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STUDY OF THE INFLUENCE OF THE EXTRACT OF PIPSISSEWA ON CELL CULTURES

Oleksiy Kovregin, Volodymyr Prokopiuk, Dmytro Lytkin, Inna Vladymyrova

The development of new diuretics of plant origin is an actual direction. Chimaphila umbellata (L.) is a perennial herb with diuretic, astringent, analgesic and other effects; and it can treat various conditions such as edema, dropsy, etc. Pipsissewa herb helps the removal of nitrogenous and chloride salts from the body due to the content of arbutin glycoside, tannins (up to 5 %).

The aim. *Evaluation of the effect of pipsissewa extract on L929 cell culture.*

Materials and methods. *Cell line L929 (fibroblasts of mouse adipose tissue) was obtained in the low-temperature bank of the Institute of Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine. Cells were cultured in DMEM medium (Bio West, France) enriched with 10 % FBS (Lonza, Germany) with 1 % antibiotic-antimycotic (Bio West, France), in a CO_2 incubator (Thermo Fisher Scientific, USA) at 37 °C in an atmosphere with 5 % CO_2. Determination of the minimum toxic concentration at which the cells remained alive was evaluated by morphological features (shape, monolayer integrity, adhesion to plastic). The study of the effect of pipsissewa extract on various cell functions was determined by the following methods: the ability to preserve morphological integrity – by the phase-contrast microscopy method, energy exchange – by the MTT test method, pinocytotic function – by the neutral red absorption method, migratory function – by the scratch test method, proliferative activity – by the doubling calculation method population*

Results. *It is proposed to use concentrations of 0.05, 0.02, 0.01, 0.005 % of pipsissewa extract for further research. After carrying out the MTT reaction, the transition of MTT to formazan was confirmed microscopically in the negative control (native cells), at PE concentrations of 0.01 % and below, and the absence of a reaction in the positive control (cells killed by ethanol) at PE concentrations above 0.02 %. When recording the parameters of the NP absorption reaction, it was determined that PE at a concentration of 0.02 % and higher sharply suppresses pinocytotic activity, despite the partial preservation of cell adhesion, reducing the concentration by two times no longer affects mitochondria. A concentration of 0.01 % reduces proliferative activity, and at a concentration of 0.005 %, no difference with the control values was found.*

Conclusions. *When studying the assessment of the effect of pipsisewa extract on L929 cell culture, a toxic effect on these cells was established when added to the culture medium at a concentration above 0.01 %. The toxic effect had a threshold effect. Migratory and proliferative functions were the most sensitive, energy, pinocytosis and preservation of morphological integrity of cells were less sensitive*

Keywords: *Chimaphila umbellata (L.), cell culture L929, proliferation, adhesion, migration*

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1. Introduction

Diuretics play a critical role in increasing urine production and promoting the elimination of water and electrolytes from the body, making them indispensable in the treatment of a wide range of diseases such as hypertension, congestive heart failure [1], kidney disorders, and some edema [2]. Diuretics are divided into different types, such as loop, thiazide, potassium-sparing, and carbonic anhydrase inhibitors, depending on their site of action in the kidney. Nevertheless, the use of these pharmacological agents is associated with possible side effects or adverse consequences [3]. Improper or excessive use of diuretics can lead to electrolyte and fluid loss, triggering compensatory mechanisms such as the renin-angiotensin system (RAS), which increases renal sodium retention in the nephron [4]. Therefore, there is an urgent need for the development of phytotherapeutic drugs that are not inferior in effectiveness and are characterized by less pronounced side effects.

Herbal remedies have long been used for the treatment of various human diseases and are a valuable source of safe and extremely effective biologically active substances [5]. The genus *Chimaphila* is a typical representative of the *Ericaceae* family, which is widely distributed [6]; it includes about five species worldwide, of which three species (one of which is endemic) can be found in China [7]. *Chimaphila umbellata* (*L.*) is a perennial herbaceous plant [8] that has diuretic, astringent, analgesic and other effects; and can treat various conditions in kidney and urinary tract diseases [6]. Pipsissewa herb helps remove nitrogenous and chloride salts from the body, normalize digestion and increase appetite, and in diabetes – lowers blood sugar [9–11].

Chimaphila umbellata (*L.*) (Fig. 1) has been studied for almost two centuries, and the first article devoted

to the phytochemistry of the plant was published in 1860. Many current studies have focused on the biotechnological advances of *C. umbellata* (L.), including its use as a natural alternative in the cosmetic, food, biofuel, and medical industries, with particular attention to its therapeutic applications [12]. The compound hemaphilin gave the name of the genus. The study also found that the amount of hemaphyllin, already present in the plant's traces, decreases from the roots to the fruits, and the latter do not produce it (Peacock 1892). *C. umbellata* (*L.*), a Eurasian plant, occurs almost throughout North America and is distributed in the cool temperate northern hemisphere [13].

Arbutin glycoside, tannins (up to 5 %), flavonoids, tannins, a bitter substance (ursone), organic acids, gums, resins, and mucus were found in the grass of the pipsisewa [14]. The herb also contains sitosterol, quinine and gallic acids, methyl ester of salicylic acid, vitamins, trace elements and other biologically active substances.

The alcohol extract *C. umbellata* (*L.*) was further chemically investigated to obtain 12 compounds including three previously unreported phenols [15]: 3′-*O*-β-D-glucopyranosyl-isohomoarbutin (**1**), 4′-*O*-β-D-glucopyranosyl-isohomoarbutin (**2**), and 5-5′-dehydro-di (2-methyl-4-hydroxy-phenyl-1-*O*-β-D-glucopyranoside) (**3**); one new cyclohexanol: (1*R*,3*R*,4*R*)-3-methylcyclohexanol β-D-glucopyranoside (**4**); and one first named new phenol: 3-[(*E*)-4-hydroxy-3-methyl-2-butenyl]-4-hydroxy-2-methylphenyl-*O*-β-D-glucopyranoside (**5**); together with: isohomoarbutin (**6**), quercetin (**7**), quercitrin (**8**), isoquercitrin (**9**), hyperoside (**10**), kaempferol (**11**), and juglanin (**12**) (Fig. 2).

Fig. 1. Appearance of *Chimaphila umbellata L.*:
a – flowering plant; *b* – single flower; *c* – flowers; *d* – flower buds; *e* – young fruits

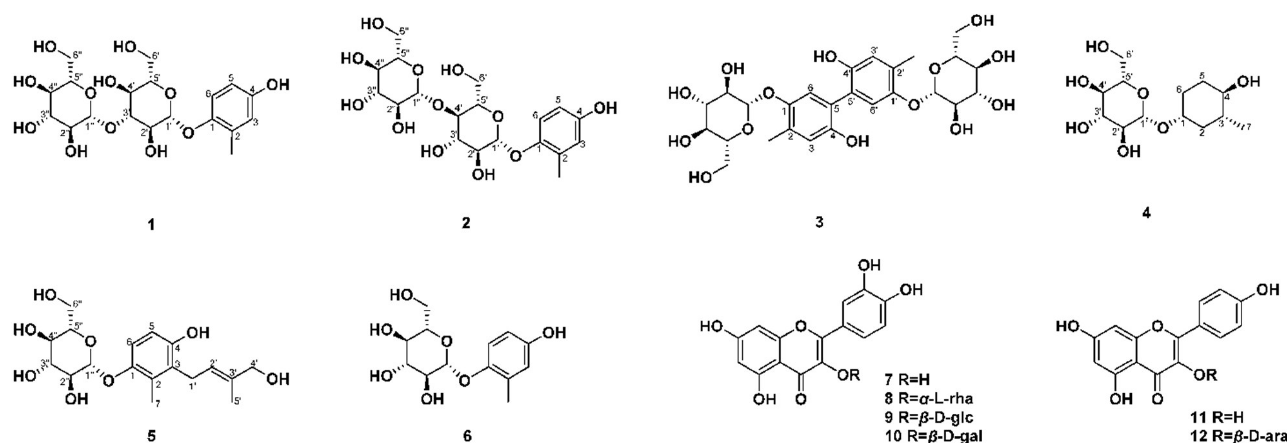

Fig. 2. Biologically active substances of *C. umbellata* (*L.*) alcohol extract

2. Planning (methodology) of research

Research on the effect of pipsissewa extract on cell cultures was carried out in two stages (Fig. 3). The first stage of the study was devoted to determining the minimum toxic concentration at which cells maintain their viability; control was carried out according to the morphological characteristics of the cells. At the second stage, the effect of pipsissewa extract on various cell functions was evaluated.

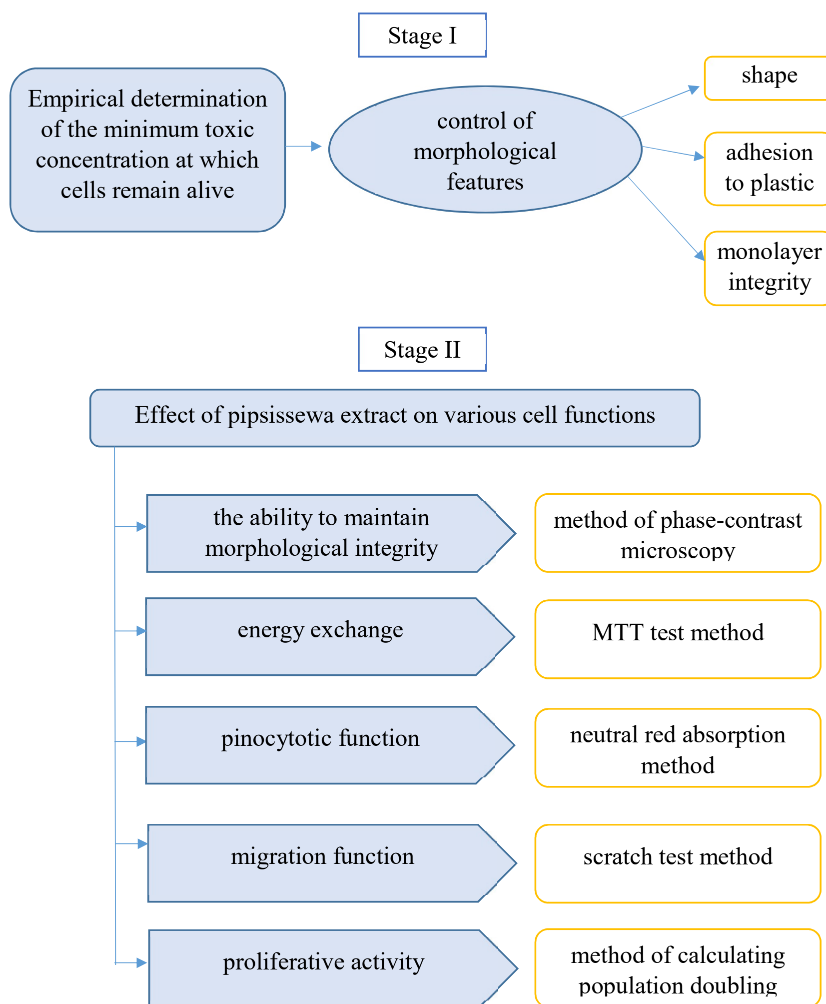

Fig. 3. Stages of planning and design of the experiment

3. Materials and methods

Pipsissewa extract was obtained based on the Research Plant "HNCLS" (Kharkiv) in the period May–June 2023.

The pipsissewa extract (PE) was obtained with 50 % ethyl alcohol according to the following stages.

The production preparation stage consisted of the preparation of premises, equipment and facilities, personnel, and checking of the necessary documentation. Sanitary training of production is carried out in accordance with the STP "Sanitary training of production and personnel".

Stage 1 "Crushing of raw materials" began with the inspection and preparation for operation of the shredders. Each (series) batch of pipsissewa grass that entered production, regardless of the availability of the supplier's quality certificate, was subject to verification in the control and analytical laboratory for compliance with the requirements of the current regulatory documentation. The raw material was used in production after receiving an analytical passport from the control and analytical laboratory, which confirmed its quality.

At stage 2 "Preparation of the extractant", 50 % ethyl alcohol was prepared from rectified ethyl alcohol, purified water, which passed initial control in accordance with the requirements of the current AND, and also added 50 % distilled ethyl alcohol, which was obtained at stage 6 "Regeneration of the extractant" of this production. The calculated amount of rectified ethyl alcohol and purified water was fed into the collector by gravity from the gauges. The extractant was stirred for 15–20 minutes and a sample was taken to determine the volume fraction of ethyl alcohol in the alcohol-water solution, which should be 50 %. If necessary, the extractant was corrected with ethyl alcohol or purified water. The obtained extractant was transferred to stage 3.

At stage 3 "Infusion and extraction", the crushed plant material was loaded into the extractor, at the bottom of which a "false bottom" lined with cotton fabric was placed. Plant raw materials were evenly distributed in the middle of the percolator and stacked with sufficient density. Ethyl alcohol 50 % was fed by gravity from the collector to prepare the extractant until a "mirror" was obtained and left to infuse for 24 hours at a temperature of 18–25 °C. At the end of the infusion time, the first extract was poured by gravity into the reactor for settling. Then the lower drain was closed and a second portion of the extractant was fed by gravity and the extraction was carried out for 24 hours at a temperature of 18–25 °C. After the indicated time, the second extract was poured into the reactor for settling. After the second extraction, the tap of the lower drain was left open and allowed to drain the extract as much as possible. The obtained extract was transferred to stage 4 "Settling". The spent meal containing the remains of the extractant was left in the extractor for regeneration of the extractant at stage 9.

At stage 4 "Settling", the extract was set in a reactor that had a hladon shell for cooling. The extract was allowed to stand for 48 hours at a temperature of 10±2 °C, which was controlled by a thermometer. At the end of the settling process, the extract was fed by gravity to stage 5 "Filtration".

At stage 5 "Filtration" the extract was fed to a notch filter using a pump. After filtering, a sample was taken for analysis and control of the finished product (extract) was carried out.

At this production, in order to rationally use raw materials, at stage 6, the regeneration of extractant resi-

dues from the meal of plant raw materials was carried out. During the extraction process, the plant material swelled, absorbing one to three parts of the extractant. Therefore, it is advisable to introduce a regeneration stage, during which, on average, up to 50 % of the extractant remaining in the meal is returned to production.

Distilled ethyl alcohol obtained at stage 6 "Regeneration of the extractant" was transferred to stage 2 "Preparation of the extractant" of this production.

The obtained pipsissewa extract was diluted with the cultivation medium to the required concentrations, filtered with an antibacterial millipore filter of 0.22 μm [16].

Cell line L929 (fibroblasts of mouse adipose tissue) was obtained in the low-temperature bank of the Institute of Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine. Cells were cultured in DMEM medium (Bio West, France) enriched with 10 % FBS (Lonza, Germany) with 1 % antibiotic-antimycotic (Bio West, France), in a CO_2 incubator (Thermo Fisher Scientific, USA) at 37 °C in an atmosphere with 5 % CO_2.

To study the shape of the cells, the confluency of the monolayer, the cells were seeded on 24-well plates (SPL, Republic of Korea) at a concentration of $1×10^5/cm^2$, after 24 hours the substance under study was added, and after another 24 hours the evaluation was carried out [17].

To assess proliferation, cells were seeded into 24-well plates of 1×105 wells, after 24 hours the test substance was added, after another 72 hours (96 hours from seeding, 72 hours of exposure), the cells were removed with trypsin from ETDA and counted using the online service: https://www.doubling-time.com/compute.php [14, 18].

To assess the migration ability of cells using the scratch test method, cells were seeded into 24-well plates of $1×10^5$ wells, after 24 hours a defect was made with a 200 μl pipette tip, the substance under study was added, after another 24 hours (48 hours from seeding, 48 hours of exposure), the width was measured defect through equal intervals from the wall to the wall of the hole [19].

The metabolic activity of the cells was assessed by the MTT test method. To do this, cells were seeded on a 96-well plate (SPL, Korea) at a concentration of $2×10^4$ per well, the study was performed the next day after seeding, after adhesion and monolayer formation. The test substance was added for 1 day. Cultivated for 24 hours in a CO_2 incubator (Thermo Fisher Scientific, USA) at 37 °C in an atmosphere with 5 % CO_2. Cells without substance and cells killed with 70 % ethanol were used as controls. After that, the medium was selected, 0.1 ml of culture medium with 15 μl of MTT at a concentration of 5 mg/ml was added, incubated for 3 hours in a CO_2 incubator (Thermo Fisher Scientific, USA) at 37 °C in an atmosphere with 5 % CO_2. After that, the medium was taken, 0.1 ml of DMSO with SDS was added and incubated for 1 hour at 37 °C. Adsorption was measured on a tablet spectrophotometer SM600 (Utrao, China) at a wavelength of 570 nm [20, 21].

Pinocytotic activity was determined by the neutral red absorption method. To conduct the test, cells were seeded on a 96-well plate (SPL, Korea) at a concentration of 2×104 per well, the study was performed the next day after seeding, after adhesion and monolayer formation. The test substance was added for 1 day. Cultivated for 24 hours in a CO_2 incubator (Thermo Fisher Scientific, USA) at 37 °C in an atmosphere with 5 % CO_2. Cells without substance and cells killed with 70 % ethanol were used as controls. After that, the medium was removed, and 0.1 ml of 50 % ethanol with 3 % acetic acid was added. Adsorption was measured on a tablet spectrophotometer SM600 (Utrao, China) at a wavelength of 540 nm [22].

DeltaOptical NIB 100 microscope (Poland), Sigeta MCMOS 3100 3.1MP camera (China) were used for visualization. ToupView V 3.7 software was used for image processing. (Hangzhou Toup Tek Photonics Co., Ltd, Hangzhou, China). The Mann-Whitney U-test, the Kruskel-Wallis test were used to assess the reliability of the difference between the comparison groups. Past V. 3.15 software (University of Oslo, Norway) was used for statistical calculations and data processing.

4. Research results

At the first stage of the study, cell cultivation was carried out in a 24-well plate with PE in concentrations of 5; 2; 1; 0.5; 0.1; 0.05; 0.02 %. Macroscopically, high concentrations changed the colour of the culture medium (Fig. 4).

Upon microscopic examination, after thawing, the cells formed a monolayer on the 2nd day and had a shape typical of the L929 line, which was preserved for a day. When PE was added at a concentration of 0.05 % or higher, complete detachment of cells was observed within 24 hours, which began after 3 hours (Fig. 5). At a concentration of 0.02 %, partial preservation of the monolayer of cells and many detached cells were observed. Considering the obtained data, it was decided to use concentrations of 0.05 for further research; 0.02, 0.01; 0.005 % extract.

After conducting the MTT reaction, the transition of MTT to formazan was confirmed microscopically in the negative control (native cells), at PE concentrations of 0.01 % and below, and the absence of a reaction in the positive control (cells killed by ethanol) at PE concentrations above 0.02 % (Fig. 6).

When recording the indicators of the MTT reaction, it was determined that PE at a concentration of 0.02 % and higher sharply suppresses the activity of mitochondria, despite the partial preservation of cell adhesion, reducing the concentration by two times no longer affects mitochondria (Fig. 7).

After conducting the NP absorption reaction, the absorption of NPs was confirmed microscopically in the negative control (native cells), at PE concentrations of 0.01 % and below, and the absence of a reaction in the positive control (cells killed by ethanol) at PE concentrations above 0.02 % (Fig. 7).

Fig. 4. Macroscopic study of PE cell cultures

When recording the parameters of the NP absorption reaction, it was determined that PE at a concentration of 0.02 % and higher sharply suppresses pinocytotic activity, despite the partial preservation of cell adhesion, a two-fold decrease in concentration no longer affects mitochondria (Fig. 8).

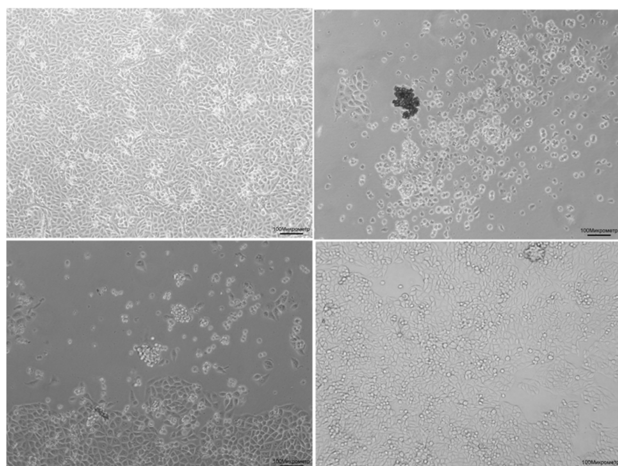

Fig. 5. Microscopic examination of L929 culture after cultivation with PE. Scale bars are 100 μm

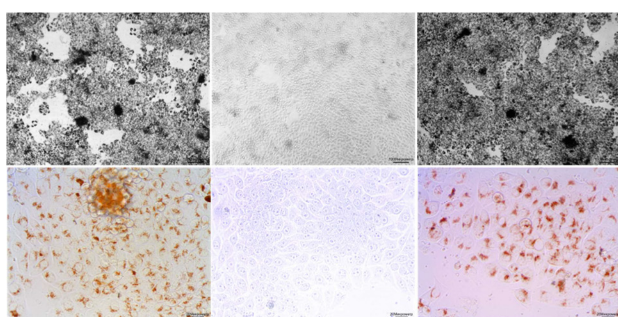

Fig. 6. Microscopic examination of the L929 culture after culturing with PE and carrying out MTT and NP absorption tests. Scale bars 100 μm (upper row) and 20 μm (lower row)

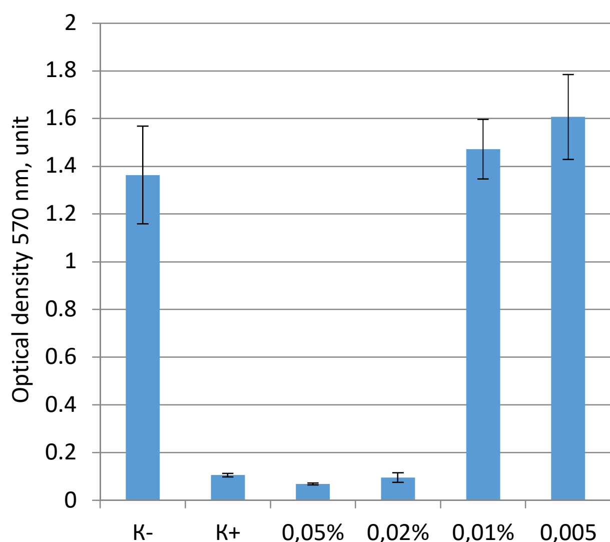

Fig. 7. Indicators of the MTT reaction of cells after cultivation with PE

When conducting the scratch test, which characterizes the ability of cells to migrate, it was demonstrated that in the control sample with native cells and in the sample with cells at a concentration of 0.01 % and 0.005 % PE, cells migrated over time, however, at higher concentrations due to the loss of the monolayer it was not possible to determine it as showy (Fig. 9). It was demonstrated that a concentration of PE of 0.01 % reduces migration indicators, and at a concentration of 0.005 %, no difference with the control values was found (Fig. 10).

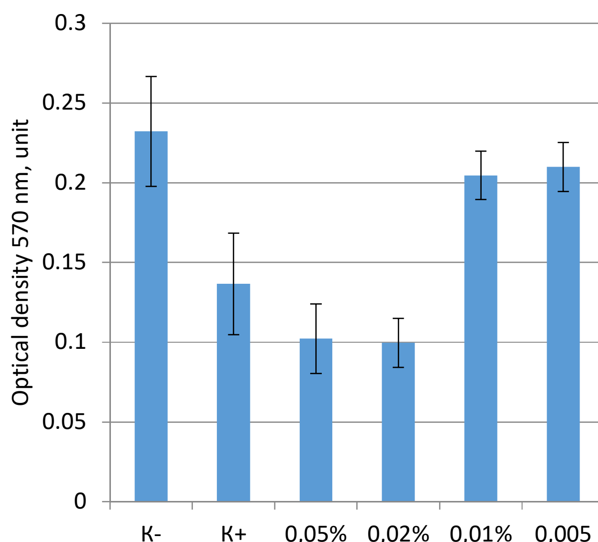

Fig. 8. Indicators of NP uptake by cells after cultivation with PE

When studying the proliferative activity of cells using the population doubling time calculation method, it was found that PE in a concentration of 0.02 % and higher in the culture medium leads to the suspension of cell division. The number of removed cells was significantly less than the number of seeded cells. A concentration of 0.01 % reduces proliferative activity, and at a concentration of 0.005 %, no difference with the control values was found (Fig. 11).

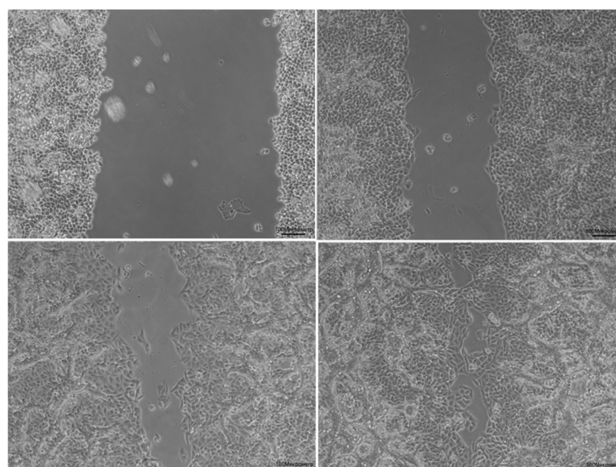

Fig. 9. Microscopic examination of the scratch test (migratory activity) of the L929 culture after cultivation with PE. Scale bars 100 μm (top row)

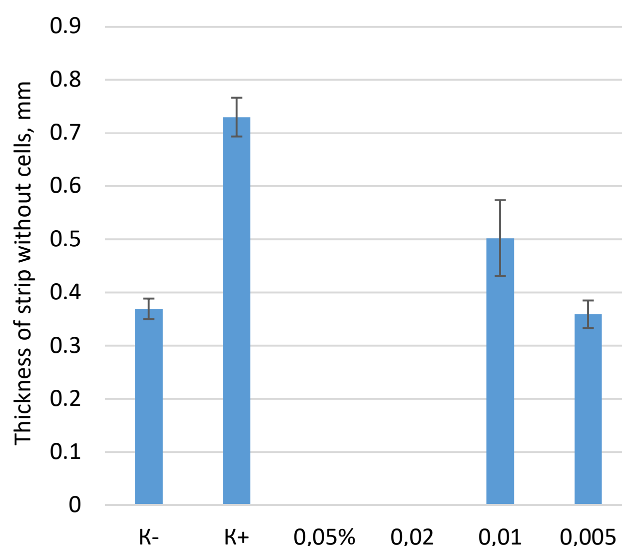

Fig. 10. Indicators of the migratory activity of cells according to scratch test data

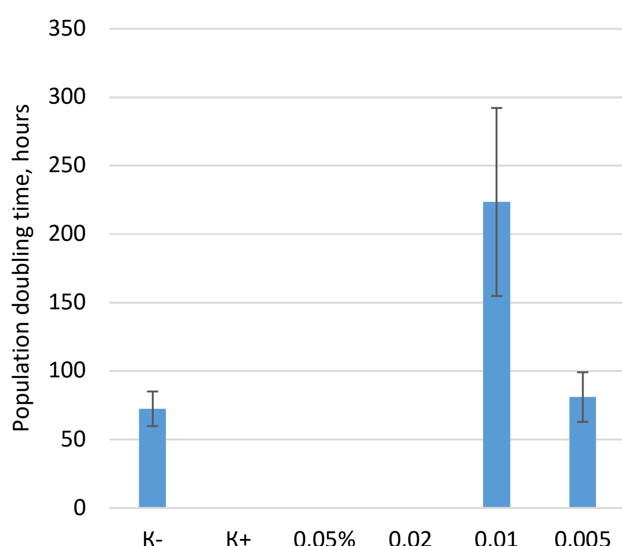

Fig. 11. Indicators of cell proliferative activity

5. Discussion of research results

In this study, the anti-proliferative and apoptotic effects of PE extract, obtained from its homeopathic mother tincture, were evaluated in human breast cancer cell lines. MTT assay was used to evaluate the dose- and time-dependent cytotoxicity of the extract in MCF-7 and HEK293 cells. The anti-proliferative effect of the extract was evaluated using the clonogenic and wound healing assays. The anti-angiogenic activity of the drug was evaluated using the chick chorioallantoic membrane assay. The mode of cell death was analyzed using Annexin V and PI staining assays through flow cytometry [23]. In addition, the expression patterns of associated genes were evaluated using immunoblot analysis.

PE exerted anti-proliferative effect in breast cancer cell line, i.e., MCF-7 cells, by inhibiting their growth and migration. The extract also demonstrated significant anti-angiogenic ability, limiting the de-novo blood vessel growth and development in chick embryos. The extract was found to increase caspaseindependent necroptosis by involving RIP1/RIP3 kinases and MLKL proteins. This finding was further confirmed by using NEC-1 (an inhibitor of necroptosis), which significantly abolished the CU extract-induced necroptotic effect.

The PE exhibits great potential in preventing breast cancer and thus warrants further investigations in vivo [23].

In this study, different dilutions of homeopathic medicines. Cell lines of prostate cancer PC-3, breast cancer MDA-MB-231, and lymphoblastic leukemia Jurkat were used as model tumour objects. Standard colorimetric MTT test and cytometric analysis of cells were used here to assess the viability and parameters of apoptosis of cells. Results Comparative assessment of the cytotoxic and apoptosis-inducing effects did not reveal significant differences in the viability of tumour cells in the groups exposed to homeopathic preparations among themselves and in comparison, with the control group. The effects of the action of homeopathic remedies are shown only in the form of trends. Some possible mechanisms of the obtained results are considered.

The results obtained in vitro indicated the absence of a direct cytotoxic effect in the studied homeopathic preparations. Despite the objective data, this does not allow us to make a final conclusion about the absence of antitumor activity of these drugs in vivo, since the possible or potential effects depend on the parameters of a living organism that are difficult to control and are often based only on hypothetical mechanisms [24].

Therefore, the obtained experimental data justify the possibility of using wintergreen extract in phytotherapy for the prevention and treatment of various diseases. Further in-depth pharmacological studies on pathological models and histochemical studies will be aimed at determining the biological markers of wintergreen extract and establishing their mechanism of action.

Practical relevance. Achieving the effect of pipsissewa extract on cell cultures provides a preliminary understanding of the possibility of using substances from this plant for preventive and therapeutic purposes. It has been established that there is no toxic effect on tissue cells, namely their biological functions and morphological integrity. Understanding that the effect of substances on a living organism occurs at the cellular level, the research is based on the use of cell lines - an alternative to acute toxicity experiments on animals.

Study limitations. The success of cell cultivation largely depends on the seeding dose. With a small number of cells, the formation of a monolayer is not observed even with long-term cultivation. With a very large number of cells, their proliferation takes place intensively, and the cells forming the monolayer age much earlier and their non-specific degeneration is observed more quickly.

Prospects for further research. Based on the results of the obtained experimental data, it is promising to study the antibacterial properties of pipsissewa extract in the presented concentrations and to study the effect of low concentrations on disease models in cell cultures and animals (toxic effect, oxidative stress, etc.).

6. Conclusions

The L929 cell line (fibroblasts of mouse adipose tissue) obtained at the Institute of Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine was used to study the effect of pipsissewa extract on cell cultures. Cells were cultured in DMEM medium (Bio West, France) enriched with 10 % FBS (Lonza, Germany) with 1 % antibiotic-antimycotic (Bio West, France) in a CO_2 incubator (Thermo Fisher Scientific, USA) at 37 °C in an atmosphere with 5 % CO_2.

Determination of the minimum toxic concentration was evaluated by the morphological features of the cells, and it was established that pipsissewa extract had a toxic effect on L929 cells when added to the culture medium at a concentration above 0.01 %. At the same time, the toxic effect had a threshold effect.

The most sensitive to the action of the extract were the migratory and proliferative functions of the cells, less sensitive – energy, pinocytosis and preservation of morphological integrity.

Conflict of interests

The authors declare that they have no conflict of interest in relation to this research, whether financial, personal, authorship or otherwise, that could affect the research and its results presented in this article.

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Data availability

Data will be made available on reasonable request.

Use of artificial intelligence

The authors confirm that they did not use artificial intelligence technologies when creating the current work.

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