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ANTIBACTERIAL ACTION OF NOVEL ZEOLITE-BASED COMPOSITIONS DEPENDS UPON DOPING WITH Ag^{+} AND Cu^{2+} CATIONS

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Recently, there is a growing interest to exploration of sorption and catalytic properties of solid nanomaterials, in particular natural zeolites, as well as to study of their antimicrobial effects with the aim of potential using them as a principal component of disinfection and degassing remedies. The purpose of this work was to study the antimicrobial action of compositions based on the Transcarpathian clinoptilolite (CL) doped with Ag^{+} and Cu^{2+} cations or Ag microparticles (MPs). These compositions were subjected to mechanochemical modification in ethanol medium and with the addition of plant (Actinidia arguta) extract used as an antioxidant. Mechanochemical treatment (MChT) of all forms of CL MPs led to their grinding which caused better contact of CL with bacterial cells, while an increased content of larger pores improved their access to the active sites on the surface of the CL MPs. Treatment of CL samples with metallic silver used as a dopant with the help of the extract of Actinidia arguta plant did not increase the antibacterial activity regardless of treatment time. Treatment of $AgNO_3$ with ethanol slightly increased the antibacterial action of the CL MPs towards Gram-positive bacteria and decreased it towards Gram-negative bacteria. The CL samples doped with copper and treated with ethanol and plant (Actinidia arguta) extract demonstrated comparable toxic action towards Bacillus subtilis regardless of grinding conditions. While such a treatment caused a significant decrease in the antibacterial activity towards Staphylococcus aureus and Pseudomonas aeruginosa strains, compared to the action of samples that were not treated with that plant extract. To address the potential biochemical mechanisms of the antibacterial action of the created zeolite-based compositions, their influence on generation of the reactive oxygen species (ROS) was studied using diphenylpicrylhydrazyl (DPH) fluorescent dye. Most versions of the CL composites demonstrated time-dependent antioxidant effect comparable with the effect of the ascorbic acid used as a positive control. Thus, the ROS generation is not the mechanism that is responsible for the antibacterial action of the created CL-based compositions. Probably, that action is explained by the peculiarities of interaction of doped CL microparticles with the surface of the bacterial cells.

Keywords: clinoptilolite-based compositions, physicochemical treatment, doping with Ag+ and Cu2+ cations, antimicrobial action.

There is an increasing interest in the use of natural zeolites as disinfecting remedies [1-8]. It was shown that providing special biological activities to zeolites may be carried out in different ways, such as heat treatment or doping with cations of transition metals. Recently, a new approach was proposed to increase the antimicrobial activity of zeolite materials that is using the mechanochemical modification, namely, the micronization [9-11].

The surface of mineral nanomaterials can be subjected to chemical activation via introducing reactive groups (carboxy, amino, hydroxy, etc.). Natural clinoptilolite (CL), in particular of the Transcarpathian origin, meets most of the requirements as for multifunctional mineral nanomaterials. This mineral contains a significant amount of surface silanol (-OH) groups. In addition, CL can be easily modified [12]. During the calcination of CL, additional surface (-OH) groups, as well as siloxane groups (Si–O–Si) and Si–O–Al groups, can be formed on its surface, which are also biologically and chemically active [9, 13-16]. Unlike typical crystal hydrates, which release a significant amount of water, when heated, natural zeolites absorb and release water without significant changes in crystal structure. At temperature range up to 200 ºC, mainly physically adsorbed water is removed from the Transcarpathian CL. At the temperature range of 200-400 ºC, chemically sorbed (ligand) water localized on the surface due to hydrogen bonds with surface OH groups is removed [17, 18]. At that temperature range, a significant proportion of associated hydroxyl groups are also removed. At temperatures above 400°C, only isolated hydroxyl groups remain on the surface of Transcarpathian CL. The process of deep dehydration and dehydroxylation of CL with the removal of chemisorbed water is practically completed at temperatures of 600-630°C [17].

In an aqueous environment, the rehydrated zeolites have some differences compared to the original ones [16]. During the rehydration of zeolites, in particular the Transcarpathian CL, that process is not completely reversible [19], therefore, thermally treated zeolites retain their biological and chemical activity in aqueous solutions and biosubstrate environments. Thermal modification of zeolites also affects the specific surface area [16, 20] that usually increases their biological and chemical activity.

Natural CL is considered to be a biologically neutral and non-toxic mineral [9, 21]. CL is the only representative of natural zeolites that is FDA approved for use in medical practice and food industry [9, 22-25]. Materials based on natural CL doped with heavy metal ions exhibit an increased biological activity [1-3, 6, 7]. In previous studies [26-34], a powerful antimicrobial action, as well as anticancer and immunomodulatory effects of compositions of the Transcarpathian CL doped with Ag^+ [26-29, 31-34] and Cu^{2+} [30] ions by sorption were found. Ag^+ and Cu^{2+} ions tend to interact with sulfhydryl groups (R-SH) present in proteins. It is believed that the antibacterial activity of the cations of these transition metals, in particular, when they are in the zeolite matrix, is in a directly proportional dependence on their affinity to sulfur-containing groups in proteins [35].

The aim of this work was to study the antimicrobial properties of compositions of the Transcarpathian CL doped with Ag^+, Cu^{2+} cations and Ag nanoparticles under mechanochemical modification in an ethanol medium and with the addition of plant (*Actinidia arguta*) extract used as an antioxidant. Components of the extract also play the role of mild reducing and stabilized reagents for doping cations in clinoptiloliute matrix – instead of stronger and more harmful inorganic reductants.

Material and Methods

CL isolated from a deposit in Sokyrnytsia of Transcarpathia region (Ukraine) was used. The mineral contained 85–90% (wt.) of the main component. The dominant impurity is alpha quartz [36]. The composition of the Transcarpathian CL in the oxide version (wt.%) is as following: SiO_2 – 67.29; TiO_2 – 0.26; Al_2O_3 – 12.32; Fe_2O_3 – 1.26; FeO – 0.25; MgO – 0.99; CaO – 3.01; Na_2O – 0.66; K_2O – 2.76; H_2O – 10.90 [37]. The Transcarpathian CL belongs to the low-silica calcium variety of CL and does not contain clay impurities [37]. Its specific surface, determined by water, is 59 $m^2 \cdot g^{-1}$ [36]. The zeolite samples were ground in a mortar with a pestle, and a fraction of grains of the required size was taken, washed with distilled water, and dried at room temperature.

Some samples of CL were calcined at the appropriate temperature for 2.5 h in a drying oven WSU200 (VEB MLW, Germany) and muffle furnace SNOL 7.2/1100 (AB Utenos Elektrotechnika, Lithuania). After heat treatment, the zeolite samples were cooled to a temperature of 20±1°C in a desiccator.

All used reagents were of the analytical grade. As dopants, $AgNO_3$, $Cu(NO_3)_2{\cdot}3H_2O$ and Ag nanoparticles were used as water suspension. They were obtained via $AgNO_3$ reduction using an extract of Actinidia arguta plant.

Preparation of extract from Actinidia arguta plant leaves. The leaves of *Actinidia arguta* Lindl. `Kyivska krupnoplidna were harvested at research plantations of M.M. Gryshko National Botanical Garden of National Academy of Sciences of Ukraine (Kyiv).

Actinidia arguta leaves extract (PE) was obtained through three times hot extracting of plant leaves with 40% ethanol in a ratio of 1:10. The obtained extract was filtered and evaporated on a Heidolph Hei-VAP Core rotary vacuum evaporator to obtain a dry extract.

Determination of flavonoids content

The total content of flavonoids was determined using the recommendations from the monograph [38].

Stock solution. Near 2 g of the powdered herbal drug was put in the cartridge of a continuous-extraction apparatus (Soxhlet type). 100 ml of heptane was added and heated under a reflux condenser until the extraction liquid was colorless. The mixture was left to cool, and heptane was discarded. 90 ml of methanol was added to continue the extraction with heating under a reflux condenser until the extraction liquid was colorless and the mixture was left to cool. The methanolic solution was transferred to a 100 ml volumetric flask, and the extraction flask was rinsed with 5-7 ml of methanol. The methanolic solutions were combined and diluted to 100 ml with methanol. 10.0 ml of this solution was diluted to 100 ml with distilled water and shaken vigorously.

Test solution. 10.0 ml of the stock solution was diluted to 100.0 ml with a 20 $g{\cdot}l^{-1}$ solution of aluminium chloride in methanol.

Solution of comparison. 10 ml of the stock solution was diluted to 100 ml with methanol. The absorbance was measured at 425 nm in the test solution after 15 min compared with the solution of comparison.

The percentage content of total flavonoids was calculated, expressed as rutin, using the following formula: i.e. taking the specific absorbance of rutin to be 370.

$(A \times 1000)/m \times 37$, where A – optical density of the test solution at 425 nm; m– mass of the herbal drug to be examined, in grams.

Determination of hydroxycinnamic acids content

Quantitative determination of the hydroxycinnamic acids (HCAs) sum in the investigated types of raw materials was carried out according to the method of quantitative determination of HCAs amount in *Orthosiphonis folium* [51].

Stock solution. 0.7 g (exact weight) of powdered material was placed in a 200 ml flask, 80 ml of ethanol (50%, v/v) was heated to reflux in a water bath for 30 min, cooled to room temperature and filtered in a volumetric flask of 100 ml through a cotton swab. The swab was washed with 10 ml ethanol (50%, v/v) to the mark and stirred.

Test solution. 1.0 ml of “stock solution” was placed in a 10 ml volumetric flask, successively adding 2 ml of a 0.5 M solution of hydrochloric acid, 2 ml of freshly prepared solution of 10 g of sodium nitrite and 10 g of sodium molybdate in 100 ml of water, 2 ml sodium hydroxide solution of dilute, bring the volume of solution with water to the mark and mix.

Solution of comparison. 1.0 ml of “stock solution” was placed in a 10 ml volumetric flask, water was added to the mark and stirred. Immediately, the optical density of the test solution was measured on a spectrophotometer at a wavelength of 505 nm in a cuvette with a thickness of 10 mm relative to the solution of comparison. The content of the sum of HCAs, calculated on the basis of rosmarinic acid, in percentages, was calculated with the formula:

$A \times 2.5/m$, where A – optical density of the tested solution at a wavelength of 505 nm; m – mass of the tested raw material, in grams.

Measurement of pro-/antioxidant activity of substances (DPH test). To determine the total pro/antioxidant activity, zeolite samples were prepared as 5 μg probes in 1.5 ml Eppendorf tubes. The substrate (fluorescent dye) 1,1-diphenyl-2-picrylhydrazyl (DPH) was prepared as a 0.01% solution in DMSO and introduced into the tubes with tested substances with periodic mixing. Immediately before starting the measurements, 100 μl aliquots of the supernatant were transferred into the wells of a 96-well plastic plate. The optical density of the solution was measured at 490 nm on a BioTek 76883 multichannel microphotometer (BioTek, USA). The measurements were performed in 4 h, 24 h (1 d), 72 h (3 d), 120 h (5 d), 168 h (7 d) time intervals. The percentage change in the optical density of the solution was determined using the formula: (%) = [(A0-A1)]/(A0)]×100. DMSO solvent was used as a zero control.

Preparation of CL samples doped with Ag^{+} and Cu^{2+} cations using mechanochemical modification. Mechanochemical processing of samples was carried out using a Pulverisette-6 planetary ball mill (Fritsch, Germany) equipped with 12 silicon nitride (Si_3N_4) balls with a diameter of 20 mm. The speed of rotation was 400 rpm, the duration of processing was 1.0-3.0 h. The medium of MChT is ethanol or a water-ethanol (1:4) mixture. The mass of CL was 5 g, the volume of added ethanol or water-ethanol mixture was 30 ml CL samples were subjected to MChT in mixtures with the addition of additives in different concentrations. The mass of the added extract from Actinidia arguta plant was twice as large as the amount of silver and copper dopant. After MChT in the ethanol phase, the samples were dried at 80°C for 24 h.

Preparation conditions and characteristics of the samples are presented in the Table 1.

X-ray diffraction and scanning electron microscope investigation. Diffraction data were obtained on a powder diffractometer STOE STADI P (STOE & Cie GmbH, Germany) (Cu Kα1-radiation, $\lambda = 1.54060$ Å, $9.500 \leq 2\theta \leq 75.50°$, step scan mode with a step size 0.015° and counting time of 100–250 s per data point). Structural characteristics are calculated using the WinCSD program package [39].

The morphology of the studied samples was investigated using scanning electron microscope (SEM) TESCAN Vega3 LMU (TESCAN, Czech Republic). The quantitative composition of the samples was studied using an energy-dispersive X-ray analyzer (EDX) (Oxford Instruments, UK).

Particle size distribution. The particle size distribution was evaluated using a Mastersizer 2000 laser diffraction analyzer (Malvern Ins., England). The distribution is characterized using the following parameters. D (0.1), D (0.5) and D (0.9) are the sizes of particles below which 10, 50 and 90% of the sample lies, respectively;

The Span is the measure of the width of the distribution: Span = [D (0.9) – D (0.1)]/D (0.5).

The narrower is the distribution, the smaller becomes the span.

FTIR spectroscopy. IR spectra in the range of 400–4,000 cm^{-1} were recorded in reflection mode using a Spectrum-One spectrometer (Perkin-Elmer, USA). Powdered mixtures of samples with dried potassium bromide were used for measurement at a ratio of sample/KBr = 1:20.

Measurement of antibacterial activity. The antibacterial effect was determined with the MTT reagent. The following strains of bacteria were used: *Staphylococcus aureus* ATCC25923, *Bacillus subtilis* ATCC31324, *Pseudomonas aeruginosa* ATCC9027, *Pseudomonas fluorescens* IMB8573. The bacterial culture in the logarithmic phase of growth in Sabouraud's medium, pH 7.2, was centrifuged for 10 min at 1.500 g, and the bacterial sediment was washed with a sterile physiological solution and resuspended. A defined volume of this suspension was injected into Sabouraud's medium in order to achieve an optical density (OD) of 0.4–0.6 at 590 nm (optical path 1.0 cm). 100 µl of each bacterial suspension was injected into Eppendorf tubes (2 ml) and then inoculated with the test sample CL. Each experiment was repeated three times. The test tubes were incubated for 4 h at 37°C. Then, 10 µl of solution (5 mg/ml) of the MTT reagent (AppliChem GmbH, Germany.) was introduced, and the incubation was continued for 1 h. Cells were collected by centrifugation for 5 min at 1,500 g, the supernatant was removed by centrifugation, and the sediment was suspended in 1 ml of DMSO. After incubation for 1 h at 37°C, the optical density of the liquid was measured at 580 nm on a ULAB 102 UV spectrophotometer (Ukraine) [41].

Measurement of pro-/antioxidant effects of created CL composites. To determine the total pro/antioxidant activity, CL samples were prepared as 5 µg portions in 1.5 ml Eppendorf tubes. The substrate DPH was prepared as a 0.01% solution in the DMSO and introduced into tubes under periodic mixing with test substances. Immediately before starting the measurements, 100 µl aliquots of the supernatant were taken into the wells of a 96-well plate. The optical density of the solution was measured at 490 nm on a BioTek 76883 multi-channel microphotometer (BioTek, USA).

Optical density measurements were performed in 4 h, 24 h (1 d), 72 h (3 d), 120 h (5 d), 168 h (7 d). The percentage change in the optical density of the solution was calculated according to the formula: (%) = [(A0-A1)]/(A0)]×100. DMSO solvent was used as a zero control [50].

Statistical analysis. Antibacterial data were presented as the mean (M) ± standard deviation (SD). Results were analyzed and illustrated with GraphPad Prism 10 (Microsoft, USA). Statistical analyses were performed using one-way ANOVA. A

T a b l e 1. List of samples of natural CL and its compositions

N sample	Pretreatment temperature of CL	Milling medium	The duration of processing, h	Dopant	PE*	Phase composition
10	–	Ethanol	1	$AgNO_3$ (2 mg Ag·g^{-1})		CL + α-SiO_2
10a	–	Ethanol	2	$AgNO_3$ (2 mg Ag·g^{-1})		CL + α-SiO_2
10b	–	Ethanol	3	$AgNO_3$ (2 mg Ag·g^{-1})		CL + α-SiO_2
11	–	Ethanol	1	–		CL + α-SiO_2
12	–	Ethanol	1	$AgNO_3$ (2 mg Ag·g^{-1})	PE (5 mg·g^{-1})	CL_{amph} + α-SiO_2
14	550°C	Air	1	$AgNO_3$ (10 mg Ag·g^{-1})	PE (20 mg·g^{-1})	CL_{amph} + α-SiO_2
16	160°C	Ethanol	1	$Cu(NO_3)_2$ (3 mg Cu·g^{-1})		CL + α-SiO_2
16a	160°C	Ethanol	2	$Cu(NO_3)_2$ (3 mg Cu·g^{-1}1)		CL + α-SiO_2
16b	160°C	Ethanol	3	$Cu(NO_3)_2$ (3 mg Cu·g^{-1})		CL + α-SiO_2
38	–	Air	1	–		
44	–	Water – Ethanol (1:4)	1	Ag nano (2 mg Ag·g^{-1})	PE (4 mg·g^{-1})	CL + α-SiO_2
44a	–	Water – Ethanol (1:4)	2	Ag nano (2 mg Ag·g^{-1})	PE (4 mg·g^{-1})	CL + α-SiO_2
44b	–	Water – Ethanol (1:4)	3	Ag nano (2 mg Ag·g^{-1})	PE (4 mg·g^{-1})	CL + α-SiO_2
48	160°C	Air	1	$Cu(NO_3)_2$ (3 mg Cu·g^{-1})	PE (6 mg·g^{-1})	CL + α-SiO_2
48a	160°C	Ethanol	1	$Cu(NO_3)_2$ (3 mg Cu·g^{-1})	PE (6 mg·g–1)	CL + α-SiO_2
48b	160°C	Ethanol	2	$Cu(NO_3)_2$ (3 mg Cu·g^{-1})	PE (6 mg·g^{-1})	CL + α-SiO_2
49	–	Air	1	–	PE (5 mg·g^{-1})	CL_{amph} + α-SiO_2

Note. *PE – plant extract; **CL_{amph} – amorphized CL

P-value of <0.05 was considered statistically significant to control and marked*.

Results and Discussion

X-ray structural and morphological studies. Natural zeolite was obtained from the Sokyrnytskya deposit in Transcarpathian region of Ukraine and consists of two main phases: CL and alpha-quartz (Fig. 1, *a*). The performed calculations confirm that CL belongs to the monoclinic syngony (space group C2/m, a = 17.655(2), b = 17.945(3), c = 7.4032(9), Å, β = 116.345 (6)°, content – 81.3(1) wt.%) and α-SiO_2 to trigonal syngony (space group P3121, a = 4.9160 (8), c = 5.406 (2) Å content – 18.7(1) wt.%), which agrees well with generally accepted data [40, 41]. Scanning electron microscope studies indicate the granular structure of natural CL (inset in Fig. 1, *a*). In order to improve the physicochemical properties of CL, including the antimicrobial activity, its samples were subjected to various treatments that have different effects on their crystalline structure and microstructure. In general, MChT of samples, especially dry (air environment) and with prior heat treatment (samples 14, 48 (Fig. 1, *b*), 49), leads to a destruction of the crystal structure of CL and its transition to an amorphous state. Under such conditions, the presence of the plant extract promotes the CL amorphization process. Instead, mechanochemical treatment in ethanol, water or their mixture (samples 10, 10a, 10b, 11, 16, 16a, 16b, 44, 44a, 44b, 48a (Fig. 1, *c*), 48b) ensures the presence of a crystalline state of CL. Moreover, the grinding time (1, 2, 3 h) does not affect significantly the degree of amorphization of CL and the crystallinity of alpha-quartz. An exception is sample 12, for which grinding in an alcohol environment, however, in the presence of a plant extract, leads to the amorphization of CL. The individual plant extract is an amorphous substance (Fig. 1, *d*). The presence of metals or their salts in the samples, due to their microquantities, does not affect significantly these processes. The samples after MChT consist of grains of different sizes and cuts, which are collected in agglomerates (insets in Fig. 1, *b*, 1, *c*). After grinding in an ethanol environment, the sample becomes denser.

Content of flavonoids and HCAs. The main groups of biologically active substances in leaves of A. arguta plant are as following [42, 43]: /i/ hydroxycinnamic acids HCAs – caffeic acid, ferulic acid and chlorogenic acid; /ii/ flavonoids – rutin, quercetin, catechins; /iii/ triterpene saponins – oleanolic acid; /iv/ alkaloids – actinidine. Particularly, rutin, quercetin and chlorogenic acid are modifiers and soft reducing agents that are involved in the formation of metal-oxygen species and metal nanoparticles at the surface [44, 45]. The total content of flavonoids and HCAs in the initial extract, prepared from the leaves, is 33.51 and 9.38%, respectively.

FTIR spectroscopy. The FTIR spectra of the extract and some of its constituents plotted in coordinates of the Kubelka-Munk equation are presented in Fig. 2. Since the spectrum of the extract is poorly resolved, in Fig. 3, it is also shown in a transmittance mode. In general, the recorded FTIR spectra confirm the results of chemical analysis. Thus, the spectrum recorded for the initial extract is represented by a set of bands characteristic of the compounds contained in the extract, primarily, the polyphenols, flavonoids and hydroxycinnamic acids. They refer to various functional groups and bonds present in the structure of these compounds [46]. For example, the band at 1.710 cm^{-1} which is present in the spectrum

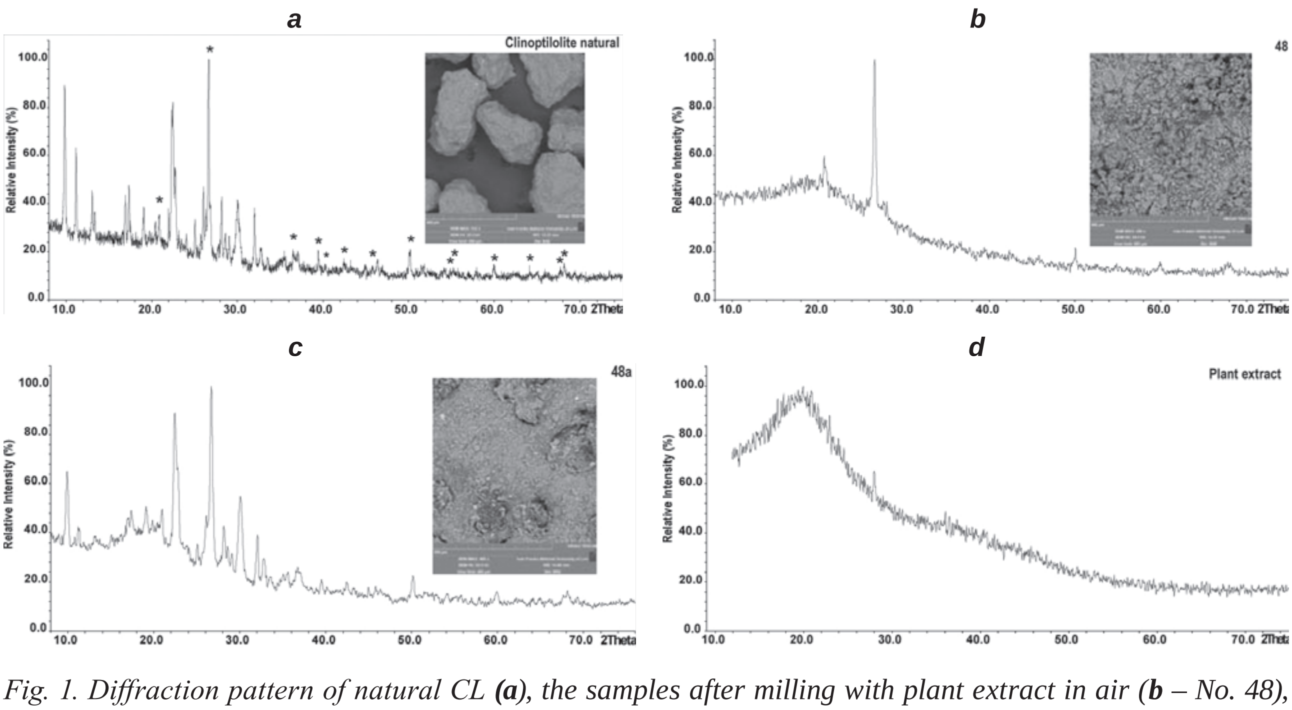

*Fig. 1. Diffraction pattern of natural CL (**a**), the samples after milling with plant extract in air (**b** – No. 48), and ethanol medium (**c** – No. 48a). Asterisks indicate the peaks from the concomitant α-quartz phase. On the insert SEM images of the corresponding samples. Diffraction pattern of plant extract (**d**)*

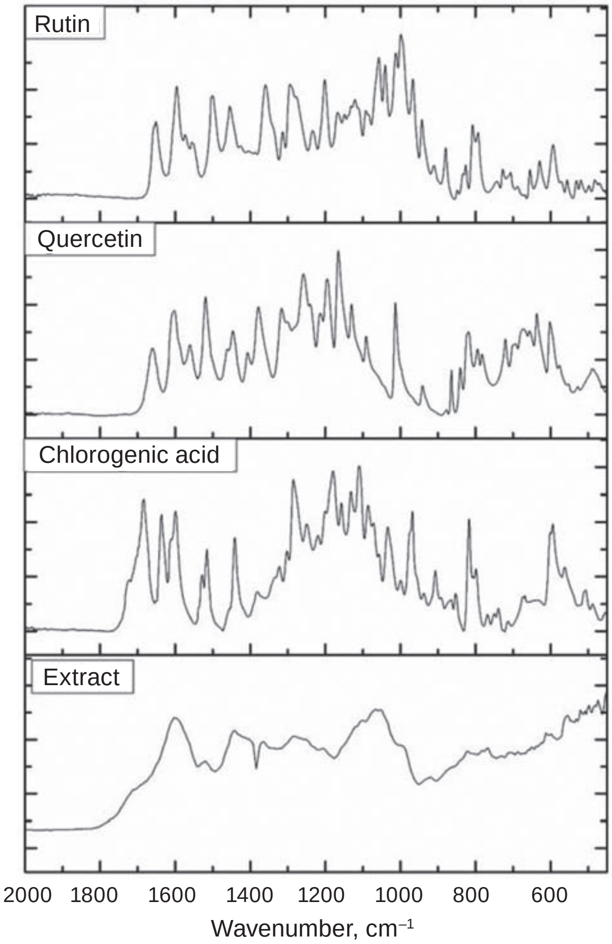

Fig. 2. FTIR spectra of plant extract and its components in coordinates of the Kubelka-Munk equation

of extract as a shoulder, is attributed to C=O in a carboxylic group of chlorogenic acid and the band at 1.598 cm^{-1} – to C=O groups in flavonoids. Also, the band at 1.391 cm^{-1} is assigned to the bending vibration of OH-groups in aromatic rings. Besides, the bands attributed to vibrations of C-O bonds are located at 1.100, 1.060 and 996 cm^{-1}.

Particle size distribution. In Fig. 4, examples of particle size distribution curves are shown. As one can see, these curves have two maxima which are usually found in milled CL [41, 47]. The first of them, seen as a shoulder, is located at 0.7-1 μm, and is imperceptible in the scale of the figures. At the same time, the shift in the main maximum is clearly visible when ethanol is used as a medium for milling. Indeed, the results of calculations based on them are listed in Table 2. MChT in ethanol leads to more significant micronization and obtaining powders in which the content of smaller size is higher compared with dry milling as indicated by the D (0.1), D (0.5) and D (0.9) values. At the same time, the width of the distribution (span), that is the polydispersity of powders, milled in ethanol, practically does not change or even increase. Therefore, the use of ethanol as a medium for milling contributes to the formation of powders with a higher content of smaller particles compared with dry milling [41].

Antibacterial activity. Previously, we have established that unmodified CL has a relatively weak antibacterial activity [26, 27, 41]. A certain corre-

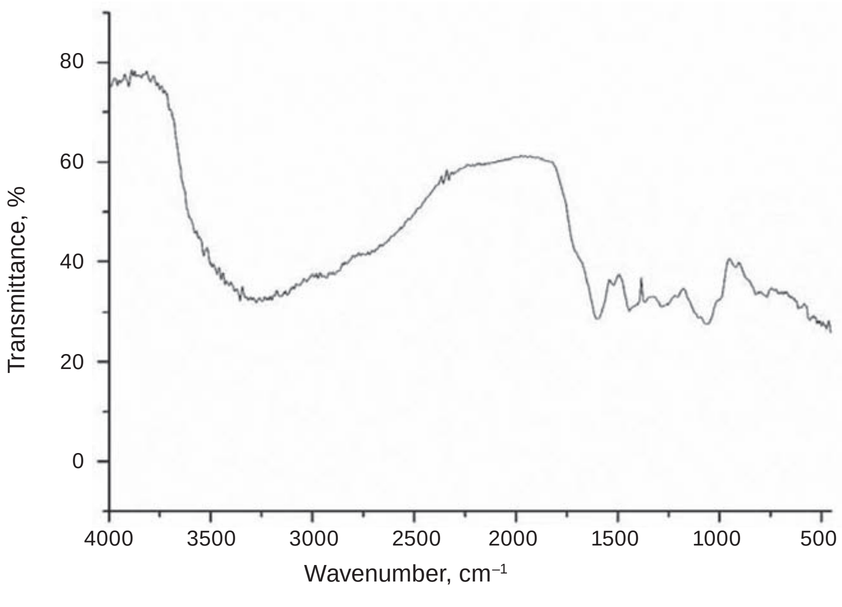

Fig. 3. FTIR spectra of plant extract in a transmittance mode

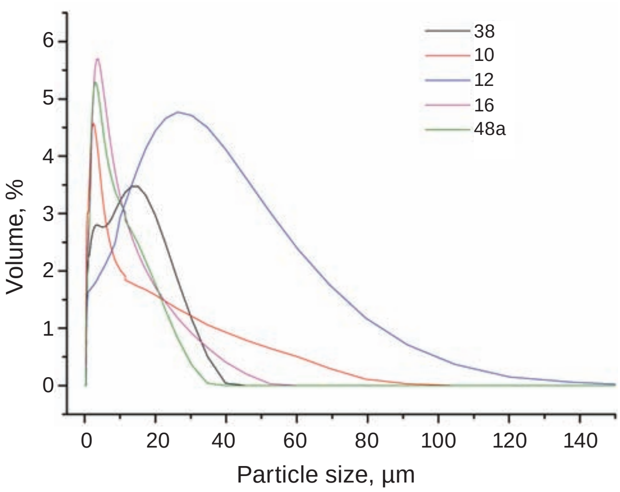

Fig. 4. The curves of the size distribution of the particles for selected samples. Numbers are same as in Table 2

Table 2. Parameters of the volume distribution of natural clinoptilolite particles by their size after milling under different conditions

N sample	D (0.1), µm	D (0.5), µm	D (0.9), µm	Span, µm
10	0.87	3.92	30.17	7.48
10a	0.92	3.75	17.21	4.34
11	1.07	4.05	21.45	5.03
12	1.38	18.75	68.16	3.62
16	1.12	4.15	15.92	3.57
16a	1.01	4.53	20.44	4.29
38	1.33	15.15	54.16	3.49
48	1.35	15.47	58.00	3.66
48a	1.24	5.07	21.67	4.03

lation was observed between the MChT conditions, on the one hand, and the antibacterial activity, on the other hand. An increase in the intensity of the formation of a more developed meso-macroporous structure and an increase in the available surface area and the content of hydroxyl and siloxane groups contribute to a slight increase in antibacterial activity. In general, thermal, chemical and mechanical modifications of the CL structure increase the antibacterial activity, although the absence of an active agent in this structure does not allow to fully establishing these relationships [41].

We tried to improve the structure of the CL in order to increase its ability to inhibit the growth and functioning of microorganisms. That was done via sorption of the transition metals, such as silver in various degrees of oxidation, as well as via chemical treatment of the obtained samples of CL. Since silver is a rather costly agent for doping, we used copper as a much cheaper analogue. We also used the green chemistry approach (flavins of plant origin (*A. arguta*) instead of using rather toxic inorganic acids.

In Fig. 5, the results of testing the antibacterial activity of various CL samples are presented. In this experiment, different species of bacteria were treated: Gram-positive bacteria (*S. aureus*, *B. subtilis*) and Gram-negative bacteria (*P. aeruginosa*, *P. fluorescenc*). Those strains were chosen considering their widespread and pathogenic risk. *S. aureus* and *P. aeruginosa* are known to be classic spread strains pathogens, while *B. subtilis* is a common strain causing rotting of food products.

As one can see in Fig. 5, CL without a dopant did not demonstrate a pronounced antibacterial activity towards treated bacterial strains regardless of the presence of ethanol (sample 11) or plant extract (sample 49), and without them (sample 38). Using the metallic silver as a dopant for the CL with the help of plant extract (samples 44, 44a, 44b) did not affect the antibacterial activity against all bacteria regardless of the treatment time – 1 h (sample 44) to 3 h (sample 44b).

Treatment of non-metallic silver with alcohol revealed a slight tendency to increase the antibacterial activity against Gram-positive bacteria and decrease the antibacterial activity towards Gram-negative ones. A reason for this effect might be an increase in the interaction point of the CL after ethanol treatment (the elevated activity is increased with the treatment time). The detected effect is more significant for Gram-positive bacteria, while a decrease in the charge of the degrees of oxidation of silver is a significant factor in the decreased antibacterial activity of the studied samples relative to Gram-negative bacteria. That might be caused by the presence of a capsule that prevents direct interaction of the bacterial membrane with active silver. The smaller is the charge of the CL agent, the smaller is its antibacterial effect, that might be caused by a reduction of silver due to the action of ethanol and the incuba-

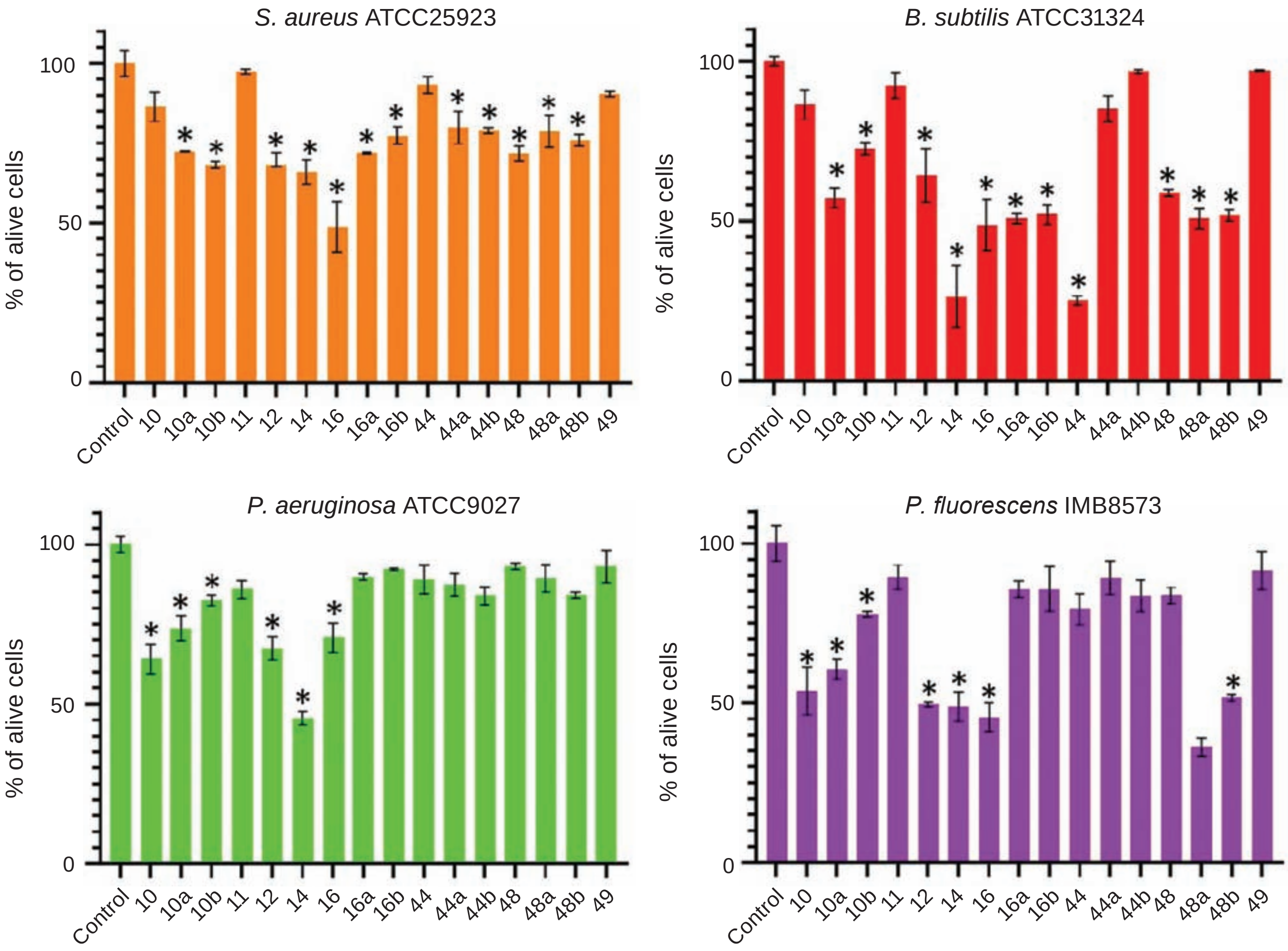

Fig. 5. Antibacterial activity of CL samples doped with silver and copper under MChT. The ordinate axis shows a percentage of alive bacterial cells, and the absciss axis shows a number of CL samples noted in Table 1

tion time. Samples of non-metallic silver treated with ethanol and plant extract (sample 12) did not possess antibacterial activity significantly higher compared to samples treated with ethanol alone (sample 10) after 1 h treatment.

CL samples doped with copper (sample 16) possessed antibacterial activity comparable to samples doped with non-metallic silver (sample 10) at the same ethanol treatment time against Gram-negative bacteria, and more pronounced activity against Gram-positive bacteria. However, an increase in the time of incubation with ethanol (samples 16a, 16b) led to a decrease in the antimicrobial activity for all treated bacteria except *B. subtilis*, which might be more sensitive to copper absorption. We assume that the reason for such effect may be the action of factors described above in relation to non-metallic silver. A reduction of copper significantly reduces its antibacterial activity that cannot be compensated by an increase in interaction points due to the effect of ethanol on the CL. The studied samples of CL with copper treated with ethanol and plant extract showed a comparable activity against *B. subtilis* regardless of the grinding conditions of the CL. This double treatment caused a significant decrease in the antibacterial activity towards strains of *S. aureus*, *P. aeruginosa*, compared to samples not treated with plant extract (sample 16).

Regarding the *P. fluorescens* strain, we found that the sample that was ground without the addition of alcohol and in the presence of the PE (sample 48) did not possess antibacterial activity. Instead, the samples ground with ethanol in the presence of PE (sample 48a) possessed an activity comparable to the CL samples doped with copper that were not treated with plant extract (sample 16). Moreover, sample 48a showed the highest antimicrobial activity towards this bacterium among all the samples tested (Fig. 5).

It might be suggested that sample grinding without ethanol in the presence of plant extract decreased the interaction of charged copper with the surface of bacteria. However, after adding ethanol during grinding for no more than 1 h, the availability of the attached CL molecules increased without a reduction of copper charge, as in samples 16a and 16b. On the other hand, the presence of PE causes a slower reduction of copper, when compared to samples 16a and 48b. Since the grinding time is the same in those samples and they differ only in the presence of PE, the detected antibacterial activity of sample 48b is much higher.

Pro-/antioxidant effects of studied clinoptilolite composites (DPH-based test). To search for the mechanisms responsible for the antibacterial action of substances, pro-/antioxidant effects of studied clinoptilolite composites were studied. That study was based on the measurement of the optical density of DPH fluorescent dye at 490 nm. DPH test permits the determination of pro- and antioxidant properties of the CL composites. We determined the characteristics of the interaction of this dye with CL microparticles. The CL compositions No. 10, 10a, 10b and 11 were evaluated respectively to a control. Compositions No. 12, 14 that contain the organic component (plant extract), as well as samples No. 16, 16a, 16b and 38 and samples No. 44, 44a, 44b, and 48, 48a, 48b, and 49 were also studied respectively to a control. Finely dispersed metallic silver (Ag^0) and ascorbic acid served as a comparison control.

The results of the above-noted measurements are presented in Fig. 6-8. As one can see, the biological activity of created CL composites strongly depends upon modifications of their microparticles that may also affect their pro-/antioxidant properties. Probably, such action might be explained by the peculiarities of the interaction of doped CL microparticles with the surface of bacterial cells [48].

At the same time, the CL sample No. 14 doped with copper and treated with ethanol and plant (Actinidia arguta) extract demonstrated the pro-oxidant effect that could explain the toxic action of that agent towards *B. subtilis* bacteria. It is known that this plant is rich in the antioxidant flavonoids [48]. Thus, the antioxidant activity of these agents might be blocked when they are immobilized in the CL composites. As shown in Figs. 6 and 8, the CL microparticles under study possess an innate antioxidant activity. The exact molecular mechanisms responsible for the potential pro-/antioxidant effects of the CL composites require additional studies.

Conclusions. CL without a dopant did not demonstrate a pronounced antibacterial activity

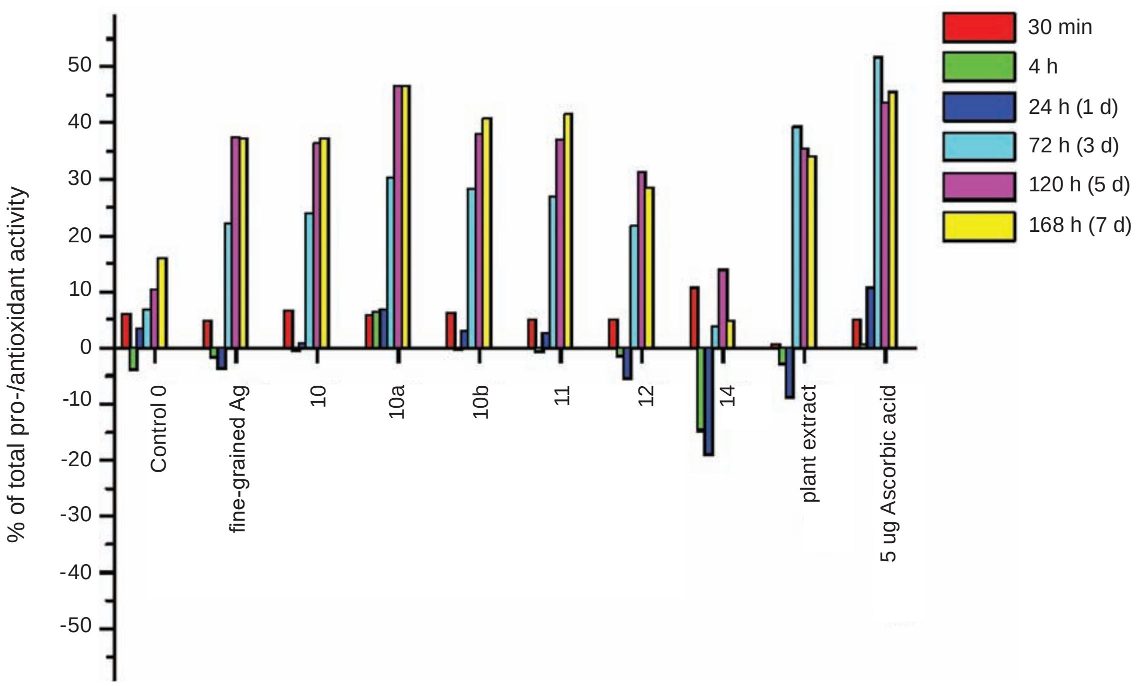

Fig. 6. Time-dependent pro-/antioxidant effects of studied clinoptilolite composites (DPH test). Numbers of composites are noted on the absciss

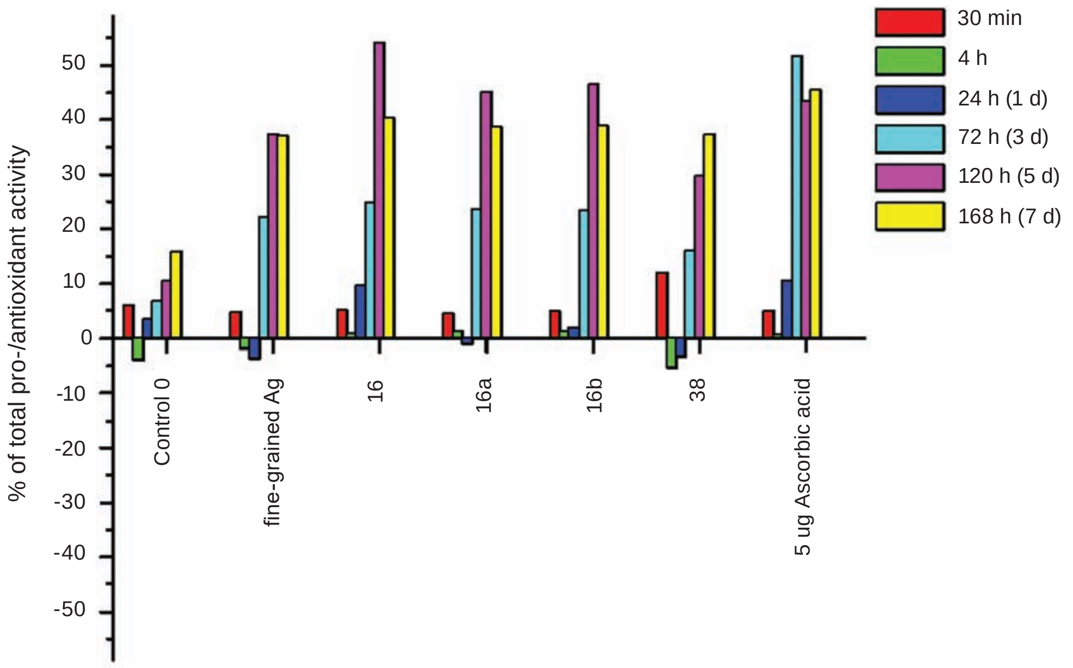

Fig. 7. Time-depend pro-/antioxidant effects of studied clinoptilolite composites (DPH test). Numbers are noted on the absciss

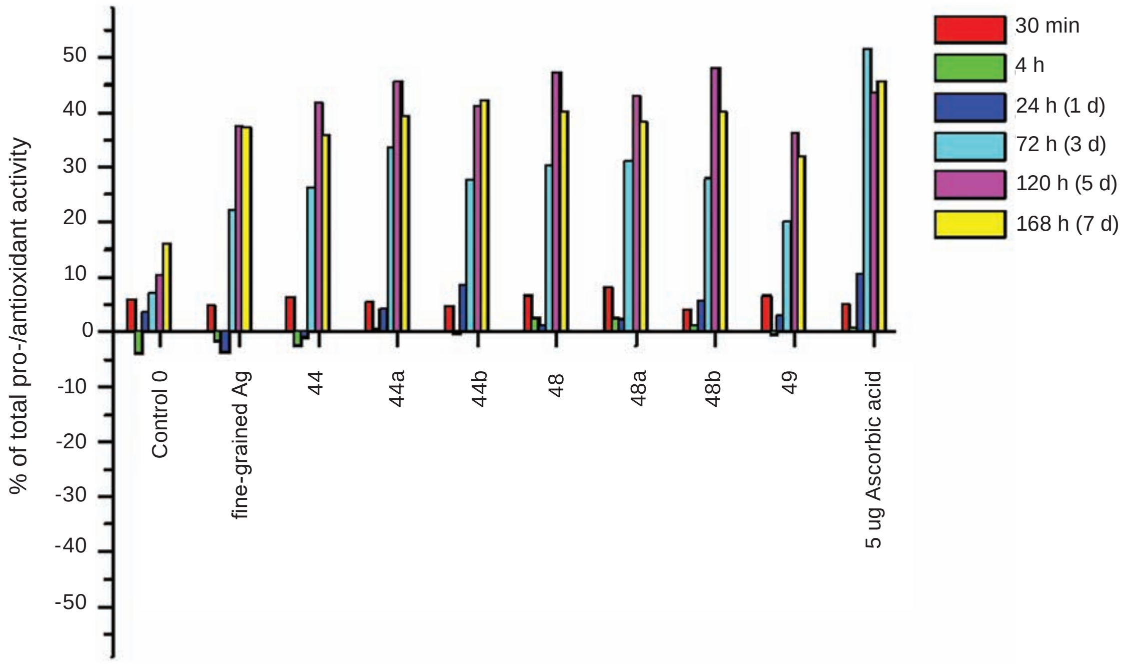

Fig. 8. Time-depend pro-/antioxidant effects of studied clinoptilolite composites (DPH test). Numbers are noted on the absciss

towards treated bacterial strains, regardless of the presence of ethanol (sample 11) or plant extract (sample 49), or without them (sample 38).

Treatment of CL samples with the metallic silver as a dopant with the extract of *Actinidia arguta* plant (samples 44, 44a, 44b) did not increase the antibacterial activity towards all treated bacteria, regardless of the treatment time (from 1 to 3 h).

Treatment of $AgNO_3$ with ethanol revealed a slight tendency to increase the antibacterial activity of CL towards Gram-positive bacteria and a decrease against Gram-negative bacteria. Samples containing oxidized silver treated with ethanol and plant extract (sample 12) did not show significant antibacterial activity compared to samples treated with ethanol alone (sample 10) after 1 h treatment.

The CL samples doped with copper and treated with ethanol and plant extract demonstrated antibacterial activity towards *B. subtilis*, regardless of grinding conditions. Such a treatment caused a significant decrease in the antibacterial activity towards *S. aureus* and *P. aeruginosa* bacteria, compared to samples that were not treated with plant extract (sample 16). At the same time, the activity of the sample modified with copper in the presence of plant extract (No 48a), towards *P. fluorescence* was the highest among all tested samples.

It was found that most versions of the CL composites demonstrate a time-dependent antioxidant effect that was comparable with the effect of the ascorbic acid used as a positive control agent. Thus, the ROS generation is not the main mechanism that is responsible for the antibacterial action of the created CL-based compositions. Probably, that action might be explained by the peculiarities of the interaction of doped CL microparticles with the surface of bacterial cells.

Conflict of interest. The authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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АНТИБАКТЕРІАЛЬНА ДІЯ НОВИХ КОМПОЗИЦІЙ НА ОСНОВІ ЦЕОЛІТУ В ЗАЛЕЖНОСТІ ВІД ДОПУВАННЯ КАТІОНАМИ Ag^+ І Cu^{2+}

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Останнім часом зростає інтерес до вивчення сорбційних та каталітичних властивостей твердих наноматеріалів, зокрема природних цеолітів, як можливих компонентів дезінфекційних засобів. Метою роботи було вивчення антимікробної дії композицій на основі закарпатського клиноптилоліту (Кл), допованого катіонами Ag^+ та Cu^{2+}. Ці композиції піддавали ся механохімічній модифікації в етанольному середовищі з додаванням екстракту листя *Actinidia arguta*, який використовувався як антиоксидант. Отримані композиціїї досліджували за допомогою рентгенівських та лазерних дифракційних аналізаторів, скануючої електронної мікроскопії, FTIR-спектроскопії. Антибактеріальну активність оцінювали за допомогою МТТ-тесту, а продукцію АФК – за допомогою дифенілпікрилгідразилового флуоресцентного барвника. Використовували наступні штами бактерій: *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*. Показано, що Кл без допанту не виявляв вираженої антибактеріальної активності стосовно оброблених штамів бактерій, незалежно від присутності етанолу чи рослинного екстракту. Зразки Кл, що містять срібло та оброблені екстрактом *Actinidia arguta*, не підвищували антибактеріальну активність. Зразки, доповані міддю та оброблені етанолом і екстрактом *Actinidia arguta*, продемонстрували токсичну дію щодо *Bacillus subtilis*, найвищий токсичний ефект проти *P. fluorescence*, але значно знизили антибактеріальну активність щодо штамів *Staphylococcus aureus* і *Pseudomonas aeruginosa* порівняно з дією зразків, необроблених рослинним екстрактом. Більшість версій композитів Кл продемонстрували залежний від часу антиоксидантний ефект, який можна порівняти з ефектом аскорбінової кислоти, яка використовувалась як позитивний контроль. Отримані дані свідчать про те, що антибактеріальна дія створених композицій на основі Кл пов'язана не з генерацією АФК, а з особливостями їх взаємодії з поверхнею бактеріальних клітин.

Ключові слова: композиції на основі клиноптилоліту, допування Ag^+ та Cu^{2+}, антимікробна дія, утворення АФК, МТТ тест.

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