UDC: 615.31/.32:579.861.2

EVALUATION OF THE INTERACTION OF SOME COMPOUNDS AGAINST PATHOGENICITY FACTORS OF *STAPHYLOCOCCUS AUREUS*

Dmitri IUNAC, Daniela COJOCARI, Greta BALAN
The discipline of microbiology and immunology,
Department of Preventive Medicine,
PI *Nicolae Testemitanu State* University
of Medicine and Pharmacy, Chisinau,
Republic of Moldova

https://doi.org/10.52556/2587-3873.2024.4(101).03

Summary

Suppression of the pathogenicity factors of Staphylococcus aureus strains by means of medicines reduces their infectious potential. The purpose of the research is to test some coordinating compounds and extracts from Spirulina platensis to evaluate their interaction against some pathogenicity factors of Staphylococcus aureus. In the present study, the antibiofilm and antilysozyme activity of three newly synthesized chemical compounds and three extracts from Spirulina platensis was experimentally tested. A greater degree of inhibition of microbial biofilms as well as antilysozyme activity was demonstrated by the combinations of biological and chemical compounds compared to the activity of these taken separately. More active or proven to be combinations of biological extracts and chemical compounds in concentrations of 50% and 75%. The study provides a potential therapeutic option for antimicrobial-resistant S. aureus bacteria by combining biological extracts and chemical compounds from different classes. The obtained results are promising and can be used for the development of future therapeutic strategies.

Keywords: *Staphylococcus aureus, chemical compounds, biological compounds, pathogenicity factors*

Rezumat

Evaluarea interacțiunii unor compuși împotriva factorilor de patogenitate ai Staphylococcus aureus

Suprimarea factorilor de patogenitate ai tulpinilor de Staphylococcus aureus prin intermediul preparatelor medicamentoase contribuie la reducere potențialului infecțios al acestora. Scopul acestei cercetări a fost evaluarea interacțiunii dintre unii compuși coordinativi și extracte din Spirulina platensis asupra unor factori de patogenitate ai Staphylococcus aureus. În cadrul acestui studiu experimental, au fost testați trei compuși chimici nou sintetizați și trei extracte din Spirulina platensis. S-a demonstrat un grad mai mare de inhibiție a biofilmelor microbiene, precum și a activității patogene, în cazul combinațiilor dinte compușii biologici ES și MX, și compușii chimici în concentrații de 50% și 75%. Aceste rezultate oferă o opțiune terapeutică potențială pentru bacteriile S. aureus rezistente la antimicrobiene, prin utilizarea combinată a extractelor biologice și a compușilor chimici din clase diferite. Studiul aduce contribuții promițătoare pentru dezvoltarea viitoarelor strategii terapeutice.

Cuvinte-cheie: *Staphylococcus aureus, compuși chimici, compuși biologici, factori de patogenitate*

Резюме

Оценка взаимодействия некоторых химических и биологических соединений против факторов патогенности Staphylococcus aureus

Подавление факторов патогенности штаммов золотистого стафилококка с помощью лекарственных препаратов снижает их инфекционный потенциал. Цель исследования — протестировать некоторые комплексные соединения и экстракты спирулина платенсис для оценки их взаимодействия с некоторыми факторами патогенности золотистого стафилококка. В настоящем исследовании были экспериментально протестированы три недавно синтезированных химических соединения и три экстракта спирулина платенсис. Большую степень ингибирования микробных биопленок, а также активность продемонстрировали комбинации биологических соединений ES и MX, а также химических соединений в концентрациях 50% и 75%. Исследование предлагает потенциальный вариант лечения устойчивых к противомикробным препаратам бактерий золотистого стафилококка путем объединения биологических экстрактов и химических соединений разных классов. Полученные результаты являются многообещающими и могут быть использованы для разработки будущих терапевтических стратегий.

Ключевые слова: *золотистый стафилококк, химические соединения, биологические соединения, факторы патогенности*

Introduction

Antimicrobial therapy of infectious diseases is a pressing public health problem, as a result of the alarming evolution of the resistance of microorganisms to available antimicrobials and the decrease in the number of new antimicrobial drugs. According to a survey conducted by the WHO, more than 80% of the global population uses antimicrobials in the therapy of various diseases. The abusive and unjustified use of antimicrobials led to the emergence and evolution of microorganisms resistant to multiple antimicrobials and emphasized the importance of developing new therapeutic alternatives [1].

The increasing worldwide incidence of staphylococcal infections resulting in therapeutic failure in recent years is explained by the acquisition of an increasing number of pathogenicity factors by these strains. Unlike "contamination" which only means the simple presence of microorganisms in the body, infection represents the conflict that occurs between the pathogen, with its means of aggression, and the macroorganism with its defense possibilities. Microbial pathogenicity is considered as a biochemical mechanism, through which microorganisms condition the appearance of the disease. Not all pathogens have equal chances to express this capacity, microbial pathogenicity being a multi-

functional complex, and infection being dependent on the microorganism-host relationship, especially on the endowment of the microorganism with various pathogenicity factors [2].

In addition to various mechanisms of resistance to antimicrobial drugs, *S. aureus* is equipped with various pathogenicity factors, responsible for the initiation of severe infectious processes. The complex of pathogenicity and persistence factors, which inactivate the antibacterial resistance mechanisms of the immune system, such as anti-lysozyme, anti-complementary, anti-interferon, etc., contribute to the adaptation and long-term survival of *S. aureus* strains in the infectious process [3, 4].

The persistence potential of microorganisms determines the duration of their presence in the macroorganism, and its suppression by means of medicines reduces the infectious potential of the microorganism [5, 6].

Research and development of alternative anti-infective strategies are indispensable to avoid therapeutic failures and the development of antimicrobial resistance. In recent years, the number of works dedicated to the research of compounds with the effect of inhibiting pathogenicity factors has increased significantly, which indicates the increased interest of researchers in this therapeutic alternative.

The **research aims** to test new chemical and biological compounds of *Spirulina platensis* to evaluate their interaction against some *Staphylococcus aureus* pathogenicity factors.

Materials and methods

Research was conducted at Discipline of microbiology and immunology, *Nicolae Testemițanu* SUMPh, from the Republic of Moldova. The strains of S. aureus used in the study were provided by the microbiological laboratory of the Timofei Moșneaga Republican Clinical Hospital, without any direct contact with patients or their personal data. Strains were isolated from various clinical biosubstrates (wound exudate, blood culture and pharyngeal exudate). 74 strains of *S. aureus were* studied and identified by classic microbiological methods and the Vitek2 Compact system (BioMerieux) based on morpho-biological, tinctorial, and biochemical properties.

Chemical and biological compounds

Three new chemical compounds (C1 – C14H19CuN7O4S, C2 – C13H16Br2CuN4S, C3 – C13H17ClCuN4S) synthesized at the Department of Inorganic Chemistry, Department of Chemistry of the State University of Moldova were included in the study. High purity "SigmaAldrich", "Acros Organics" or "Alfa Aesar" reagents were used as precursors for the synthesis of chemical compounds.

The biological compounds used were the extracts, the biologically active complexes - ES, ES1 and MX, which were obtained by biotechnological means from the strain of the cyanobacteria *Spirulina platensis* CNMN CB-02, from the National Collection of Non-pathogenic Microorganisms of the Institute of Microbiology and Biotechnology of Academy of Science of the Republic of Moldova.

ES spirulina extract is an amino acid/oligopeptide complex that contains non-essential and essential amino acids in the free state and combined oligopeptides, biologically functionalized macro- and microelements.

ES1 spirulina extract represents a synergistic combination of the amino acid/oligopeptide complex, phospholipids, sulfated polysaccharides, proteins, biologically functionalized macro- and microelements, derived from spirulina.

MX spirulina extract represents a myxoxanthophyll carotenoid pigment obtained from *Spirulina platensis* biomass in a concentration of 0.214 mg/ml in an aqueous solution of 80% ethyl alcohol.

Biofilm production test

For the quantitative testing of biofilm production by *S. aureus* strains, the microtitration method was used [7]. Test cultures were grown in trypticase soy broth and adjusted to 0.5 McFarland turbidity standard. 200µl of bacterial suspension were added to each of the three wells of the 96-well plate. Only 200 µl of soy trypticase broth was added to the negative control wells. The plates were covered and incubated aerobically for 24 hours at 37°C. After the expiration of the time, each well was emptied and rinsed five times with 250 µl of sterile physiological solution, after which it was allowed to dry at room temperature. Then, the plate was stained for 30 min with 200 µl of 0.1% crystal violet solution. To remove excess stains, the plates were rinsed under running tap water and dried at room temperature; the dye bound to the adherent cells was resolubilized with 160 µl of 33% glacial acetic acid solution. The results were read with an ELISA reader and the optical density (OD) of each well was measured at a wavelength of 570 nm (A570).

The isolates were classified into four categories: non-adherent, optical density lower than 0.056; weakly adherent ($0.056 < DO \leq 0.112$), moderately adherent ($0.112 < DO \leq 0.222$) and strongly adherent, optical density greater than 0.222.

Determination of antilysozyme activity

The tested strain was cultured on agar slants for 18-24 hours at 37°C. Then the strain was replanted in peptone broth and incubated for 6 hours at 37°C. The optical density of the culture in the peptonate

broth was adjusted to 0.15, which corresponds to 1x108 CFU/ml.

In parallel, the lysozyme suspension was prepared in peptone broth with a concentration of 12.5 µg/ml. The use of a higher concentration of lysozyme inhibits the growth of microorganisms, and lower concentrations do not allow the phenomenon to be tested.

100 µl of broth with lysozyme in a concentration of 12.5 µg/ml and 25 µl of microbial suspension were added to the wells of the immunoenzymatic analysis plate. 100 µl of peptone broth and 25 µl of microbial suspension were added to the control wells (two in number). The plates were thermostated for 4 hours. After 2 hours and 4 hours of incubation, the optical density was measured. The results were read with the ELISA reader and the optical density (OD) was measured at 600 nm wavelength (A600).

The distribution of strains according to the degree of highlighting of the phenomenon was carried out according to the following criteria: low degree of expression (K<0.49); medium level of expression (within the limits of 0.5≤ K ≥2.49) and high level of expression (K>2.5), where K – coefficient of antilysozyme activity of the tested strain [8].

Studying the effect of chemical and biological substances on the expression of pathogenicity factors of microorganisms

To study the effect of chemical and biological substances on the expression of pathogenicity factors, 24-hour microbial cultures were co-incubated with the test compound at concentrations of 75.0%, 50.0% and 25.0% MIC, established by microtitration in broth, in a ratio of 1:9 at 37°C for 1-18 hours. To exclude the possibility of the action of the tested substances on the components of the techniques used, the cultures were subjected to centrifugation for 15 min at 3000 rpm, and the supernatant was separated from the cells. As a control, the same microorganism cultures were used, incubated under the same conditions in Müeller-Hinton broth. Pathogenicity factors were determined according to the methods described above. The index of enzyme activity (IAE) was calculated as follows [9]: IAE = diameter of enzyme activity zone of the tested strain/diameter of the colony zone of the tested strain.

Results

Initially, we determined the biofilm-forming capacity of clinical strains of *S. aureus*. 46 (62.2%; 95% CI 61.6-62.8) of the tested strains formed detectable biofilm, and 28 (37.8%; 95% CI 37.6-38.0) did not produce biofilm. Strongly adherent biofilm was formed by 18 (39.1%; CI 95% 37.25-40.95) of the strains, moderately adherent biofilm – 20 (43.5%; CI 95% 41.89-45.11) and weakly adherent biofilm – 8 (17.4 %; 95% CI 14.89-19.91).

Later, we determined the influence of chemical and biological compounds in different concentrations, on the ability to form biofilms (Figure 1).

According to the results obtained, under the action of chemical and biological compounds in a concentration of 25% and compounds ES1 and C3 in a concentration of 50%, the microorganisms produced a strong biofilm (DO>0.220). A better antibiofilm action was demonstrated by these compounds in a concentration of 75%, where the microorganisms mainly produced a moderate bio-

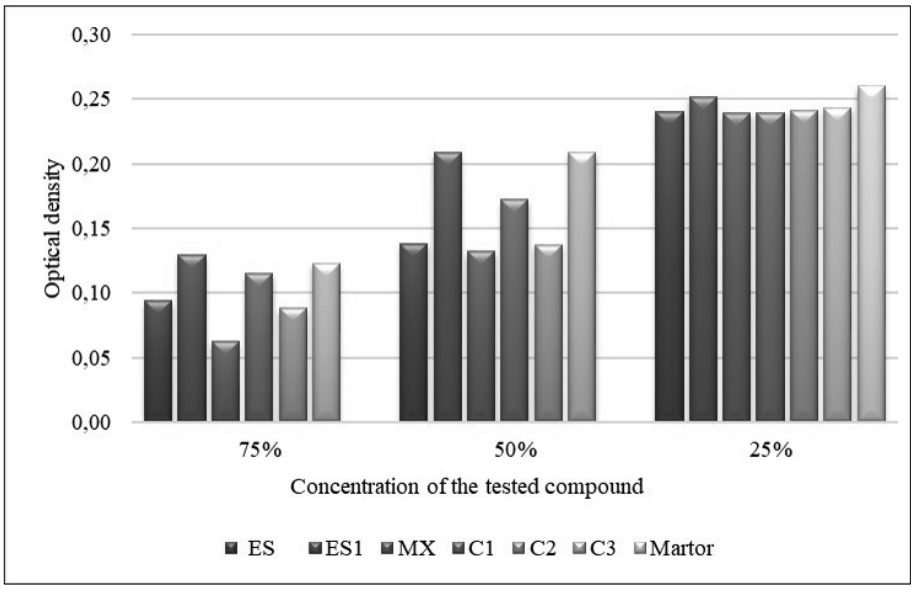

Figure 1. *Degree of biofilm formation under the action of chemical and biological compounds*

film (0.220<DO<0.112), with the exception of the biological compound MX, under whose action the strains produced a weak biofilm (0.112≤DO<0.056). *S. aureus* strains produced weak biofilm under the action of the biological compound ES and the chemical compound C2.

The action of chemical and biological compounds on the ability of *S. aureus* strains to form biofilms was also studied in combination (Figure 2).

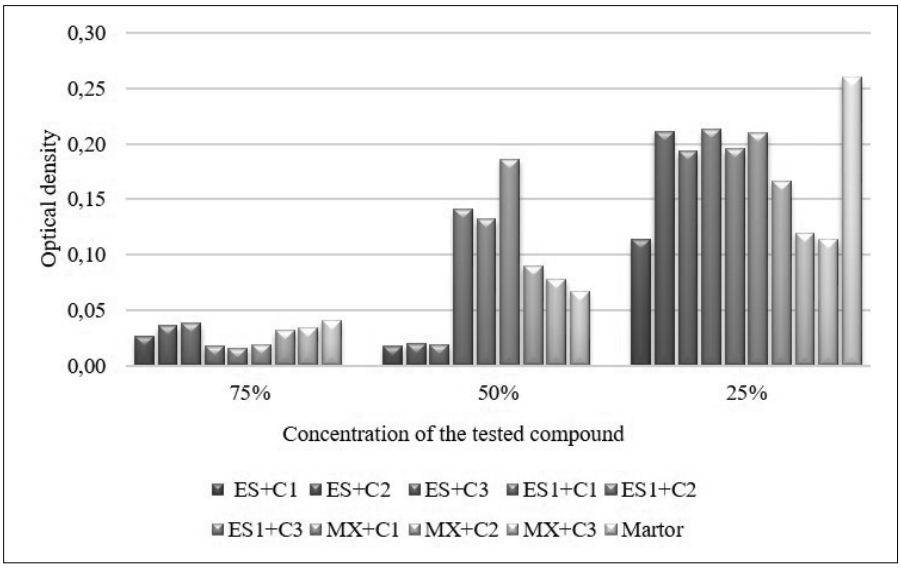

Figure 2. *Degree of biofilm formation under the action of chemical and biological compounds in combination*

A greater degree of inhibition of microbial biofilms was demonstrated by the combinations of biological compounds ES and MX, and chemical compounds in concentrations of 50% and 75%. The microbial strains, under the influence of these combinations, lost their ability to form biofilms or formed a weak biofilm, except for the 50% ES + C2 combinations; MX + C1 and the combinations of the chemical compounds with the biological compound ES1, under whose influence the bacteria formed a moderate biofilm.

At the action of the combinations in a concentration of 25%, the strains produced, in most cases, a strong biofilm, with the exception of the combinations between the biological compound MX and the chemical compounds as well as the combinations ES + C1, ES + C3 and ES1 + C2, where the microorganisms formed moderate biofilm.

Another pathogenicity factor analyzed in the present study was the antilysozyme activity of the isolated strains. Lysozyme, a universal resistance factor of the macroorganism, destroys peptidoglycan, predominantly in gram-positive bacteria, by increasing the permeability of the outer membrane and lipopolysaccharides. Therefore, protection against this enzyme is one of the important tactics adopted by staphylococci to ensure their long-term survival in the host organism [10].

Of the 74 *S. aureus* strains, 71 (95.9; CI 95% 95.6-96.2) showed antilysozyme activity and only 3 (4.1%; CI 95% 4.0-4.2%) were inactive. The vast majority of clinical strains - 29 (40.8%; CI 95% 40.6-41.0) - presented an average degree (K0.5-2.49) of expression of antilysozyme activity, 23 (32.4%; CI 95% 32.2-32.6) – high grade (K>2.5) and 19 (26.8%; CI 95% 26.6-27.0) – low grade (K<0.49).

After analyzing the results of testing the action of chemical and biological compounds on antilysozyme capacity, we found that both chemical and biological compounds inactivated the antilysozyme activity of the tested strains, compared to the control strains, which showed a high degree of antilysozyme activity (K 2.62-2.74). Under the action of biological and chemical compounds in a concentration of 25%, the antilysozyme activity of *S. aureus* strains was maintained at a high level. The use of the tested compounds in concentrations of 50% and 70% led to a decrease in the degree of expression of antilysozyme activity - average degree of expression (Figure 3).

When using chemical and biological compounds in combination (at concentrations of 50% and 70%), a small degree of expression of antilysozyme activity was recorded in *S. aureus* strains, except for the combination between 50% ES1 and 50% C3, in which the degree of expression of antilysozyme activity was medium (Figure 4).

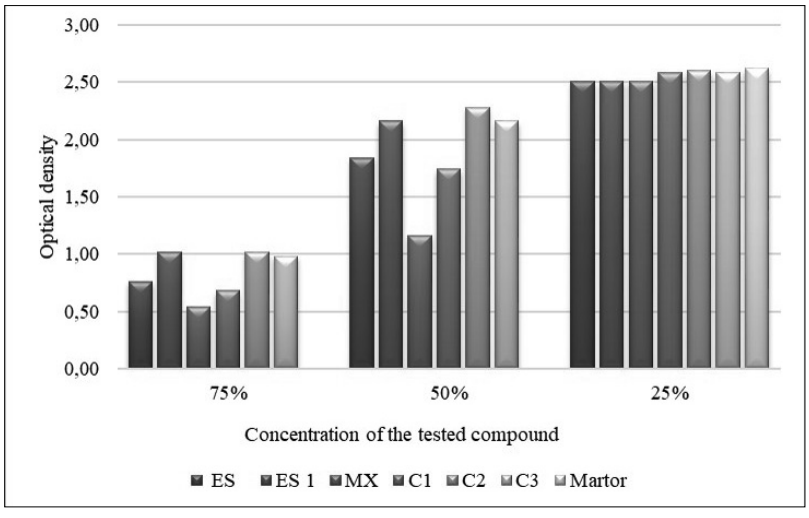

Figure 3. *Antilysozyme activity of microorganisms under action chemical and biological compounds*

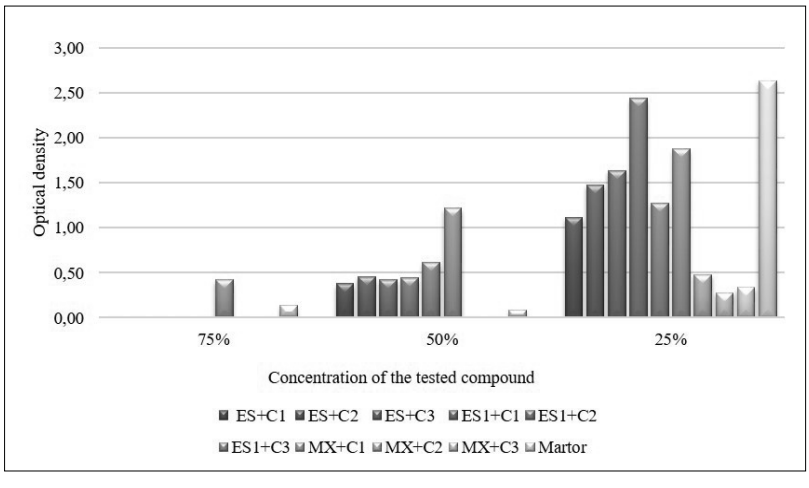

Figure 4. *Antilysozyme activity of microorganisms under the action of chemical and biological compounds in combination*

The combinations of chemical compounds with the biological compound MX presented the best effect on the expression of the antilysozyme activity of the tested microbial strains. The combinations MX + C1 and MX + C2 at the concentrations of 50% and 75% reduced the antilysozyme activity of the microbial strains to zero.

Discussions

An important pathogenicity factor of *S. aureus* is the formation of biofilms which, once established, create premises for the persistence of microorganisms and the chronicity of the process, which leads to the limitation of antibacterial therapy. Combating biofilms requires the use of high doses of antimicrobials and diversification of strategy. Unfortunately, many of the existing antimicrobials do not inhibit the formation of biofilms, especially if they are formed by antimicrobial-resistant microorganisms. To overcome this problem, it is proposed to study the antibiofilm effect of some compounds of natural origin (eg animal, vegetable, fungal, bacterial, etc.) or synthetic [11].

The production of pathogenicity factors is controlled by regulatory mechanisms (QS, Eng. quorum sensing), and interference with these mechanisms could influence the production of some virulence factors. One of the challenges in disrupting this system is that a microorganism can be endowed with multiple QS systems of the same class. Therefore, complete inhibition of QS systems with monotherapy-based treatments is difficult to achieve [2].

In response to the challenge, the ability of combination therapy to suppress QS systems in *S. aureus* strains was tested. Following the studies, it was found that combined therapy clearly inhibits the expression of pathogenicity factors, unlike treatments based on the use of a single preparation. This finding suggested the innovative idea of using combinatorial therapy, using compounds with vari-

ous mechanisms of action, to exert influence on the various QS systems of the microbial agent [12, 13].

Conclusions

In recent years, scientists have directed their research towards the development of combined antimicrobial drugs in search of new therapeutic alternatives in the treatment of infectious diseases. The present study demonstrated that some pathogenicity factors of *S. aureus*, such as antilysozyme activity and the ability to form biofilms, were more strongly reduced when using chemical and biological compounds in combination. A more significant inhibition of pathogenicity factors was shown when combining the myxoxanthophyll carotenoid pigment obtained from *Spirulina platensis* biomass with chemical compounds. The synergism of antimicrobial compounds of biological and chemical origin is one of the current fields of medical research, developing a new perspective in the development of active molecules against multi-resistant microorganisms to antimicrobials.

Declaration of conflict of interest

The authors declare no conflict of interest.

Bibliography

1. WHO. Ten threats to global health in 2019 [online], 2019. Available at: https://www.who.int/emergencies/ten-threats-to-global-health-in-2019
2. BĂLAN G., BURDUNIUC O., LOZAN-TÎRȘU C., GRUMEZA M. Factorii de patogenitate a microorganismelor – semnificația lor în patologia infecțioasă. Chișinău: Tipografia „Print-Caro". 2018, 72 p. ISBN 978-9975-56-615-5.
3. BALAN G. The bacterial strains isolated from trophic ulcers their persistence factors. In: *The Moldovan Medical Journal*. 2019;62(4):36-48. ISSN 2537-6373.
4. IUNAC D., GALBEN L., FERDOHLEB A., BALAN G. Mecanisme de rezistență la antimicrobiene a tulpinilor de *Staphylococcus aureus*: sinteză narativă. In: *Sănătate Publică, Economie* și *Management* în *Medicină*. 2023;95(2), p. 38-43. ISSN 1729-8687.
5. JUNG S., RYU C., KIM J. Bacterial persistence: Fundamentals and clinical importance. In: *J Microbiol.* 2019;57(10):829-835. doi:10.1007/s12275-019-9218-0.
6. REBELO J., DOMINGUES C., MONTEIRO F., et al. Bacterial persistence is essential for susceptible cell survival in indirect resistance, mainly for lower cell densities. In: *PLoS ONE*. 2021;16(9):1-23: doi.org/10.1371/journal.pone.0246500.
7. Sudhir K., Subba T. An Improved Crystal Violet Assay for Biofilm Quantification in 96-Well Microtitre Plate. In: *BioRxiv*. 2017:1-10. ISSN 2692-8205. doi: https://doi.org/10.1101/100214
8. BUKHARIN O., VALYSHEV A., ELAGINA N., et al. The photometric determination of the antilysozyme activity of microorganisms. In: *Mikrobiol Epidemiol Immunobiol.* 1997;4:117-120. ISSN 0372-9311.
9. ЛАБИНСКАЯ А.С., ЕЩИНА А.С., БЛИНКОВА Л.П. Общая и санитарная микробиология с техникой микробиологических исследований. Москва: Медицина, 2021, 588 с. ISBN: 978-5-8114-7723-4.
10. SHENG L., WANG J., HUANG M., et al. The changes of secondary structures and properties of lysozyme along with the egg storage. In: *International Journal of Biological Macromolecules*. 2016;92:600-606. doi:10.1016/j.ijbiomac.2016.07.068.
11. GOEL N., FATIMA F., KUMAR S., et al. Antimicrobial resistance in biofilms: Exploring marine actinobacteria as a potential source of antibiotics and biofilm inhibitors. In: *Biotechnology Reports*. 2021;30(26):30-40. doi: 10.1016/j.btre.2021.e00613
12. FONG J., ZHANG C., YANG R., et al. Combination therapy strategy of quorum quenching enzyme and quorum sensing inhibitor in suppressing multiple quorum sensing pathways of *P. aeruginosa*. In: *Sci. Rep*. 2018;8:1155-1166. ISSN 2045-2322.
13. BĂLAN G. *Compuși noi cu acțiune asupra microorganismelor izolate din ulcere trofice: tz. de doct. hab.* în *medicină*. Chișinău, 2022. 283 p.

Autor corespondent:
Dmitri Iunac, asistent universitar,
Disciplina de Microbiologie și Imunologie,
Departamentul Medicină Preventivă,
tel: 069932866,
e-mail: dmitri.iunac@usmf.md