CZU: 615.322:582.232:579.61

MODIFICATION OF SOME BIOCHEMICAL PARAMETERS OF BACTERIAL CULTURES UNDER THE INFLUENCE OF CHEMICAL COMPOUNDS AND SPIRULINA EXTRACTS

Dmitri IUNAC, Daniela COJOCARI, Elena ZARICIUC, Valeriu RUDIC, Greta BALAN

The discipline of microbiology and immunology, Department of Preventive Medicine, PI *NicolaeTestemitanu* State University of Medicine and Pharmacy, Chisinau, Republic of Moldova

https://doi.org/10.52556/2587-3873.2025.1(103).06

Summary

Establishing the particularities of action of antimicrobial compounds is an imperative both from the point of view of the appreciation of curative effects, and from the point of view of promoting the pharmaceutical product from the idea to the drug implemented in therapeutic practice. A comprehensive study was conducted, which included three newly synthesized chemical compounds and two spirulina extracts as the study object. To study the change in biochemical parameters under the influence of these compounds, three microbial strains were used. All experimental results obtained were subjected to the usual statistical analysis with the application of descriptive statistics and inferential statistics tools. Spirulina extracts do not have a direct toxic effect on the strains of P. aeruginosa, E. coli, S. aureus, a fact confirmed by keeping the level of malondialdehyde and lactate dehydrogenase released at the level of the control. Chemical compounds applied in minimum inhibitory concentrations produce pronounced toxic effects in the bacterial cultures studied: increasing the amount of the malonic dialdehyde; increasing activity of the released lactate dehydrogenase; decreasing activity of primary antioxidant enzymes. The combined action of the compounds on the investigated parameters is more effective, because in this case the minimum inhibitory concentration of the compounds is 2-4 times lower in comparison with that of the separately used compounds. Spirulina extracts did not exert a toxic effect on the bacterial strains, in contrast to the chemical compounds which, in minimal inhibitory concentrations, produced pronounced toxic effects. When combining chemical compounds with biological ones, we found their synergistic effect.

Keywords: *chemical compounds, spirulina extracts, bacteria, biochemical parameters*

Rezumat

Modificarea unor parametri biochimici ai culturilor bacteriene sub influența compușilor chimici și a extractelor de spirulină

Stabilirea particularităților de acțiune ale compușilor antimicrobieni reprezintă o prioritate atât din punctul de vedere al aprecierii efectelor curative, cât și din perspectiva promovării produsului farmaceutic de la idee până la medicamentul implementat în practica terapeutică. A fost realizat un studiu amplu, care a avut ca obiect de studiu trei compuși chimici nou-sintetizați și două extracte de spirulină. Pentru a studia modificarea parametrilor biochimici sub influența acestor compuși, au fost utilizate trei tulpini microbiene. Toate rezultatele experimentale obținute au fost supuse analizei statistice uzuale, cu aplicarea instrumentelor de statistică descriptivă și statistică inferențială. Extractele de spirulină nu au un efect toxic direct asupra tulpinilor de P. aeruginosa, E. coli, S. aureus, fapt confirmat prin menținerea nivelului de malondialdehidă și lactat-dehidrogenază eliberate la nivelul controlului. Compușii chimici aplicați în concentrații minime inhibitorii produc efecte toxice pronunțate în culturile bacteriene studiate: creșterea cantității de malondialdehidă; creșterea activității lactat-dehidrogenazei eliberate; scăderea activității enzimelor antioxidante primare.

Acțiunea combinată a compușilor asupra parametrilor investigați este mai eficientă, deoarece în acest caz concentrația minimă inhibitorie a compușilor este de 2-4 ori mai mică în comparație cu cea a compușilor utilizați separat. Extractele de spirulină nu au exercitat un efect toxic asupra tulpinilor bacteriene, spre deosebire de compușii chimici care, în concentrații minime inhibitorii, au produs efecte toxice pronunțate. Atunci când am combinat compușii chimici cu cei biologici, am observat un efect sinergic al acestora.

Cuvinte-cheie: *compuși chimici, extracte de spirulină, bacterii, parametri biochimici*

Резюме

Изменение некоторых биохимических параметров бактериальных культур под воздействием химических соединений и экстрактов спирулины

Установление особенностей действия антимикробных соединений является приоритетом как с точки зрения оценки лечебных эффектов, так и с точки зрения продвижения фармацевтического продукта от идеи до лекарственного препарата, внедренного в терапевтическую практику. Было проведено комплексное исследование, в котором объектом изучения выступили три синтезированных химических соединения и два экстракта спирулины. Для изучения изменений биохимических параметров под влиянием этих соединений использовались три микробных штамма. Все полученные экспериментальные результаты были подвергнуты стандартному статистическому анализу с применением методов описательной и аналитической статистики. Экстракты спирулины не оказывают прямого токсического воздействия на штаммы P. aeruginosa, E. coli, S. aureus, что подтверждается сохранением уровня малонового диальдегида и лактатдегидрогеназы на уровне контроля. Химические соединения, применяемые в минимальных ингибирующих концентрациях, вызывают выраженные токсические эффекты в исследуемых бактериальных культурах: увеличение количества малонового диальдегида; повышение активности высвобождаемой лактатдегидрогеназы; снижение ак-

тивности первичных антиоксидантных ферментов. Совместное действие соединений на исследуемые параметры является более эффективным, поскольку в этом случае минимальная ингибирующая концентрация соединений в 2–4 раза ниже по сравнению с отдельно применяемыми веществами. Экстракты спирулины не оказывали токсического воздействия на бактериальные штаммы, в отличие от химических соединений, которые в минимальных ингибирующих концентрациях вызывали выраженные токсические эффекты. При комбинировании химических соединений с биологическими был выявлен их синергетический эффект.

Ключевые слова: *химические соединения, экстракты спирулины, бактерии, биохимические параметры*

Introduction

The level of oxidative stress caused by different exogenous actions on living cells can be measured by direct quantification of free radicals and reactive molecules, or by indirect methods using biological markers of the degradation of cellular structures. Biological markers of stress can be quantified more simply from a technical point of view, they are much more stable, compared to reactive species, and the data obtained reflect not so much the current situation as the induced changes, which are often irreversible.

Biodegradation products of three classes of macromolecular biological compounds – nucleic acids, proteins and lipids – are often used as stress markers. 8-hydroxydeoxyguanosine is the most commonly used marker of oxidative DNA damage, and oxidative protein damage can be assessed by measuring the products of protein carbonylation and nitration (3-nitrotyposines). Reactive oxygen species can also cause the formation of advanced glycation end products (AGE) and advanced oxidation protein products (AOPP) [1-5]. However, the most commonly used and most accurate markers of oxidative stress are considered the end products of lipid oxidative degradation [6-9]. Lipid peroxidation occurs with the production of several secondary products, two of which are major – malonic dialdehyde (DAM) and 4-hydroxyl-2-nonenal. DAM, arises from the breakdown of large polyunsaturated fatty acids and the metabolism of arachidonic acid during the synthesis of thromboxane A2. The reaction of DAM with thiobarbituric acid (TBA) at pH 3.5 forms a DAM-TBA adduct that is detected spectrophotometrically at 532 nm, a reaction known as the barbituric acid reagent assay. Highlighting the change in the content of malonic dialdehyde in the cell mass is successfully applied to identify stress conditions or the toxic effects of different substances. This molecule can be considered a universal marker, which can be quantified in different cell types, including prokaryotes, and in the case of testing pharmaceuticals it can be an indicator of the acumen of their use as therapeutic remedies.

Another significant molecule for revealing toxic effects of xenobiotics on living cells is the enzyme lactate dehydrogenase (LDH). It is a key enzyme that converts pyruvate to lactate and regenerates NAD + for the continuation of glycolysis [7, 15]. In addition to its role in fermentative metabolism, LDH is also involved in microbial virulence. Maintaining an adequate level of LDH activity in microbial cells is the condition for their resistance to stress, as it determines the ability to maintain redox balance. In the absence of LDH, bacteria have been shown to be susceptible to various stress factors, including substances with antimicrobial action. LDH deficiency impairs the ability of microorganisms to colonize host organisms [12-14]. Thus, the LDH enzyme is important for the metabolism and virulence of pathogenic microorganisms. LDH activity is strictly manifested inside living cells, and in case of toxic effects, resulting in changes in the permeability of the membrane and cell wall, or their damage, it can be released in the extracellular environment. Under these conditions, the LDH activity detected outside the cells can be considered as a demonstration of the toxic effects on the cells.

Antioxidant protection is achieved by antioxidant systems, among which the key role is played by antioxidant enzymes. Superoxide dismutase, catalase and peroxidase are three primary antioxidant enzymes, the activity of which ensures the efficient elimination of free radicals and the protection of living cells from the harmful effects of oxidative stress. More than that, in the case of pathogens, these enzymes are often synthesized and eliminated in the extracellular environment, so as to ensure the protection of the microbiological pathogen from the immune system of the attacked macroorganism, which is also manifested by the elimination of local free radicals, at the level of host interaction with the pathogen [15, 16].

The aim of this study is to evaluate the impact of newly synthesized chemical compounds and spirulina extracts on key biochemical parameters in bacterial cultures, in order to assess their antimicrobial potential.

Material and methods

This research represents an *in vitro* experimental study designed to evaluate the effects of newly synthesized chemical compounds and *Spirulina* extracts on key biochemical parameters of reference bacterial cultures.

Chemical compounds and Spirulina extracts

Chemical compounds: new chemical compounds (C1 – $C_{13}H_{16}Br_2CuN_4S$; C2 – $C_{14}H_{19}CuN_7O_4S$; C3 – $C_{10}H_{14}CuN_4O_5S_2$) synthesized at the Department of Inorganic 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.

In this study, we employed chemical compounds at their minimum inhibitory concentrations (MICs), determined by the double dilution method using reference bacterial strains. For compound C1, the MICs were *E. coli* – 15.6 µg/mL, *P. aeruginosa* – 125 µg/mL, and *S. aureus* – 0.98 µg/mL. For compound C2, the MICs were *E. coli* – 7.81 µg/mL, *P. aeruginosa* – 125 µg/mL, and *S. aureus* – 0.98 µg/mL. For compound C3, the MICs were *E. coli* – 3.91 µg/mL, *P. aeruginosa* – 15.6 µg/mL, and *S. aureus* – 0.12 µg/mL.

The biological compounds: extracts, biologically active complexes - ES and MX were obtained by biotechnological means from the strain of the cyanobacterium *Spirulina platensis* CNMN CB-02 (spirulina), from the National Collection of Non-pathogenic Microorganisms of the Institute of Microbiology and Biotechnology.

ES spirulina extract – amino acid/oligopeptide complex containing non-essential (glycine, alanine, serine, cysteine, tyrosine, aspartic acid, glutamic acid, proline) and essential (arginine, phenylalanine, histidine, isoleucine, leucine, lysine, methionine, threonine) amino acids, tryptophan, valine), in free state and combined in oligopeptides (up to 10 kDa), biologically functionalized macro- and microelements. For the *in vitro* tests, the ES extract in alcoholic solution was used, with an extract concentration of 10 mg/ml and an alcohol concentration of 50%.

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

Dealcoholized *Spirulina* extracts were obtained with a rotary evaporator HL/G3 Heidolph (Schwabach, Germany) and used.

Bacterial strains

Three reference bacterial strains from the American Type Culture Collection (ATCC) were used in this study: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 25923. The strains were pre-cultured in tryptic soy broth (TSB). Subsequently, all strains were cultivated on species-specific media, including Tryptic Soy Agar, Mannitol Salt Agar, MacConkey Agar, and Peptone Agar (BioMérieux, France)."

Determination of biochemical parameters

Malonic dialdehyde determination test. The test allows the monitoring of one of the final products of lipid peroxidation – malonic dialdehyde. This compound is a recognized marker of oxidative stress and directly indicates the level of lipid degradation in cells. The test is based on the reaction of DAM with thiobarbituric acid and the quantification of the product of this reaction. 1 ml of 20% trichloroacetic acid was added to 100 mg of bacterial biomass. The mixture is mortared cold. The homogenous mass obtained is subjected to 12.000 g for 5 minutes. 0.4 ml of supernatant was transferred into two identical test tubes with rolled stoppers. In one of the test tubes, which serves as a control, 0.4 ml of 20% trichloroacetic acid was added. In the other test tube, 0.4 ml of 0.5% thiobarbituric acid was added. The tubes were placed in the water bath preheated to 98°C, where they were kept for 30 minutes, after which they were cooled to room temperature. After cooling the absorbance was read at two wavelengths - 532 nm and 600 nm [17].

Determination of released lactate dehydrogenase activity. Quantification of lactate dehydrogenase activity was performed in the culture fluid. The reaction mixture containing 0.5 ml of 100 mM pyruvate, 5 mg of NADH in 20 ml of 500 mM potassium phosphate buffer solution with pH 7 was prepared; 100 μL of culture liquid was added to this mixture. The absorbance was read immediately and over 5 min at the wavelength of 340 nm. LDH activity was expressed in international units per liter (U/L). One unit expresses the amount of lactate dehydrogenase, which reduces 1 μM of NAD per minute [17].

Determination of superoxide dismutase activity. Superoxide dismutase (SOD) activity was determined by the method, the principle of which consists in the use of xanthine and xanthine oxidase (XOD) in order to generate the superoxide radical. The latter reacts with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) forming the red colored formazan [17].

Determination of glutathione peroxidase activity. Glutathione peroxidase activity was determined by the method, which is based on the oxidation reaction of glutathione (GSH) by cumene hydroperoxide catalyzed by glutathione peroxidase (GPX). In the presence of glutathione reductase (GR) and NADPH oxidized glutathione (GSSG) is immediately converted to the reduced form, and at the same time the oxidation of NADPH to NADP+ occurs. The decrease in absorbance at 340 nm is proportional to glutathione reductase activity [17].

Determination of catalase activity. The colorimetric test for determining the CT activity applied in

the study is based on the quantification of the H_2O_2 that remains in the reactant mixture after the action of the catalase enzyme has been completed. In the reactant mixture, catalase converts hydrogen peroxide into water and oxygen, after which the enzymatic reaction is stopped with sodium azide. Quantify the amount of H_2O_2 remaining in the reaction mixture. The determination is based on the redox reaction between 3,5-dichloro-2-hydroxybenzenesulfonic acid (which is a phenolic compound) and 4-aminoantipyrine in the presence of hydrogen peroxide and peroxidase (RP). As a result of this reaction, a quinone red dye (N-(4-antipyryl)-3-chloro-5-sulfonatep-benzoquinone-monoimine) is obtained, the absorbance of which is measured at 520 nm. The calculation was made using the calibration curve [17].

Statistical data analysis

All experimental results obtained were subjected to the usual statistical analysis with the application of descriptive statistics tools (calculation of arithmetic means, standard deviations), and inferential statistics (significance tests). The calculation of statistical indicators was carried out using the capabilities of MS Excel. Student's test was applied as a test of significance.

Results

The level of toxicity of individual chemical and biological compounds and in combinations was assessed by the amount of malonic dialdehyde in the biomass of the studied microorganisms. The obtained results can be seen in figures 1-3. We can mention that in all three bacterial cultures the same pattern of the response to the action of the tested agents was observed: the natural compounds did not change the DAM content, while the chemical compounds, both individually and in combination with the natural ones, produced an increase significant of the amount of DAM, which depends on the compound and the bacterial species.

Thus, in the case of the *P. aeruginosa* strain, the natural extracts (myxoxanthophyll and the spirulina extract, which correspond to experimental variants 1 and 2) did not change the level of DAM in the biomass (figure 1). Although the average values obtained for these experimental variants are 13.8 and 17.3% respectively lower than in the control, the statistical analysis showed an insufficient level of significance ($p=0.054$ and $p=0.078$ respectively). The three chemical compounds applied one by one (experimental variants 3,6,9), as well as in combination with myxoxanthophyll (variants 5,8,11) or spirulina extract (variants 4,7,10) produced a significant increase in DAM (for all cases $p<0.001$). The DAM values in the experimental samples are quite homogeneous and denote an increase of 2.2 – 2.5 times compared to the control.

In the case of the *E. coli* strain, the natural compounds produced a decrease in the amount of DAM compared to the control by more than 20% (figure 2). In both cases the difference is statistically true ($p=0.00359$ in the case of myxoxanthophyll and $p=0.00897$ in the case of ES extract). The chemical compounds C2 and C3, both applied individually and in combination with MX and ES produced a doubling of the amount of DAM, while the compound C1 appeared to be more toxic, producing a 3-fold increase in the amount of DAM, both alone, as well as in combination with spirulina extracts.

The strain of *S. aureus* reacted according to the same principle as that of *E. coli* (figure 3), only that the effect of natural compounds to decrease the level of DAM in this case is missing. The DAM values under the action of myxoxanthophyll and spirulina extract are at the level of the control. And for this strain, the toxic effect was more pronounced in the case of the C1 compound. We note that the increase in the amount of DAM was greater in the case of *S. aureus*. Thus, the compounds C2 and C3 produced an increase of 2.5-2.7 times compared to the control (compared to 1.9-2.0 times in *E. coli*), and the compound C1 induced an increase of 3.9-4.2 times the amount of DAM (compared to 3.0-3.2 for *E. coli*).

The level of change in the selective permeability of the cell membrane and the integrity of the cell envelopes in the case of the studied bacterial cells was assessed by the activity of lactate dehydrogenase released in the extracellular environment. This enzyme, typical of the intracellular space, is absent in living, physiologically active cells, and appears only in case of their lysis, either for physiological reasons (for example, programmed death) or for exogenous reasons, induced by the presence of toxic substances.

The results obtained in the test carried out with reference to the level of LDH activity released by the three bacterial cultures can be seen on figure 4.

In the case of controls, the activity of this enzyme varies from one culture to another, and is 38 units in the case of *E. coli* and 155 units per liter in the case of *S. aureus*. This difference, as in fact also for malonic dialdehyde, may be caused both by the natural differences between these two cultures and by the different sensitivity to DMSO, which is the solubilizing agent used to dissolve the chemical compounds used in the study.

All 3 bacterial cultures responded differently to the action of chemical compounds applied individually, or in combination with natural ones, but as in the case of malonic dialdehyde, both myxoxanthophyll

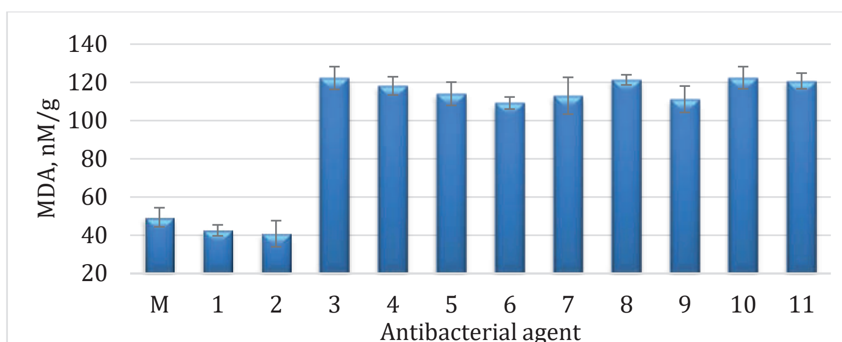

Figure 1. *The influence of the tested compounds on the amount of malonic dialdehyde in the cell mass of P. aeruginosa. M – control, 1 – myxoxanthophyll (MX), 2 – spirulina extract (ES), 3 – C2; 4 – C2+ES; 5 – C2+MX; 6 – C3; 7 – C3+ES; 8 – C3+MX; 9 – C1; 10 – C1+ES; 11 – C1+MX*

C3+ES; 8 – C3+MX; 9 – C1; 10 – C1+ES; 11 – C1+MX

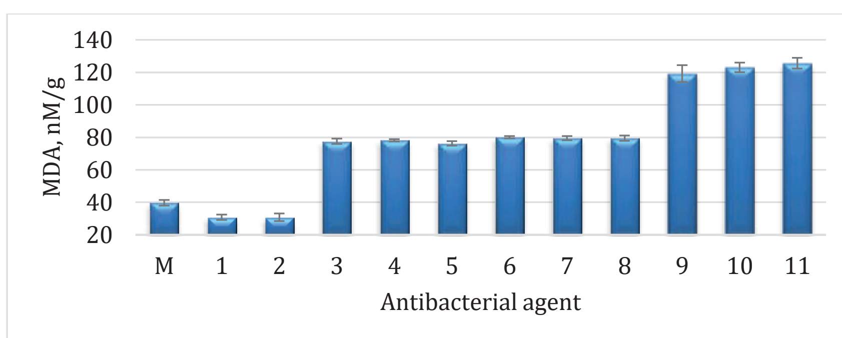

Figure 2. *The influence of the tested compounds on the amount of malonic dialdehyde in the cell mass of E. coli (antibacterial agents – as in figure 1)*

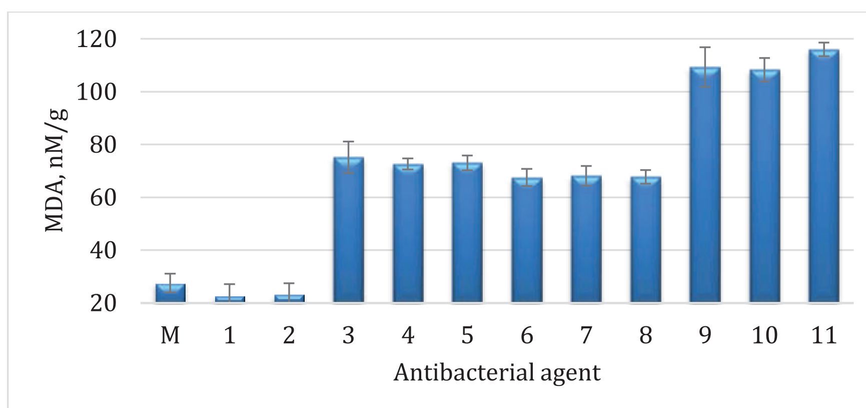

Figure 3. *The influence of the tested compounds on the amount of malonic dialdehyde in the cell mass of S. aureus (experimental variants – as in figure 1)*

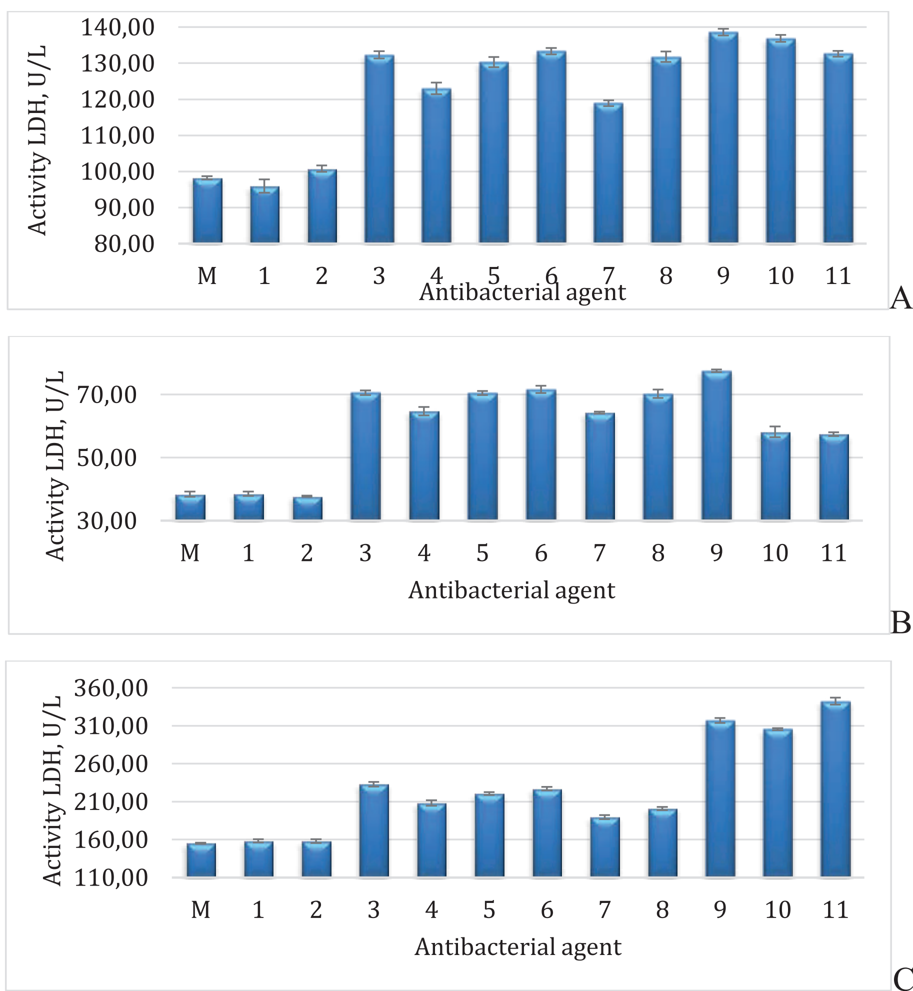

Figure 4. *Influence of the tested compounds on the activity of lactate dehydrogenase released by (A) P. aeruginosa (B) E. coli and (C) S. aureus, (experimental variants – as in figure 1)*

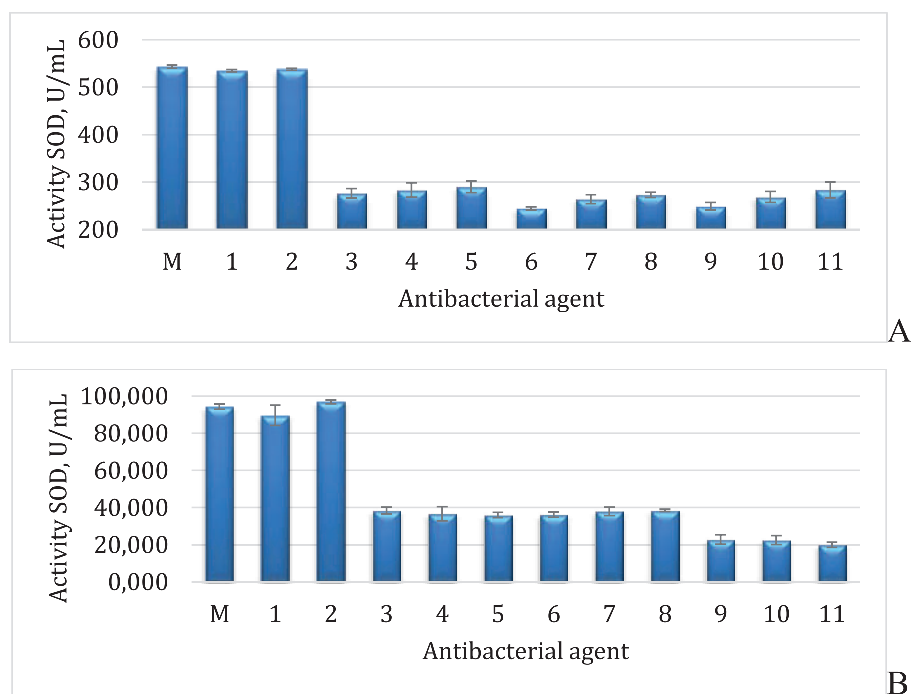

Figure 5. *Influence of tested compounds on superoxide dismutase activity in (A) biomass and (B) culture liquid in P. aeruginosa, (antibacterial agents – as in figure 1)*

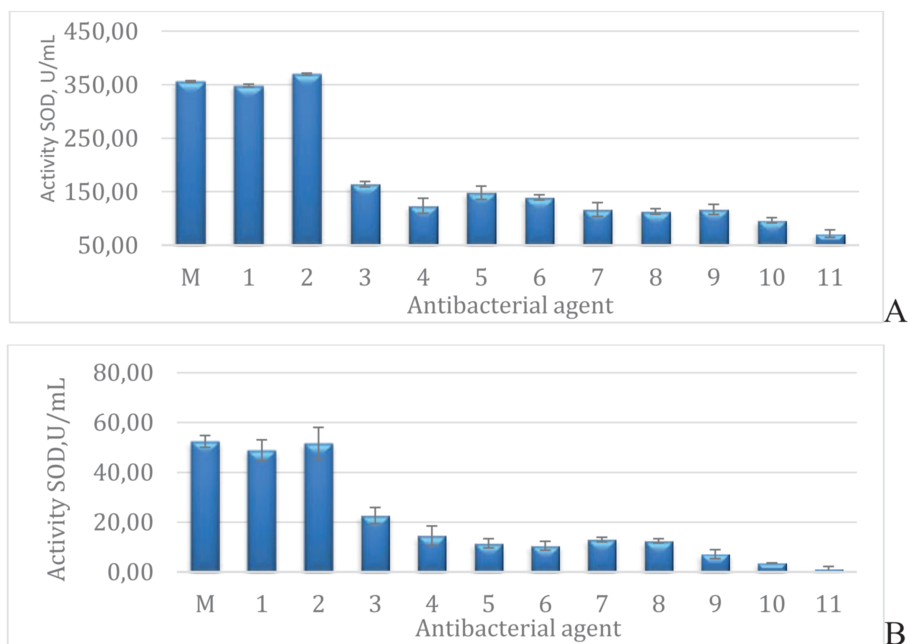

Figure 6. *Influence of tested compounds on superoxide dismutase activity in (A) biomass and (B) culture liquid in E.coli, (antibacterial agents – as in figure 1)*

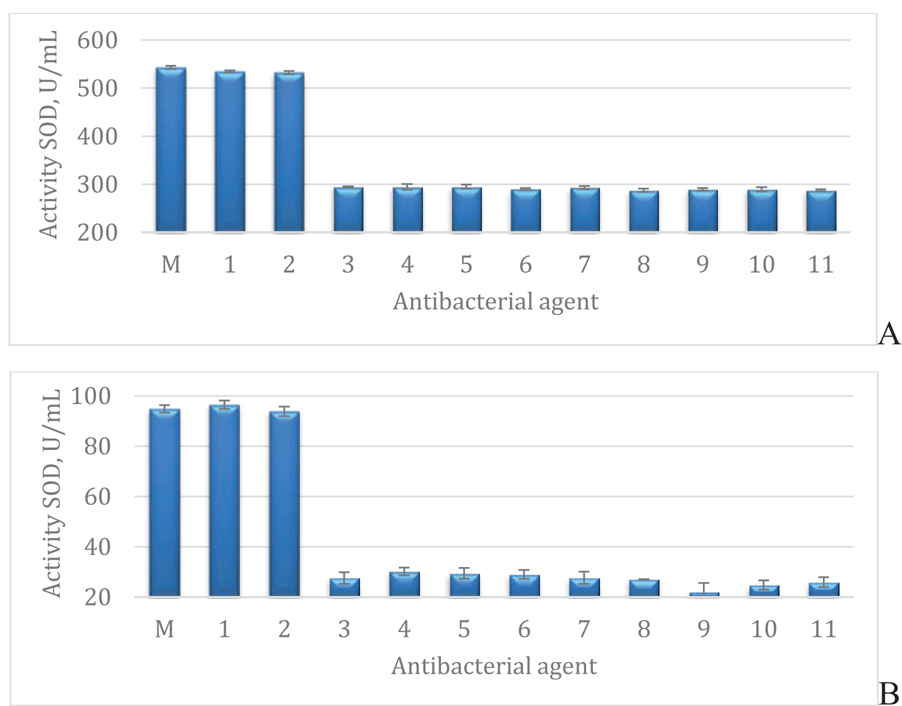

Figure 7. *Influence of tested compounds on superoxide dismutase activity in (A) biomass and (B) culture liquid in S. aureus, (antibacterial agents – as in figure 1)*

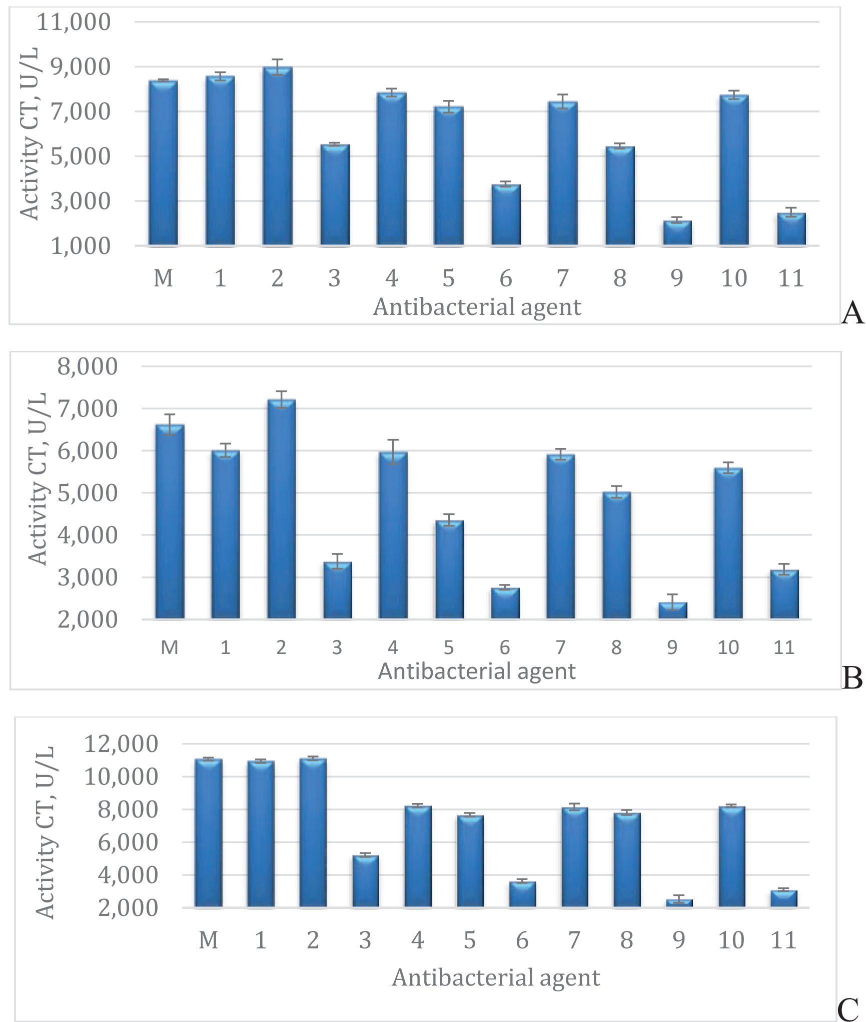

Figure 8. *Influence of tested compounds on catalase activity in (A) P. aeruginosa, (B) E. coli and (C) S. aureus, (antibacterial agents – as in figure 1)*

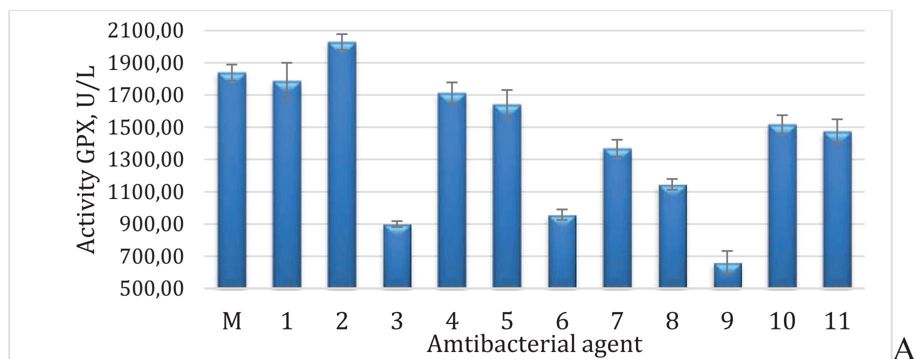

Figure 9. *Influence of tested compounds on glutathione peroxidase activity in (A) P. aeruginosa, (B) E. coli and (C) S. aureus, (antibacterial agents – as in figure 1)*

and spirulina extract did not change the activity of LDH released in the culture liquid.

The activity of lactate dehydrogenase released by the culture of *P. aeruginosa* under the influence of chemical compounds and their combinations with myxoxanthophyll and ES was significantly higher compared to the control (by 1.21-1.39 times, p<0.001). It is interesting to note that in the case of the three chemical compounds applied in combination with ES, a decrease in toxicity is observed compared to the action of the individual compounds. Although these differences are small, their statistical significance allows us to assume a mitigation of the deteriorating effects observed at the level of experimental variants. Thus, the C2 compound caused an increase in the activity of LDH released by *P. aeruginosa* by 34.7%, while in the variant with the C2+ES combination this increase is by 25.2% (p=0.0005). The same is observed in the case of the C3 compound, which leads to a 35.7% increase in the released LDH activity, and in combination with ES the increase is 21.0%. In the case of the C1 compound, myxoxanthophyll has the effect of reducing the action of the chemical compound on the LDH released by *P. aeruginosa* (decrease from an increase of 41.0% to one of 35.0%). In both cases the differences are statistically true. However, we must take into account the fact that the amount of chemical compound in the experimental variants where they are used alone is 4 times lower compared to the variants, where they are applied in combination with natural compounds. As a result, at the current level of research we still cannot draw conclusions about the individual role of each component in the manifestation of the antibacterial effect, it being certain that these interactions are interesting from a theoretical point of view, but also from an application point of view.

Under the influence of chemical and natural compounds (except for extracts used individually) in *E. coli* the increase of LDH activity in the extracellular environment was 1.5-2.0 times depending on the experimental variant. And in this bacterial culture, the natural extracts seem to have an effect of mitigating the effects of the chemical compounds applied individually, especially in the case of the C1 compound, where a quarter of the toxic effect manifested by the compound is annihilated in the variants with myxoxanthophyll and with ES.

S. aureus reacts according to the same pattern, both to the action of antimicrobial agents applied individually and in the appropriate combinations. As with *E. coli*, the most pronounced toxic effect is the compound C1, both individually and in combination. Myxoxanthophyll, however, in this case, it seems that on the contrary, it amplifies the effect generated by the compound. Thus, under the influence of the chemical compound, the released LDH activity increases 2.0 times, while in the case of the C1+MX combination this increase is 2.2 times, the difference being statistically significant (p=0.0007).

The activity of antioxidant enzymes, on the contrary, in case of severe stress, decreases, which leads to the inability of cells to protect themselves from the harmful action of free radicals, which are formed as a result of the interaction with the toxic xenobiotics applied. In the case of the bacterial cultures studied, the first line of antioxidant protection is formed by the primary antioxidant enzymes, which remove the superoxide radical (superoxide dismutase) and hydrogen peroxide, which is formed as a product of the dismutation reaction (catalase and glutathione peroxidases). SOD activity was measured both in cells and in the culture fluid, and CT and GPX activity – only in the cell mass, their levels in the culture fluid being below the detection limit of the applied methods, and physiologically insignificant.

Figure 5 shows the results obtained when testing the action of antibacterial agents on *P. aeruginosa*. Myxoxanthophyll and ES spirulina extract do not produce significant changes in the level of SOD activity, either intracellular or released into the growth medium. At the same time, in all experimental variants SOD activity - in biomass practically 2 times (by 46.7-54.9%), and in the culture liquid by 59.2-78.8%.

C3 and C1compounds most strongly reduce intracellular SOD activity, while SOD activity in the culture fluid is identically reduced by C2 and C3 compounds, while C1 is more active compared to the first two. In the case of superoxide dismutase activity, no significant differences were revealed between the experimental variants in which the chemical compounds were applied individually and those where they were combined with the spirulina extracts.

In the case of the *E. coli* strain, the physiological level of SOD activity is lower, compared to *P. aeruginosa*, but the effects of the tested compounds on this parameter are very similar (figure 6). At the same time, the decrease in SOD activity from the value point of view is even more pronounced, especially in the case of the culture liquid, where the C1compound and its combinations practically reduce the activity of the enzyme to zero. In bimass, SOD activity is reduced under the influence of antibacterial agents by 53.8 – 79.9%, and in the culture liquid – by 56.8 – 97.7%. Combinations of chemical compounds with natural ones appear to be more effective in blocking SOD activity both inside cells and the released enzyme. Taking into account the fact that the concentration of compounds in combinations is 2-4 times lower compared to the variant of their

individual use, we can assume a synergistic effect of the applied components, which is to be studied in a special way.

According to the physiological level of intra- and extracellular SOD activity, the culture of *S. aureus* is closer to *P. aeruginosa* (figure 7). The response to antibacterial agents is also very similar in these two strains. And in the case of *S. aureus*, intracellular SOD activity is reduced to a lesser extent compared to extracellular SOD. Thus, SOD activity in biomass in all experimental variants, except for those in which individual natural compounds were applied, decreased by 45-47%, and that in the culture liquid - by 69.8-77.9%. The obtained values are very close, there are no significant differences between them. In the case of the application of myxoxanthophyll and ES spirulina extract, the activity of superoxide dismutase both in cells and in the extracellular space is kept at the level of the control sample.

The inactivation of SOD is a harmful effect for aerobic organisms, they become unable to cope even with normal physiological processes, which result in the formation of the superoxide radical, not to mention the pathological ones, generated by the presence of toxic substances in the environment. Decreased extracellular SOD activity for pathogenic microorganisms is an indication of decreased pathogenicity. Thus, the low activity of extracellular antioxidant enzymes makes it impossible to annihilate the reactive oxygen species released by the immunocompetent cells of the microorganism attacked by the infection. In the case of antibacterial agents, such an action is very beneficial, being oriented towards the inactivation of pathogenic organisms, reducing the degree of pathogenicity through mechanisms different from those manifested by classic antibiotics, thus making it possible to overcome the resistance of microorganisms to known therapeutic agents.

Oxygen peroxide is a reactive molecule with a pronounced harmful effect, thanks to its relatively long lifetime compared to other free radicals. In living cells, the annihilation of this substance is the responsibility of several enzymes, the main ones being catalase and peroxidase. Catalase activity in bacterial cultures treated with the antibacterial agents under study are shown in figure 8.

As for other parameters evaluated above, the application of myxoxanthophyll and ES spirulina extract individually did not result in a statistically significant change in catalase activity in the three bacterial cultures studied. The chemical compounds applied individually very significantly decreased CT activity, and their combination with myxoxanthophyll or ES extract partially annihilated, and in some cases practically completely, this effect. In the case of *P. aeruginosa*, for example, the compound C2 caused a 34% reduction in CT activity, and in combination with ES the CT activity is very close to that of the control, being only 6.4% lower (p=0.0021). And myxoxanthophyll has an effect of preserving CT activity, but less, in this case persisting a decrease of 14.0% (p=0.00065). The same applies to this compound and its combinations with natural extracts and against *E. coli* and *S. aureus* strains. In the case of *E. coli*, under the action of C2, CT activity decreases by 49%, in the case of the combination with ES the difference is much smaller - only 9.8%, and in the case of the combination with MX the decrease in CT activity is 34.2%. In *S. aureus*, CT activity is reduced by this compound by 52.9%, and in the case of the combination with ES - only by 25.6, and MX - by 30.7%. Practically according to the same model, the effects of the other two chemical compounds are manifested when they are applied individually or in combination with the two spirulina extracts. The C3 compound reduces the activity of the *P. aeruginosa* strain by 55.23%, that of *E. coli* – by 58.3% and that of *S. aureus* – by 67.17%. At the same time, the combination of this compound with ES reduces this difference up to 10.6 -26.4%. In the case of this compound and myxoxanthophyll gives results very close to those offered by ES. Only in the case of the culture of *P. aeruginosa* in the case of MX the difference remains greater in the case of the C3+MX combination – 35%.

The most significant reduction in CT activity was induced by compound C1, by 63.6–77.4%. In the case of *P. aeruginosa*, the C1+ES combination practically restores CT activity to normal, which is only 7.72% lower compared to the control. Instead, the C1+MX combination in the case of this culture, although the concentration of the compound is 4 times lower than in the case of the chemical compound applied individually, produces a decrease in catalase activity by 70.2% compared to the control. And in the case of *E. coli* the results are very similar. The compound applied alone produces a 63.6% decrease in CT activity, in combination with myxoxanthophyll - by 51.8%, and together with the ES extract - by only 15.5%. For *S. aureus* culture these figures are: 77.1% reduction in CT activity for compound applied alone; of 72.0% in the case of the C1+MX combination and of 25.7% in the case of the C1+ES combination.

Another enzyme involved in the degradation of hydrogen peroxide is glutathione peroxidase. The results obtained for this parameter can be seen in figure 9. And in the case of this enzyme, the natural compounds did not cause significant changes in the GPX activity, and only in the case of the *P. aeruginosa* ES extract was a statistically significant increase with 10.3% (p=0.0047) of the activity of this enzyme. Otherwise, the response pattern of the researched cultures was very close to that observed for catalase.

In the same bacterial culture, the compound C2 produced a reduction of GPX activity by 51.1%, and in the case of its combination with ES and MX, the difference in the level of enzyme activity compared to the control was 6.9 and 10.8% respectively. The C3 compound reduced GPX activity in *P. aeruginosa* by 47.9%, and in the case of the combinations – by 25.5% (ES) and 37.7 (MX). Most significantly, GPX activity in *P. aeruginosa* was reduced by the compound C1 – by 64%. In the case of combinations, the reduction is much more modest – 17.5-19.9%.

GPX activity in *E. coli* was reduced by the three compounds applied individually by 52.6–64.7%, and the combinations produced smaller but significant reductions – by 14.1–28.1%.

Against the bacterial culture *S. aureus*, the studied compounds manifested similar to those described above. Thus, the compound C2 applied alone produced a reduction of GPX activity by 46.6%, in combination with ES – by 10.2%, and in combination with myxoxanthophyll – by 20%. The C3 compound applied individually reduced glutathione peroxidase activity by 44.39%, and in combination with ES and MX – by 11.22 and 11.96%, respectively. The C1 compound produced the most pronounced reduction in GPX activity – by 68.43%. And in the case of its combination with ES spirulina extract and Mixoxantofila, a high degree of inhibition of glutathione peroxidase activity was observed - by 42.54 and 39.7% respectively.

Thus, we found that the activity of these two key antioxidant enzymes involved in the detoxification of cells by removing hydrogen peroxide is strongly affected by both the individual chemical compounds applied, as well as by their combinations and the extract from spirulina and myxoxanthophyll obtained from the same cyanobacterium. In addition to the significant decrease in intracellular superoxide dismutase activity and that released in the culture liquid, the effects of the studied antibacterial agents result in the blocking of the first line of antioxidant protection of pathogen cells, which makes them vulnerable to the action of the protective factors of the macroorganism, being a useful quality for a future therapeutic agent.

Discussions

The success of pathogenic microorganisms in generating infections within the host organism is directly proportional to their ability to counteract the effects of exogenous oxidative stress, which is determined by the activation of immune defense mechanisms in the affected macroorganism. The cells of the host immune system (especially macrophages) are characterized by high activity of the specific enzyme NADH-oxidase, which, through its catalytic transfer of electrons from NADH to oxygen, produces the superoxide radical. The dismutation of the superoxide radical, catalyzed by superoxide dismutase, results in the formation of hydrogen peroxide. H_2O_2 molecules react intensely with proteins containing Fe(II), causing irreversible modifications such as carbonylation and the formation of protein aggregates [18,19]. Amino acids such as cysteine, methionine, and tryptophan are particularly vulnerable to the oxidative action of hydrogen peroxide, which can lead to both reversible changes, expressed by the formation of sulfenic acid and thiolation, and irreversible changes, such as the formation of sulfinic and sulfonic acids.

Thus, in response to the action of various reactive oxygen species (ROS) in bacterial cells, radical modifications of the proteome occur, which may not necessarily be harmful to the bacteria. Post-translational modifications trigger the activation of cellular protection mechanisms through specific signal transduction pathways [20]. Oxidative stress in bacterial cultures can also be induced by interactions with solutions containing metal ions. For example, in cultures of *Staphylococcus aureus*, silver (I) ions induce oxidative stress, manifested by a reduction in the radical-scavenging capacity of the biomass. The intensity of oxidative stress increases proportionally with the concentration of ions in the environment [19]. Under the action of glabridin in *S. aureus* cultures with multiple antibiotic resistance, a substantial increase in hydrogen peroxide and nitric oxide radicals has been observed. The concentration of these reactive species rises with increasing doses of glabridin, and they subsequently damage DNA, lipids, and proteins [21].

Exogenous oxidative stress in bacterial cultures is characterized by an active rise in ROS levels. Initially, there is an overaccumulation of superoxide radicals, which are then enzymatically converted into hydrogen peroxide and, ultimately, the most damaging free radical – the hydroxyl radical. To survive, bacteria activate detoxification mechanisms mediated by antioxidant enzymes, the most important being superoxide dismutase, catalase, and peroxidase. Superoxide dismutase plays a particularly important role in protecting bacterial DNA [22]. For example, *E. coli* produces cytoplasmic superoxide dismutase variants Mn-SOD (sodA) and Fe-SOD (sodB), which protect DNA and proteins from oxidation, as well as the periplasmic Cu/Zn-SOD (sodC), which protects cell wall components and the cytoplasmic membrane from oxidative damage. The active production of regulatory factors under oxidative stress has been documented for most pathogenic microorganisms: in *Salmonella* species, the soxRS regulon is induced; in *Pseudomonas* species, the formation of redox-

cycling compounds encoded by the pqrCBAR gene is triggered; and in *Bacillus* species, the induction of perR and ohr occurs in response to oxidative stress [22].

Exposure of *S. aureus* cultures to vancomycin or ciprofloxacin is associated with significantly higher levels of hydroxyl radicals compared to untreated bacterial cells. In these conditions, a close correlation is observed between the increase in hydroxyl radical content and the reduced expression of katA, as well as decreased catalase activity. Researchers suggest that bactericidal antibiotics acting on *S. aureus* cultures modulate catalase expression, which in turn influences free radical formation [23, 24]. Thus, antibacterial substances acting on pathogenic microorganisms induce oxidative stress, characterized by free radical accumulation, reduced total antioxidant capacity, and decreased expression and activity of protective antioxidant enzymes. Monitoring these processes in bacterial biomass can provide valuable insights into both the efficacy of tested substances and their possible mechanisms of action against pathogenic microorganisms.

In addition to these general mechanisms, it is important to highlight the particularities of the extracts tested in this study. The amino acid/oligopeptide complex (ES) differs fundamentally from the carotenoid fraction (MX), not only in chemical composition but also in biological activity. ES, being rich in free amino acids and short peptides, may modulate bacterial metabolism and redox balance without exerting a direct bactericidal effect. In contrast, MX is dominated by the carotenoid myxoxanthophyll, whose antioxidant potential is expressed mainly through radical scavenging and stabilization of cellular membranes. This duality suggests that while synthetic chemical compounds act primarily through direct bactericidal mechanisms, *Spirulina*-derived extracts contribute by modulating oxidative stress responses and preserving enzymatic activity.

Recent studies support this interpretation: phycocyanin and phycoerythrin mixtures from *Spirulina* exhibited MIC values of approximately 3.12 µg/mL against *E. coli* and 1.56 µg/mL against *S. aureus*, demonstrating notable antimicrobial activity [25]. A comprehensive review highlighted that *Arthrospira* (*Spirulina*) extracts display potent strain-specific antimicrobial activity, with MICs as low as 2–15 µg/mL and inhibition zones up to 50 mm—comparable or even superior to standard antibiotics [26]. Furthermore, ethanolic extracts of *Spirulina platensis* exhibited strong antibacterial effects against several skin pathogens, including *S. aureus*, MRSA, and *P. aeruginosa*, and effectively inhibited biofilm formation and eradication [27]. The growing interest in *Spirulina*'s antibacterial potential is supported by recent reports confirming notable activity against pathogenic strains of *S. aureus*, *E. coli*, and *Vibrio* spp. [28].

Conclusions

ES spirulina extract and myxoxanthophyll do not have a direct toxic effect on bacterial cultures *P. aeruginosa*, *E. coli*, *S. aureus*, a fact confirmed by maintaining the level of malondialdehyde and lactate dehydrogenase released at the level of the control. Likewise, these two natural compounds do not disrupt the activity of primary antioxidant enzymes.

The chemical compounds C2, C3 and C1 applied in minimally inhibitory concentrations produce pronounced toxic effects in the bacterial cultures studied: increase in the amount of malonic dialdehyde - end product of lipid peroxidation; increase in the activity of released lactate dehydrogenase – evidence of cell membrane damage; decrease in the activity of primary antioxidant enzymes - a fact that demonstrates the inability to maintain the redox balance in the cell.

The effects of combinations of chemical compounds with ES spirulina extract and myxoxanthophyll on the investigated parameters demonstrate their effectiveness as potential antibacterial agents, given that the concentrations of the chemical compounds are 2-4 times lower compared to those applied in the case of their harmful use.

The chemical compounds C2, C3 and C1 and the natural ones ES and MX act synergistically, through mechanisms not highlighted in this study, but which give them great prospects as antimicrobial agents with low degree of toxicity and low potential for resistance formation.

References

1. BALAN G. Compuşi noi cu acţiune asupra microorganismelor izolate din ulcere trofice: tz. de doc. hab. în med. Chișinău, 2022. 283p. Retrieved June 02, 2024, from http://www.cnaa.md/files/theses/2022/58401/greta_balan_thesis.pdf.
2. CEPOI L., RUDI L., CHIRIAC T., et al. Dialdehida malonică – un potențial marcher al toxicității nanoparticulelor în mediul acvatic. In: *One Health and Risc Management*. 2020, 1(1), 64-71. doi:10.5281/zenodo.3701197.
3. CHOROMAŃSKA M., KLIMIUK A., KOSTECKA-SOCHOŃ P. et al. Antioxidant Defence, Oxidative Stress and Oxidative Damage in Saliva, Plasma and Erythrocytes of Dementia Patients. Can Salivary AGE be a Marker of Dementia? In: *International Journal of Molecular Sciences*. 2025, 18(10). doi:10.3390/ijms18102205.
4. CHOUCHANI E., JAMES A., FEARNLEY I. et al. Proteomic approaches to the characterization of proteint hiol modification. In: *Curr. Opin. Chem. Biol.* 2011, 15, 120-128. doi: 10.1016/j.cbpa.2010.11.003.
5. CUI X., GONG J., HAN H. et al. Relationship between free and total malondialdehyde, a well-established

marker of oxidative stress, in various types of human biospecimens. In: *Journal of Thoracic Disease.* 2018, 10(5), 3088-3197. doi:10.21037/jtd.2018.05.92.
6. DEMASI M., AUGUSTO O., BECHARA E. et al. Oxidative Modification of Proteins: From Damage to Catalysis, Signaling, and Beyond. In: *Antioxid Redox Signal.* 2021, 35(12), 1016-1080. doi:10.1089/ars.2020.8176.
7. GASPAR P., AL-BAYATI F., ANDREW P. et al. Lactate Dehydrogenase Is the Key Enzyme for Pneumococcal Pyruvate Metabolism and Pneumococcal Survival in Blood. In: *Infection and Immunity.* 2014, 82(12), 5099-5109. doi:10.1128/iai.02005-14.
8. GRYSZCZYŃSKA B., FORMANOWICZ D., BUDZYŃ M. et al. Advanced Oxidation Protein Products and Carbonylated Proteins as Biomarkers of Oxidative Stress in Selected Atherosclerosis-Mediated Diseases. In: *BioMed Research International.* 2017, 1-9. doi:10.1155/2017/4975264.
9. GUDUMAC V., TAGADIUC O., RÎVNEAC V. et al. Investigaţii biochimice. Elaborare metodică. Micrometode. Vol. II. Chișinău: Elena V.I. SRL, 2010. 104 p.
10. GULEA A., USATAIA I., GRAUR V. et al. Synthesis, Structure and Biological Activity of Coordination Compounds of Copper, Nickel, Cobalt, and Iron with EthylN'-(2-Hydroxybenzylidene)-N-prop-2-en-1-ylcarbamohydra-zonothioate. In: *Russian Journal of General Chemistry.* 2020, 90(4), 630-639. doi:10.1134/S107036322004012X.
11. LAOUAMI S., MESSAOUDI K., ALBERTO F. et al. Lactate dehydrogenase A promotes communication between carbohydrate catabolism and virulence in *Bacillus cereus.* In: *J. Bacteriol.* 2011, 193, 1757-1766. doi:10.1128/JB.00024-11.
12. LISANNE C., JOOP D., ROBIN P. et al. Advanced glycation end products: An emerging biomarker for adverse outcome in patients with peripheral artery disease. In: *Atherosclerosis*. 2016, 254, 291-299. doi:10.1016/j.atherosclerosis.2016.10.012.
13. MARTÍNEZ S., AIASSA V., SOLA C., BECERRA M. Oxidative stress response in reference and clinical Staphylococcus aureus strains under Linezolid exposure. In: *Journal of Global Antimicrobial Resistance.* 2020, 22, 257-262. doi:10.1016/j.jgar.2020.02.032.
14. MIRLOHI M., YAGHOOTI H., SHIRALI S. et al. Increased levels of advanced glycation end products positively correlate with iron overload and oxidative stress markers in patients with β-thalassemia major. In: *Ann Hematol.* 2018, 97, 679-684. doi:10.1007/s00277-017-3223-3.
15. NEVES A., RAMOS A., SHEARMAN C. et al. Metabolic characterization of Lactococcus lactis deficient in lactate dehydrogenase using in vivo 13C-NMR. In: *Eur. J. Biochem.* 2000, 267, 3859-3868. doi:10.1046/j.1432-1327.2000.01424.x.
16. NUR I., MUNNA M., NOOR R. Study of exogenous oxidative stress response in Escherichia coli, *Pseudomonas* spp., *Bacillus* spp., and *Salmonella s*pp. In: *Turk J Biol.* 2014, 38, 502-509. doi:10.3906/biy-1312-93.
17. PERRONE A., GIOVINO A., BENNY J., MARTINELLI F. Advanced Glycation End Products (AGEs): Biochemistry, Signaling, Analytical Methods, and Epigenetic Effects. In: *Oxidative Medicine and Cellular Longevity.* 2020, 1-18. doi:10.1155/2020/3818196.
18. PIACENZA L., PELUFFO G., ALVAREZ M. et al. Trypanosoma cruzi Antioxidant Enzymes As Virulence Factors in Chagas Disease. In: *Antioxidants & Redox Signaling.* 2013, 19(7), 723-734. doi:10.1089/ars.2012.4618.
19. RANA N., SAUVAGEOT N., LAPLACE J. et al. Redox balance via lactate dehydrogenase is important for multiple stress resistance and virulence in *Enterococcus faecalis*. In: *Infect. Immun.* 2013, 81, 2662-2668. doi:10.1128/IAI.01299-12.
20. RICHARDSON A., LIBBY S., FANG F. A nitric oxide-inducible lactate dehydrogenase enables *Staphylococcus aureus* to resist innate immunity. In: *Science.* 2008, 319, 1672-1676. doi:10.1126/science.1155207.
21. SAVAGE D., HILT J., DZIUBLA T. *In vitro* methods for assessing nanoparticle toxicity. In: *Methods in Molecular Biology.* 2019, 1894, 1-29. doi:10.1007/978-1-4939-8916-4_1.
22. SINGH V., PAL A., DOROKAR M. A polyphenolic flavonoid glabridin: Oxidative stress response in multidrug-resistant Staphylococcus aureus. In: *Free Radical Biology and Medicine.* 2015, 87, 48-57. doi:10.1016/j.freeradbiomed.2015.06.016.
23. TSIKAS D. Assessment of lipid peroxidation by measuring malondialdehyde (MDA) and relatives in biological samples: Analytical and biological challenges. In: *Analytical Biochemistry.* 2017, 524, 13-30. doi:10.1016/j.ab.2016.10.021.
24. WANG G. Improved Methods for Classification, Prediction and Design of Antimicrobial Peptides. In: *Methods Mol Biol.* 2015, 1268, 43-66. doi:10.1007/978-1-4939-2285-7_3.
25. AHMADI R., ABDOLMALEKI P., BAKHSHI B. et al. Synergistic antioxidant and antimicrobial activities of phycocyanin and phycoerythrin from *Spirulina platensis*. In: *Int J Biol Macromol*. 2024,270:137145. doi:10.1016/j.ijbiomac.2024.137145.
26. ILIEVA I., TENEVA I., STOYANOV P. et al. Antimicrobial properties of the cyanobacterium *Arthrospira* (Spirulina): a review. In: *Microorganisms.* 2024, 12(2):404. doi:10.3390/microorganisms12020404.
27. RUNGJIRAPHIRAT S., CHAIJAROENKUL W., NABANGCHANG K. Antibacterial activity of ethanolic and methanolic extracts of *Spirulina platensis* against multidrug-resistant skin pathogens and biofilm formation. In: *Biology (Basel)*. 2025, 14(5):502. doi:10.3390/biology14050502.
28. SABAT P., SAMANTA S., DAS R. et al. Antibacterial potential of *Spirulina* sp. against pathogenic bacteria: insights into bioactive compounds and mechanisms. In: *Bioresour Bioprocess*. 2025, 12(1):57. doi:10.1186/s40643-025-00861-0.

Autor corespondent:
Dmitri Iunac, asistent universitar,
Disciplina de microbiologie și imunologie,
Departamentul Medicină Preventivă,
IP USMF *Nicolae Testemițanu,*
tel: +37369932866,
e-mail: dmitri.iunac@usmf.md