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INFLUENCE OF A NEW COMBINED PRODUCTION BASED ON DENSE CARROT ROOT EXTRACT AND QUERCETIN ON THE MORPHOLOGICAL AND PROLIFERATIVE PROPERTIES OF L929 LINE FIBROBLASTS IN CELL CULTURE

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At the first stage of studying the pharmacological properties of substances, the general cytotoxic effect is determined based on the assessment of viability, morphological integrity and functional activity of cells. One of the standard cell lines often used in cytotoxicity tests is the L929 line.
Studying the ability of a combined lipid-lowering agent based on dense carrot root extract and quercetin to influence the morphological and functional properties of fibroblasts of the L929 line in cell culture was ***the aim*** *of this study.*
Materials and methods. *Determination of the cytotoxicity of the drug under study was carried out in a cell culture of the L929 line at a concentration of 40, 100, and 200 mg/ml. The safety of cells in the resulting suspension was assessed using staining with a 0.4 % trypan blue solution. Cell morphology and nuclear-cytoplasmic ratio were assessed in fixed culture preparations stained with hematoxylin-eosin. To assess the migration and proliferative activity of a monolayer culture, the "scratch" test was used.*
Results. *When L929 cells were incubated in a nutrient medium with the addition of the product, their viability and morphological properties were preserved; images of a monolayer of the culture of these cells under phase-contrast microscopy coincided with the quantitative analysis data. The absence of changes in the nuclear-cytoplasmic ratio was further evidence of the absence of toxic effects of the studied drug in relation to fibroblast culture. According to the experiment results, the migration and proliferative activity of L929 cells under the influence of the drug did not differ significantly from intact cells.*
Conclusions. *The results of the toxicological study showed that the combined product based on dense carrot root extract and quercetin does not have a cytotoxic effect on the L929 cell line culture and can be recommended for further preclinical research*
Keywords: *L929 cell line, cytotoxic effect, dense carrot root extract, quercetin*

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1. Introduction

Pharmaceutical safety is an essential factor in the development of every medicament [1]. Recently, there has been increasing debate around the use of laboratory animals for scientific purposes, including in developing medicines. Animal studies used to predict the toxic effects of drugs have been widely criticised not only by anti-vivisectionists and clinicians but also by toxicologists themselves. It concerns both moral standards and the scientific significance of the results obtained [2]. Due to fundamental differences in the anatomy, physiology, pathology, and metabolism of humans and animals, data on the effectiveness and toxicity of drugs obtained in animal models are often not adequately confirmed in the clinic.

Therefore, it is quite justified to develop adequate alternative research methods [3], which involve replacing animals with cultures of cells, tissues, etc.

Researchers increasingly use human and animal cell cultures as biological test systems due to their ease of cultivation, controllability, and greater reproducibility compared to in vivo test systems. In addition, reducing time and economic costs plays an important role. The main advantage of cultured cells is the possibility of intravital visual observation of cells that remain viable throughout the experiment using a microscope [4]. The only caveat is the requirements for standardising the quality of cell and tissue culture.

The study of the biological activity of substances, regardless of the further purpose of their use, as a rule, at the first stage involves an assessment of their toxicity. Methods for assessing toxicity that are alternative to classical tests on experimental animals, namely models using cell cultures, are increasingly used in biochemical and toxicological studies [5]. Such methods allow, in addition to solving ethical problems associated with the massive use and death of experimental animals, to significantly reduce the cost and the time of preliminary research of new drugs, primarily at the stage of their preclinical trials. Another advantage of in vitro models is the ability to work directly on human cell cultures, which makes the

data obtained more adequate when applied to the human body. In addition, using cell cultures makes it possible to determine the nature of the biological activity of the compounds under study directly at the cellular level [6, 7].

In 1959, microbiologist R. Burch and zoologist W. Russel in London published «The Principle of Humane Experimental Technique,» a book outlining generally accepted principles for the humane use of animals for research and educational purposes. In it, they formulated the concept of the three R's (Replacement, Reduction, Refinement) [8]. Today, the concept of the "three R's" is at the forefront of scientific research.

If we define general cytotoxicity as an adverse effect on the structure and properties of cells, affecting their survival, proliferation, and functioning, then at the cellular level, we can distinguish three main mechanisms of substances' toxic effects: damage to cell membranes, disruption of metabolic processes, and dysregulation of ionic composition and cell division [9].

The use of alternative methods for studying acute toxicity requires an understanding and accurate interpretation of the concept of types of cytotoxic action [10]:

– general cytotoxicity, characterised by the same sensitivity to the action of a substance, regardless of the cell type;

– selective cytotoxicity, a distinctive feature of which is the dependence of sensitivity to a toxic agent on the type of cell;

– specific functional cytotoxicity, which occurs if the xenobiotic does not directly affect the cell, but its effect is critical at the level of the whole organism.

Currently, a sequence of three main stages of screening studies to predict acute toxicity in vitro is proposed [11]. At the first stage, the overall cytotoxic effect is determined based on the assessment of inhibition of cell proliferation. The best object for this is an undifferentiated cell line capable of transformation and rapid division [12].

L929 is a continuous mouse fibroblast cell line commonly used for in vitro cytotoxicity studies due to its reproducible biological responses and growth rate, according to the international standard ISO 10993-5 [13].

The carrot (Daucus carota L.) is a cultivated subspecies of wild carrot (Daucus carota). Carrots have anti-inflammatory, wound-healing, soothing, vasodilating, antispasmodic, myotropic effects, mainly on the muscles of the coronary vessels. Compounds that may act as antioxidants include flavonoids and alkaloids.

Several studies have shown that some carrot metabolites are able to induce powerful cytotoxic effects, specifically in cancer cells, by interfering with important cellular pathways. Carrot metabolites have been demonstrated to modulate different proteins involved in cell proliferation, apoptosis, epithelial-to-mesenchymal transition, and inflammation, all critical processes responsible for cancer progression and metastasis. Among these compounds, acetylenic oxylipins, such as falcarinol (FaOH) and falcarindiol (FaDOH), are upregulated in response to fungal diseases, acting as natural pesticides in carrots [14], and exhibit a diverse range of biological activities in mammals.

It has been observed that extracts from carrots containing the highest concentration of falcarinol exhibited the highest inhibitory effect on Caco-2 cell growth, supporting the higher cytotoxic potency of falcarinol compared to falcarindiol [15].

Individual studies have been conducted on the toxicity of carrot dihydroisocoumarins in vitro on Chinese hamster cells [16, 17].

Flavonoids are particularly important in the prevention of cardiovascular diseases, as they exhibit antioxidant properties and inhibit the formation of free radicals. The most important flavonoids is quercetin, which exhibits an antioxidant effect due to complex formation with heavy metals and is second only to synthetic ubiquinone in activity among antioxidants of plant origin.

Studies have shown that quercetin has hypolipidemic activity, the ability to increase the level of high-density lipoproteins, hypoglycemic, hypotensive, cytoprotective, anti-infective/antiviral and detoxifying effects, the ability to modulate the level of nitric oxide in damaged tissues and blood and borer [18, 19].

In addition to its antioxidant properties, quercetin exhibits anti-tumour and apoptosis-inducing effects. Quercetin has growth-inhibitory effects on cells derived from breast, lung, and liver cancers via signalling pathways such as the PI3K/Akt/mTOR, Wnt/β-catenin, and MAPK/ERK1/2 pathways [20].

For a long time, the use of the beneficial pharmacological properties of quercetin was limited by its low bioavailability, caused by poor solubility in biological fluids of the body, which prevented the rapid achievement of target cells and penetration through the phospholipid bilayer of the membrane into the cells to the affected subcellular structures.

In this regard, scientists are constantly making attempts to improve its solubility and bioavailability, which is expected to significantly expand the list of medical indications for the use of this natural bioflavonoid.

Recently, special attention has been paid to the production and use of solid dispersions. Since the properties of medicinal substances often determine the technology of the dosage form, the method of solid dispersions allows, in some cases, to use them as an equivalent to the drug substance with improved biopharmaceutical characteristics. This opens up the opportunity to improve the quality of existing pharmaceutical forms or create qualitatively new dosage forms for a given medicinal substance.

Currently, the National University of Pharmacy has already obtained a standardised solid dispersion of quercetin with PEO and neusilin, which, together with voglibose, is included in the composition of a new combined antidiabetic agent under the code name "Glykverin".

Taking into account the previous experience of creating a solid dispersion with quercetin at the Department of Factory Technology of Medicines of the NUPh, Professor Inna Kovalevskaya, under the leadership of the head of the department, Professor Elena Ruban, using a new technological approach, a new drug was obtained based on carrot root extract of a thick and solid dispersion of quercetin with colidon-30 and MKTs-102 for the

prevention and treatment of atherosclerosis and myocardial lesions of various origins.

The chemical composition of dence carrot root extract contains a complex of biologically active substances: phenolic compounds, flavonoids, carotenoids α- and β-carotene, lutein, zeaxanthin, anthocyanins, riboflavin, thiamine, ascorbic acid, niacin and tocopherol and others, which determine a wide range of their pharmacological action.

The optimal composition and conditionally therapeutic dose of 200 mg/kg of this agent were determined in the previously conducted pharmacological screening, which showed an increase in the hypolipidemic effect of quercetin solid dispersion when using colidon-30 with MKTs-102 compared to native quercetin and reliable hypolipidemic properties of the effect of dence carrot root extract. The pronounced hypotriglyceridemic and hypocholesterolemic effect of the combined agent allowed us to assume the additive summation of the effects of quercetin and dence carrot root extract [21].

Studying the ability of a combined lipid-lowering agent based on dense carrot root extract and quercetin to influence the morphological and proliferative properties of fibroblasts of the L929 line in cell culture was the **aim** of this study.

2. Planning (methodology) of research.

Studies were carried out in accordance with Good Laboratory Practice [22] and Good Cell Culture Practice standards on the L929 cell line.

The study protocol describing the different stages of the present research work is presented in the following flow chart (Fig. 1):

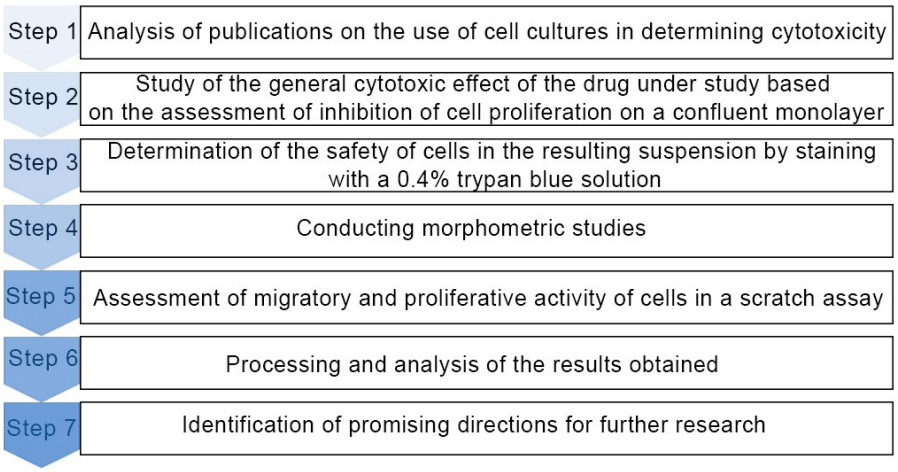


Fig. 1. Study protocol

3. Material and methods

To obtain samples of the medicinal product, we used a thick extract of carrot roots, which was obtained at the Department of Chemistry of Natural Compounds of the NUPh under the supervision of Professor Victoria Kislichenko from carrot roots by extraction using the maceration method with 80 % ethyl alcohol in a raw material and extractant ratio of 1:5 at a water bath heating temperature of 60±5 °C with subsequent concentration on a rotary vacuum evaporator. The extract is a viscous mass of dark brown colour with a characteristic carrot smell. Residual moisture is 17 % [23]. The qualitative composition of the extract was investigated by chromatographic methods, and the quantitative content of polyphenols, hydroxycinnamic acids, and flavonoids, and the sum of steroidal compounds in the extract was determined by spectrophotometric method. The method of high-performance liquid chromatography in carrot seed root extract thick identified 3 phenolic compounds, the total content of which was 12,068.09 mg/kg [24].

To obtain a solid dispersion, a quercetin substance containing 97.5 % to 101.5 % quercetin in its composition, based on dry matter, produced by the Public Joint Stock Company "Scientific and Production Center "Borshahiv Chemical and Pharmaceutical Plant" (Ukraine), was used. The solid dispersion was obtained by dissolving quercetin and a carrier in 90 % ethanol, the remaining solvent was removed by absorption.

The cells were stored in a cryobank of the Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine at a temperature of –196 °C. For experiments, cells were thawed and seeded into culture flasks on nutrient medium DMEM/F12 (Biowest, France) with the addition of 10 % fetal calf serum and antibiotics: benzylpenicillin (200 U/ml (Arterium, Ukraine), streptomycin (40 μg/ml ("Arterium", Ukraine) and amphotericin (5 μg/ml "Biowest", France). Cultivation was carried out at 37ºC in an atmosphere with 5 % CO_2 for 4 passages.

To determine the cytotoxicity of the test agent, cells of the 4th passage were seeded in plastic Petri dishes (SPL LifeSciences, Korea) at a concentration of 1×10^5 cells/ml. Various concentrations of the drug under study were added to the confluent monolayer of cells based on the dense carrot root extract and a solid dispersion of quercetin with colidon-30 and MCC-102 (DCRESDQ) (40, 100, 200 mg/ml), diluted in the nutrient medium DMEM /F12. Incubation was carried out for 40 min at 37ºC in an atmosphere with 5 % CO_2. After this, the solution was washed three times with fresh nutrient medium, and the cells were detached from the surface using a mixture (1:1) of 0.5 % trypsin (Sigma, USA) and Versene (PAA, USA). Kept for 5 minutes at a temperature of 37 °C. After the cells were detached from the surface, they were collected into tubes (15 ml) and washed once with DMEM/F12 medium by centrifugation.

As controls, an intact culture and a cell culture containing an appropriate concentration of quercetin (200 mg/ml) in the nutrient medium were used.

3. 1. Determination of cell safety

The safety of cells in the resulting suspension was assessed by staining with a 0.4 % trypan blue solution [25],

which was added to the cell suspension in a 1:1 ratio. This dye penetrates the damaged membrane of dead cells, staining them blue, while living cells (with an intact membrane) are not stained. Staining with tripan blue allows you to quickly assess the viability of a cell culture, which does not require special expensive reagents and equipment. Cells in the samples were counted using a Goryaev chamber. Safety was determined as the ratio of the number of unstained (living) cells to their total number, expressed as a percentage. Cell observation and microphotography were carried out using an inverted microscope AmScope XYL-403 (China).

3. 2. Morphometric studies

Cell morphology and nuclear-cytoplasmic ratio (NCR) were assessed by [26] in fixed culture preparations stained with hematoxylin-eosin. The micrograph of the cell culture was analysed using the AxioVisionRel. program 4.8 (CarlZeiss, Germany). NCR was calculated using the formula:

$$NCR=An/Ac$$

where *NCR* is the nuclear-cytoplasmic ratio, *An* is the area of the nucleus, *Ac* is the area of the cytoplasm.

3. 3. Assessment of cell migration and proliferative activity in "scratch assay"

Scratch assay was used to assess the migratory and proliferative activity of a monolayer culture [27]. A scratch on the monolayer was made with a plastic tip from a dispenser with a diameter of 0.8 mm. After this, the cell culture was rinsed three times with a nutrient medium to remove detached and damaged cells and returned to culture conditions. Using an AmScope XYL-403 inverted microscope, microphotographs of the initial scratch (0 hours) and its changes after 24 and 48 hours were taken. The area of the scratch was evaluated using the program "Axio Vision Rel. 4.8". The results were presented as the ratio of the area of the scratch at 24 or 48 hours to the area of the initial scratch, expressed as a percentage.

3. 4. Statistical analysis

Data are presented as median with first and third quartiles. The number of experiments was n=4. Each point was processed in 2 parallel samples. Determination of the significance of differences in the obtained data in the compared samples was carried out using the Mann-Whitney (U) test. Differences were considered statistically significant at $p<0.05$.

4. Results

A micrograph of intact (untreated) cells is shown in Fig. 2.

Cells of the L929 line, when grown under adhesive conditions, had a polygonal shape with one or more short protrusions. The cells had a clearly visible nucleus with one or more nucleoli. Light-colored vacuoles were visible in the cytoplasm of some cells. Also, several (2–3) cells at the mitotic stage, as well as oval or round cells, were observed in the field of view.

L929 cells treated with quercetin at a concentration of 200 mg/ml for 40 minutes did not change their morphology and did not lose their ability to adhere. After incubation, a monolayer of cells with a characteristic morphology was present (Fig. 3, *a*, *b*).

Cells of the L929 line were treated with the studied combined agent based on the dense carrot root extract and solid dispersion of quercetin with colidon-30 and MCC-102 (DCRESDQ) at concentrations of 40, 100 and 200 mg/ml within 40 minutes they also did not change their morphology and did not lose their ability to adhesion (Fig. 4, *a*–*c*).

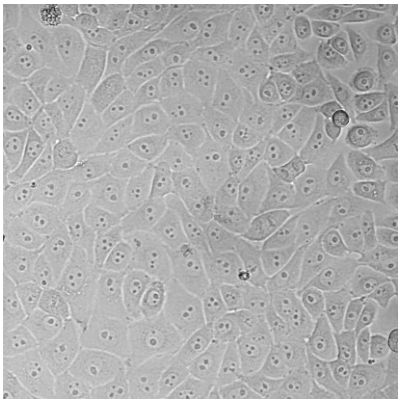
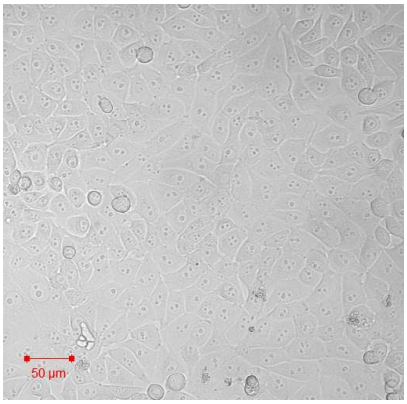
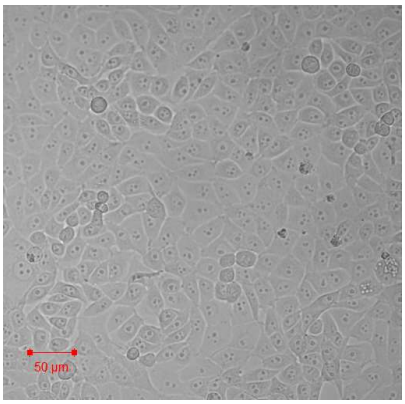


Fig. 2. Monolayer of intact cell culture of the L929 line. Intravital phase-contrast microscopy

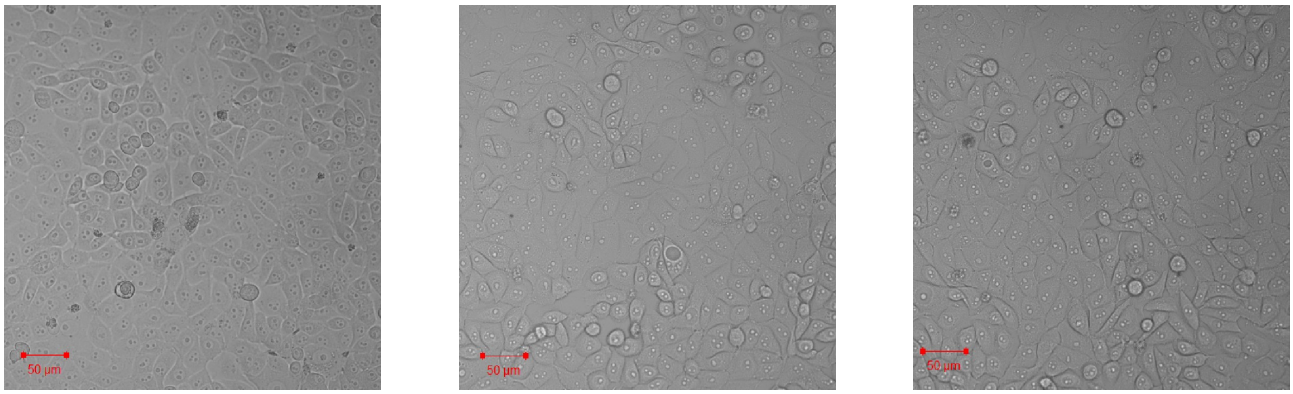


a



b

Fig. 3. Monolayer of L929 cell culture treated for 40 min. quercetin at a concentration of 200 mg/ml; *a* – cells before incubation; *b* – cells after incubation. Intravital phase-contrast microscopy



a *b* *c*

Fig. 4. Monolayer of L929 cell culture treated for 40 min. DCRESDQ at concentrations of 40, 100 and 200 mg/ml. Intravital phase-contrast microscopy. DCRESDQ concentration: *a* – 40 mg/ml; *b* – 100 mg/ml; *c* – 200 mg/ml

Quantitative analysis of cell safety after incubation is presented in Table 1. It is noteworthy that there are no significant differences in the indicator between samples incubated in the presence of quercetin or various concentrations of DCRESDQ and the intact control.

At the next stage of work, NCR was determined to assess the effect of DCRESDQ on L929 cells (Table 2). An intact cell culture under the culture conditions used was characterised by an NCR index of 0.35 (0.32; 0.39). When cells were treated with quercetin or DCRESDQ at concentrations of 40, 100 and 200 mg/ml, NCR did not change significantly compared to the intact control.

Table 1

Indicators of safety of L929 cells after incubation of quercetin and DCRESDQ for 40 minutes

Processing conditions	Concentration, mg/ml	Cell safety, %
Intact control	–	99.2 (98.8; 99.6)
Quercetin	200	82.1 (81.1; 85.5)
DCRESDQ	40	98.6 (97.9; 99.2)
	100	98.4 (97.3; 99.3)
	200	98.8 (98.2; 99.4)

In the "scratch" test, it was found that in an intact cell culture, approximately 50 % closure of the scratch occurs 24 hours after its application, while after 48 hours there is about 30 % of the growth surface unfilled with a monolayer. As follows from the data in Table 3, incubation in the presence of quercetin or various concentrations of DCRESDQ did not significantly affect the ability of L929 cells to migrate and proliferate.

Table 2

Nuclear-cytoplasmic ratio in the L929 cell line cultured with different concentrations of DCRESDQ

Index	Intact control	Quercetin	DCRESDQ, mg/ml		
			40	100	200
NCR	0.35 (0.32; 0.39)	0.35 (0.31; 0.41)	0.36 (0.29; 0.39)	0.34 (0.30; 0.37)	0.35 (0.32; 0.39)

Table 3

"Scratch" area (%) in the L929 cell line cultured with different concentrations of DCRESDQ

Intact control		Quercetin		DCRESDQ, mg/ml					
				40		100		200	
24 hours	48 hours	24 hours	48 hours	24 hours	48 hours	24 hours	48 hours	24 hours	48 hours
49 (43.49; 51.13)	30.19 (24.95; 32.19)	50.05 (47.74; 53.67)	29.51 (27.48; 32.55)	51.22 (48.08; 53.48)	32.14 (28.95; 37.25)	49.64 (48.06; 53.22)	28.67 (27.76; 34.93)	51.27 (48.83; 56.81)	30.24 (28.88; 36.33)

5. Discussion

Determination of toxicity parameters is one of the main stages of preclinical research of new drugs. It should be noted that the acute toxicity of both components of the combination drug under study has been studied in vivo and covered in the scientific literature. In particular, in the USA, quercetin is generally recognised as safe (GRAS) by the Food and Drug Administration [28], it does not exhibit acute and chronic toxicity in vivo [29], the results of some clinical trials showed no signs of toxicity or side effects [30]. Carrot extracts also show no clinical signs of toxicity or mortality when administered intragastrically to rats at a high dose of 2000 mg/kg [31].

The above allowed us to assume that these substances have no toxic effect when combined as part of the studied combination drug DCRESDQ and limit ourselves to studying its possible cytotoxic properties in vitro.

When L929 cells were incubated in a nutrient medium with the addition of both quercetin substance and DCRESDQ, their viability and morphological properties were preserved; images of monolayer culture of these cells under phase-contrast microscopy coincided with the data of quantitative analysis.

In the body, cell size is highly variable, but the ratio of the nuclear area to the cytoplasmic area is a fair-

ly constant indicator [32]. This ratio is an important factor determining the biosynthetic ability of a cell and the implementation of its cell cycle [33]. In an unchanged cell, the increase in DNA, nuclear volume, and cytoplasm are clearly coordinated. Deviation from normal for a certain type of NCR cell is an unfavorable sign (intoxication, aging, apoptosis, oncotransformation, etc.) [34]. The absence of changes in the NCR was further evidence of the absence of the toxic effect of DCRESDQ concerning fibroblast culture.

The monolayer "scratch" test (scratch assay) is a generally accepted and informative method for assessing the migratory and proliferative activity of cells in vitro [35]. This test is based on the fact that cells migrate to an area with lower cell density, after which they proliferate [36]. Migration-proliferative processes continue until the cell density at the "scratch" site reaches the normal density of the monolayer. According to the results of our experiment, the migration and proliferative activity of L929 cells under the influence of DCRESDQ did not differ from intact cells.

Practical relevance. The results experimentally justify the expediency of further research on the drug as a potential drug with antiatherogenic and cardioprotective properties for the prevention and pharmacocorrection of cardiovascular pathology.

Study limitation. Specific toxicity has not been studied.

Further research prospects. Study of the selective cytotoxic effect of the test substance, including based on data on impaired membrane excitability, determination of hepatotoxicity, and the role of biotransformation in the toxic effect of the test substance on the cell.

6. Conclusions

The results of the toxicological study showed that the combined product based on the dense carrot root extract and solid dispersion of quercetin with colidon-30 and MCC-102 does not have a cytotoxic effect on the cell culture of the L929 line and can be recommended for further preclinical research in order to create a drug with antiatherogenic and cardioprotective properties for the prevention and pharmacocorrection of heart and vascular diseases.

Conflict of interest

The authors declare that they have no conflict of interest related 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 at a 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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