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REPORT ON SCIENTIFIC AND RESEARCH WORK
UNDER RESEARCH IMPLEMENTATION AGREEMENT
№ 127–2023 dated 01/11/2023

«Virucidal, bactericidal, and fungicidal action of 35% hydrogen peroxide»

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LIST OF TERMS, SYMBOLS AND ABBREVIATIONS

HAdV-2	-	Human adenovirus type 2
IAV	-	Influenza A virus
MTT	-	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
CPE	-	Cytopathic effect of the virus
CC_{50}	-	50% cytotoxic concentration
$TCID_{50}$/ml	-	Median tissue culture infectious dose

The purpose of the research work:

To study the virucidal, bactericidal and fungicidal action of 35% hydrogen peroxide produced by HIDROJEN PEROKSIT SANAYI, batch 6749, production date 11.09.2023.

The sample was provided to D.K. Zabolotny Institute of Microbiology and Virology of NAS of Ukraine under the Act No./127-2023 of sample collection dated 07.11.2023 by HIDROCHIM SRL. The sample was taken by Andrey Gritaenko, Director of HIDROCHIM SRL.

Virucidal effect of hydrogen peroxide 35%

Tests of the virucidal effect of hydrogen peroxide 35% produced by HIDROJEN PEROKSIT SANAYI were carried out in accordance with the Methodological Recommendations "Determination of the virucidal effect of disinfectants" approved by the Ministry of Health of Ukraine No. 231 of 08.04.2009.

CHAPTER 1 MATERIALS AND METHODS

Cultivation of cells and their preparation for experiments

To propagate adenovirus and conduct research, we used a culture of transplantable cells included in the catalog of the European collection of cell and animal cultures – Vero (kidney cells of an African green monkey), obtained from the Cell Bank of the R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, National Academy of Sciences of Ukraine (Kyiv, Ukraine).

To propagate influenza virus and conduct research MDCK cell culture (canine kidney cells) was used, which was obtained from the L.V. Gromashevsky Institute of Epidemiology and Infectious Diseases, National Academy of Medical Sciences of Ukraine (Kyiv, Ukraine).

Cells were grown in sterile plastic flasks (Sarstedt, Germany) in nutrient medium consisting of 45% DMEM (Sigma, USA), 45% RPMI 1640 (Sigma, USA), and 8-10% heat-inactivated for 30 min at 56°C fetal bovine serum (FBS) (Sigma, USA), antibiotic gentamicin (100 μg/ml). Cells were subcultured when a monolayer was formed, that is, they were disaggregated from the surface using 0.02% Versen solution (Sigma, USA) and 0.025% trypsin-EDTA solution (Sigma, USA), resuspended in a nutrient medium, and their concentration in the suspension was adjusted to $2x10^5$ cells/ml. A subcultivation ratio was determined after counting the number of cells in the Goryaev chamber using an inverted microscope (Carl Zeiss Jena, Germany) with a magnification of 70x. The

cell suspension in a volume of 200 µl was introduced into the wells of a 96-well plate (Sarstedt, Germany). Plates with cells were cultured in a thermostat at 37°C and 5% CO_2. After 24 hours during cultivation, the state of the cell monolayer in plates was monitored using a light inverted microscope (with a magnification of 70x). Cells were included in the research if they formed about 90% of the monolayer and in the absence of bacterial and fungal growth.

A mixture of 50% DMEM (Sigma, USA) and 50% RPMI 1640 (Sigma, USA) without serum was used as a supportive medium for Vero cells.

Viruses and their preparation for research

The reference strain human adenovirus type 2 (HAdV-2) was obtained from the virus museum of the Institute of Medical Microbiology of the Budapest University of Medical Sciences (Budapest, Hungary).

Influenza A virus (H1N1) strain A/FM/1/47 (IAV) was obtained from the L.V. Gromashevsky Institute of Epidemiology and Infectious Diseases, National Academy of Medical Sciences of Ukraine (Kyiv, Ukraine).

After 1 day of growth of Vero cells in 650 ml plastic flasks and the formation of a monolayer, the cells were washed with Hanks' solution (Sigma, USA) and introduced with a virus in a small amount of medium (enough to cover the monolayer) with a multiplicity of 0.01 - 0.001 PFU/cell. Adsorbed for 1 hour at room temperature, then the necessary amount of supporting medium was added and the cells were incubated at 37°C for 4-5 days until the appearance of intensive viral replication on the cells. To isolate the virus from the cellular material, 3-fold freezing and thawing cycles were carried out until the cells were completely destroyed, the cellular dentrite was removed by centrifugation at 2000 rpm within 10 min. The titers of the virus in the supernatant were determined using the MTT assay, the samples were poured into cryotubes of 1 - 5 ml and stored at -70°C. The studies used HAdV-2 with an infectious titer of 7.7 $\log_{10}$ $TCID_{50}$/ml.

After the formation of monolayer in MDCK cell culture the cells were washed with the phosphate buffered saline pH = 7.4 (PBS). The IAV suspension in PBS was added in the amount enough to cover the monolayer. After the incubation for 1 hour at 37°C and 5% CO_2, the supernatant was removed and the supporting medium was added (49% DMEM (Sigma, USA), 49% RPMI 1640 (Sigma, USA), 2% FBS (Sigma, USA) and 1 µl/ml TPCK-treated trypsin (Sigma, USA)). Cells were incubated for 24-72 h at 37 °C and 5% CO2 until the clear signs of CPE. The supernatant was collected and undergone 3-fold freezing and thawing cycles with a consistent centrifugation at 3000

rpm for 30 min at 4°C. The titers of the virus in the supernatant were determined using the gentian violet.

Study of the cytotoxic effect of 35% hydrogen peroxide using MTT

The MTT assay is based on the functioning of the mitochondrial dihydrogenase system of intact cells, which under normal conditions process the artificial substrate MTT (3, (4,5-dimethyltriazol-2-yl)-2,5-diphenyltetrozolium bromide) into formazan. The reaction products can be determined by a quantitative spectrophotometric method. The conversion of MTT into formazan dose-dependently decreases with the death of cells under the influence of toxic substances or a virus.

Cells were grown in 96-well plates. After 24 hours of cell growth, the nutrient medium was replaced with a medium containing tenfold or twofold dilutions of the tested agent (from 0.35% to 0.000035%). In the control cells, the medium was replaced with a fresh one without the addition of the agent. For each concentration at least 3-4 wells with cells were used. Plates were kept in a thermostat at 37°C in a 5% CO_2 atmosphere for 2-3 days. The state of the cell monolayer was monitored using an inverted light microscope (x70 magnification).

MTT substrate (Sigma, USA) was dissolved in sterile PBS at room temperature to a concentration of 5 mg/ml. 20 µl of the filtered MTT solution was added to wells of a plate and incubated with cells for 2-4 hours at 37°C. After incubation, the medium was removed, 150 µl of 96° ethanol was added to the cells to dissolve the formazan crystals. The results were analyzed spectrophotometrically on a Multiskan FC reader (Thermo Fisher Scientific, USA) at a wavelength of 538 nm.

Based on the dose-dependent curves using Microsoft Excel software, the value of peroxide dilution/concentration that causes 50% inhibition of cell population growth (CC_{50} index) was determined.

Study of the virucidal effect of 35% hydrogen peroxide

The purpose of the study is to determine the direct effect of the agent on the extracellular virus.

Undiluted adenovirus (with a titer of 7.7 $\log_{10}$ TCID50/ml) was mixed with an equal volume of hydrogen peroxide at a concentration of 1.4%, 0.7% and 0.35% (respectively, the concentration of hydrogen peroxide with the virus was 0.7 %, 0.35% and 0.175%) and incubated at 20°C for 15, 30 and 60 min. To prevent the manifestation of the cytotoxic effect of the disinfectant on cells,

which is used to determine the residual infectious activity of the virus by its cytopathic effect, the disinfectant must be neutralized before it is introduced into the cell monolayer. A 5% solution of sodium thiosulfate (Sigma, USA) was used to neutralize hydrogen peroxide. After the end of the exposure, an equal amount of 5% sodium thiosulfate solution was added to the virus–hydrogen peroxide mixture and left for 5 min, after which successive tenfold serial dilutions of the obtained experimental virus samples and controls were prepared in a supportive nutrient medium. Vero cells were infected with tenfold serial dilutions of virus-containing material (hydrogen peroxide–virus–neutralizer suspension) at 50 µl per well.

As controls were used:

1. Cell culture control - uninfected cells (minimum 10 wells of the plate) with a supporting medium;

2. Virus control - instead of hydrogen peroxide, a supporting medium without FBS was used;

3. Control of the virus in a mixture with a protein load - instead of hydrogen peroxide, a supporting medium with FBS was used;

4. Control of the toxicity of the disinfectant mixture with neutralizer;

5. Control of the complete neutralization of the disinfectant - formaldehyde was used as a reference disinfectant (to perform this control, one part of the virus-containing liquid was mixed with 4 parts of Hanks' solution and 5 parts of 1.4% formaldehyde solution).

Adsorption of adenovirus on cells was carried out at 37°C for 1 hour, after which 150 µl of the supporting medium was added to the virus-containing material. The plate was kept in 5% CO_2 at 37°C until the appearance of a pronounced cytopathic effect of the virus (4 days).

To determine the virucidal activity against the influenza virus, the undiluted virus (with a titer of 8.14 $\log_{10}$ $TCID_{50}$/ml) was mixed with an equal volume of hydrogen peroxide at a concentration of 0.7% (correspondingly, the concentration of hydrogen peroxide with the virus was 0.35%) and incubated at 37°C for 5, 15 and 30 min. Next, MDCK cells were infected with tenfold serial dilutions of the virus-containing material at 50 µl per well. As a control, a suspension of the virus was used, which was maintained under similar conditions in a mixture with a medium without FBS. Adsorption was carried out at 37°C for 1 h, after which the supernatant was removed from the wells and 150 µl of supporting medium was added (49% DMEM (Sigma, USA), 49% RPMI 1640 (Sigma, USA), 2% ETS (Sigma, USA), penicillin-streptomycin 100 µg/ml, (Biowest, France), TPCK-trypsin 2 µg/ml). Cells not infected with the virus were used as control cells. The plate was

kept in an atmosphere of 5% CO_2 at 37°C until the appearance of a pronounced cytopathic effect of the virus (2-3 days).

The analysis was performed using the MTT method and staining with gentian violet (4 mg/ml). The results were analyzed spectrophotometrically on a Multiskan FC reader (Thermo Scientific, USA) at a wavelength of 538 nm. Using the obtained optical densities, the % inhibition of cell viability under the action of the virus (or % of the viral CPE per cell) was determined by the formula:

% inhibition of cell viability or % CPE = 100 – (A*100/B),

where A – the average value of the optical density of the sample, a B – the average value of the optical density of the cell control.

The dilution of the virus, that reduces the optical density of the sample compared to the optical density of cell control by 50% was determined, which is the titer of the virus and is expressed in TCID50/ml.

The virucidal effect of the agent was determined by reduction the virus titer and its infectivity according to the formula:

General virucidal effect = A – B,

where A – virus titer in the control, a B – virus titer in the sample.

CHAPTER 2 RESEARCH RESULTS

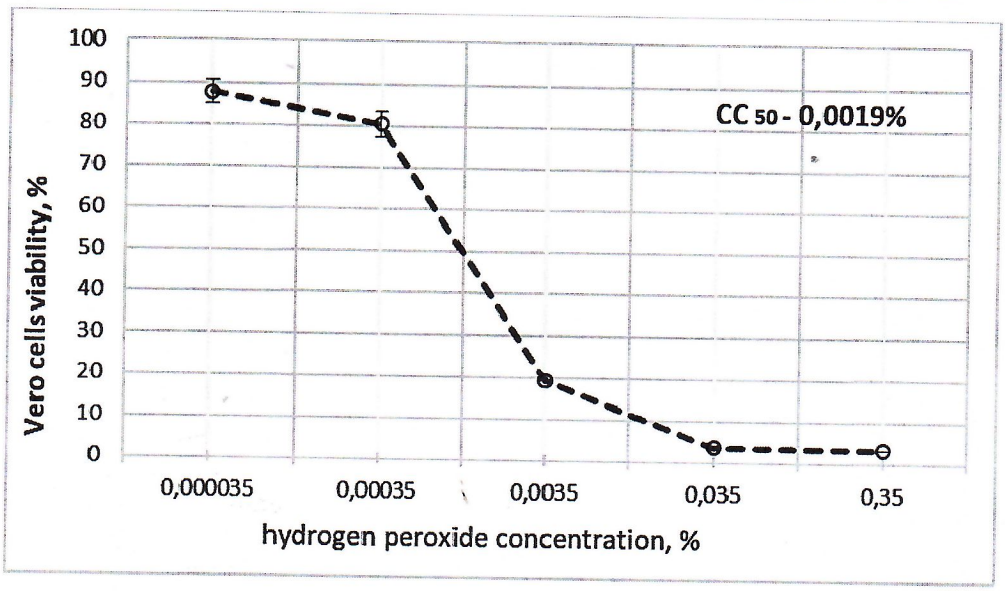
Study of the cytotoxic effect of hydrogen peroxide

The study of the substance toxicity is a key point in its research as a potential means of treatment or antiseptic. A classic method for determining the effect of the investigated substances on the physiological state of cells, namely on the activity of mitochondria, is the MTT assay. It demonstrates a direct correlation between the viability of cells *in vitro* and the ability of the mitochondrial dehydrogenase system to convert MTT into formazan, which is significantly inhibited under the influence of toxic substances.

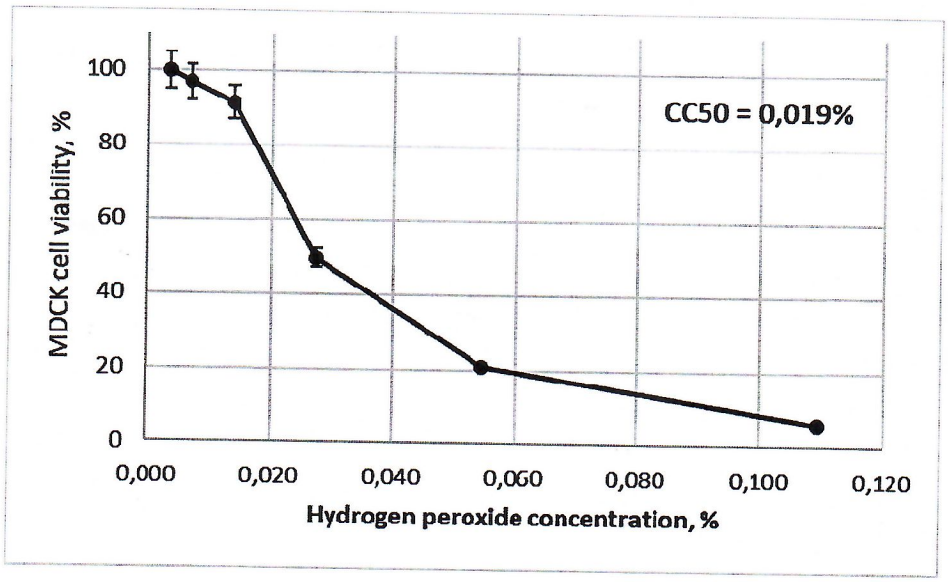
Cell lines sensitive to human adenovirus type 2 and influenza A virus were used to study the cytotoxic effect of hydrogen peroxide. The toxicity of the solution in cell cultures was determined by adding it to the supporting medium in concentrations of 0.000035 - 0.35% (for Vero) and 0.003% - 3.5% (for MDCK), each concentration was analyzed in three replicates with mandatory inclusion of control samples (without the added substance). Plates with cells and hydrogen peroxide were incubated at 37°C in an atmosphere of 5% CO_2. After 72 hours of exposure, MTT solution was

added and the optical density in the wells was determined by the spectrophotometric method. The value of the optical density in the wells with control cells not treated with the agent was taken as 100% and the percentage of viable cells was determined for each concentration of the substance (Fig. 1). Hydrogen peroxide is highly toxic to the Vero cell culture, since in concentrations of 0.0035 - 0.35% it reduces its mitochondrial activity by 81 - 97% compared to cell control. When using a 0.00035% solution of the agent, cell viability was within 80%. It was determined that the CC50 indicator for hydrogen peroxide is 0.0019%.

A similar effect is observed for MDCK cell culture (Fig. 1, B): at peroxide concentrations of 0.35-0.05% the share of viable cells does not exceed 20%. Further, as the concentration decreases, mitochondrial activity increases. CC50 of the studied solution for MDCK is 0.019%.



A



B

Figure 1. Cytotoxic effect of hydrogen peroxide on Vero (A) and MDCK (B) cell cultures

A study of the cytopathic effect of sodium thiosulfate was also conducted, which was subsequently used to neutralize the toxic effect of peroxide on cells. It was shown that at a concentration of the neutralizer solution of 1.2% and below, cell viability was within 46-59% (Fig. 2). Its CC50 is 0.9%.

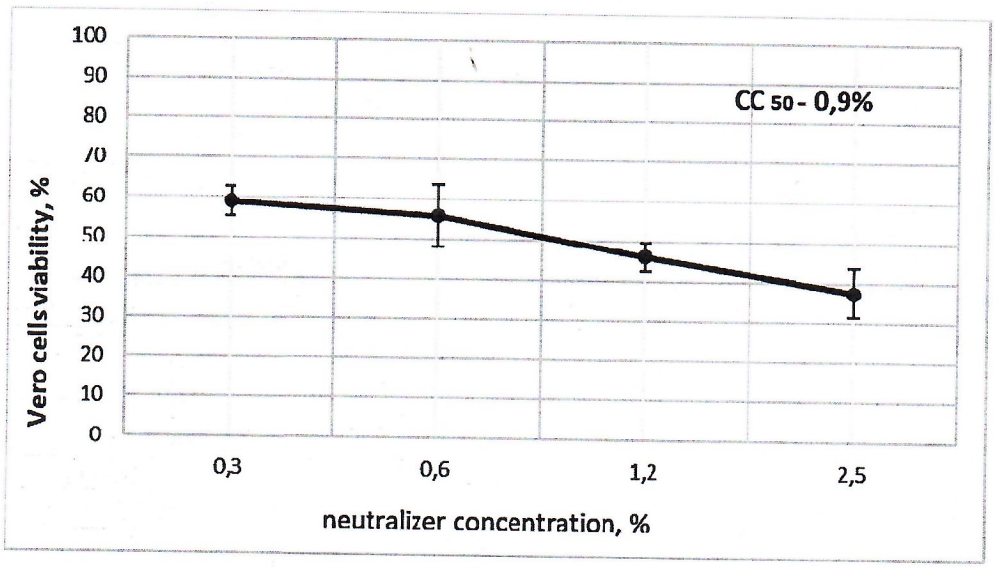


Figure 2. Cytotoxic effect of the neutralizer

Study of the virucidal effect of hydrogen peroxide

The main indicator of the efficiency of antiseptics and disinfectants in given concentrations and duration of exposure in evaluation (certification) studies is the complete absence of signs of virus reproduction under the conditions of compliance with the required titer (4-7 in $\log_{10}$ $TCID_{50}$/ml), or a decrease in the viral infectious titer at least by 4 $\log_{10}$ $TCID_{50}$/ml compared to the control. In screening studies (for the search for new antiseptics) or expansion of the properties of already existing ones, the minimum value of the decrease in the infectious titer of the test virus during exposure, which indicates the presence of a virucidal effect, is considered to be 2 $\log_{10}$ $TCID_{50}$/ml.

Generally accepted when analyzing the virucidal activity of a disinfectant using a suspension method, it is dissolved in a supporting medium for cell cultures, or mixed with an appropriate volume of medium so that the final concentration of the agent in the solution is 2 times higher than recommended in the instructions for using the agent. This is due to the fact that during the tests, the solution of the studied antiseptic is mixed with the virus-containing suspension in equal proportions, thereby its concentration is reduced by 2 times. However, taking into account the revealed high toxicity of hydrogen peroxide on Vero cell culture, and the inability of the neutralizer to affect the cell-damaging effect of high concentrations of the agent, the virucidal activity of 1.4%, 0.7% and 0.35% hydrogen peroxide solutions was studied (i.e. with virus, the final concentrations of the agent were 0.7%, 0.35% and 0.175%, respectively).

The infectious titer of human adenovirus type 2 after exposure to the substance was determined by its cytopathic effect on Vero cells. The analysis of the results using the MTT dye was carried out after 4 days at the maximum manifestation of the CPE of the virus.

Although the methodological recommendations for determining the virucidal effect of disinfectants suggest using 0.5-1% solutions to neutralize the toxic effect of the agents, we used a 5% solution of sodium thiosulfate, since a lower concentration did not neutralize the lysing effect of hydrogen peroxide on cells at all (since we observed 100% cell death). Even when using 1.4%, 0.7%, and 0.35% hydrogen peroxide, the 5% neutralizer restored Vero cell viability by 15-28% (toxicity control of the disinfectant-neutralizer mixture).

As can be seen from Figures 3 and 4, 0.175% and 0.35% hydrogen peroxide solutions do not show a significant virucidal effect regardless of the time of exposure to the virus, because the decrease in the infectious titer of HAdV-2 did not exceed 1.6 $\log_{10}$. When using hydrogen peroxide in a final concentration of 0.7% and its exposure time with the virus for 15 and 30 minutes, a slight

decrease in the infectious titer was also found (by ≈1.7 log_{10}) (Fig. 5). On the other hand, with an increase in the exposure time of HAdV-2 and the agent to 1 hour, the virucidal effect increased to 2.6 log_{10}. The use of a reference disinfectant of 0.7% formaldehyde, regardless of the exposure time with the virus, leads to complete inactivation of HAdV-2.

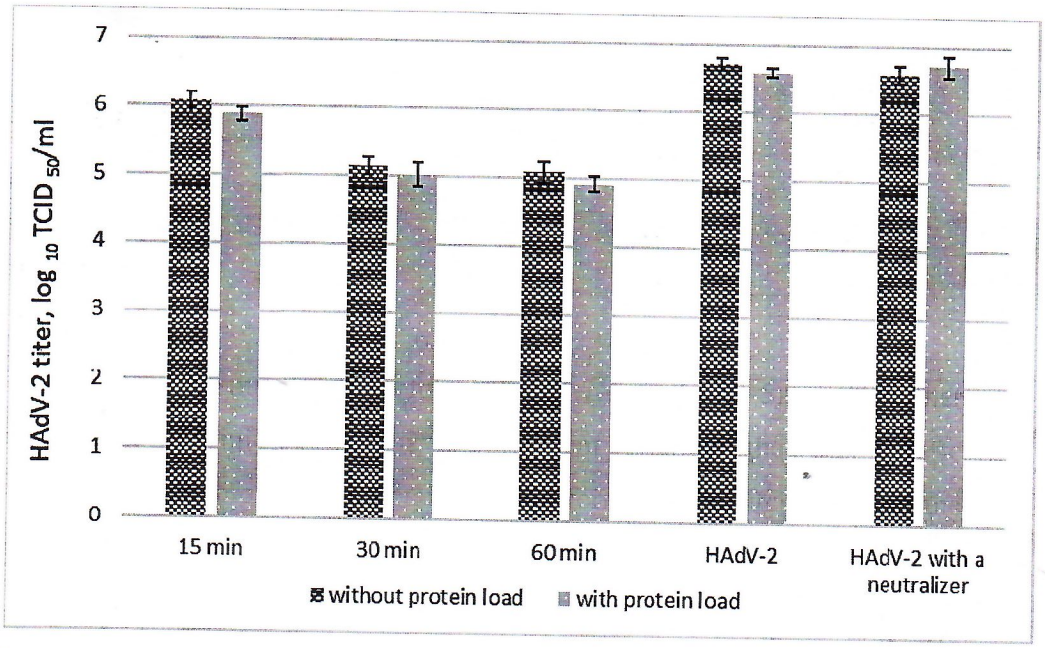


Figure 3. The effect of 0.175% hydrogen peroxide solution on HAdV-2 infectious titer at different exposure duration

It should be noted that the data obtained in the study of the virucidal effect of hydrogen peroxide were conducted without protein load and in its presence are similar, which indicates that the protein load does not affect the antiviral properties and effectiveness of the studied concentrations of the agent.

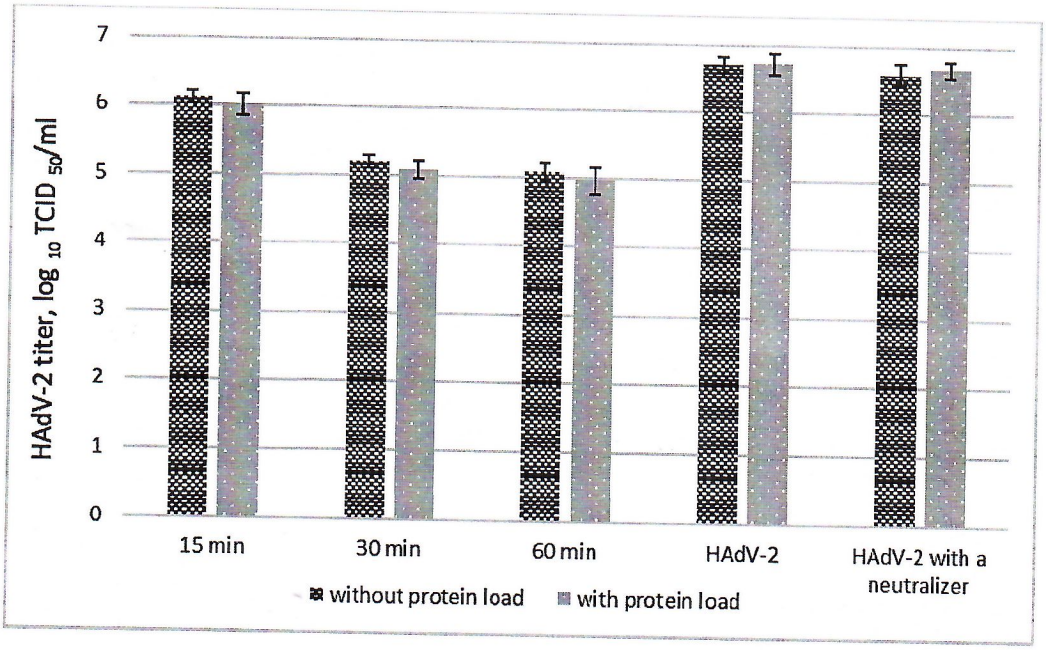


Figure 4. The effect of 0.35% hydrogen peroxide solution on HAdV-2 infectious titer at different exposure duration

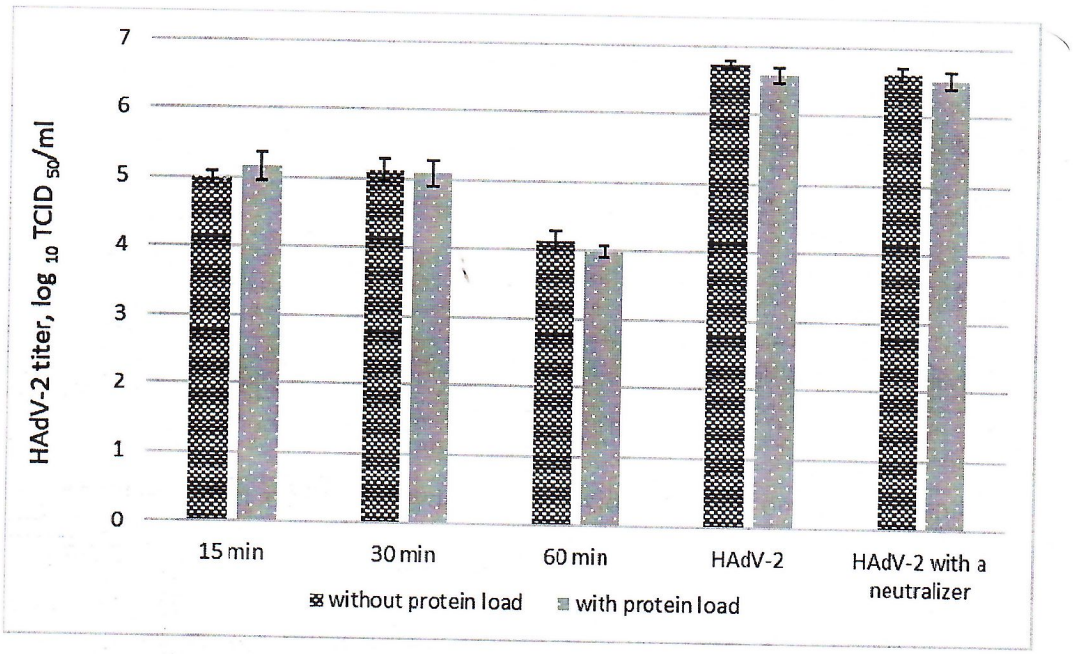


Figure 5. The effect of 0.7% hydrogen peroxide solution on HAdV-2 infectious titer at different exposure duration

Determination of the virucidal effect on the influenza A virus was carried out at a final disinfectant concentration of 0.35% (before contact with the viral suspension it was 0.7%). It can be seen from Figure 6 that the hydrogen peroxide solution shows a pronounced effect at the given studied concentration. Thus, in 5 minutes of incubation, the infectious titer of the virus decreases by

3.48 log_{10}, compared to the control ($TCID_{50}$/ml = 8.13 log_{10}). After 15 and 30 minutes of exposure, the effect is even better – the decrease in titer is by 5.05 and 5.01 log_{10}, respectively.

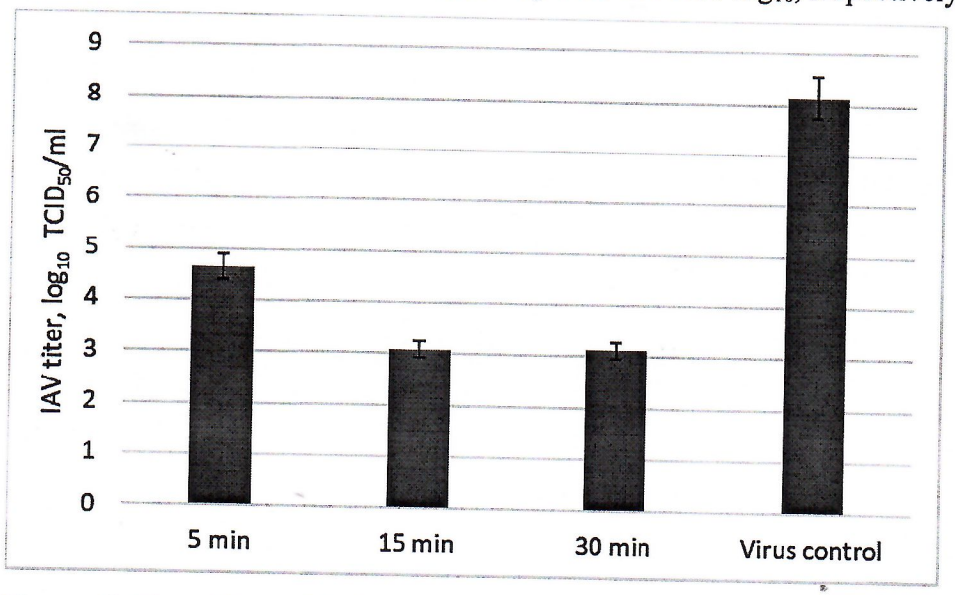


Figure 6. The effect of hydrogen peroxide solution on the infectious titer of influenza A virus

CONCLUSION:

A study of the antiviral activity of hydrogen peroxide (HIDROJEN PEROKSIT SANAYI) showed that its 0.7% solution exhibits a pronounced virulicidal effect in relation to human adenovirus type 2, since in 60 minutes of exposure to the virus, its titer decreases by ≈2.6 log_{10} compared to the control values. Moreover, the solution shows a clear virulicidal effect against influenza A virus in >5 log_{10} reduction of the infectious titer compared to the virus control.

Determination of the main bactericidal activity of hydrogen peroxide according to EN 1040:1997 IDT

MATERIALS AND METHODS

The objects of research were hydrogen peroxide and its dilution (0.54%, 1.09% and 2.18%).

DESCRIPTION OF THE TEST

Preparation of suspensions of bacteria.

Test cultures were used in the research *Staphylococcus aureus* ATCC 6538 and *Pseudomonas aeruginosa* ATCC 9027. Test suspensions *Staphylococcus aureus* and *Pseudomonas aeruginosa* were prepared with the concentration of cells $1,5\text{-}5\times10^8$ CFU/cm^3.

The content of viable microorganisms was additionally monitored by diluting the test suspensions in 10^6 and 10^7 times with a dilution solution which consists of, g/l: tryptone – 1.0; NaCl – 8.5; distilled water – 1000 cm^3. The resulting dilutions were added to a Petri dish in two replicates and added 12-15 cm^3 melted and cooled to 45±1°C medium which consist of, g/l: tryptone – 15.0; soybean peptone -5.0; agar – 15.0; distilled water – 1000 cm^3. Microorganisms were cultured at the temperatures (37±1)°C during 24 hours.

Validation of the method with dilute solution neutralization.

An aqueous solution of polysorbate was chosen as a neutralizing agent 80 (30 g/dm^3).

Test cell suspensions *Staphylococcus aureus* and *Pseudomonas aeruginosa* diluted in 10^6 and 10^5 times with a dilution solution which consists of, g/l: tryptone – 1.0; NaCl – 8.5; distilled water – 1000 cm^3. To control the content of viable microorganisms, 1 cm^3 was taken from the resulting suspension, transferred sterilely to a test tube and diluted 10 times with dilution solution. The resulting dilutions were added to a Petri dish in two replicates and added 12-15 cm^3 melted and cooled to 45±1°C medium which consist of, g/l: tryptone – 15.0; soybean peptone -5.0; agar – 15.0; distilled water – 1000 cm^3. Microorganisms were cultured at the temperatures (37±1)°C during 24 hours.

An aqueous solution of polysorbate 80 (30 g/dm^3) was taken with a pipette in an amount of 8.0 cm^3 and added to two test tubes. The first one was used to control toxicity, the second one - to control the neutralization efficiency of the dilute solution. After that, 1 cm^3 of sterile distilled water was added to the first test tube, and a solution of hydrogen peroxide was added to the second test tube. Then, 1 cm^3 of the test suspension diluted in 10^5 – 10^6 was added to each of the tubes, mixed

and left for (30 ± 1) min. From each tube, 1 cm^3 was taken in duplicate, placed in a Petri dish and added 12-15 cm^3 melted and cooled to 45±1°C medium which consist of, g/l: tryptone – 15.0; soybean peptone -5.0; agar – 15.0; distilled water – 1000 cm^3. Microorganisms were cultured at the temperatures (37±1)°C during 24 hours.

Bactericidal activity tests.

Test solutions of hydrogen peroxide were prepared with concentrations of (2.18%, 1.09% and 0.54%). A pipette was used to take 8.0 cm3 of the test solution of hydrogen peroxide and put it into a test tube, after that, 1.0 cm^3 of sterile distilled water and 1.0 cm^3 of test suspension with a cell concentration of $1.5\text{-}5.0\times10^8$ CFU/cm^3. Then immediately turn on the timer and stir the mixture. The activity of the products was determined over the period of interaction of the components, which was chosen to be 30 min ± 10 s.

Immediately before the end of the selected interaction period, the mixture was stirred, sampled with a 1.0 cm^3 pipette, and transferred to a test tube containing 8.0 cm^3 of neutralizing agent (polysorbate 80 (30 g/dm^3)) and 1.0 cm^3 of sterile distilled water. After 5 min ± 10 s, two samples of 1.0 cm3 of the mixture were taken and each 1.0 cm3 sample was transferred to a separate Petri dish and added 12-15 cm^3 melted and cooled to 45±1°C medium which consist of, g/l: tryptone – 15.0; soybean peptone -5.0; agar – 15.0; distilled water – 1000 cm^3. Microorganisms were cultured at the temperatures (37±1)°C during 24 hours.

The concentration of the biocide that reduced the content of viable fungal cells by 10^5 times was considered effective.

RESULTS

VERIFICATION OF METHODOLOGY AND VALIDATION OF THE METHOD WITH DILUTE SOLUTION NEUTRALIZATION

Test microorganism	Viable microorganisms content, CFU/cm^3			
	Test fungal suspension (N)	Diluted fungal suspension (N_v)	Control of neutralizing agent toxicity (N_x)	Control of dilute solution neutralization efficiency (N_y)
Staphylococcus aureus	$3{,}1\times10^8$	$3{,}0\times10^3$	$1{,}8\times10^2$	$1{,}5\times10^2$
Pseudomonas aeruginosa	$2{,}8\times10^8$	$2{,}8\times10^3$	$2{,}2\times10^2$	$1{,}9\times10^2$
For two test strains: value of N is in the range: $1.5\times10^8 - 5.0\times10^8$ CFU/cm^3; value of N_v is in the range: $6.0\times10^2 - 3.0\times10^3$ CFU/cm^3; value of $N_x \geq 0.05\ N_v$ value of $N_y \geq 0.05\ N_v$				
The absence of toxicity and neutralization efficiency was confirmed during the testing of the neutralizing agent under product test conditions with a test concentration of 2.18%.				

TEST RESULTS

Test microorganism	The content of viable microorganisms N_a, CFU/cm^3 in the test mixture at the indicated concentrations			Indicator of reduction in the level of viability of microorganisms at the specified test concentrations of the product		
	0.54%	1.09%	2.18%	0.54%	1.09%	2.18%
Staphylococcus aureus	$3{,}2\times10^2$	45,1	1,6	$1{,}0\times10^5$	$6{,}9\times10^5$	$1{,}9\times10^7$
Pseudomonas aeruginosa	$2{,}0\times10^2$	32,2	1,6	$1{,}4\times10^5$	$8{,}7\times10^5$	$1{,}7\times10^7$
The concentration of the biocide that reduced the content of viable fungal cells by 10^4 times (1.09%) was considered effective.						

CALCULATION OF TEST UNCERTAINTY

Test microorganism	The content of viable microorganisms N_a, CFU/cm^3 in the test mixture (0.54%)										Average value	Standard deviation, δ	Extended test uncertainty, $U = 2\times\delta$
	1	2	3	4	5	6	7	8	9	10			
Staphylococcus aureus	252	232	184	255	246	196	231	263	255	228	324,2	6,7	13,4
Pseudomonas aeruginosa	200	212	201	195	215	186	224	231	216	205	208,5	4,5	9,0
Test microorganism	The content of viable microorganisms N_a, CFU/cm^3 in the test mixture (1.09%)										Average value	Standard deviation, δ	Extended test uncertainty, $U = 2\times\delta$
	1	2	3	4	5	6	7	8	9	10			
Staphylococcus aureus	42	46	50	51	43	46	38	51	45	39	45,1	1,5	3,0
Pseudomonas aeruginosa	33	28	31	29	31	37	34	32	32	35	32,2	0,9	1,8
Test microorganism	The content of viable microorganisms N_a, CFU/cm^3 in the test mixture (2.18%)										Average value	Standard deviation, δ	Extended test uncertainty, $U = 2\times\delta$
	1	2	3	4	5	6	7	8	9	10			
Staphylococcus aureus	1	3	0	2	1	2	3	2	1	1	1,6	0,25	0,5
Pseudomonas aeruginosa	2	1	2	1	1	2	2	1	1	3	1,6	0,2	0,4

CONCLUSION

The studied sample of hydrogen peroxide is effective against *Staphylococcus aureus* and *Pseudomonas aeruginosa* in a concentration of at least 1.09%.

Determination of the main fungicidal activity of hydrogen peroxide according to EN 1275:1997 IDT.

MATERIALS AND METHODS

The objects of research were hydrogen peroxide and its dilution (0.54%, 1.09% and 2.18%).

DESCRIPTION OF THE TEST

Preparation of suspensions of fungal spores.

Test cultures were used in the research *Candida albicans* ATCC 885-653 and *Aspergillus niger* ATCC 16404. Test suspensions *Candida albicans* and *Aspergillus niger* were prepared with the concentration of cells $1.5\text{-}5.0\times10^7$ CFU/cm^3. The concentration was checked using a Goryaev camera – 200 CFU in 5 random squares of the chamber correspond to the concentration 1.0×10^7 CFU/cm^3.

The content of viable microorganisms was additionally monitored by diluting the test suspensions in 10^5 and 10^6 times with a dilution solution which consists of, g/l: tryptone – 1.0; NaCl – 8.5; distilled water – 1000 cm^3. The resulting dilutions were added to a Petri dish in two replicates and added 12-15 cm^3 melted and cooled to 45±1°C medium which consist of, g/l: malt extract -30.0; soybean peptone -3.0; agar – 15.0; distilled water – 1000 cm^3. Microorganisms were cultured at the temperatures (30±1)°C during 48 hours.

Validation of the method with dilute solution neutralization.

An aqueous solution of polysorbate was chosen as a neutralizing agent 80 (30 g/dm^3).

Test cell suspensions (spores) *Candida albicans* and *Aspergillus niger* diluted in 10^5 and 10^4 times with a dilution solution which consists of, g/l: tryptone – 1.0; NaCl – 8.5; distilled water – 1000 cm^3. To control the content of viable microorganisms, 1 cm^3 was taken from the resulting suspension, transferred sterilely to a test tube and diluted 10 times with dilution solution. The resulting dilutions were added to a Petri dish in two replicates and added 12-15 cm^3 melted and cooled to 45±1°C medium which consist of, g/l: malt extract -30.0; soybean peptone -3.0; agar – 15.0; distilled water – 1000 cm^3. Microorganisms were cultured at the temperatures (30±1)°C during 48 hours.

An aqueous solution of polysorbate 80 (30 g/dm^3) was taken with a pipette in an amount of 8.0 cm^3 and added to two test tubes. The first one was used to control toxicity, the second one - to control the neutralization efficiency of the dilute solution. After that, 1 cm^3 of sterile distilled water

was added to the first test tube, and a solution of hydrogen peroxide was added to the second test tube. Then, 1 cm^3 of the test suspension diluted in 10^4 - 10^5 was added to each of the tubes, mixed and left for (30 ± 1) min. From each tube, 1 cm^3 was taken in duplicate, placed in a Petri dish and added 12-15 cm^3 melted and cooled to 45±1°C medium which consist of, g/l: malt extract -30.0; soybean peptone -3.0; agar – 15.0; distilled water – 1000 cm^3. Microorganisms were cultured at the temperatures (30±1)°C during 48 hours.

Fungicidal activity tests.

Test solutions of hydrogen peroxide were prepared with concentrations of (2.18%, 1.09% and 0.54%). A pipette was used to take 8.0 cm3 of the test solution of hydrogen peroxide and put it into a test tube, after that, 1.0 cm^3 of sterile distilled water and 1.0 cm^3 of test suspension with a cell concentration of $1.5\text{-}5.0\times10^7$ CFU/cm^3. Then immediately turn on the timer and stir the mixture. The activity of the products was determined over the period of interaction of the components, which was chosen to be 30 min ± 10 s.

Immediately before the end of the selected interaction period, the mixture was stirred, sampled with a 1.0 cm^3 pipette, and transferred to a test tube containing 8.0 cm^3 of neutralizing agent (polysorbate 80 (30 g/dm^3)) and 1.0 cm^3 of sterile distilled water. After 5 min ± 10 s, two samples of 1.0 cm3 of the mixture were taken and each 1.0 cm3 sample was transferred to a separate Petri dish and added 12-15 cm^3 melted and cooled to 45±1°C medium which consist of, g/l: malt extract -30.0; soybean peptone -3.0; agar – 15.0; distilled water – 1000 cm^3. Microorganisms were cultured at the temperatures (30±1)°C during 48 hours.

The concentration of the biocide that reduced the content of viable fungal cells by 10^4 times was considered effective.

RESULTS

VERIFICATION OF METHODOLOGY AND VALIDATION OF THE METHOD WITH DILUTE SOLUTION NEUTRALIZATION

Test microorganism	Viable microorganisms content, CFU/cm³			
	Test fungal suspension (N)	Diluted fungal suspension (N_v)	Control of neutralizing agent toxicity (N_x)	Control of dilute solution neutralization efficiency (N_y)
Candida albicans	2.2×10^7	1.2×10^3	1.1×10^2	1.0×10^2
Aspergillus niger	1.7×10^7	1.0×10^3	6.8×10^1	8.2×10^1
For two test strains: value of N is in the range: $1.5\times10^7 - 5.0\times10^7$ CFU/cm³; value of N_v is in the range: $6.0\times10^2 - 1.5\times10^3$ CFU/cm³; value of $N_x \geq 0.05\ N_v$ value of $N_y \geq 0.05\ N_v$				
The absence of toxicity and neutralization efficiency was confirmed during the testing of the neutralizing agent under product test conditions with a test concentration of 2.18%.				

TEST RESULTS

Test microorganism	The content of viable microorganisms N_a, CFU/cm³ in the test mixture at the indicated concentrations			Indicator of reduction in the level of viability of microorganisms at the specified test concentrations of the product		
	0.54%	1.09%	2.18%	0.54%	1.09%	2.18%
Candida albicans	2.4×10^2	63	4	0.92×10^4	3.5×10^4	5.5×10^5
Aspergillus niger	5.9×10^2	78	12	0.29×10^4	2.2×10^4	1.42×10^5
The concentration of the biocide that reduced the content of viable fungal cells by 10^4 times (1.09%) was considered effective.						

CALCULATION OF TEST UNCERTAINTY

Test microorganism	The content of viable microorganisms N_a, CFU/cm³ in the test mixture (0.54%)										Average value	Standard deviation, δ	Extended test uncertainty, U = 2×δ
	1	2	3	4	5	6	7	8	9	10			
Candida albicans	278	254	233	265	195	280	203	236	198	240	238.2	10.4	20.8
Aspergillus niger	612	578	540	605	582	560	604	610	573	599	586.3	6.1	12.2
Test microorganism	The content of viable microorganisms N_a, CFU/cm³ in the test mixture (1.09%)										Average value	Standard deviation, δ	Extended test uncertainty, U = 2×δ
	1	2	3	4	5	6	7	8	9	10			
Candida albicans	60	73	52	54	66	81	50	62	59	69	62.6	3.0	6.0
Aspergillus niger	86	75	80	81	68	79	77	81	78	77	78.2	1.5	3.0
Test microorganism	The content of viable microorganisms N_a, CFU/cm³ in the test mixture (2.18%)										Average value	Standard deviation, δ	Extended test uncertainty, U = 2×δ
	1	2	3	4	5	6	7	8	9	10			
Candida albicans	2	7	4	5	2	4	4	2	1	8	3.9	0.7	1.4
Aspergillus niger	7	16	12	11	8	14	15	12	12	10	11.7	0.9	1.8

CONCLUSION

The studied sample of hydrogen peroxide is effective against *Candida albicans* and *Aspergillus niger* in a concentration of at least 1.09%.

CONCLUSION

Virucidal, bactericidal and fungicidal effect of 35% hydrogen peroxide produced by HIDROJEN PEROKSIT SANAYI, batch 6749, production date 11.09.2023, was studied.

It was found that the sample is effective against Human adenovirus serotype 2 and Influenza A virus (H1N1), A/FM/1/47 in a concentration of at least 0.70%; *Staphylococcus aureus* and *Pseudomonas aeruginosa* in a concentration of at least 1.09%; *Candida albicans* and *Aspergillus niger* in a concentration of at least 1.09%.