine the antimicrobial effects of combined PSs application and red-spectrum LED irradiation on microbial growth, microorganisms were divided into several groups to independently assess the effects of PSs, LED irradiation, and their combined application. The objects of study were clinical isolates of *Staphylococcus aureus* (n = 5), *Candida albicans* (n = 5), *Escherichia coli* (n = 5), and *Enterococcus faecalis* (n = 5), as well as reference strains *S. aureus* ATCC 25923, *C. albicans* ATCC 2091, and *E. coli* ATCC 25922.

Results and discussion. The combined effect of the PSs methylene blue and azure with red-spectrum LED irradiation led to a reduction in the growth intensity of the studied microbial strains by an average of 34.1-72.5% compared with the control groups. The degree of antimicrobial effect observed with the combined use of PSs and LED significantly exceeded that of the photosensitizers applied individually. A 0.1% aqueous solution of methylene green did not exhibit antimicrobial activity either when used alone or in combination with LED irradiation, whereas brilliant green resulted in complete inhibition of microbial growth.

Conclusions. The developed aPDT method using the PSs methylene blue and azure demonstrated a significant antimicrobial effect, which was on average 17.8–52.7% higher compared with the use of the PSs alone. Considering its high efficacy and the absence of risk for the development of microbial resistance, the aPDT method may be recommended for the complex treatment of purulent-inflammatory processes caused by opportunistic microorganisms.

Key words: opportunistic microorganisms, antimicrobial photodynamic therapy, antibiotic resistance, low-intensity irradiation.

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ПРОТИМІКРОБНА АКТИВНІСТЬ КОМПЛЕКСНОГО ЗАСТОСУВАННЯ ФОТОСЕНСИБІЛІЗАТОРІВ ТА СВІТЛОДІОДНОГО ВИПРОМІНЮВАННЯ ЧЕРВОНОГО СПЕКТРУ

Вступ. Нераціональне і надмірне застосування антибіотиків у клінічній практиці, а також широке використання протимікробних засобів поза межами медицини зумовлює пришвидшення темпів поширення антимікробної резистентності, що в свою чергу зумовлює пошук ефективних немедикаментозних засобів боротьби з інфекційними агентами.

Метою роботи було дослідити комплексний вплив фотосенсибілізаторів (ФС) – 0,1% водних розчинів азуру, метиленового синього, метиленового зеленого, брильянтового зеленого та світлодіодного випромінювання червоного спектру на ріст умовно-патогенних мікроорганізмів.

Матеріали і методи. При дослідженні сумісного впливу ФС та світлодіодного випромінювання червоного спектру на інтенсивність росту мікроорганізмів, останні було поділено на кілька груп для вивчення ступеню впливу вказаних факторів окремо. Об'єкти дослідження – клінічні ізоляти *S. aureus* (n = 5), *C. albicans* (n = 5), *E. coli* (n = 5) та *E. faecalis* (n = 5), а також колекційні штами *S. aureus* ATCC 25923, *C. albicans* ATCC 2091 та *E. coli* ATCC 25922.

Результати досліджень та їх обговорення. Комплексний вплив ФС метиленового синього та азуру зі світлодіодним випромінюванням червоного спектру зумовлював зниження інтенсивності росту досліджуваних штамів мікроорганізмів в середньому на 34,1-72,5%, порівняно з контрольними групами. Ступінь антимікробного впливу при сумісному застосуванні ФС та LED суттєво перевищував дію ФС при окремому їх застосуванні. 0,1% водний розчин метиленового зеленого не проявляв протимікробної активності ані при самостійному застосуванні, ані в комплексі з LED, тоді як брильянтовий зелений зумовлював повне інгібування росту мікроорганізмів.

Висновки. Розроблена методика аФДТ із використанням ФС метиленового синього та азуру володіє суттєвим протимікробним ефектом, який в середньому на 17,8–52,7% вищий у порівнянні з окремим застосуванням ФС. Зважаючи на високу ефективність та відсутність ризику виникнення резистентності мікроорганізмів, метод аФДТ може бути рекомендований для комплексного лікування гнійно-запальних процесів, зумовлених умовно-патогенними мікроорганізмами.

Ключові слова: умовно-патогенні мікроорганізми, антимікробна фотодинамічна терапія, антибіотикорезистентність, низькоінтенсивне випромінювання.

Introduction. In just about 100 years since their discovery, antibiotics have radically transformed modern medicine, saved millions of human lives, and contributed to an increase in average human life expectancy by nearly 23 years. They have become an essential component in the treatment of infectious diseases and have enabled the performance of life-saving therapeutic interventions and complex medical procedures, including surgical operations, organ transplantation, chemotherapy, and intensive care treatment [1–3].

At the same time, antimicrobial resistance (AMR) represents a natural and inevitable biological phenomenon resulting from the intrinsic ability of microorganisms to acquire genetic mutations and exchange resistance determinants through horizontal gene transfer [4]. However, the emergence and rapid dissemination of resistant pathogens have been significantly accelerated by the irrational and excessive use of antibiotics in clinical practice. The situation is further exacerbated by the extensive application of antimicrobial agents beyond human medicine, particularly in agriculture and animal husbandry, which contributes to the selection and spread of resistant microorganisms across human, animal, and environmental reservoirs [5, 6].

Despite the implementation of multiple strategies aimed at addressing antimicrobial resistance (AMR) over recent decades, the spread of resistance continues to show no signs of slowing [7, 8]. Consequently, the investigation of novel approaches for combating infectious agents remains highly relevant. Among emerging alternatives, antimicrobial photodynamic therapy (aPDT) has gained considerable attention due to several advantages,

including its non-invasive nature, minimal adverse effects, activity against a broad spectrum of Gram-positive and Gram-negative bacteria as well as fungi and viruses, and its potential to reduce pharmacological burden on the patient [9–11]. This method involves the use of a photosensitizer (PS) followed by irradiation with low-intensity light of an appropriate wavelength. This initiates a photodynamic reaction, leading to the generation of reactive oxygen species [9, 12, 13].

Several aspects concerning the effects of low-intensity radiation on the biological properties of microorganisms, as well as the role of radiation parameters such as coherence and polarization during combined application with photosensitizers, require further in-depth investigation.

The aim of this study was to investigate the combined effects of photosensitizers – 0.1% aqueous solutions of azure, methylene blue, methylene green, and brilliant green in combination with red-spectrum light-emitting diode (LED) radiation on the growth of opportunistic microorganisms.

Methodology and Methods. The investigated microorganisms were standardized as follows: 24-hour agar cultures or 5–6-hour broth cultures of both clinical and reference strains were suspended in a liquid nutrient medium and adjusted to an optical density equivalent to 0.5 McFarland standards. The resulting suspensions were then further diluted by a factor of 1.25×10^5.

To evaluate the combined effects of PSs and low-intensity radiation, as well as the individual contributions of each factor, the studied microorganisms were divided into several experimental groups (table 1).

The first (control) group consisted of the tested microorganisms, whose standardized inoculum was subcultured onto Petri dishes containing nutrient medium without any additional treatment. The microorganisms in the second group were exposed to low-intensity irradiation for 10 minutes. The volume of the irradiated suspension was 1 ml. For the third group, 100 μL of the photosensitizer (PS) was added to 900 μL of the inoculum, followed by a 10-minute dark incubation period, after which the suspension was plated onto Petri dishes. In the fourth group, the PS was added in the same proportion as in the third group. After a 10-minute dark incubation, the samples were exposed to low-intensity light of the appropriate wavelength for 10 minutes.

Table 1

Groups of microorganisms used to investigate the combined effects of photosensitizers and low-intensity radiation on their growth intensity

Group 1	Group 2	Group 3	Group 4
Microbial inoculum subcultured onto nutrient medium	Microbial inoculum exposed to low-intensity radiation (10 min)	Microbial inoculum treated with photosensitizer (PS) in a 9:1 ratio	Microbial inoculum treated with PS followed by exposure to low-intensity radiation of the appropriate wavelength (10 min)

The study objects included clinical isolates of *Staphylococcus aureus* (n = 5), *Candida albicans* (n = 5), *Enterococcus faecalis* (n = 5), and *Escherichia coli* (n = 5), collected from periodontal pockets of patients with chronic generalized periodontitis. In addition, reference strains *S. aureus* ATCC 25923, *C. albicans* ATCC 2091, and *E. coli* ATCC 25922 (Selectrol®, UK) were used. Studies involving reference strains were performed in five independent replicates to ensure statistical reliability. Clinical isolates were identified using standard bacterioscopic and bacteriological methods [14]. For the final identification of pure cultures of microorganisms, a biochemical method using test systems STAPHYtest 16, CANDIDAtest 21, and ENTEROtest 16 («PLIVA-Lachema a.s.», Czech Republic) was employed.

Red and near-infrared LED irradiation was generated using the *Medolight Red Bioptron Light Therapy System* (Zepter, Switzerland, 2017). The device generates radiation with wavelengths of 640 ± 30 nm and 880 ± 30 nm at a power density of 5.35 mW/cm² at a distance of 0-1 cm, and operating in five emission frequency modes: 0 Hz, 10 Hz, 600 Hz, 3000 Hz, and 8000 Hz. Irradiation of standardized microbial inoculum was carried out in sterile Petri dishes at a fixed distance of 1 cm from the light source with exposure of 10 min and frequency 8000 Hz. The volume of each irradiated inoculum was 1 ml.

Statistical analysis of the obtained data was performed by calculating the mean values and standard deviations. Differences between control and experimental groups of microorganisms were evaluated using analysis of variance (ANOVA) followed by Tukey's post hoc test. Differences were considered statistically significant at $p < 0.05$.

Results and discussion. In previous studies [15] the optical absorption spectra of the indicated photosensitizers (PS) in the visible and near-infrared ranges were determined. Accordingly, the absorption maximum of azure was 621 nm, methylene blue – 664 nm, methylene green – 631 nm, and brilliant green – 624 nm, which corresponds to the red spectral region and, consequently, to the range of the red LED radiation source (640 ± 30 nm).

A pronounced antimicrobial effect of the combined application of a 0.1% aqueous solution of methylene blue and LED irradiation on the growth of all tested microorganisms was demonstrated (table 2).

The addition of methylene blue caused a reduction in the number of colonies of clinical isolates of *S. aureus* by an average of 24.8% compared with group 1 ($p = 0.0026$). The combined effect of the photosensitizer and red-spectrum LED irradiation reduced the growth intensity of the specified *S. aureus* strains by an average of 51.0% compared with the control ($p < 0.0001$).

In the case of the reference strain *S. aureus* ATCC 25923, the growth intensity in group 2 was, on average, 16% higher than in the control ($p = 0.0063$). The number of colonies in groups 3 and 4 was, on average, 19.0% ($p = 0.0021$) and 34.3% ($p < 0.0001$) lower, respectively, compared with group 1 (Fig. 1).

The growth intensity of groups 3 and 4 of the studied strains *C. albicans*, *E. coli*, and *E. faecalis* was, on average, 18.8-28.5% and 34.4-48.9% lower, respectively, compared with the control. At the same time, the difference

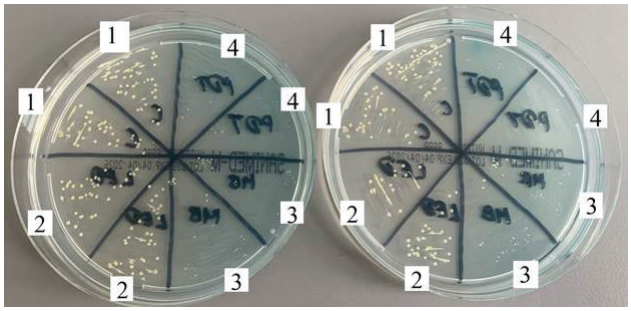

Fig. 1. Growth of *S. aureus* after subculturing onto nutrient media. 1 – control; 2 – culture exposed to red-spectrum LED irradiation; 3 – addition of methylene blue; 4 – addition of methylene blue followed by red-spectrum LED irradiation

in the number of colonies between groups 1 and 3 of *C. albicans* was not statistically significant ($p \geq 0.1163$).

It should be noted that in all cases the intensity of microbial growth in group 4 was statistically significantly lower compared with microorganisms of groups 1-3 ($p < 0.05$).

The combined application of a 0.1% aqueous solution of azure and red-spectrum LED-radiation also demonstrated a pronounced antimicrobial effect against all tested strains. Quantitative and statistically processed data on microbial growth intensity are presented in the table 3.

The addition of a 0.1% aqueous solution of azure to microorganisms in group 3 reduced their growth intensity by an average of 18.2-41.9% compared with the control groups. The combined effect of azure and LED irradiation decreased the number of colonies of the studied strains by an average of 34.1-72.5% compared with the control. Figure 2 shows the growth of a clinical isolate of *S. aureus* in different groups.

The microbial growth intensity in group 4 was on average 17.8-52.7% lower compared with microorganisms in group 3. In the experimental series involving the combined application of red-spectrum LED irradiation and azure, the separate application of irradiation alone did not produce a statistically significant effect on the number of microbial colonies compared with the control groups.

The results of studies using 0.1% aqueous solution brilliant green demonstrated a pronounced antimicrobial effect of this substance and complete inhibition of the growth of the tested microorganisms when applied separately (fig. 3).

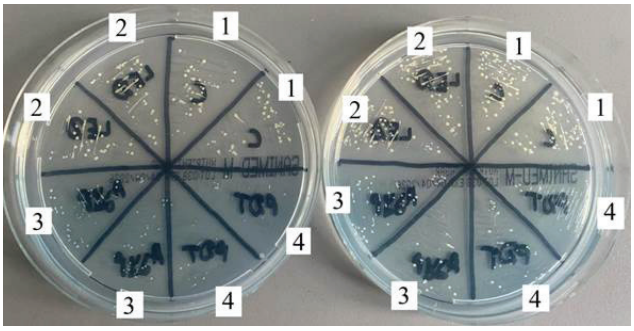

Fig. 2. Growth of *S. aureus* after subculturing onto nutrient media. 1 – control; 2 – culture exposed to red-spectrum LED irradiation; 3 – addition of azure; 4 – addition of azure followed by red-spectrum LED irradiation.

The application of a 0.1% aqueous solution of methylene green as a PS did not result in statistically significant changes in the growth intensity of the investigated microorganisms, either following its standalone administration or upon combined exposure with red-spectrum LED irradiation (fig. 4).

Thus, the combined effect of 0.1% aqueous solutions of methylene blue and azure with red-spectrum LED irradiation demonstrated significant antimicrobial activity against all tested clinical and reference microbial strains. The most pronounced antimicrobial effect was observed when the photosensitizer azure was applied against *E. faecalis*.

Table 2

Number of microbial colonies following combined application of methylene blue and red-spectrum LED irradiation (x ± SD)

Species of microorganisms	Group 1	Group 2	Group 3	Group 4
S. aureus, clinical isolates (n = 5)	57.4 ± 5.9 a	62.4 ± 5.9 a	43.2 ± 4.5 b	30.6 ± 3.7 c
S. aureus ATCC 25923 (n = 5)	60.0 ± 4.9 b	69.6 ± 3.2 a	48.6 ± 2.9 c	39.4 ± 3.4 d
C. albicans, clinical isolates (n = 5)	31.4 ± 4.6 a	37.8 ± 5.2 a	26.6 ± 4.0 a	20.6 ± 2.9 b
C. albicans ATCC 2091 (n = 5)	25.6 ± 4.8 a	30.4 ± 3.2 a	20.8 ± 5.1 a	16.0 ± 4.7 b
E. coli, clinical isolates (n = 5)	43.8 ± 4.1 b	54.0 ± 3.4 a	33.8 ± 3.1 c	26.2 ± 3.0 d
E. coli ATCC 25922 (n = 5)	37.8 ± 3.6 b	46.4 ± 2.4 a	29.8 ± 2.5 c	22.6 ± 2.9 d
E. faecalis clinical isolates (n = 5)	63.8 ± 6.1 a	69.6 ± 4.0 a	45.6 ± 4.9 b	32.6 ± 5.1 c

Note: Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test. Means within a row sharing different superscript letters (a–d) differ significantly at $p < 0.05$

Table 3

Number of microbial colonies following combined application of azure and red-spectrum LED irradiation (x ± SD)

Species of microorganisms	Group 1	Group 2	Group 3	Group 4
S. aureus, clinical isolates (n = 5)	52.8 ± 5.8 a	57.8 ± 5.5 a	43.2 ± 5.3 b	34.8 ± 5.3 c
S. aureus ATCC 25923 (n = 5)	61.6 ± 4.3 a	67.0 ± 5.0 a	48.2 ± 3.7 b	39.6 ± 2.9 c
C. albicans, clinical isolates (n = 5)	32.4 ± 4.2 a	36.4 ± 5.0 a	26.0 ± 3.5 b	19.6 ± 4.0 c
C. albicans ATCC 2091 (n = 5)	29.2 ± 4.6 a	31.0 ± 3.8 a	23.0 ± 3.5 b	17.2 ± 3.1 c
E. coli, clinical isolates (n = 5)	45.0 ± 5.5 a	49.2 ± 5.8 a	36.8 ± 5.4 b	29.0 ± 4.2 c
E. coli ATCC 25922 (n = 5)	58.6 ± 6.6 a	63.0 ± 6.8 a	47.4 ± 4.8 b	38.0 ± 3.8 c
E. faecalis clinical isolates (n = 5)	64.0 ± 8.0 a	69.4 ± 7.1 a	37.2 ± 7.0 b	17.6 ± 2.9 c

Note: Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test. Means within a row sharing different superscript letters (a–c) differ significantly at $p < 0.05$

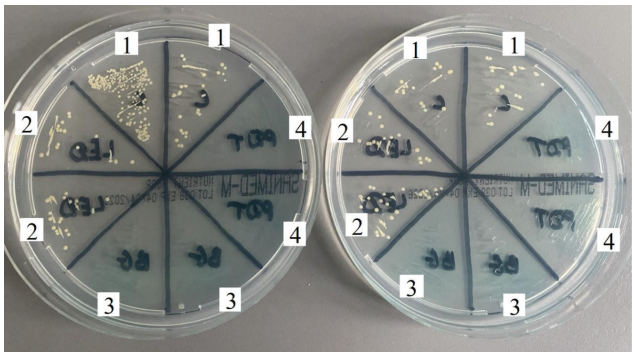

Fig. 3. Growth of *S. aureus* after subculturing onto nutrient media. 1 – control; 2 – culture exposed to red-spectrum LED irradiation; 3 – addition of brilliant green; 4 – addition of brilliant green followed by red-spectrum LED irradiation.

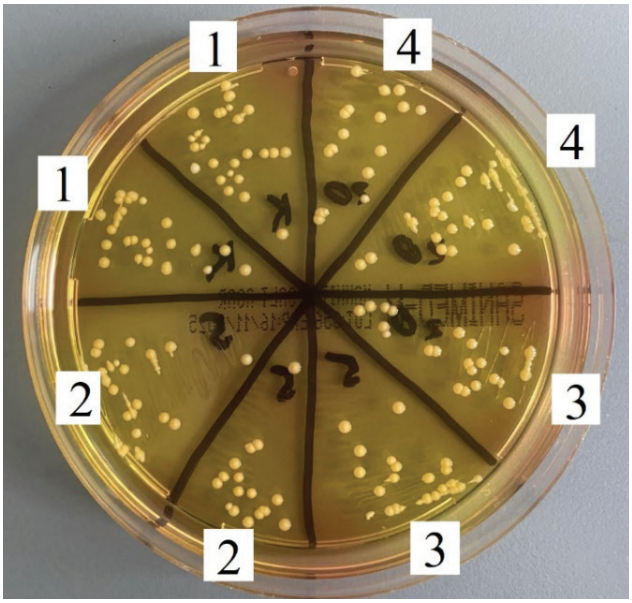

Fig. 4. Growth of *S. aureus* after subculturing onto nutrient media. 1 – control; 2 – culture exposed to red-spectrum LED irradiation; 3 – addition of methylene green; 4 – addition of methylene green followed by red-spectrum LED irradiation

APDT necessitates a specific light source to activate the PS, typically through exposure to low-intensity visible light at a defined wavelength. Most photosensitizers are optimally activated by red light in the range of 630-700 nm, which corresponds to a tissue penetration depth of approximately 0.5 to 1.5 cm [16]. In the presence of molecular oxygen, the activated PS generates reactive oxygen species. These species initiate a cascade of photochemical and biological processes that result in irreversible cellular damage and subsequent microbial cell death [17]. This mechanism of action precludes the development of microbial resistance, thereby supporting the consideration of aPDT as a promising adjunctive approach in the management of infectious diseases.

Light-induced inactivation of pathogens has been shown to be more effective against Gram-positive bacteria, largely due to their relatively simpler cell wall architecture, which facilitates deeper penetration of the PS [18]. Evidence also suggests that aPDT may contribute to mitigating COVID-19, either through the treatment of infected individuals or via the development of functional photoactive materials. Such applications include self-disinfecting textiles and surfaces, as well as systems designed for the decontamination of water and air [19].

Over recent decades, the majority of research on antimicrobial photodynamic therapy (aPDT) has primarily focused on the properties of the photosensitizer (PS). However, the characteristics of the light source are of equal importance [20]. In our previous studies [15, 21, 22], various light sources were employed, including LED, polarized incoherent low-energy radiation (PILER), and low-power lasers. In all cases, pronounced antimicrobial and antibiofilm effects were observed. These findings suggest that, within aPDT, the critical parameters of low-power radiation are the wavelength and power density. Such evidence may broaden the potential applications of aPDT while reducing the reliance on pharmacological interventions and, consequently, the overall medicinal burden on the organism.

Given its broad-spectrum activity – namely a pronounced antimicrobial effect against Gram-positive and Gram-negative bacteria as well as *Candida albicans* – the proposed aPDT

method may be considered for inclusion in the complex treatment of pathological processes caused by opportunistic microorganisms, in particular generalized periodontitis and purulent-inflammatory diseases of the skin and soft tissues.

Conclusions. A 10-minute exposure of microbial inocula to LED irradiation alone did not produce a statistically significant inhibitory effect on microbial growth and, in some cases, resulted in a mild stimulatory effect, evidenced by an increase in colony counts on Petri dishes by an average 16.0-23.6% relative to the control group. The addition of the photosensitizers methylene blue and azure reduced the growth intensity of the studied microorganisms by 18.2–41.9% compared with the control. The combined application of these photosensitizers with LED irradiation further decreased microbial growth intensity by an average of 34.1–72.5%. The number of microbial colonies in group 4 was, on average, 17.8–52.7% lower compared with group 3.

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