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## ПОШИРЕННЯ ВІРУСУ ОГІРКОВОЇ МОЗАЇКИ НА ОВОЧЕВИХ КУЛЬТУРАХ В УКРАЇНІ

***Аналізували відібрані в агроценозах різних регіонів України рослини родин Cucurbitaceae та Solanaceae з вірусоподібними симптомами на наявність вірусу огіркової мозаїки (ВОМ). За результатами імуноферментного аналізу встановлено, що серед 126 тестованих зразків овочевих культур – 38 зразків містили антигени ВОМ. ВОМ широко розповсюджений в агроценозах Вінницької, Запорізької, Київської, Одеської, Полтавської та Черкаської областей. Було отримано кДНК гену капсидного білку українського ізоляту ВОМ розміром 500 bp.***

***Ключові слова: родини Cucurbitaceae та Solanaceae, імуноферментний аналіз, вірус огіркової мозаїки.***

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## РАСПРОСТРАНЕНИЕ ВИРУСА ОГУРЕЧНОЙ МОЗАИКИ НА ОВОЩНЫХ КУЛЬТУРАХ В УКРАИНЕ

***Анализировали отобраные в агроценозах различных регионов Украины растения семейств Cucurbitaceae и Solanaceae с вирусоподобными симптомами на наличие вируса огуречной мозаїки (ВОМ). По результатам иммуноферментного анализа установлено, что среди 126 тестируемых образцов овощных культур – 38 образцов содержали антигены ВОМ. ВОМ широко распространены в агроценозах Винницкой, Запорожской, Киевской, Одесской, Полтавской и Черкасской областей. Было получено кДНК гена капсидного белка украинского изолята ВОМ размером 500 bp.***

***Ключевые слова: семейства Cucurbitaceae и Solanaceae, иммуноферментный анализ, вирус огуречной мозаики.***

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# CYTOTOXICITY AND ANTIVIRAL ACTIVITY OF NOVEL FLUORIC COMPOUNDS

***The cytotoxicity and antiviral nucleoside activity of fluoric compounds in EBV model systems were investigated in vitro. The apoptosis-stimulating effect was found for compound SBIO-6, that makes it perspective for further research in the area of antitumor analysis. The results can be used in computer modeling of the structure-biological activity relationships of substances and will be used for development of new highly efficient antiviral agents.***

***Key wods: antiviral nucleoside activity, compound SBIO-6, antitumor analysis.***

**Introduction.** Epstein-Barr virus (EBV) belonging to *Gammaherpesvirinae* subfamily, *Herpesviridae* family, is a lymphotropic DNA virus able to infect cells of lymphatic system [13]. Etiological role of EBV has been confirmed for such clinical human diseases as infectious mononucleosis (initial stage of infection), Burkitt's lymphoma, nasopharyngeal carcinoma, lymphoproliferative disorders [8, 9, 11]. After onset of the initial infection in human organism, herpesviruses are capable of latent infection and may be reactivated when effected by various factors, most commonly in case of immune deficiency state of a human organism. Having successfully invaded human body, the virus induces life-long persistence in its cells. Disorders of human immune system lead to virus activation with following manifestation of clinical symptoms. Prominent increase in occurrence of herpetic diseases among adults and children necessitates comprehensive research of herpetic infections and development of efficient methods of prophylaxis and treatment.

Today, chemotherapy using acyclic nucleosides is most developed. This group of preparations includes synthetic analogues of natural nucleosides forming DNA molecules of every biological species on our planet. Four preparations (acyclic analogues of nucleosides) sharing similar structure are known as main antiherpetic medicine agents: acyclovir, valacyclovir, penciclovir and famciclovir. Their efficiency has been tested and confirmed in randomized clinical trials. Every such preparation interrupts viral DNA synthesis during virus reproduction but has no effect on latent virus and extracellular virus particles, virions [1, 3, 12]. Therapy of herpetic infections remains challenging for physicians of various specializations. Novel potent preparations and therapy schemes which are being designed not only should be efficient and safe in the long term treatment, but also reasonably priced and available for wide rage of patients.

**This work was aimed** at cytotoxic and antiviral nucleoside activity of fluoric compounds in EBV model system *in vitro*.

**Materials and methods.** Epstein-Barr virus (EBV). Suspension of lymphoblastoid cells B95-8, obtained from the Institute of Virology of RAMS (Mocsow) in 1991, was used as a source of the virus. For virus accumulation, producer cells were cultivated without changing the media with the suspension density of $1x10^6$ cells/ml for 10 days. TPA (12-O-tetradecanoylphorbol-13-acetate) (Sigma, USA) was used as EBV inducer and added to B95-8 cell culture according to the manufacturer's recommendations. The virus was isolated from cells by Walls-Crawford method [4]. We have used following established cell cultures from European Collection of Animal Cell Cultures: 1) B95-8 (leucocytes of marmoset) cells which are transformed by Epstein-Barr virus (EBV) and produce it chronically were used as the source of EBV; and 2) Raji – non-differentiated lymphoblastoid human B cells isolated from Burkitt's lymphoma. Lymphoblastoid cells were

thermostatically cultivated in 50 ml and 250 ml plastic culture matrasses at 5% of $CO_2$. Growth media contained RPMI 1640 (90%), embryonic bovine serum (10%), antibiotic (streptomycin + penicillin, 100 mcg/ml each), and L-glutamine (2 mmol/l). Cells were passaged each 3 days by adding fresh media to the initial cell culture down to planting dose of 500000 cells per 1 ml.

We studied 4 compounds synthesized at the Institute of Organic Chemistry of NASU. Their structural formulas are given on Figure 1.

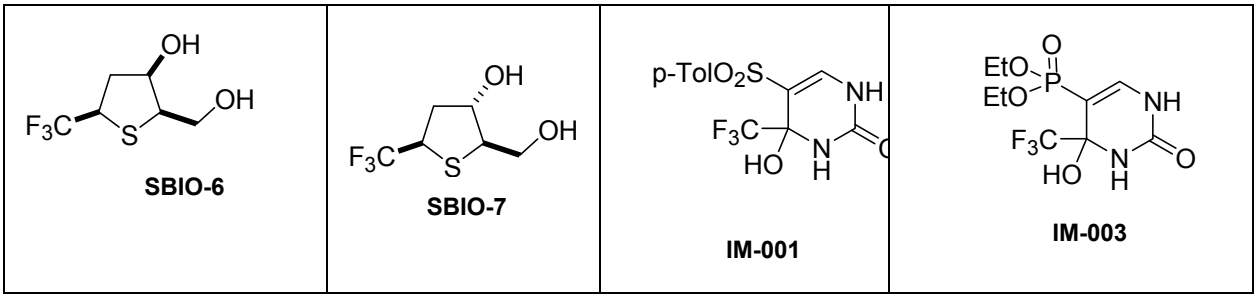


**Figure 1. Structure of studied compounds**

'Cymevene' (ganciclovir) (Hoffmann-La Roche, Switzerland) was used as a reference preparation. Studied preparations were initially diluted with DMSO. Working dilutions of the preparations were made using growth media containing RPMI-1640 and L-glutamine (Sigma, USA), embryonic bovine serum (Sigma, USA), penicillin (100 mcg/ml), and streptomycin (100 mcg/ml). samples were filtered through syringe membrane filters (Sarstedt, Germany) with pores of 0,22 mcm in diameter. Vital capacity of cells was assessed using MTT method [10]. MTT substrate (Sigma, USA) was diluted with sterile phosphate buffer (pH 7,2) at room temperature to the concentration of 5 mg/ml. 20 mcl of filtered MTT solution were deposited in wells of 96-well plates and incubated with cells for 2-4 hours at 37°C. After the incubation, the media was removed and 150 mcl of 96% ethanol was added into each well with cells to dilute the formazan crystals. Optical density was determined spectrophotometrically at the wavelength of 570 nm using 'Dynatech' plate reader (Switzerland). Further, linear regression algorithm of Microsoft Excel for Pentium Pro [2] was used when establishing the preparation concentration which inhibited vital capacity of cells by 50% ($CC_{50}$) as compared to the control. Polymerase chain reaction was used to determine the reproduction rate of Epstein-Barr virus in analyzed cells. The reaction was carried out by 'DNA Laboratory' company using 'AmpliSens-100-R' PCR test systems. To visualize apoptotic cells, they were stained with Hoechst 33342 dye (Sigma, USA). Hoechst 33342 dye is characterized with DNA tropism and able to bind DNA at A-G pairs, allowing to determine apoptotic cells in just 6-8 hours after receiving apoptotic stimulus. For this analysis, the cells were washed using buffered physiological solution by centrifuging at 1,500 rpm for 5 min. Further, cell precipitate was incubated with 100 mcl of Hoechst 33342 dye at final dilution of 0,1 mg/ml for 30 min at 37°C. Cells previously washed in buffered physiological solution were then resuspended in 50% glycerin with 4% paraformaldehyde and deposited onto glass slides. Further, the cell preparation was covered with cover glass and soldered with paraffin. Analysis for apoptotic cells in these preparations were carried out using fluorescent microscope ML-2 (Lumam, Russia) at x900 magnification.

**Results and discussion.** Cytotoxic effect of studied compounds in Raji cell culture was determined by applying the compounds into the cells at certain concentrations. Each concentration was repeated four times with obligatory use of control samples where the compounds were missing. The compounds were used at concentrations 2000, 1000, 500, 250, 125 and 62,5 mcg/ml. Plates with cell culture were incubated at 37°C and 5% of $CO_2$. After 48 hour exposure to a compound, the percentage of viable cells in all samples was determined by staining with trypan blue and by using MTT method as described above. The resulting effects of the compounds are presented in Table 1. When analyzing these results spectrophotometrically, optical density for wells with 'control' cells (not treated with the compounds) was used as 100%. In turn, rates of decrease of vital capacity of cells relative to control values are given in Table 1. These figures are directly proportional to a percentage of dead cells.

***Table* 1. Influence of studied compounds on vital capacity of Raji cells**

| Concentration, mcg/ml | Studied compounds | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | SBIO-6 | | SBIO-7 | | IM-001 | | IM-003 | |
| | % of dead cells | | | | | | | |
| | *Trypan blue* | *MTT test* | *Trypan blue* | *MTT test* | *Trypan blue* | *MTT test* | *Trypan blue* | *MTT test* |
| 62,5 | 9 | 13 | 0 | 0 | 9 | 0 | 13 | 0 |
| 125 | 21 | 21 | 6 | 0 | 12 | 0 | 12 | 5 |
| 250 | 46 | 58 | 12 | 1 | 14 | 2 | 13 | 7 |
| 500 | 78 | 100 | 11 | 5 | 50 | 50 | 11 | 15 |
| 1000 | 75 | 100 | 55 | 56 | 89 | 80 | 23 | 35 |
| 2000 | 92 | 100 | 90 | 100 | 100 | 100 | 54 | 71 |
| Correlation coefficient | 0,819 | -0,98 | 0,979 | -0,98 | 0,923 | -0,90 | 0,956 | -0,99 |
| $CC_{50}$ (mcg/ml) | 600 | 200 | 1000 | 1000 | 750 | 800 | 1900 | 1400 |
| $CC_{50}$ (averaged for two methods) (mcg/ml) | 400 | | 1000 | | 775 | | 1650 | |

Results obtained using two different methods demonstrate a difference in the cytotoxic effect of the preparations. Cytotoxicity index represented as a percentage of dead cells by trypan blue staining was generally higher than that provided by MTT method. It may be explained by a fact that mitochondrial dehydrogenase system of a dead cell stops

converting the substrate following a decrease of cell's functional activity and alterations of internal balance. The cell membrane, however, at that point is still intact and impermeable for trypan blue dye. Using correlation analysis, we have established a compound concentration decreasing vital capacity of cell population by 50% – i.e., index of cytotoxic concentration, $CC_{50}$ [6]. Results presented in the table show that SBIO-6 compound was characterized with $CC_{50}$ of 592 (trypan blue staining) and 220 mcg/ml (MTT method) and thus was the most toxic. $CC_{50}$ values for IM-001 and IM-003 compounds exceeded 1000 mcg/ml providing evidence that these compounds were non-toxic for Raji cell culture and were potent agents for assessing their antiEBV activity.

Antiviral activity of test agents was determined by a level of inhibition of EBV reproduction in Raji cells using semiquantitave PCR for a range of compound concentrations (10, 50, 100 mcg/ml), where each was repeated three times. Ganciclovir was used a reference preparation.

For infection with EBV, cells were precipitated in sterile tubes by centrifuging at 1,500 rpm for 10 min. The obtained precipitate was washed twice using RPMI 1640 media without serum for maximum removal of embryonic bovine serum which prevents virus adsorption on a cell. Resultant precipitate was diluted in minimal volume of media, and then virus preparation was added. Virus adsorption was carried out at 37°C for 1 h. Afterwards the cell were washed twice as described above and the growth media with embryonic bovine serum (5%) was added down to planting dose of 500000 cells per 1 ml.

After virus adsorption and dilution of cell culture to 500000 cells/ml, studied compounds were added at concentrations 10, 50, 100 mcg/ml (in RPMI 1640 media). Studied samples were collected in 48 h. This time interval is considered optimal for both growth dynamics of Raji cell culture and EBV reproduction cycle. Virus DNA accumulation levels in infected cells treated with the compounds (applied at a range of concentration) was determined in comparison to control infected cells where virus DNA accumulation constituted 100% (Fig.2).

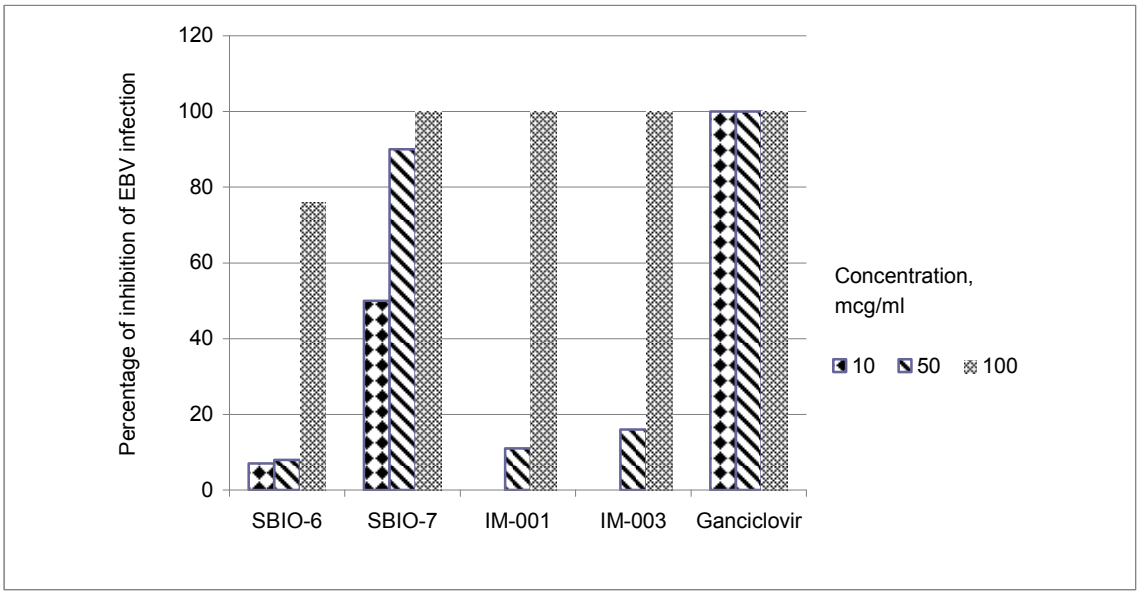


**Figure 2. Activity of fluoric compounds against Epstein-Barr virus**

Presented results allow considering studied compounds as perspective antiviral substances active against Epstein-Barr virus.

We have also evaluated the ability of studied compounds of inducing apoptosis in EBV-transformed Raji cells. Advances in fundamental knowledge on molecular mechanisms of virus-induced oncogenesis, functions and interactions among viral and cellular oncogenes, mechanisms for evading apoptotic death of virus-infected cells leading to tumor growth favored the development of novel approach for selection of antiviral preparations capable of not only efficient inhibition of virus reproduction but also stimulating recovery or elimination of infected cell, in particular by apoptosis, for treating virus-associated neoplasms [5, 7, 14].

One of the approaches to therapy of EBV-positive tumors presumes 'switching' the latent form of EBV infection (which is typical for the majority of EBV-positive tumor cells) into cytolytic form. This approach may be clinically beneficial as lytic EBV infection leads to cell destruction. Another therapeutical advantage is based on the induction of expression of EBV 'lytic' genes (viral thymidine kinase and DNA polymerase) which are expressed exclusively during the lytic cycle of the virus and are capable of converting nucleoside analogues into their respective cytotoxic forms. For instance, many various chemotherapeutical preparations (arginine butyrate, 5-fluorouracil (5-FU), cis-platinum, taxol, gemcitabine, doxorubicin, and sodium butyrate) have been tested in combination with ganciclovir for their ability to 'switch' EBV infection from latent form to lytic cycle in tumor cells. Gemcitabine and doxorubicin activated transcription from promoters of two pre-early virus genes, *BZLF1* and *BRLF1*, in EBV-negative B cells. Ganciclovir led to an increase in cell death initially induced by gemcitabine or doxorubicin in virus-transformed lymphoblastoid cells, and prevented the development of lytic form of viral infection [15].

Research conducted on the determination of apoptotic cell after application of studied compounds at the range on concentrations confirmed apoptosis-stimulating effect for SBIO-6 compound. The results are presented on Fig.3.

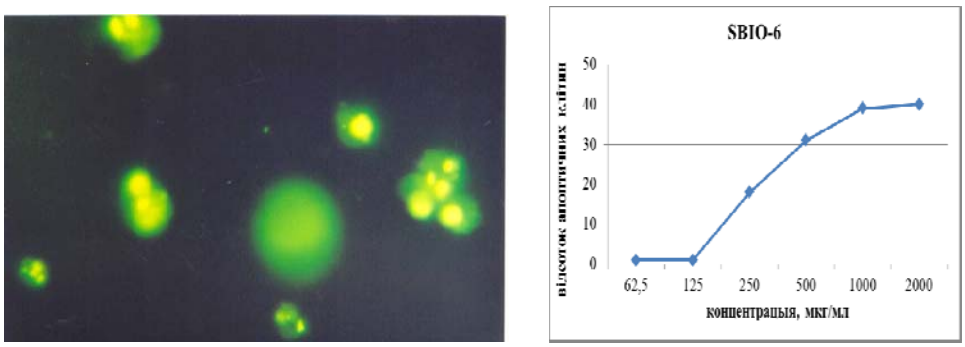


**Figure 3. Detection of apoptotic Raji cells after adding SBIO-6 compound at 1000 mcg/ml (Hoechst 33342 dye)**

As can be seen from Fig.3, initial 'active' concentration for this compound was 250 mcg/ml which resulted in 18% of apoptotic cells. Further increase of compound concentration led to subsequent increase in number of apoptotic cells.

Apoptosis modulators influence tumor cells and hence represent efficient mean for treating cancer patients. This approach is promising because it allows increasing tumor cells' sensibility to effects of cytotoxic factors. In turn, this allows decreasing doses for chemo- or radiotherapy and reducing death rates for normal (non-tumor) cells. In addition, apoptosis modulators may induce lethal damage and elimination of tumor cells resistant to conducted therapeutical agents. There are grounds to assume that only combined (complex) therapy of tumor growth may 'guarantee' maximum damage of malignant transformed cells, diminish their resistance to chemotherapy agents, prevent dissemination of tumor (metastasis), and also reduce toxic side effects of preparations/means of chemo-, radiotherapy and other ways of cancer treatment.

Fluoric nucleoside analogues are used as chemotherapeutic preparations for combating many viral infections induced, in particular, by Hepatitis C virus and HIV. However, use of such preparations for treating EBV infection is not developed at the time. This work describes novel concept in treating EBV-associated disorders, i.e. use of fluoric nucleoside analogues for preventing virus replication and subsequent virus liquidation. In spite of unknown mechanisms of action of the preparations under study, their indexes of antiviral activity and low cytotoxicity *in vitro* make them perspective agents for designing antiviral compounds targeting EBV infections.

**Conclusions.** Here we confirmed cytotoxic effect of studied compounds SBIO-6, SBIO-7, IM-001, and IM-003 in lymphoblastoid Raji cell culture. Value of $CC_{50}$ for these compounds constituted 350-3700 mcg/ml. We have assessed antiherpetic activity of studied compounds toward Herpes simplex virus type 1 (HSV-1) and type 2, and Epstein-Barr virus. Analysis of antiviral activity of the compounds demonstrated absent or negligibly low activity against HSV-1 and notable activity against EBV. When applied at the concentration of 100 mcg/ml, SBIO-7, IM-001, and IM-003 compounds totally (100%) inhibited EBV reproduction. Determination of apoptotic cells resulting from the application of studied compounds at a range of concentrations revealed apoptosis-stimulating effect for SBIO-6 which may be perspective for future research as an antitumor agent. Obtained results may be used for computer-assisted modeling of structure functional relations for recommended compounds and for prognostic design of novel highly active antiviral agents.

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## ЦИТОТОКСИЧНІСТЬ ТА ПРОТИВІРУСНА АКТИВНІСТЬ НОВИХ ФТОРОВМІСНИХ СПОЛУК

***Досліджена цитотоксичність та антивірусна активність фторовмісних нуклеозидних сполук у модельній системі ВЕБ в системі in vitro. Виявлено апоптозстимулюючий ефект для сполуки SBIO-6, що може бути перспективою для подальших досліджень його в напрямку протипухлинного аналізу. Отримані результати можуть бути використані при комп'ютерному моделюванні взаємозв'язку між структурою і біологічною активністю речовин і будуть застосовуватися для створення нових високоактивних противірусних засобів.***

***Ключові слова: фторовмісні нуклеозидні сполуки, сполука SBIO-6, протипухлинний аналіз.***

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## ЦИТОТОКСИЧНОСТЬ И ПРТИВОВИРУСНАЯ АКТИВНОСТЬ НОВЫХ ФТОРСОДЕРЖАЩИХ СОЕДИНЕНИЙ

*Исследована цитотоксичность и антивирусная активность фторсодержащих нуклеозидных соединений в модельной системе ВЭБ в системе in vitro. Выявлено апоптозстимулирующий эффект для соединения SBIO-6, что может быть перспективой для дальнейших исследований его в направлении противоопухолевого анализа. Полученные результаты могут быть использованы при компьютерном моделировании взаимосвязи между структурой и биологической активностью веществ, и будут применяться для создания новых высокоактивных противовирусных средств.*

*Ключевые слова: фторсодержащие нуклеозидные соединения, соединение SBIO-6, противоопухолевый анализ.*

**UDK 578.4/578.81+632.3.01/08+579.64**

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# LOOKING FOR KILLERS: BACTERIOPHAGES AGAINST PHYTOPATOGENIC BACTERIA

***The samples of rotten tomatoes and papers were collected from different regions of Ukraine. Phytopathogenic microorganisms – causative agents of plant diseases were extracted and introduced into culture for strain identification. The presence of bacteriophages in the samples was determined by using agar overplayed method and TEM (transmission electron microscopy).***

***Key words: phytopathogenic microorganisms, bacteriophages.***

**Introduction.** Bacterial pathogens are significant factors which reduce yields of agriculturally important plants worldwide. Among these bacteria the most important are *Pseudomonas syringae*, *Pectobacterium carotovorum,* and bacteria from *Xanthomonadaceae* family.

A variety of approaches have been developed to minimise the impact of bacterial plant diseases on the quantity and economy of food production. Currently, phytopathogens are controlled through management programs, which mostly relay on application of bactericides (copper-based). However, irrational use of these compounds leads to evolution of bacteria and emergence of new, highly resistant forms of phytopathogens. Besides chemical compounds are often accumulated in plants/soils and pose environmental hazards. Antibiotics (e.g., tetracycline and streptomycin) have been utilized in agriculture to control phytopathogenic bacteria also. Extensive use of antibiotics in agriculture has led to selection of resistant bacterial strains [11]. Moreover, resistance genes have been spread to other bacteria, including human and animal pathogens or nonpathogenic bacteria present in the environment [7].

Due to these agrichemical disadvantages, biological control of plant bacteria has attracted attention of many scientists and bacteriophages propose more advantages then other biological agents [6]. Bacteriophages are very specific, even to bacterial pathovars and strains; they don't cause lysis of microbial cells, represented on plants and don`t influence on soil normoflora. Besides, phages are natural components of ecosystems and always persist in host population [4]. In nature bacteriophages coexist with the host microorganisms in balance, so there is no necessity of searching them elsewhere or produce them in the laboratory by synthesis *de novo*. We just need to isolate bacteriophages from environment were specific host is present, investigate their biological properties and convert these viruses into the weapon against their hosts. Bacteriophages can also be coupled with the application of other control strategies (antagonistic bacteria, biocides etc.) for increased pressure on the pathogen [16].

The first works, that showed the potential of bacteriophages in control of phytopathogenic microorganisms, were published in 1924. Mollman and Hemstreet demonstrated that phages lysates prevent rotting of cabbage, caused by pathogenic microorganism *Xanthomonas campestris pt. campestris* [13]. Then many scientists explored phages antimicrobial activity on important agricultural plants, such as rice, pepper, tomatoes and etc. [9]. Despite the promising early works, phage therapy preparation did not prove to be a reliable and effective means of controlling phytobacteria. The main reason of this is the development of antibiotics and biocides. During the last decades of of the 20$^{th}$ century, bacteriophages were re-evaluated as antimicrobial agents [6]. In 2005 fist commercial phage preparation was recommended in the US for usage on crops to control infection caused with two phytopatogenic bacteria – *Xanthomonas campestris pt. vesicatoria* and *Pseudomonas syringae pt. tomato* [8].

Success of application of "AgriPhage$^{TM}$" (OmnyLytics) stimulated the development of new phage-based preparation against the most harmful phytobacteria worldwide. Ukraine, as agricultural country, faced with problem of crop yield losses due to bacterial infections also [5] and is interested in the development of bacteriophage preparations. However, situation is complicated with the absence of information about distribution of phytopathogenic bacteria in Ukraine. Isolation and identification of actual bacterial strains should be conducted prior to the development of bacteriophage preparations. Hence, the objectives of this study were isolation of bacteria and their bacteriophages from samples of infected plants.

**Materials and methods.** Samples of rotten vegetables – tomato (*Solanum lycopersicum* L.) and sweet pepper (*Capsicum anuum* L.) were collected from distinct regions of Ukraine (15 samples of tomato and 10 samples of sweet pepper from Kirovograd-, Cherkassy-, Sumy-, Kherson-, Kiev regions). Liquid medium of Luria-Bertani (baktotrypton – 1%, yeast extract – 0,5%, NaCl – 1%) was used for bacteria cultivation and bacteriophage enrichment. Miller agar (peptone – 1%, yeast extract 0,5%, NaCl – 1%, agar 1.4%) was used for propagation of bacteriophages and their hosts, whereas 1,4% agar and 0,7% agar were used for the hard and soft-agar layers, respectively, in phage plating. All bacterial isolates were maintained on clippings of Miller's agar [1].

The samples of tomatoes and sweet peppers with symptoms of rotting were sterilized by 72% ethanol. Then small pieces of diseased tissues were cut off with sterile knife and placed into LB-broth for enrichment of bacteriophages.For bacteria isolation, the sap was taken from cut surface using microbial loop and plated on Miller's [2].

Other bacteria (*Pectobacterium carotovorum, Pectobacterium amylovorum, Pseudomonas syringae pt. tomato, Xanthomonas campestris pt. campestris 117* and *125, Serratia marcescens IMBG291*) tested for phage sensitivity were obtained from culture collection of Laboratory of mi-