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ORIGINAL ARTICLE

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Cellular, genomic and transcriptomic effects of secondary metabolites of the Hybrid Butterbur on the HeLa cell line

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ABSTRACT

Purpose of the study. To evaluate the cellular, genomic (gene copy number) and transcriptomic (gene expression) effects of *P.hybridus* (L.) secondary metabolites when they affect the HeLa cell line.
Materials and methods. The isolation of secondary metabolites from plant material and its identification were carried out by preparative chromatography. The composition was determined using mass spectrometric analysis, and the final verification of structural formulas was carried out by nuclear magnetic resonance at the Department of Natural Compounds, the Faculty of Chemistry of the Southern Federal University. The subsequent phase of the study was conducted using both cultural and molecular methods. HeLa cells were cultivated under standard conditions in a MEM medium. Once the confluence level was reached 75–80 %, the nutrient medium was replaced with the introduction of the studied compounds (at a concentration of 4 micrograms/ml) and cultivated for 72 hours. Cell mortality was determined using a NanoEnTek JuliFl counter (Korea) in the presence of 0.4 % trypan blue. The assessment of apoptosis following secondary metabolite exposure was conducted on a BD FACSCanto II flow cytometer using the FITC Annexin V Apoptosis Detection Kit I. The level of replication and expression of the genes responsible for apoptosis was assessed by digital droplet PCR (ddPCR).
Results. The following compounds were isolated and verified, and were assigned the following sequence numbers to facilitate their use in the experiment: No. 2 – 2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one, No. 3 – 5-(hydroxymethyl) furan-2-carbaldehyde, No. 5.3 – 2,2,8-trimethyldecahydroazulene-5,6-dicarbaldehyde, *P. hybridus* (L.) At the stage of cell death assessment, it was found that the greatest effect was achieved in the compound under ordinal No. 2. However, the evaluation of the copy number and expression of the *CASP8, CASP9, CASP3, BAX, BCL2, TP53, MDM2, CDKN1B, CDK1, CCND1, CCND3*, and *RB1* genes by DD-PCR revealed the presence of apoptosis initiation in tumor cells at the molecular level under the action of compounds No. 2 and No. 5.3 obtained from *P. hybridus* (L.).
Conclusion. The outcomes were multifeatured. Only compound 2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one exhibited a pronounced cytostatic effect out of all compounds utilized in the experiment. Concurrently, the compound 2,2,8-trimethyldecahydroazulene-5,6-dicarbaldehyde was found to induce an increase in the expression of the *CASP3, CASP8, TP53*, and *BAX* genes.

Keywords: secondary plant metabolites, apoptosis, gene expression, copy number variation, HeLa cell line, digital droplet PCR

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3.1.6. Онкология, лучевая терапия
ОРИГИНАЛЬНАЯ СТАТЬЯ

Клеточные, геномные и транскриптомные эффекты вторичных метаболитов Белокопытника гибридного на клеточную линию HeLa

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РЕЗЮМЕ

Цель исследования. Оценить клеточные, геномные (копийность генов) и транскриптомные (экспрессия генов) эффекты вторичных метаболитов *P. hybridus* (L.) при их воздействии на клеточную линию HeLa.
Материалы и методы. Выделение вторичных метаболитов из растительного материала и его идентификацию проводили методом препаративной хроматографии, определение состава – с помощью масс-спектрометрического анализа, окончательную верификацию структурных формул – методом ядерно-магнитного резонанса на кафедре природных соединений химического факультета ФГАОУ ВО «Южный федеральный университет». Следующую часть исследования выполняли с использованием культуральных и молекулярных методов. Культивирование HeLa проводили в стандартных условиях в среде MEM. При достижении 75–80 % уровня конфлюэнтности заменяли питательную среду с внесением исследуемых соединений (в концентрации 4 мкг/мл) и культивировали 72 ч. Смертность клеток определяли на счетчике NanoEnTek JuliFl (Корея) в присутствии 0,4 % трипанового синего. Оценку апоптоза после воздействия вторичных метаболитов проводили на проточном цитофлюориметре BD FACSCanto II с помощью FITC Annexin V Apoptosis Detection Kit I. Оценку уровня копийности и экспрессии генов, ответственных за апоптоз, выполняли методом цифровой капельной полимеразной цепной реакции (ПЦР) (DD-PCR).
Результаты. Были выделены и верифицированы следующие соединения, которым для упрощения использования в эксперименте были присвоены следующие порядковые номера: № 2 – 2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one, № 3 – 5-(hydroxymethyl)furan-2-carbaldehyde, № 5.3 – 2,2,8-trimethyldecahydroazulene-5,6-dicarbaldehyde *P. hibridus* (L.). На этапе оценки клеточной гибели было обнаружено, что наибольший эффект достигается у соединения под порядковым № 2. Однако оценка показателей копийности и экспрессии генов *CASP8, CASP9, CASP3, BAX, BCL2, TP53, MDM2, CDKN1B, CDK1, CCND1, CCND3* и *RB1* методом DD-PCR выявила наличие инициации апоптоза в опухолевых клетках на молекулярном уровне под действием соединений под № 2 и № 5.3, полученных из *P. hibridus* (L.).
Заключение. Получены результаты разнонаправленного характера. Из всех использованных в эксперименте соединений выраженный цитостатический эффект выявлен только у соединения 2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one. В то же время при использовании соединения 2,2,8-trimethyldecahydroazulene-5,6-dicarbaldehyde выявлено увеличение экспрессии генов *CASP3, CASP8, TP53, BAX*.

Ключевые слова: вторичные метаболиты растений, апоптоз, экспрессия генов, копийность генов, клеточная линия HeLa, цифровая капельная ПЦР

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Конфликт интересов: все авторы заявляют об отсутствии явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи

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INTRODUCTION

Cervical cancer is one of the main causes of female mortality. Every year, more than 528,000 new cases of breast cancer and more than 266,000 deaths from this disease are detected [1, 2]. The HeLa cell line is a very convenient and simple object for conducting model experiments *in vitro*. This cell line was obtained on February 8, 1951 from a cervical tumor of a patient named Henrietta Lacks at the Baltimore hospital [3]. In our study, we used this cell line to evaluate the cytotoxic effect of the organic compounds of plant origin that we obtained.

Plants synthesize a huge number of secondary metabolites, and in fact it is these metabolites that form the basis of many commercial pharmaceuticals, as well as herbal medicines. Many secondary metabolites, such as alkaloids, terpenoids and phenylpropanoids, are being considered for drug development [4].

Secondary metabolites of plants are structurally diverse compounds that do not directly participate in the growth, development and reproduction of plants, but more often perform a protective function. These compounds with different chemical structures can act as potential multi-purpose anticancer agents [5]. For the first time in history, the term secondary metabolite was proposed by the German biologist Albrecht Kessel in 1891. when he gave a lecture "On the chemical composition of cells" for the Berlin Society of Physiologists, in which he said: "I propose to call compounds that are important for each cell primary, and compounds that are not present in any plant cell secondary" [6]. Currently, the secondary metabolites of plants are divided into several large groups. Terpenoids (isoprenoids) cover more than 40,000 structures and form the largest class of all known plant metabolites. They represent a class of hydrocarbons, i. e. products of biosynthesis of the general formula (C_5H_8) n, with a carbon skeleton that is a derivative of isoprene $CH_2=C(CH_3)–CH=CH_2$. Alkaloids are characterized as heterocyclic compounds containing a nitrogen molecule in a heterocycle and count about 21,000 compounds. Phenolic compounds are aromatic compounds with a benzene ring containing at least one hydroxyl group [7].

The species selected in our work for the isolation of secondary metabolites is the hybrid *Petasites hibridus* (L.) Gaertn., B. Mey. & Scherb is a herbaceous perennial plant of the *Asteraceae* family, found in the European territory of Russia, and, in particular, in the Krasnodar Territory and the Republic of Adygea. The reasons for the interest in this species are that various representatives of the genus *Petasites*, including *P. hibridus* (L.) itself, contain compounds with cytotoxic effects on tumor cells of various nosologies [8]. So in the Japanese White-collar *Petasites japonicus* (Siebold & Zucc.) Maxim. sesquiterpene I and sesquiterpene II were detected, which showed cytotoxic effect against both human astrocytoma U-251MG tumor cells, as well as against the MDA-MB-231 breast cancer cell line [9].

Various methodological approaches are used to study the effect of secondary plant metabolites on tumor cells, including cytometry and flow cytofluorometry, model experiments on cell cultures and molecular genetic studies. The latter include the assessment of the level of replication and gene expression. CNV (copy number variation) is a type of genetic polymorphism that leads to a change in the number of certain genetic loci and, as a result, a change in the expression of these genes and their products – proteins and non-coding RNAs [10]. Studies of the effect of secondary plant metabolites on the expression and replication of genetic loci regulating apoptosis and the cell cycle in cervical cancer are currently few, so this aspect requires additional study. This is what this work is dedicated to.

The study purpose was to evaluate the cellular, genomic (gene replication) and transcriptomic (gene expression) effects of secondary metabolites of *P. hibridus* (L.) when they are exposed to the HeLa cell line.

MATERIALS AND METHODS

Extraction of metabolites. The primary plant material was collected and determined with the participation of the staff of the Department of Botany of the Academy of Biology and Biotechnology of the D. I. Ivanovsky Southern Federal University. Isolation and verification of secondary metabolites of *P. hibridus* (L.) were carried out by employees of the Department of Natural and High Molecular Weight Compounds of the Faculty of Chemistry of the Southern Federal University. Tetrachloroethylene was used as a solvent for primary extraction, which was poured into mechanically purified and crushed rhizomes.

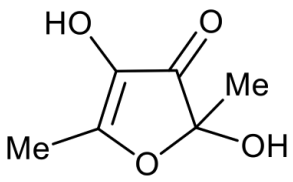
The primary extraction process lasted for four months. To extract tetrachloroethylene from vegetable raw materials, the decantation method was used, followed by concentration of the solution by distillation of the solvent in a distillation unit. Tetrachloroethylene was used as a solvent to reduce the amount of polar compounds (mono- and disaccharides, amino sugars, etc.). The next step was the separation of the resulting concentrated solution using column chromatography using silica gel as a sorbent on column 20*2. Various eluents were used: first, tetrachloroethylene, which allowed to obtain 10 fractions of various colors, from colorless to light yellow. Then methylene chloride was used, which gave 10 more fractions. After that, the eluent was changed, and the column was filled with a mixture of methylene chloride and alcohol in a ratio of 10/1, which led to the production of two more fractions. All fractions were concentrated by evaporation on a rotary evaporator.

The method of high-performance liquid chromatography with mass detection was used to identify the isolated compounds. The mass spectra were analyzed using NIST 2011 biotechnology, which confirms the results of studies with alkaloids and other biologically active compounds.

Fractions containing higher fatty acids, nitrogenous bases of nucleic acids and their glycosides were excluded from further work. In addition, the previously isolated fractions were further purified using column chromatography, and the purified compounds were identified using nuclear magnetic resonance (^{1}H NMR). The identification of purified fractions using NMR made it possible to determine the purity and confirm the structure of compounds previously assumed using mass detector chromatography. The following main names have been identified for experimental use: No. 2 – 2,4-dihydroxy-2,5-dimethylfuran-3(2H)-oh, No. 3 – 5-(hydroxymethyl)furan-2-carbaldehyde, No. 5.3 – 2,2,8-trimethyldecahydroazulene-5,6-dicarbaldehyde (Fig. 1).

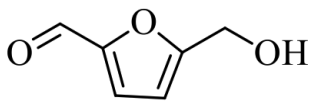
Assessment of biological effects

The biological effect of the isolated compounds was evaluated on the HeLa CCL2 cell line. The cell line was obtained from the biobank of the National Medical Research Centre for Oncology, which works in accordance with the recommendations on the organization of the structure of biorepositories and the ethical requirements of the latest edition of the ISBER Best Practices and based on the ISO 9001 standard [11]. The cells were cultured at 37 °C and 5 % CO_2 in a nutrient medium Igla MEM (BioloT) containing 10 % fetal serum from cows (HyClone, USA), up to a number of 1 × 10^6 cells. When 80 % confluence was achieved, the nutrient medium was replaced with a similar one with the addition of 4 micrograms / ml of furfural and azulene derivatives to the test samples, and without the addition of the studied substances in the negative control. The exposure time was 72 hours. After that, the cells were removed from culture vials with 0.1 % trypsin solution. The number of living and dead cells was determined using an automatic NanoEnTek JuliFl counter (Korea) with 0.4 % trypan blue staining. Cells removed from culture vials were preserved in an RNA



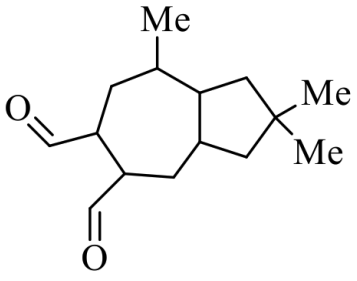
2,4-dihydroxy-2,5-dimethylfuran-3(2*H*)-one (**2**)

Chemical Formula: $C_6H_8O_4$
Molecular Weight: 144,13



5-(hydroxymethyl)furan- -2-carbaldehyde (**3**)

Chemical Formula: $C_6H_6O_3$
Molecular Weight: 126,11



2,2,8-trimethy|decahydroazulene-5,6-dicarbaldehyde (**5.3**)

Chemical Formula: $C_{15}H_{24}O_2$
Molecular Weight: 236,3499

Fig. 1. Structural formulas of three compounds isolated from the hybrid *P. hibridus* (L.) protein

medium (IntactRNA Eurogene). Cellular apoptosis was assessed on a BD FACSCanto II flow cytofluorometer using the FITC Annexin V Apoptosis Detection Kit I. Cells stored in an RNA medium were divided into two equal aliquots, from which total DNA/RNA preparations were extracted using the commercial DNA-sorb-B and Trizol kit, respectively.

Molecular methods

The evaluation of copy number variations and gene expression was performed by digital drip PCR using the QX200™ ddPCR™ EvaGreen Supermix kit (Bio-Rad, USA). The Droplet Digital polymerase chain reaction system (ddPCR™) was developed for high-precision absolute quantitative analysis of target sequences of nucleic acids encapsulated in discrete droplets of water-oil emulsion determined by volumetric method. Using a droplet generator, each sample of the studied locus was divided into 20,000 droplets in three repeats. Amplification was carried out to the end point (40 cycles) on the C1000 Touch Thermal Cycler Bio-Rad.

After the amplification was completed, a QX200 Bio-Rad reader was placed on the sample plate, which counted droplets giving fluorescent positive and negative signals to calculate the concentration of target DNA and ctDNA. The principle of measuring the level of copyness and expression indicators using digital drip PCR technology was to directly count events via the FAM channel. In positive droplets containing at least one copy of the target DNA, the droplet reader shows fluorescence, unlike negative droplets in which amplification did not occur. QuantaSoft v1 software.7.4 measures the number of positive and negative droplets in each sample, and then applies an algorithm for calculating the Poisson distribution function to determine the initial concentration of target DNA molecules in units of "copies/μl" (Fig. 2).

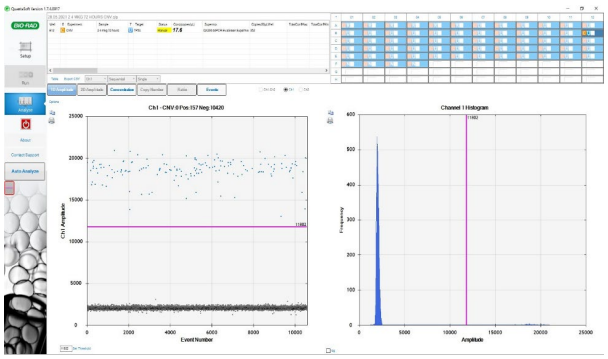


Fig. 2. Screenshot of the QuantaSoft v1 software.7.4 during the result processing

The level of CNV and gene expression was calculated as follows. According to the formula, the concentration of each studied locus / concentration of the reference locus × the number of copies of the reference loci in the genome (as a rule 2).

Statistical data processing

Statistical data processing was carried out using the Statistica 19.0 program (StatSoft Inc., USA). To assess the significance of the differences, a single-factor analysis of variance was used (the critical level of statistical significance was $p < 0.05$).

STUDY RESULTS

At the first stage of the study, the purification and verification of compounds that can exhibit cytotoxic effects on tumor cells of various nosologies was carried out. The identification of the isolated compounds was performed by mass spectometry and nuclear magnetic resonance (NMR); during it, 2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one was verified, which was assigned the serial number 2,5-(hydroxymethyl)furan-2-carbaldehyde with the serial number 3, as well as 2,2,8-trimethyldecahydroazulene-5,6-dicarbaldehyde with the serial number 5.3. All three compounds are isolated from the rhizomes of *P. hibridus* (L.). The data obtained by evaluating the cytotoxic effect of the compounds involved in the experiment on the NanoEnTek JuliFl cell counter are presented in Table 1.

As can be seen from the data presented in Table 1, all compounds according to the results of the trepan blue test had an approximately equivalent effect on HeLa tumor cells; in experimental samples, the number of dead cells exceeded the control by 1.97–2.44 times. The images below, obtained using an inverted microscope (Leica DM IL LED), show a comparison of a control sample of the HeLa cell line with a sample treated with compound No. 2. After exposure, a violation of the monolayer in the experimental sample is seen associated with weaker cell attachment or lysis, and a large number of cells are "scalded" (Fig. 3).

The following images show the effect achieved when exposed to compound No. 3 in comparison with the control, a dense monolayer of cells is observed, at the same time a large number of scalding cells (Fig. 4).

Figure 5 shows a comparison of the control of Hela cells with cells that were exposed to compound 5.3, in the experimental sample there is a dense monolayer of cells and an increase in the number of scalding cells that exceeds that observed in the control.

The results of the evaluation of the antitumor effect of the metabolites used by us are also confirmed by the data of flow cytofluorometry presented below.

The most pronounced cytotoxic effect was shown by (2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one) at No. 2 at a concentration of 4 micrograms /ml at exposure for 72 hours. The remaining compounds used did not have such an effect according to flow cytofluorometry (Fig. 6–8, table 2).

As can be seen from Figure 6, 72-hour incubation with (2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one) had a cytostatic effect on HeLa cells, expressed in an increase in the number of cells in the state of early apoptosis from 7.2 to 13.3 %, and late apoptosis from 5.8 to 8.3 %. The total number of cells in the state of apoptosis after exposure (2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one) increases 1.6 times.

Based on the data in Fig.7, it follows that a 72-hour incubation with 5-(hydroxymethyl)furan-2-carbaldehyde did not have a cytotoxic effect on HeLa cells, the difference with the control in early apoptosis changed from 7.2 to 7.3 %, and late apoptosis – from 5.8 to 6.2 %. The total number of cells in the state of apoptosis after exposure to 5-(hydroxymethyl) furan-2-carbaldehyde increases by 0.5 times.

Figure 8 demonstrates that 72-hour incubation with 5-(hydroxymethyl)furan-2-carbaldehyde also did not have a cytotoxic effect on HeLa cells, the difference with the control in early apoptosis changed from 7.2 to 7.5 %, and late apoptosis – from 5.8 to 6.8 %. The total number of culture cells in the state of apoptosis after exposure to 2,2,8-trimethyldecahydroazulene-5,6-dicarbaldehyde increased 1.3 times.

Digital drip PCR was used to evaluate changes in CNV and expression (CNV/EXP) indices under the influence of secondary metabolites from *P. hibridus* (L.) isolated by us. When exposed to 2,2,8-trimethyldecahydroazulene-5,6-dicarbaldehyde at a concentration of 4 micrograms/ml exposure for 72 hours, there were increases in the level of expression of *CASP3* in relation to the control by 28.28 times ($p < 0.05$), and *CASP8* by 46.71 times ($p < 0.05$). At the same time, the expression of the *CASP9* locus increased by 3.43 times ($p < 0.05$). Exposure to 5-(hydroxymethyl) furan-2-carbaldehyde at a concentration of 4 micrograms/l and an exposure of 72 hours had the following effect: the expression level of *CASP3* increased by 4.57 times relative to the control ($p < 0.05$), it also increased the expression level of *CASP8* by 10.48 times ($p < 0.05$). When using 2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one at a concentration of 4 micrograms/ml exposure for 72 hours, the expression of the *CASP3* locus relative to the control increased by 3.95 times ($p < 0.05$), and *CASP8* by 3.38 times ($p < 0.05$). At the same time, the indicators of the copy level (CNV) of the *CASP8*, *CASP9*, and *CASP3* loci did not undergo major changes (Fig. 9).

At the same time, the assessment of changes in the levels of copy number variability (CNV) and expression (EXP) at the *TP53* and *MDM2* loci showed the following results. The compound 2,2,8-trimeth-

Table 1. The number of living and dead HeLa cells after exposure to isolated secondary metabolites after staining with trypan blue

Compounds, concentrations	72 hours, living cells	72 hours, dead cells
Control	93.52 %	6.48 %
№ 2, 4 μg /ml	87.23 %	12.77 %
№ 3, 4 μg /ml	86.66 %	13.34 %
№ 5.3, 4 μg /ml	84.16 %	15.84 %

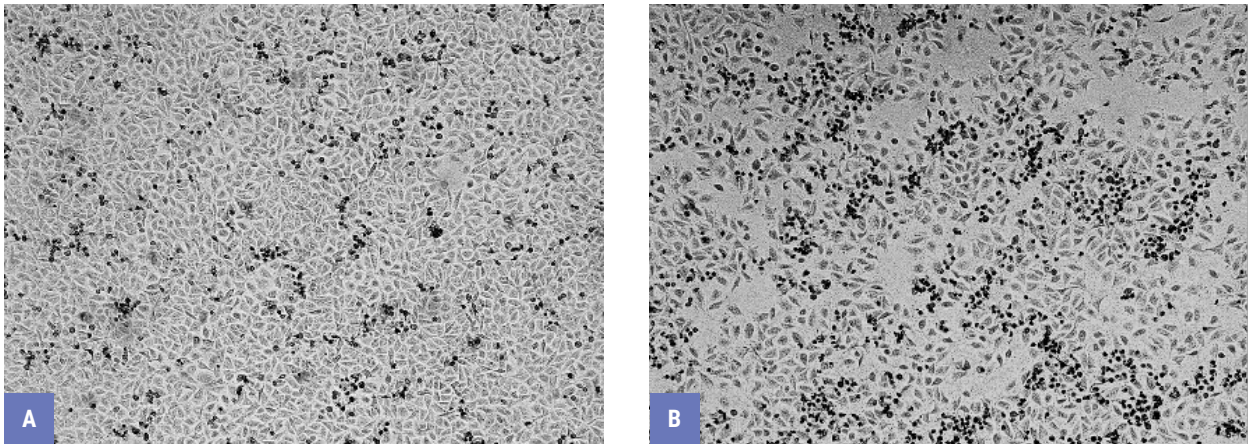


Fig. 3. HeLa cells after exposure to 2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one. A – control specimen; B – experimental specimen

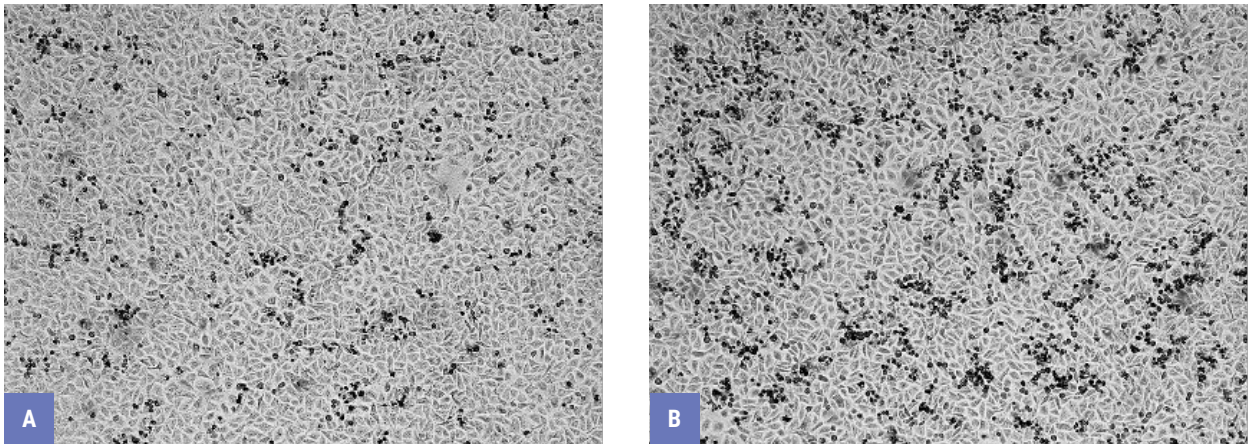


Fig. 4. HeLa cells after exposure to terpenoid 5-(hydroxymethyl)furan-2-carbaldehyde. A – control specimen; B – experimental specimen

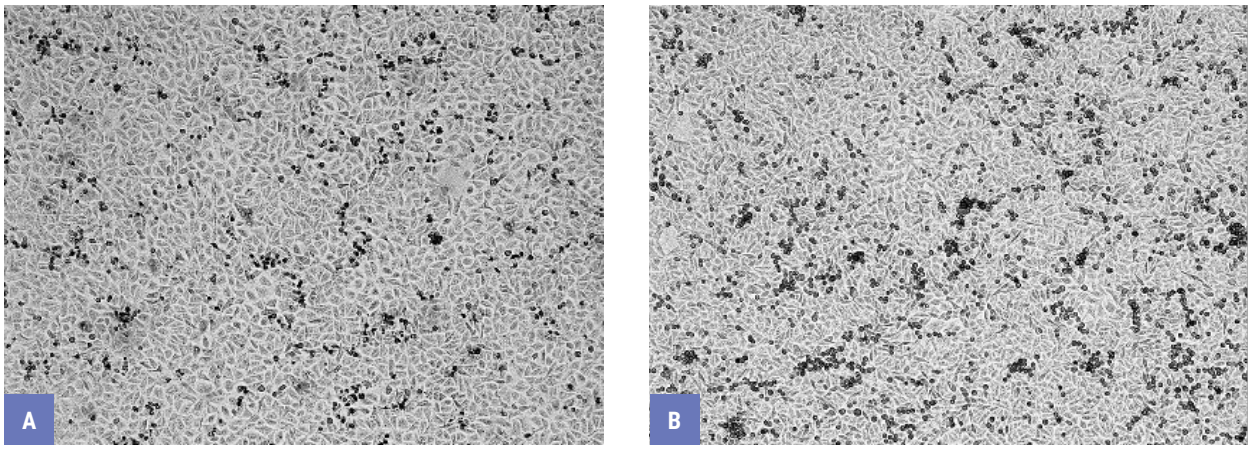


Fig. 5. HeLa cells after incubation with the terpenoid 2,2,8-trimethyldecahydroazulene-5,6-dicarbaldehyde. A – control specimen; B – experimental specimen

yldecahydroazulene-5,6-dicarbaldehyde at an exposure of 72 hours and a concentration of 4 micrograms/ml increased the *TP53* copy level by 1.05 times ($p < 0.05$), and *MDM2* decreased by 0.26 times ($p < 0.05$) relative to the control. The difference between them was 4 times. In addition, 2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one increased *TP53* expression level by 1.46 times ($p < 0.05$) at exposure of 72 hours and concentration of 4 micrograms/ml, while *MDM2* decreased by 0.88 times ($p < 0.05$). The difference was 1.66 times (Fig. 10).

The following data were obtained when evaluating changes in the level of replication and expression of the *BAX* and *BCL2* loci. The terpenoid 2,2,8-trimethyldecahydroazulene-5,6-dicarbaldehyde increased the level of CNV of the I locus by 0.9 times relative to the control ($p < 0.05$), the level of *BCL2* decreased by 0.13 times ($p < 0.05$). The difference between them

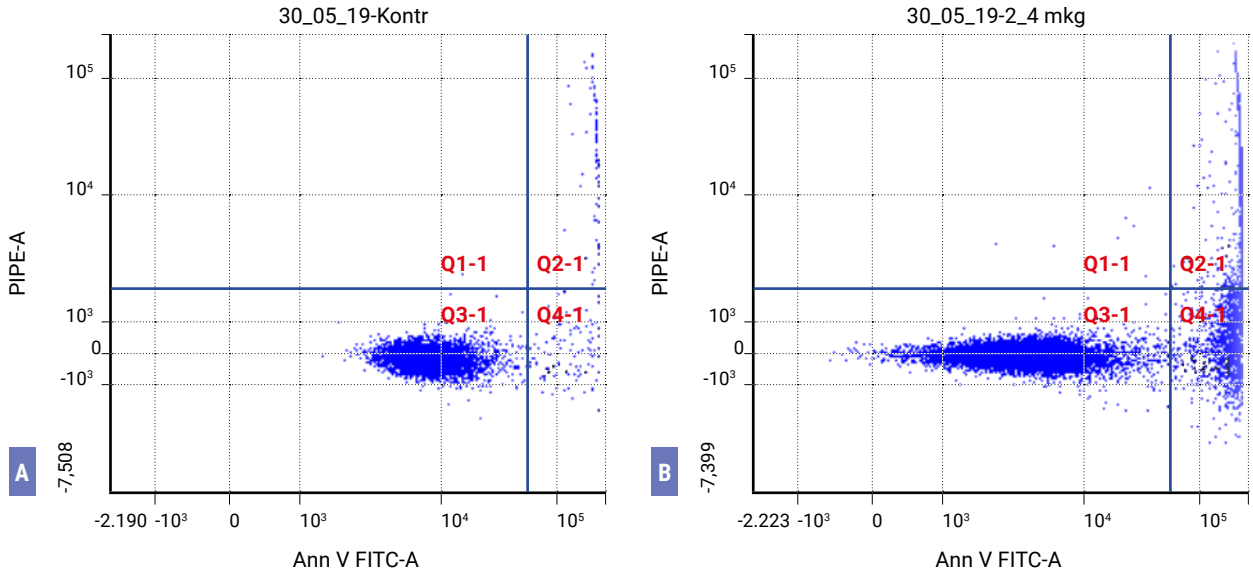


Fig. 6. Effect of (2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one) on necrosis/apoptosis of the HeLa cell line: A – control specimen; B – experimental specimen, (Q3-1 – living cells, Q4-1 – early apoptosis, Q2-1 – late apoptosis/necrosis, Q1-1 – dead cells)

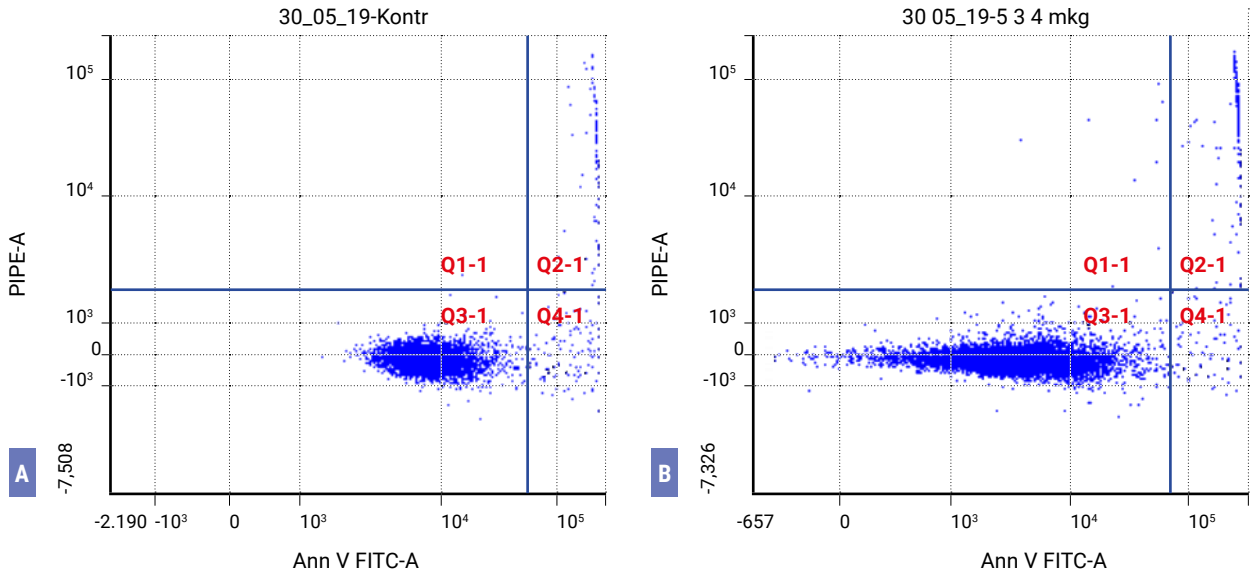


Fig. 7. Exposure to 5-(hydroxymethyl)furan-2-carbaldehyde on necrosis/apoptosis of the HeLa cell line: A – control specimen; B – experimental specimen, (Q3-1 – living cells, Q4-1 – early apoptosis, Q2-1 – late apoptosis/necrosis, Q1-1 – dead cells)

was 6.92 times. Also, when exposed to the same compound, the expression level of *BAX* increased by 1.73 times ($p < 0.05$), and *BCL2* decreased by 1.19 times ($p < 0.05$). The difference between them was 1.45 times in favor of an increase in *BAX* (Fig. 11).

The furan and azulene derivatives of *P. hibridus (L.)* metabolites used in our study changed the level of replication and expression of *CDKN1B*, *CDK1*, *CCND1*, *CCND3* and *RB1* loci as follows. Thus, 2,2,8-trimethyldecahydroazulene-5,6-dicarbaldehyde increased *CCND3* expression by 20.66 times relative to the control ($p < 0.05$), RB1 expression increased by 7.35 times ($p < 0.05$) at an exposure of 72 hours and a concentration of 4 micrograms/ml. In turn 5-(hydroxymethyl) furan-2-carbaldehyde with an exposure of 72 hours and a concentration of 4 micrograms/ml also increased the expression level of *CCND3* by 5.23 times ($p < 0.05$). At the same time, 2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one at similar concentrations and exposures increased the *CCND3* copy level by 3.48 times ($p < 0.05$), and the expression level increased by 2.42 times relative to the control ($p < 0.05$). At the same time, 2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one at the point of 4 micrograms/ml with an exposure of 72 hours increased the expression level of the *RB1* locus relative to the control by 4.51 times ($p < 0.05$) (Fig. 12).

DISCUSSION

Since the early 2000s, many works have been published worldwide on the search for new compounds of natural origin, including plant origin ones, with cytostatic or cytotoxic effects on tumor cells of various diseases [12]. In our study, we conducted not only a model experiment to assess the level of cytotoxic

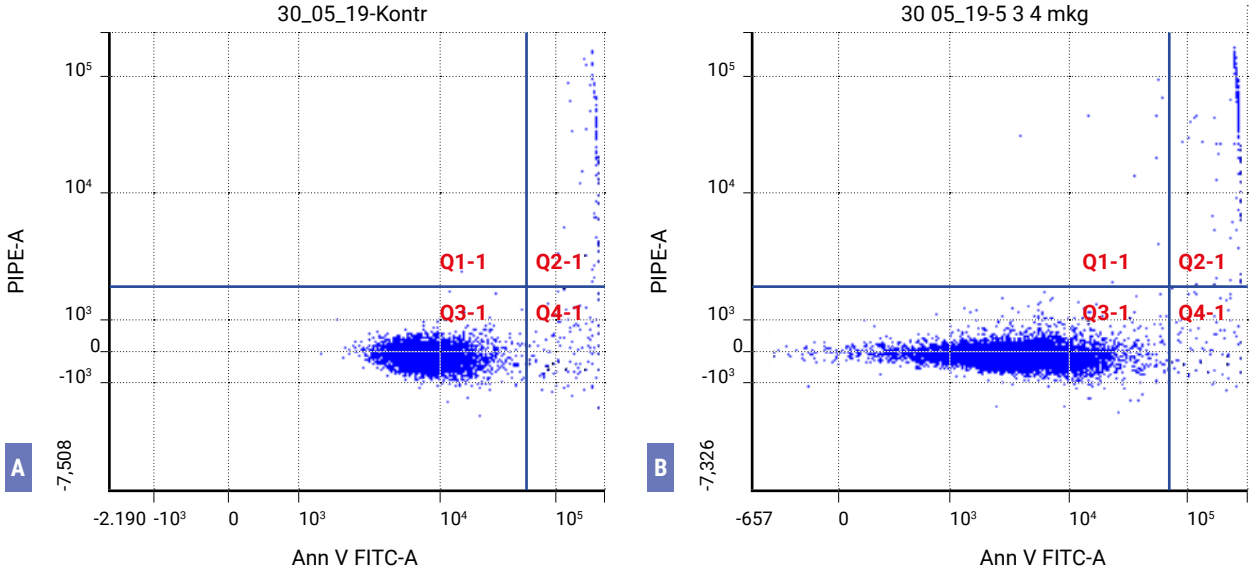


Fig. 8. The effect of 2,2,8-trimethyldecahydroazulene-5,6-dicarbaldehyde on necrosis/apoptosis of the HeLa cell line: A – control specimen; B – experimental specimen, (Q3-1 – living cells, Q4-1 – early apoptosis, Q2-1 – late apoptosis/necrosis, Q1-1 – dead cells)

Table 2. The number of HeLa cells in a state of apoptosis after exposure to isolated secondary metabolites (72 hours exposure)

Compound	Concentration, μg /ml	Alive cells Q3-1	Early-stage apoptosis Q4-1	Late-stage apoptosis / necrosis Q2-1	Dead cells Q1-1
Control		87.0 %	7.2 %	5.8 %	0 %
No. 2	4	78.3 %	13.3 %	8.3 %	0 %
No. 3	4	91.1 %	2.5 %	6.3 %	0 %
No. 5.3	4	94.2 %	2.6 %	3.2 %	0 %

effect of the *P. hibridus* (L.) secon dary metabolites that we obtained, but also used the digital drip PCR method to register molecular genetic changes in loci responsible for suppressing tumor growth and apoptosis in HeLa tumor cells.

The data obtained in the study show mixed results. The most pronounced change in the expression level of the *CASP8* and *CASP3* loci was revealed when exposed to 2,2,8-trimethyldecahydroazulene-5,6-dicarbaldehyde. Cytosolic caspases are cysteine-asparagine proteases, which are the main family of proteins involved in the transmission of cell death signals. Caspases are divided into three groups: initiatory, inflammatory and effector. They are directly involved in the initiation of apoptosis. As is known, the *CASP8* protein, which is an initia-

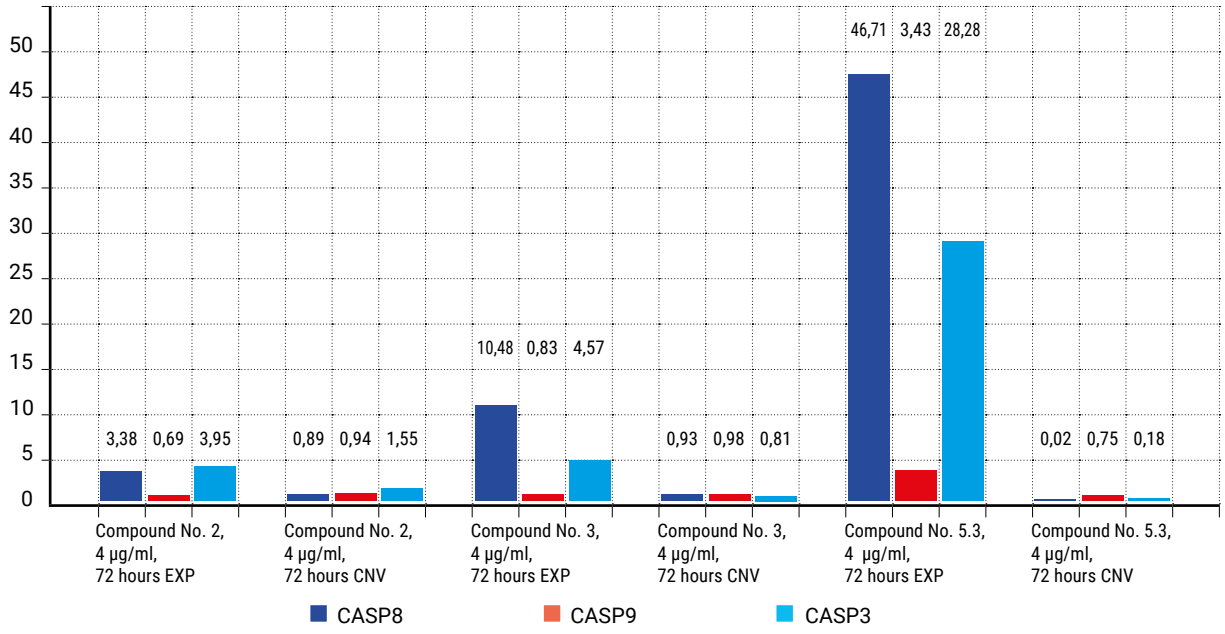


Fig. 9. Changes in the level of replication and expression of the *CASP8*, *CASP9*, and *CASP3* loci under the action of 2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one (No. 2), 5-(hydroxymethyl)furan-2-carbaldehyde (No. 3) and 2,2,8-trimethyldecahydroazulene-5,6-dicarbaldehyde (No. 5.3)

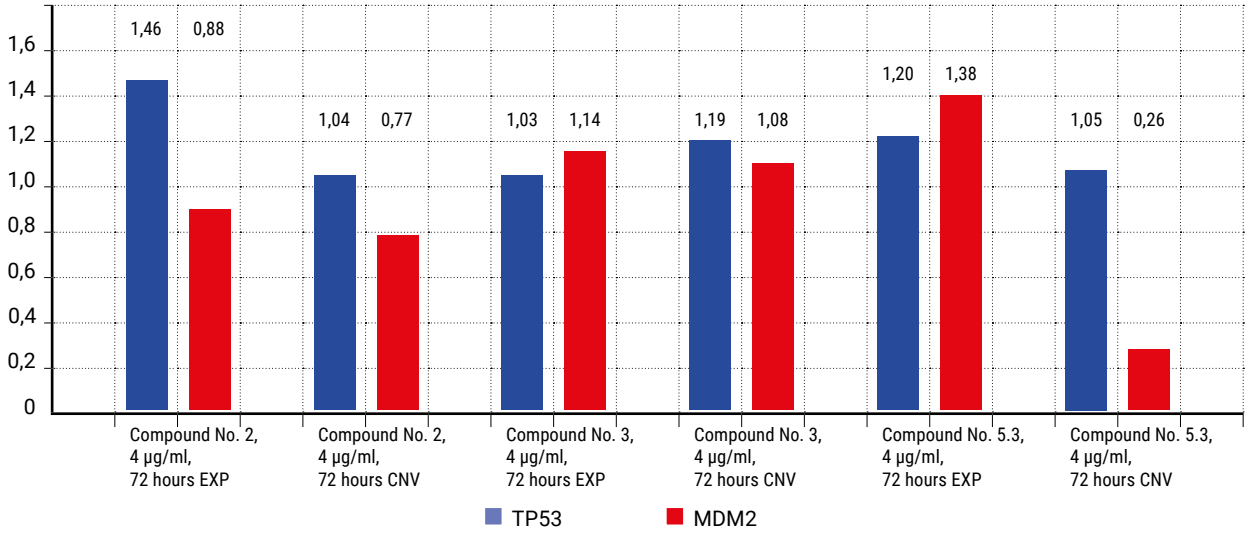


Fig. 10. Changes in the level of replication and expression of *TP53*, *MDM2* loci when exposed to 2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one (No. 2), 5-(hydroxymethyl) furan-2-carbaldehyde (No. 3) and 2,2,8-trimethyldecahydroazulene-5,6-dicarbaldehyde (No. 5.3)

tor, is associated with tumor necrosis factor (TNF) located on the cell surface, as well as the FAS ligand (FasL), and induces apoptosis (CD95). Activation of the *CASP8* protein via the external apoptosis pathway triggers BID-mediated activation of BAX and BAK proteins on the outer membrane of mitochondria, which leads to the release of cytochrome C and subsequent activation of CASP9, which, in turn, activates CASP3 and CASP7, thereby performing the process of apoptosis along the mitochondrial pathway [13]. It should be noted that when exposed to 5-(hydroxymethyl)furan-2-carbaldehyde showed a change in the expression level of *CASP8* and *CASP3* of a similar profile.

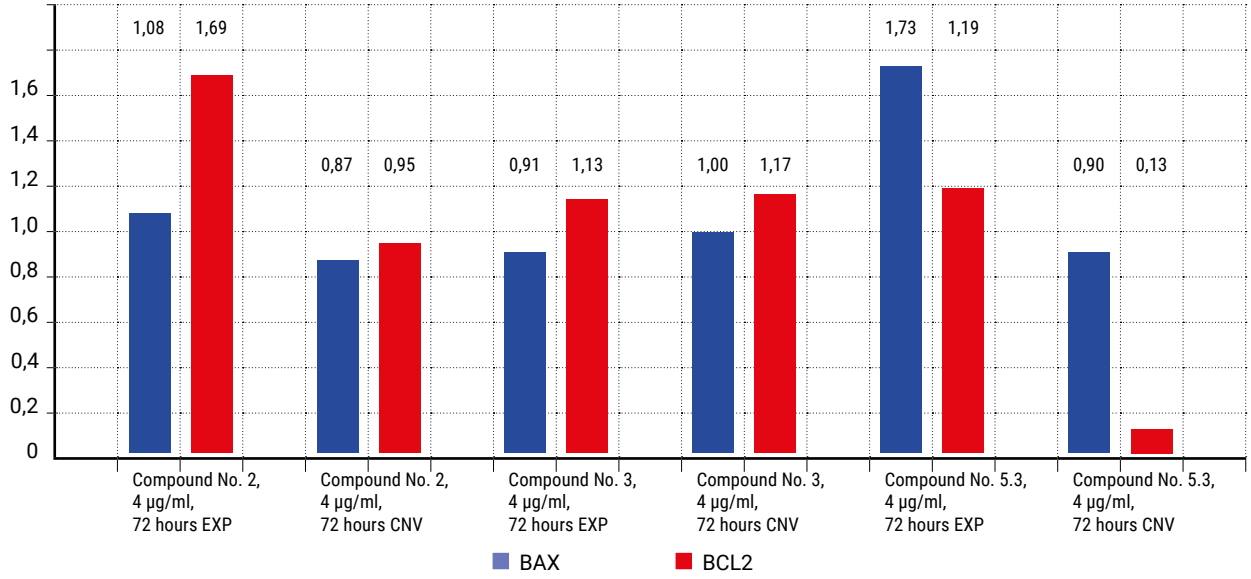


Fig. 11. Changes in the level of replication and expression of *BAX*, *BCL2* loci when exposed to 2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one (No. 2), 5-(hydroxymethyl) furan-2-carbaldehyde (No. 3) and 2,2,8-trimethyldecahydroazulene-5,6-dicarbaldehyde (No. 5.3)

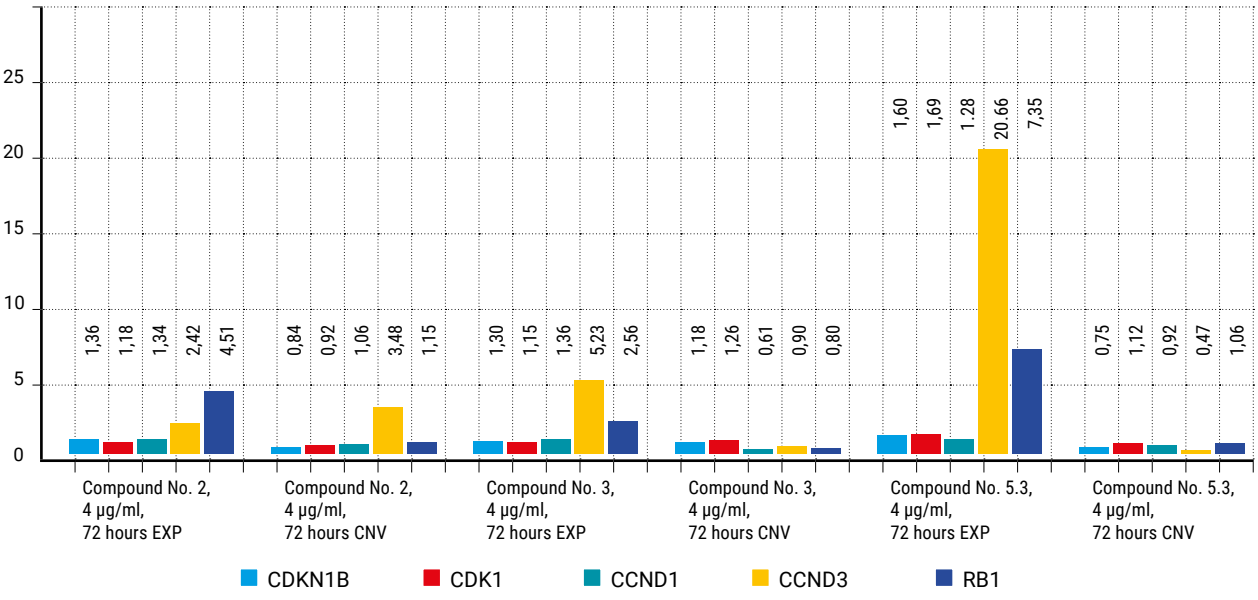


Fig. 12. Changes in the level of replication and expression of *CDKN1B*, *CDK1*, *CCND1*, *CCND3*, *RB1* loci under the influence of 2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one (No. 2), 5-(hydroxymethyl)furan-2-carbaldehyde (No. 3) and 2,2,8-trimethyldecahydroazulene-5,6-dicarbaldehyde (No. 5.3)

At the same time, 2,2,8-trimethyldecahydroazulene-5,6-dicarbaldehyde increased the expression level of *TP53* and significantly reduced the expression of *MDM2*, which may indicate a specific targeting of the action of this compound. It should be borne in mind that *TP53* has tumor suppressive activity, which largely explains its ability to induce cell death, including apoptosis, through transcription-dependent and transcription-independent mechanisms [14]. In addition, the nuclear protein p53 transcriptionally activates the expression of many pro-apoptotic genes of the *BCL-2* family, such as *NOXA*, *PUMA*, *BID*, *BAD*, *BIK*, *BAX*, etc., whereas it inactivates the expression of anti-apoptotic *BCL-2*, *BCL–Xl* and *MCL1*, leading to mitochondrial apoptosis [15]. The relationship between changes in the expression level of *TP53* and *BAX* loci was also reflected in the results obtained. As in the case of the *TP53*/*MDM2* locus bundle, exposure to the terpenoid 2,2,8-trimethyldecahydroazulene-5,6-dicarbaldehyde affected the expression level of *BAX*/*BCL2* loci.

The changes in the expression level of CCND3 and RB1 loci under the influence of 2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one were also revealed. This compound is the only one used in our study, that led to a decrease in the expression level of the CCND3 locus relative to RB1 (the expression level of RB1 was almost 2 times higher than the expression level of CCND3). As is known, d-type cyclins (d1, d2 and d3) are cell cycle regulators that activate cyclin-dependent kinases cdk4 and cdk6, which are often overexpressed in malignant neoplasms. The CCND3 gene product interacts with the Rb tumor suppressor protein and participates in its phosphorylation. CDK4 activity is associated with this CCND3, which is necessary for the transition of the cell cycle to the G2 phase. Inhibition of CCND3 and cyclin-d-cdk4/6 kinase in tumor cells with a high content of retinoblastoma rb1 protein causes cell cycle arrest. However, reducing only the level of rb1 in tumor cells does not lead to a stop in proliferation [16]. The data on the change in the expression of CCND3 and RB1 are consistent with the data of objective control from photographs obtained using an inverted microscope and data from flow cytofluorometry.

CONCLUSION

The study made it possible to establish the multidirectional effect of secondary metabolites of *P. hibridus* (L.) on the death and apoptosis of HeLa cells. The data obtained by digital drip PCR revealed a maximum increase in the expression of genes responsible for regulating apoptosis (*CASP3*, *CASP8*, *TP53*, *BAX*) under the action of 2,2,8-trimethyldecahydroazulene-5,6-dicarbaldehyde, as well as a change in the expression of *CCND3* and *RB1* genes under the influence of 2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one. At the same time, according to cytometry and flow cytofluorometry, a more pronounced proapoptogenic (cytotoxic) effect was detected in 2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one. It should be noted that in our work, the expression index reacted most actively to the studied substances, which, in some cases, was dissonant with both gene replication and the level of mortality and apoptosis of tumor cells. Perhaps chemical modifications of the compounds used by us will have a more pronounced effect both at the molecular genetic level and at the cellular level.

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Burov O. N. – isolation and verification of compounds from plant material;
Bondarenko E. S. – cytofluorimetric analysis;
Sagakyants A. B. – analysis of the cytofluorometry results;
Kutilin D. S. – manuscript editing;
Dzigunova Yu.V. – collection and determination of plant material;
Novikova I. A. – design of the bibliography, manuscript editing;
Przhedetskiy Yu. V. – statistical data processing.