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MODIFICATION OF OXIDATIVE STRESS INDICES IN CULTURES OF PATHOGENIC MICROORGANISMS UNDER THE INFLUENCE OF NOVEL CHEMICAL COMPOUNDS

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Keywords: *antimicrobial activity, oxidative stress, chemical compounds, copper, reference strains.*

Introduction. *It is known that excessive formation of hydrogen peroxide in the microbial cultures under the action of chemical compounds with antibacterial effects is the first stage in the formation of oxidative stress. Thus, it became appropriate to conduct a study that would make it possible to control the level of oxidative stress induced by chemical compounds in cultures of pathogenic microorganisms.*

Material and methods. *The objects of the in vitro study were Cu(II) coordination compounds; Co(II), Zn(II) and aromatic propenones synthesized at the Department of Inorganic Chemistry of the State University of Moldova. Antimicrobial activity was tested on 5 reference strains. The level of oxidative stress was controlled using the hydrogen peroxide test, and the level of lipid peroxidation was determined indirectly by monitoring the product of peroxidation, namely the malondialdehyde.*

Results. *Under the action of new chemical compounds with antimicrobial properties on reference cultures of Staphylococcus aureus ATCC 25923, Bacillus cereus ГИСК 8035, Escherichia coli ATCC 25922, Shigella sonnei ATCC 25931 and Salmonella enterica (Salmonella abony ГИСК 03/03y) in cultures that create a state of oxidative stress, confirmed by the accumulation of hydrogen peroxide and lipid peroxidation products.*

Conclusions. *Thus, the process of lipid peroxidation, which follows the pattern of chain reactions, is one of the reactions that lead to the death of cell culture. The level of hydrogen peroxide formed under the action of the tested compounds was also monitored.*

Cuvinte cheie: *activitate antimicrobiană, stres oxidativ, compuși chimici, cupru, tulpini de referință.*

MODIFICAREA INDICATORILOR STRESULUI OXIDATIV ÎN CULTURILE DE MICROORGANISME PATOGENE SUB INFLUENȚA COMPUȘILOR CHIMICI NOI

Introducere. *Este cunoscut faptul că formarea excesivă a peroxidului de hidrogen în culturile de microorganisme, sub influența compușilor chimici cu efecte antibacteriene, constituie primul pas în generarea stresului oxidativ. Astfel, a devenit oportună realizarea unui studiu care ar monitoriza nivelul stresului oxidativ indus de către compușii chimici în culturile de microorganisme patogene.*

Material și metode. *În calitate de obiecte de studiu in vitro au servit compușii coordinativi ai Cu (II); Co (II), Zn (II) și propenonele aromatice, sintetizate la Catedra de chimie anorganică de la Universitatea de Stat din Moldova. Efectele antimicrobiene au fost testate pe 5 tulpini de referință. S-a monitorizat nivelul stresului oxidativ prin testul de determinare a peroxidului de hidrogen și a fost stabilit indirect nivelul de peroxidare a lipidelor, prin monitorizarea produsului peroxidării – dialdehida malonică.*

Rezultate. *Sub acțiunea compușilor chimici noi cu proprietăți antimicrobiene asupra culturilor de referință Staphylococcus aureus ATCC 25923, Bacillus cereus ГИСК 8035, Escherichia coli ATCC 25922, Shigella sonnei ATCC 25931 și Salmonella enterica (Salmonella abony ГИСК 03/03y) în culturi se creează o stare de stres oxidativ, confirmat prin acumularea peroxidului de hidrogen și a produselor peroxidării lipidelor.*

Concluzii. *Astfel, procesul de peroxidare a lipidelor, care decurge după modelul reacțiilor în lanț, este una dintre reacțiile care conduc la moartea culturii celulare. De asemenea, a fost monitorizat și nivelul peroxidului de hidrogen format sub influența compușilor testați.*

INTRODUCTION

The success of pathogenic microorganisms in generating infections in the host organism is directly proportional to their ability to counteract the effects of exogenous oxidative stress, which is determined by the activation of the immune defense mechanisms of the affected macroorganism. The cells of the host immune system are characterized by a high activity of the specific enzyme NADH-oxygenase, which, following its catalytic activity in the transfer of electrons from NADH to oxygen, forms a superoxide radical. The superoxide radical dismutation reaction, catalyzed by superoxide dismutase, ends with the formation of hydrogen peroxide. H_2O_2 molecules react intensively with proteins containing Fe(II), causing their irreversible changes, like carbonylation and formation of protein aggregates (1, 2).

The amino acids cysteine, methionine, and tryptophan are especially vulnerable to the oxidative action of hydrogen peroxide, which can lead to both reversible changes, expressed in formation of sulfonic acid and thiol, and irreversible changes leading to formation of sulfuric and sulfonic acids (3).

Thus, in response to the action of various reactive oxygen species (ROS), bacterial cells undergo a radical modification of the proteome, which is not necessarily harmful to bacteria. Post-translational change leads to the activation of cellular defense mechanisms due to the activation of some specific signal transduction pathways (4).

Oxidative stress in bacterial cultures can be caused by the interaction of cells with solutions containing metal ions. For example, in the bacterial culture of *Staphylococcus aureus,* silver (I) ions cause oxidative stress, expressed in a decrease in the ability to reduce radicals in the biomass. The intensity of oxidative stress increases is higher to the increase in the concentration of ions in the environment (5). Exogenous oxidative stress in bacterial cultures occurs with an active increase in the amount of ROS. At the first stage, the superoxide radical is superaccumulated, which is subsequently converted by enzymatic reactions into hydrogen peroxide and the most dangerous free radical, the hydroxyl radical. To survive, bacteria activate detoxification mechanisms mediated by antioxidant enzymes, the most important of which are superoxide dismutase, catalase, and peroxidase. Superoxide dismutase is very actively involved in the defense reactions of bacterial DNA (6, 7, 8, 9).

Lipids are the structural and functional basis of biological membranes, and their oxidation leads to mechanical damage to biological barriers and functional membranes, which affects the process of cell communication with the environment, as well as the normal metabolic reactions. Accumulation in the reaction medium of lipid degradation end products indicates irreversible changes in cells, very often incompatible with their vital activity. Malondialdehyde is one of the end products of the lipid chain oxidation process, whereas its level is a marker of the oxidative stress, experienced by the cell.

Thus, substances with an antibacterial effect, acting on the pathogenic bacterial cultures, may cause an oxidative stress associated with the accumulation of free radicals, a decrease in the total antioxidant capacity, and a decrease in the expression and activity of protective antioxidant enzymes. Monitoring of these processes in bacterial biomass can provide useful information both on the effectiveness of the tested substances and the possible mechanisms of their action on pathogenic microorganisms.

Establishing the particularities of new antimicrobial compounds action is important both in terms of assessing the curative effects and in terms of promoting the pharmaceutical product from the idea to the drug implemented in therapeutic practice.

The aim of the study: thus, it became appropriate to conduct a study that would elucidate some changes in oxidative stress in pathogenic microorganisms that reflect the antioxidant status of pathogenic cultures under the influence of newly selected chemical compounds as substances with high antibacterial potential.

MATERIAL AND METHODS

Coordinating Cu (II) compounds were included as objects of in vitro study; Co (II), Zn (II) and aromatic propenones were synthesized at the Department of Inorganic Chemistry (State University of Moldova). High purity Sigma-Aldrich reagents were used as synthetic precursors, which were tested for antimicrobial properties.

Antimicrobial activity was tested on the following

reference strains: *Staphylococcus aureus* ATCC 25923 (*Staphylococcus aureus* subsp. aureus (ATCC® 25923™), *Bacillus cereus* ГИСК 8035, *Escherichia coli* ATCC 25922, *Shigella sonnei* (Levine) *Salmonella enterica subsp. enterica serovar abony* (former name *Salmonella abony* ГИСК 03/03y).

The hydrogen peroxide test covers the area of oxidative stress monitoring. The hydrogen peroxide content is determined in accordance with the method developed by Bellincampi et al. in 2000 (10). The method is based on the oxidation of Fe^{2+} ions with hydrogen peroxide to form Fe^{3+} ions. The latter form compounds stained with xylene orange.

100 mg of biomass is ground with 1 ml of ultra-hot acetone (-18°C). The homogenate is centrifuged for 10 minutes up to 12,000 g, 0.25 ml of xylene orange is added to 0.25 ml of the supernatant (Preparation: 260 µl of concentrated sulfuric acid is diluted with a small volume of distilled water, 9.5 mg of Mohr's salt ($FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$) is added. 7.6 mg of xylene orange is dissolved in another water amount. Both solutions are mixed, 1.822 g of sorbitol is added and the volume is adjusted to 50 ml. The control sample contains 0.25 ml acetone and 0.25 ml xylene orange.

Samples are kept for 45 min. at room temperature. The reaction mixture is centrifuged for 5 min. at 10,000 g, after which the optical density is measured at a wavelength of 560 nm. The calculations were performed using a calibration curve obtained for the concentration range from 200 to 1500 ng of hydrogen peroxide per ml (11). To obtain the value of hydrogen peroxide in µM/g dry matter, the following formula was used:

$$C=((K \cdot V \cdot X)/m))/880, \text{ where}$$

C is the concentration of H_2O_2 in µM/g of substance; K is the concentration of H_2O_2 determined based on the calibration curve in ng/ml; V is the volume of the extract; X - dilution of the extract; m is the dry weight of the sample; 880 is the transfer coefficient of ng of hydrogen peroxide in µM.

The lipid peroxidation level is determined indirectly by observing the product of peroxidation, malondialdehyde (MDA). The amount of MDA is determined by the accumulation of its reaction product with thiobarbituric acid (12).

To 100 mg of biomass add 1 ml of 20% trichloroacetic acid and triturate in the cold. Centrifuge the homogenate for 5 min. at 12,000 g, transfer 0.4 ml of the supernatant to 2 stoppered tubes. 0.4 ml of 20% trichloroacetic acid is added to the first tube – this tube serves as a control one. Add 0.4 ml of 0.5% thiobarbituric acid to another tube. Samples are then incubated in a water bath at 100°C for 30 min, then cooled at room temperature. Measurements are carried out by a spectrophotometer at a wavelength of 532 nm and 600 nm to correct for nonspecific absorption (13).

To calculate the amount of MDA, the extinction coefficient e=155 $mM^{-1}cm^{-1}$ is used. The calculation formula is as following:

$$C = ((\Delta E/155) \cdot X \cdot V)/m, \text{ where}$$

C is the concentration of MDA in mM/g dry matter; ΔE is the difference in optical density at 532 and 600 nm; 155 - extinction coefficient (see above); X - sample dilution; V is the volume of the extract, dry weight.

RESULTS

The results reflecting MDA and H_2O_2 levels in the *Staphylococcus aureus* ATCC 25923 reference culture are shown in figure 1.

The level of hydrogen peroxide in the culture treated with furacillin increased by 17.2% compared to the level in the control sample, and the quantitative increase in malondialdehyde was 44.3%. They differed from the control ones and were statistically significant, which confirms the antibacterial effect of the reference antiseptic. In experimental variants, obtained by processing the culture of staphylococcus with selected new chemical compounds, the level of hydrogen peroxide exceeded the control sample up to 37.7%. For the two compounds tested (compound 8 and 9), the difference with the control sample was not statistically significant.

The level of malondialdehyde during treatment with furacillin in the culture of staphylococcus increased by 44.3% compared with the control cultures, whereas in cultures treated with appropriate doses of new chemical compounds, by 50.4-106.1% compared with the control samples. All experimental samples differ from the control sample by a 99% confidence interval. Thus, the results of the MDA test confirm the presence of a pronounced oxidative stress state in the staphylococcus culture under the action of new chemical compounds with antibacterial effects.

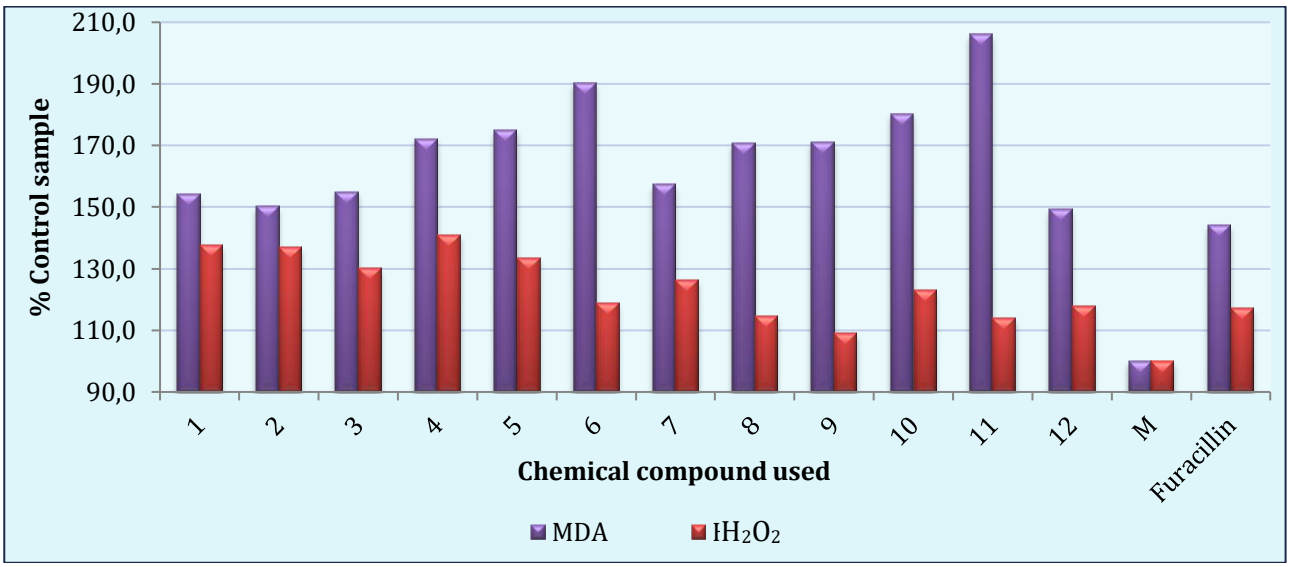

Figure 1. The amount of malondialdehyde and hydrogen peroxide in the standard culture of *Staphylococcus aureus* ATCC 25923 under the action of selected new chemical compounds: 1 – $C_{38}H_{38}Cu_2N_{14}O_{10}S_4$; 2 – $C_{42}H_{42}Cu_2N_{14}O_{12}S_4$; 3 – $C_{46}H_{46}Cu_2N_{18}O_{10}S_6$; 4 – $C_{46}H_{42}Cu_2N_{18}O_{10}S_4$; 5 – $C_{15}H_{19}ClCuN_4O_2S$; 6 – $C_{15}H_{19}CuN_5O_5S$; 7 – $C_{15}H_{17}ClCuN_4OS$; 8 – $C_{15}H_{19}CuN_5O_5S$ (2,5); 9 – $C_{15}H_{19}CuN_5O_5S$ (3,4); 10 – $C_{15}H_{19}CuN_5O_5S$ (2,4); 11 – $C_{18}H_{22}Cl_2Cu_2N_8S_2$; M – uncultivated bacteria.

The results of the H_2O_2 and MDA tests obtained in the experiments on the reference culture of *Bacillus cereus* ГИСК 8035 are shown in figure 2.

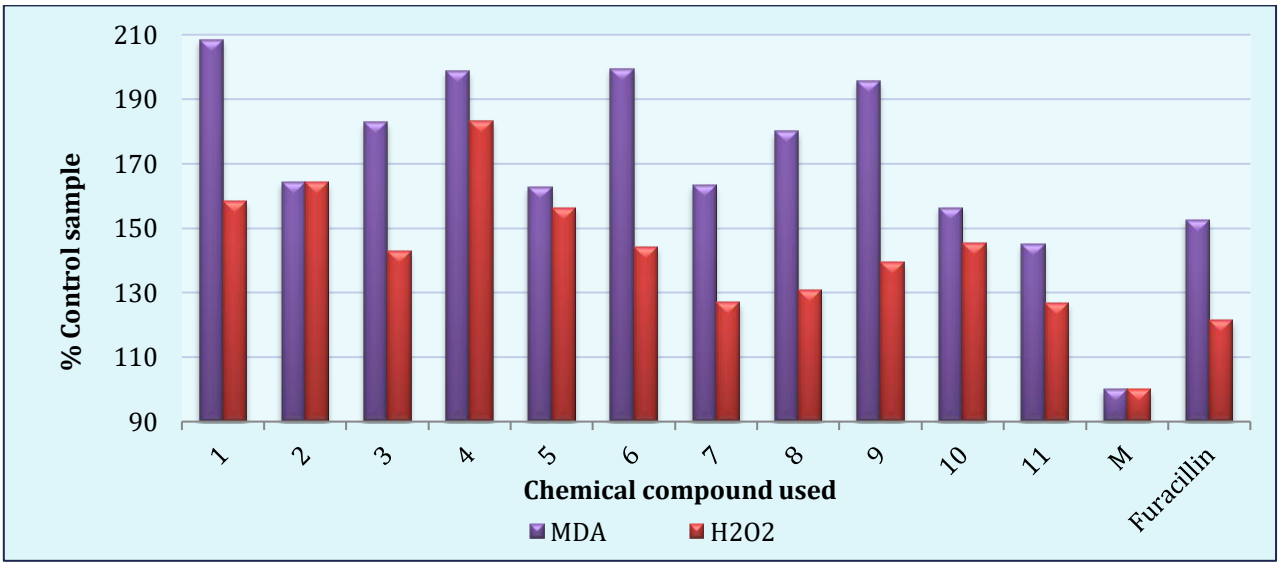

Figure 2. The amount of malondialdehyde and hydrogen peroxide in the standard culture of *Bacillus cereus* ГИСК 8035 under the action of the new selected chemical compounds:1 – $C_{38}H_{38}Cu_2N_{14}O_{10}S_4$; 2 – $C_{42}H_{42}Cu_2N_{14}O_{12}S_4$; 3 – $C_{46}H_{46}Cu_2N_{18}O_{10}S_6$; 4 – $C_{46}H_{42}Cu_2N_{18}O_{10}S_4$; 5 – $C_{15}H_{19}ClCuN_4O_2S$; 6 – $C_{15}H_{19}CuN_5O_5S$; 7 – $C_{15}H_{17}ClCuN_4OS$; 8 – $C_{15}H_{19}CuN_5O_5S$ (2,5); 9 – $C_{15}H_{19}CuN_5O_5S$ (3,4); 10 – $C_{15}H_{19}CuN_5O_5S$ (2,4); 11 – $C_{18}H_{22}Cl_2Cu_2N_8S_2$; M – uncultivated bacteria.

Unlike staphylococcal culture, *Bacillus cereus* has a statistically true increase in all the studied compounds compared with the control sample of the hydrogen peroxide content in the cell lysate. In case of furacillin, the increase is 21.4%, and in case of new tested compounds, the values exceeded the control ones by 26.8-58.3%. At the same time, the content of malondialdehyde signi-

ficantly increases in comparison with the control sample. In case of furacillin, the MDA amount increased by 52.4%, whereas in newly tested compounds, the lipid peroxidation was 45.0-95.5% more intense than in the control one.

The reference culture *Shigella sonnei ATCC 25931* was similar to that of *Bacillus cereus.* The results are shown in figure 3.

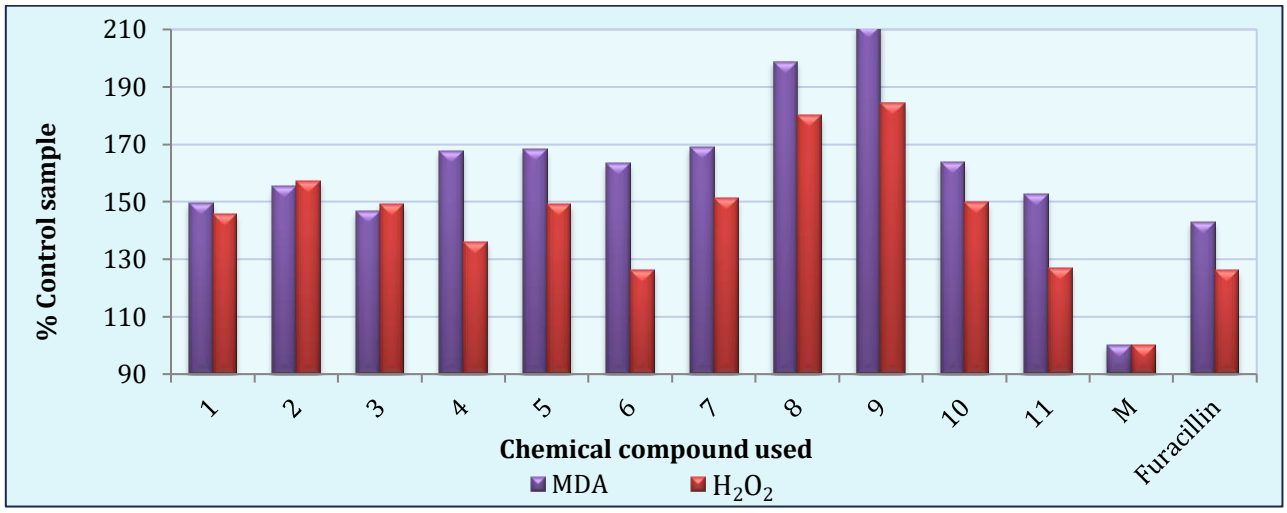

Figure 3. The amount of malondialdehyde and hydrogen peroxide in the standard culture of *Shigella sonnei* ATCC 25931 under the action of the new selected chemical compounds: 1 – $C_{38}H_{38}Cu_2N_{14}O_{10}S_4$; 2 – $C_{42}H_{42}Cu_2N_{14}O_{12}S_4$; 3 – $C_{46}H_{46}Cu_2N_{18}O_{10}S_6$; 4 – $C_{46}H_{42}Cu_2N_{18}O_{10}S_4$; 5 – $C_{15}H_{19}ClCuN_4O_2S$; 6 – $C_{15}H_{19}CuN_5O_5S$; 7 – $C_{15}H_{17}ClCuN_4OS$; 8 – $C_{15}H_{19}CuN_5O_5S$ (2,5); 9 – $C_{15}H_{19}CuN_5O_5S$ (3,4); 10 – $C_{15}H_{19}CuN_5O_5S$ (2,4); 11 – $C_{18}H_{22}Cl_2Cu_2N_8S_2$; M – uncultivated bacteria.

The level of hydrogen peroxide in the biomass of *Shigella sonnei* ATCC 25931 increased by 26% under the action of furacillin, and by 26.3-57.3% under the action of new tested chemical compounds. The differences between the values of the experimental samples and the control sample for hydrogen peroxide were true at a confidence interval of 99% (compounds 7-9) and 95% for the rest of samples. The level of malondialdehyde in the biomass of *Shigella sonei* increased by 42.9% when treated with furacillin compared with the control, and by 49.6-211.0% in the newly tested chemical compounds.

Compounds with maximum activity belong to the group of Cu(II) compounds with 4-(dimethylphenyl)thiosemicarbazone-2-formylpyridine. In this case, the process of lipid peroxidation and the accumulation of end products are almost doubled. This indicates a pronounced oxidative stress, which is confirmed by both the antimicrobial activity test and the ABTS test, showing a sharp decrease in the antioxidant capacity of the cultures.

Out of a number of newly tested chemical compounds, six new compounds showed antibacterial activity at a sufficiently high level compared to the *Escherichia coli* ATCC 25922. The results reflecting the accumulation of lipid peroxidation products and hydrogen peroxide in the corresponding culture under the action of antibacterial agents are shown in figure 4.

Under the action of furacillin, the level of hydrogen peroxide increased by 39%, and malondialdehyde - by 52% compared to the control sample. While in the mixtures of new chemical compounds selected in all variants, there was a significant increase in the content of hydrogen peroxide (up to 62%) and malonic dialdehyde (up to 81%). The differences between each of the experimental samples and the control sample were statistically true at 95% confidence interval.

Figure 5 depicts the results, showing changes in MDA and H_2O_2 levels in *Salmonella enterica* culture under the action of new antibacterial compounds.

Under the action of furacillin, there was an increase in the level of hydrogen peroxide by 35.7% compared with the control, and by 52.4% in MDA. Under the action of the tested compounds, the

content of hydrogen peroxide was higher by 62.3 and 65.2%, and the content of MDA is 50.5 and 76.4% higher than in the control sample.

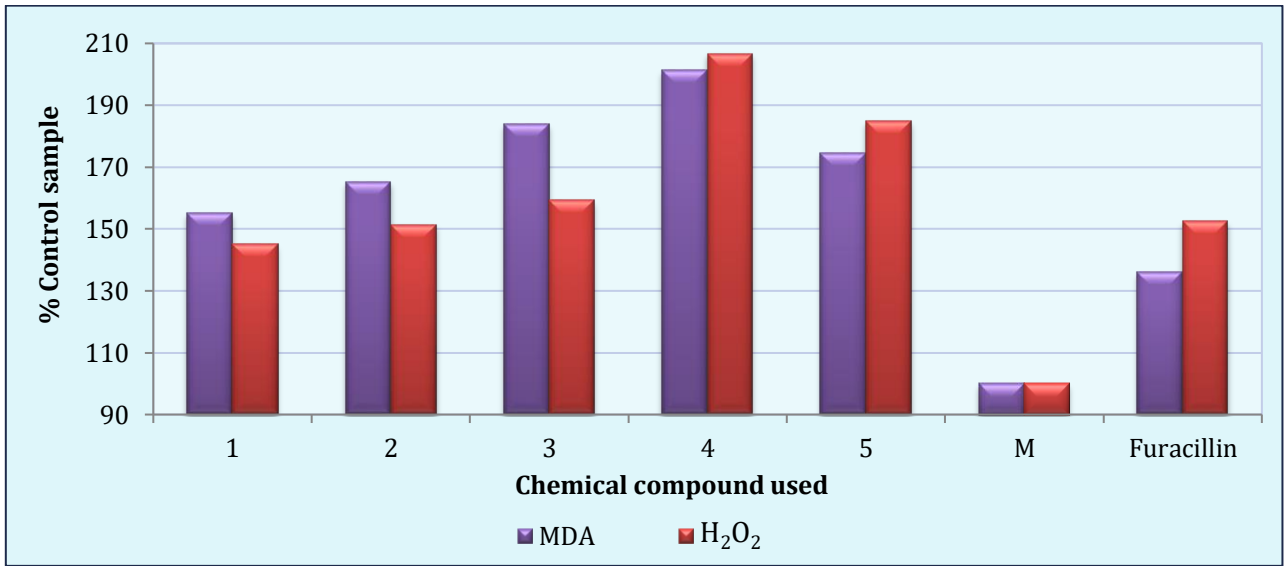

Figure 4. The amount of malondialdehyde and hydrogen peroxide in the standard culture of *Escherichia coli* ATCC 25922 under the action of the new selected chemical compounds: 1 – $C_{38}H_{38}Cu_2N_{14}O_{10}S_4$; 2 – $C_{44}H_{40}Cl_2Cu_2N_{14}O_4S_6$; 3 – $C_{46}H_{46}Cu_2N_{18}O_{10}S_6$; 4 – $C_{46}H_{42}Cu_2N_{18}O_{10}S_4$; 5 – $C_{18}H_{22}Cl_2Cu_2N_8S_2$; M – uncultivated bacteria.

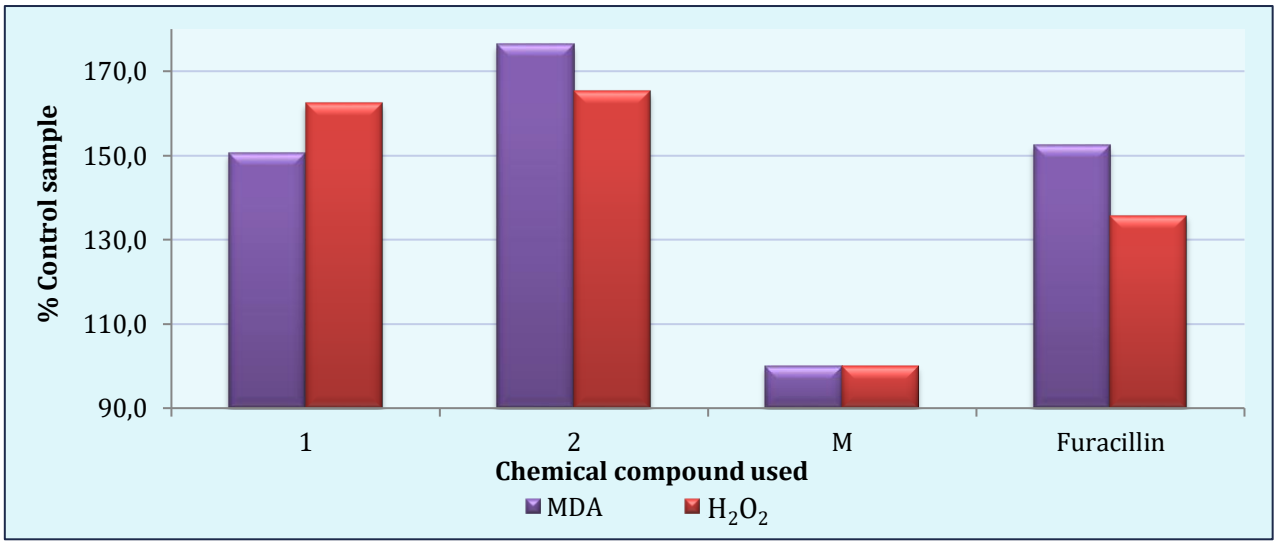

Figure 5. The amount of malondialdehyde and hydrogen peroxide in the standard culture of *Salmonella enterica (S. abony ГИСК 03/03 y)* under the influence of selected new chemical compounds: 1 – $C_{38}H_{38}Cu_2N_{14}O_{10}S_4$; 2 – $C_{44}H_{40}Cl_2Cu_2N_{14}O_4S_6$; M – uncultivated bacteria.

DISCUSSIONS

Dintre toate patologiile ce fac parte din grupul SSN, conform cercetării efectuate, am identificat că insuficienţa VAo şi insuficienţa VTs au fost caracteristice preponderent pentru SpA, date similare fiind descrise în studiul lui Roldan C. şi There are numerous publications that describe various changes in the biochemical content of pathogenic microorganisms cells subjected to the toxic action of antimicrobial preparations. The bibliographic study shows that one of the general and common mechanisms for virtually all antimicrobial prepa rations is the induction of oxidative stress in the

cells of the pathogen, expressed in the accumulation of free radicals (13, 14, 15).

According to the study results on the action of new chemical compounds on five reference cultures included in the study, a significant increase in lipid peroxidation products was found, which indicates an intense oxidative stress by accumulation of hydrogen peroxide.

Based on the foregoing, we consider it appropriate to quantify the parameters that express the antioxidant status of cells in cultures of pathogenic microorganisms exposed to new chemical compounds and those that were not subjected to treatment. Evaluation of product changes can serve as a tool to assess the effectiveness of new substances as antimicrobial products.

CONCLUSIONS

1. The level of hydrogen peroxide in the reference strain of *Shigella sonnei* ATCC 25931 increased under the action of the newly tested chemical compounds by 26.3-57.3%. The level of malondialdehyde increased by 49.6-211.0% when tested with the new chemical compounds.
2. Under the action of the new chemical compounds tested in all reference cultures, there was a significant increase in the accumulation of lipid peroxidation products, which described irreversible oxidative changes and confirmed the state of deep oxidative stress in reference cultures.
3. In the reference culture of *Staphylococcus aureus* ATCC 25923, treated with selected new chemical compounds, a level of hydrogen peroxide was obtained that exceeded the control sample by up to 37.7%. The level of malondialdehyde in cultures treated with appropriate doses of new chemical compounds was 50.4-106.1% compared to the control.
4. The values of the reference culture of *Bacillus cereus* ГИСК 8035, when tested with the new compounds, exceeded the control sample by 26.8-58.3%, and the malonaldehyde content was 45.0-95.5% more intense than in the control one.
5. The level of hydrogen peroxide in the reference strain *Shigella sonnei* ATCC 25931 increased by 26.3-57.3% under the action of the new tested chemical compounds. The level of malondialdehyde increased by 49.6-211.0% when tested with new chemical compounds.
6. In the culture of *Escherichia coli* ATCC 25922, under the action of selected new chemical compounds (in all variants), an increased level of hydrogen peroxides up to 62% and of malondialdehyde up to 81% was recorded.
7. *Salmonella enterica* (*S. abony ГИСК 03/03 y*), under the action of the newly tested chemical compounds, showed a higher content of hydrogen peroxide by 62.3 and 65.2%, as well as a higher content of MDA by 50.5 and 76.4% than in the control culture.
8. Under the action of the new chemical compounds with antimicrobial properties on the reference cultures of *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ГИСК 8035, *Escherichia coli* ATCC 25922, *Shigella sonnei* ATCC 25931 and *Salmonella enterica* (*Salmonella abony* ГИСК 03/03y), oxidative stress occurred, being confirmed by the accumulation of hydrogen peroxide and lipid peroxidation products.

CONFLICT OF INTERESTS

Nothing to declare.

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