PROCEEDINGS OF THE YEREVAN STATE UNIVERSITY

Chemistry and Biology 2023, **57**(3), p. 238–247

Biology

THE CYTOTOXIC PROPERTIES OF *ALCHEMILLA SMIRNOVII* EXTRACT ON THE HELA CANCER CELLS VIA DOWNREGULATION OF ARGINASE ACTIVITY

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Plants of the genus Alchemilla are well known for their pharmacological effects. *Alchemilla smirnovii* Juz., also known as lady's mantle possessed high growth-inhibiting properties against cancer cells according to earlier reports. We aimed to explore the anticancer modulatory effect of *A. smirnovii* extract toward 5-fluorouracil and elucidate the possible mechanism of its cytotoxic action in the HeLa cancer cell model. Synergistic interactions of plant extract with the chemotherapeutic agent were assessed by MTT assay. Based on obtained data the ethanol extract of *A. smirnovii* exhibits synergistic effects, enhancing the growth-inhibiting properties of 5-fluorouracil when used together. Additionally, the extract leads to a reduction in arginase activity and an elevation in the level of MDA. This implies that *A. smirnovii* induces oxidative stress, and can potentially cause apoptosis in cancer cells.

https://doi.org/10.46991/PYSU:B/2023.57.3.238

Keywords: *Alchemilla smirnovii*, HeLa, anticancer effect, 5-fluorouracil, arginase.

Introduction. According to the published data, the death rate for all cancers was 197.93 per 100.000 people in the Republic of Armenia in 2020, which is already higher than the death rate caused by cardiovascular diseases (194.68 per 100.000 people) (World Health Ranking 2020). Cervical cancer is one of the most common cancers in women, so current research is being conducted on the HeLa cancer cell line [1]. The risk of cancer may be reduced by arginine amino acid due to its beneficial effect on regulating nutrient metabolism and T cells [2].

Notably, L-arginine, as a substrate of arginase, is also the sole substrate of nitric oxide synthase (NOS), which metabolizes arginine to L-citrulline and nitric oxide (NO). NO plays a dual role in tumor growth depending on its concentration. At low to moderate concentrations, NO stimulates cancer cell progression, prevents apoptosis, and enhances angiogenesis and metastasis [3].

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Under pathological conditions, increased arginase expression/activity can lead to the uncoupling of NOS and the formation of the superoxide anion O^{2-}, which can react with NO to form peroxynitrite ($ONOO^-$) [4]. O^{2-} and $ONOO^-$ are highly reactive and can damage intracellular macromolecules, including polyunsaturated fatty acids (PUFAs) and nucleic acids. Oxidation of PUFAs by reactive oxygen species (ROS) leads to lipid peroxidation, in which peroxidized PUFAs, together with their breakdown products (e.g., 4-hydroxy-2-nonenal (4-HNE)), can act as signaling molecules that stimulate inflammation, apoptosis, or ferroptosis [5].

For a long time, natural and herbal products have been considered precise sources of healing, used in traditional medicine to treat various diseases, including infections and malignant diseases [6]. Clinical experience has proven that certain herbs and their bioactive compounds are effective against a variety of cancers through various mechanisms, effectively improving the quality of life of patients without significant side effects [7]. Earlier in our work, we screened the cytotoxic properties of ethanolic extracts of 10 plant species on cancer cell lines of various origins. Based on the findings, five of the tested plant extracts showed strong growth-inhibiting properties. *Alchemilla smirnovii* Juz., also known as lady's mantle (general name of the genus) expressed one of the highest growth-inhibiting properties against A549 (human lung adenocarcinoma) and HeLa (human cervical carcinoma) cancer cells [8].

Plants of the genus *Alchemilla* are well known for their pharmacological effects. They have been widely used in folk medicine to treat various medical conditions including diabetes, multiple sclerosis, anemia, ulcers, hernias, gynecological and abdominal disorders, purulent wounds, rashes, purulent wounds, eyelid inflammations, etc. [8, 9]. Many biological activities of the plant species within the genus have been reported in the literature including anticancer, antibacterial, antifungal, and antiviral properties [9]. Based on ethnopharmacological and literature data, as well as promising cytotoxicity results according to a screening of *A. smirnovii* ethanol extract on different cancer cell lines we decided to continue elucidating the anticancer potential of this plant and explore the possible mechanism of its action. The anticancer modulatory effect of *A. smirnovii* extract on anti-cancer drug 5-fluorouracil (5-FU) was also interesting. 5-FU is an analog of uracil and is widely used in chemotherapy therapy [10]. However, this drug has many side effects, and over time, the body's cells become resistant to it [11]. We hypothesized that the combination of 5-FU and a plant extract can suppress the side effects of chemotherapy drugs and exhibit a possible synergistic effect to reduce the therapeutic concentration of the drug.

Materials and Methods.

Chemicals and Reagents. All chemicals and reagents were purchased from "Sigma-Aldrich GmbH" (Taufkirchen, Germany).

Plant Material Collection, Identification, and Extraction. The *Alchemilla smirnovii* Juz. plant aerial parts were harvested from the Tavush Region of Armenia (1800–2400 *m* a. s. l.) during the flowering period (June–July). Identification of plant materials was done at the Department of Botany and Mycology, YSU (Armenia) by Dr. Narine Zakaryan. A plant sample was deposited to the Herbarium of YSU and a voucher specimen number was provided. 50 *mg* DW/*mL* plant crude extract was

prepared by maceration technique using pure ethanol (96%) at a 10:1 solvent-to-sample ratio (v/w) [12]. The percent yield of the extract was 30.67 ± 2.31% [8].

Cell Culture. HeLa (human cervical carcinoma) cells have been maintained in Dulbecco's Modified Essential Medium (DMEM) supplemented with 10% Bovine serum and 1× Pen/Strep. Cells have been seeded in tissue culture-treated 96-well plates at a maximum density of $2 \cdot 10^5$ cells/cm^2. The cells were propagated at 37°C in an atmosphere of 5% CO_2 in a CO_2 incubator ("Biosan S-Bt Smart Biotherm", Latvia).

MTT Cytotoxicity Assay. The MTT test [13] was performed to assess the inhibition of growth of HeLa cells exposed for 4, 24, or 72 *h* to different concentrations (0.5, 0.25, and 0.125 *mg* DW/*mL*) of the *A. smirnovii* extract. Treatments were performed as four technical replicates. Three independent replicates of each treatment were carried out. Cytotoxicity was expressed as percent growth inhibition of cells exposed to tested plant extract compared to control cells treated with the appropriate volume of solvent only (1% ethanol in the final mixture), whose growth was regarded as 100%.

Arginase and NOS Activity, NO and MDA Quantity Assay. HeLa cells were seeded in 24-well ($5 \cdot 10^4$ cells per well) plates and incubated for 24 *h*. After incubation, the medium in wells (450 μL) was refreshed. The cells were treated with 50 μL control or test compounds with the following final concentrations: phosphate-buffered saline (PBS), 1% ethanol (Control, HeLa), 5-FU (40 μM), AS (0.125 and 0.25 mg/*mL*). Each test sample (50 μL) was added to five different passages of HeLa cells, which were triplicated [14]. After 24 *h* incubation, the medium without cells was used for the determination of nitrite anions and malondialdehyde (MDA) amounts. Cells from each group were collected (trypsinized, neutralized, centrifuged), lysed on ice with Lysis buffer, collected in a centrifuge tube, and further lysed for 10 *min*. After centrifugation at 13 000 *g* for 10 *min* at 4°C, the supernatant was collected. The levels of Nitrite anions, MDA, Arginase, and NOS were quantified according to the methods described below [15, 16].

NO Quantity Measurement. NO levels in the cell culture medium were determined as nitrite anions. Griess assay was used for measurement as described before [17]. 100 μL Griess reactant was added to 100 μL of each sample. The supernatants were transferred to the tubes containing pellets of cadmium and incubated at room temperature for 12 *h* to convert nitrate to nitrite. The samples' absorbance was measured at λ = 550 *nm* and the NO quantity was calculated based on a standard curve prepared with $NaNO_2$.

MDA Assay. MDA quantity in the cell culture medium was determined with a colorimetric assay using the Ohkawa thiobarbituric acid-malondialdehyde method [18].

Determination Arginase Activity. The modified Diacetyl Monoxime colorimetric method was employed to assess the arginase activity in cell lysates [19].

Determination of NOS Activity. Nitric oxide synthase activity (μmol citrulline/*mg* protein) in cell lysates was measured by the conversion of L-arginine to L-citrulline [20]. 100 μL of cell lysates was added to 200 *mL* of reaction mixture 50 *mmol*/*L* Tris buffer, pH 7.4, containing 10 *mmol*/*L* dithiothreitol (DTT), 10 μmol/*L* THB4, 10 μg/*mL* calmodulin, 1 *mmol*/*L* NADPH, 4 μmol/*L* flavin adenine

dinucleotide (FAD), 4 *μmol/L* flavin mononucleotide (FMN), and 2 *μmol/L* L-arginine. The assay was carried out at 37°C, and it was terminated with 2 *mL* of ice-cold stop buffer (20 *mmol/L* CH_3COONa, pH 5.5, containing 2 *mmol/L* EDTA, 0.2 *mmol/L* EGTA, and 1 *mmol/L* L-citrulline). Assays were systematically performed with Ca^{2+} (1 *mmol/L* $CaCl_2$) or without Ca^{2+} to measure total versus Ca^{2+}-independent NOS activities. The Ca^{2+}-dependent NOS activity was calculated as total NOS activity minus Ca^{2+}-independent NOS activity. All assays were performed in duplicate on aliquoted samples (to avoid freezing/thawing cycles). The results were normalized for protein content.

Statistical Analysis. The obtained results were presented as the mean values with standard errors (M±SD). Statistical analyses were performed using GraphPad Prism 8 software (San Diego, USA), and a significance level of $p < 0.05$ was deemed statistically significant.

Results.

Cytotoxic Properties of A. smirnovii Extract and 5-Fluorouracil. At first, we separately investigated the growth-inhibiting effects of 5-fluorouracil and *A. smirnovii* aerial part extract on HeLa cells. The plant extract suppressed the growth of HeLa cells in all three exposure times: 4, 24, and 72 *h*. During 24 *h* exposure time it expressed significant growth inhibit properties even at the lowest tested concentrations 0.125 *mg* DW/*mL* (Fig. 1, A) ($p < 0.05$). For 4 *h* and 72 *h* exposure times the extract exhibited strong growth-inhibiting properties only at the highest tested concentration 0.5 *mg* DW/*mL*. 5-FU expressed high cytotoxic properties on HeLa cells even at the lowest tested concentration and the longest exposure time (10 *μM* and 72 *h*) (Fig. 1, B). For 24 *h* and 4 *h* exposure times 5-FU possessed considerable cytotoxicity on HeLa cells starting from 20 *μM* and 80 *μM* concentrations respectively. The IC_{50} values of 5-FU and *A. smirnovii* extract were determined for HeLa cells for only 24 *h* and 72 *h* exposure times. For 4 *h* exposure time, it was not possible to calculate IC_{50} values for both *A. smirnovii* extract and 5-FU. The IC_{50} values for 5-FU were 90.81 *μM* (24 *h*) and 12.09 *μM* (72 *h*). The IC_{50} values for as extract were 0.3661 *mg* DW/*mL* (24 *h*) and 12.09 *mg* DW/*mL* (72 *h*) respectively (Fig. 1, C) ($p < 0.05$). For modulatory studies, 0.25 *mg* DW/*mL* concentration of plant extract and 10 *μM* concentration of 5-FU were chosen.

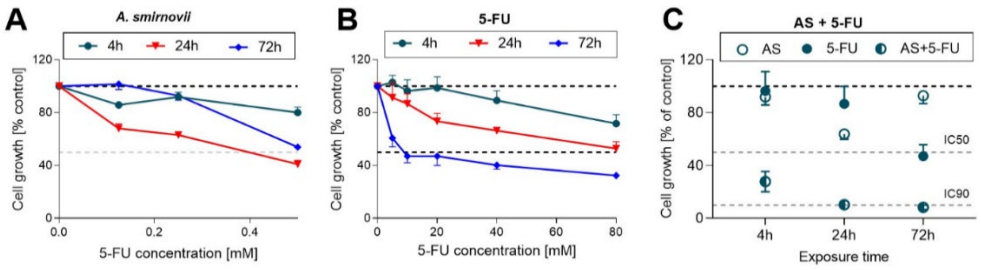

Fig. 1. Inhibition of growth of HeLa cells by *A. smirnovii* extract (A), 5-fluorouracil (5-FU) (B), and combination of *A. smirnovii* (AS) extract and 5-FU (C) (subinhibitory concentrations of plant extract and 5-FU were used: 0.25 *mg* DW/*mL* and 10 *μM*, respectively) determined by MTT test ($n = 3$, $p < 0.05$).

Synergistic Effect of A. smirnovii Ethanol Extract on 5-Fluorouracil against the Growth of HeLa Cervical Cancer Cells. Subinhibitory concentrations of the plant extract and 5-FU were used during the evaluation of their combined effect on the growth of HeLa cells. Based on obtained data the plant extract significantly increased the cytotoxic effect of 5-FU by acting synergically in all tested exposure times. Even at tested lowest exposure time the extract and 5-FU did not express any noticeable inhibiting properties their combination reduced the growth of HeLa cell by more than 70% (Fig. 1, C). For longer exposure times the combination inhibited the cell growth by more than 90%.

Changes of Quantity MDA and NO, and Arginase and NOS Activity in the Conditions of Different Concentrations of the A. smirnovii on HeLa Cells. In the second phase of the research work various components were determined in HeLa cells after treatment of cells with the plant extract to understand the possible mechanisms of anticancer action of *A. smirnovii* extract. Particularly, the effect of the *A. smirnovii* extract was studied on the change in the activity of arginase, and NOS enzymes, and the change in the quantities of nitrite ions and malondialdehyde. These components were chosen taking into account their central role in tumor development. 5-FU was used as a positive control.

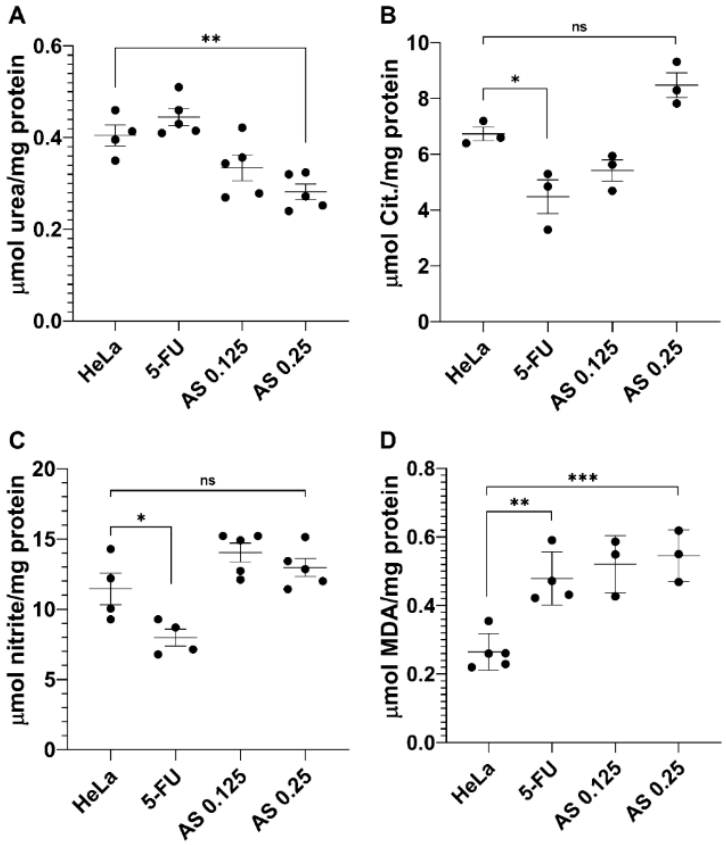

Fig. 2. Evaluation of changes in the activity of arginase, NOS enzymes, nitrite ions, and MDA after the treatment of 0.125 *mg* DW/*mL* and 0.25 *mg* DW/*mL* concentration of the *A. smirnovii* for 24 *h* ($n = 3$, $p<0.05$).

The results showed that a statistically significant decrease in arginase activity was observed in the group treated with the 0.25 *mg*/*mL* concentration of *A. smirnovii* extract compared to the control group (Fig. 2, A). There was no significant change in the quantity of NO and NOS (Figs. 2, C, B). In the case of MDA, an increase in its quantity was observed at concentrations of 0.25 *mg*/*mL* and 0.125 *mg*/*mL*, 2 times, compared to the control group (Fig. 2, D).

Discussion. In our earlier research works, we reported high-growth inhibiting properties of *A. smirnovii* aerial part ethanolic extract on different cancer cell lines. We also showed the high DPPH radical scavenging activity of *A. smirnovii* extract and emphasized its potential as a source of antioxidant compounds [8].

Therefore, it was interesting to explore the possible mechanisms of cytotoxic properties of this plant. Particularly, we studied changes in the amount of NO and MDA, as well as the activity of NOS and arginase which play an important role in cancer development. On the other hand, using plant extracts or derived compounds to modulate the anticancer effectiveness of chemotherapy is a promising strategy for overcoming drug resistance and reducing the side effects of drugs [15]. In recent years more and more attention has been given to the exploration of the anticancer effects of combining herbal extracts with classical chemotherapeutic compounds.

As part of this approach, we investigated the modulating activity of sub-inhibitory concentrations of *A. smirnovii* extracts on fluorouracil. Thus, when we explored the cytotoxicity of the 5-fluorouracil combined with *A. smirnovii* ethanol extract, the decrease in HeLa cell growth was significantly higher compared to their exposure separately, which confirms the synergism of the combination. Evaluation of the combined effect of the herb and chemotherapeutic agent at 4, 24, and 72 *h* showed a higher anti-proliferative effect. Considering the separate effect of the herb and drug on the HeLa cervical cancer cells at 4, 24, and 72 *h*, it was found that the highest anti-cancer effect of the herb was observed at 24 *h*.

Chronic irritation, infection, or inflammation can cause different types of cancers. In this process, a critical role is the arginase enzyme because it can regulate polyamine metabolism [16]. High levels of polyamines are associated with an increase in tumorigenesis. Therefore, the inhibition of arginase can effectively decrease the amounts of polyamines during cancer, thereby restraining tumor growth and having an antiproliferative effect [16]. Accordingly, boosted catalytic enzymatic activity of arginase leading to increased polyamine production represents a potential mechanism for arginase working as a pro-tumor factor [3]. In cancer cells, high levels of reactive oxygen species can result from increased metabolic activity, mitochondrial dysfunction, peroxisome activity, increased cellular receptor signaling, oncogene activity, increased activity of oxidases, cyclooxygenases, lipoxygenases, and thymidine phosphorylase, or through crosstalk with infiltrating immune cells [21]. Recent data showed that inflammation is a critical component of tumor progression. Macrophages induce the generation of ROS within tumor cells through the secretion of various stimuli, such as TNFα [1]. The production of ROS by neutrophils and macrophages as a mechanism to kill tumor cells is well established. Furthermore, during inflammation processes, activated macrophages also generate nitric oxide which reacts with superoxide to produce peroxynitrite radicals that are

similar in their activity to hydroxyl radicals and contribute to tumor cell apoptosis [21].

Experiments carried out on cell medium and lysate showed that at 0.25 *mg* DW/*mL* a concentration of *A. smirnovii* there was a decrease in the activity of the arginase enzyme; in the case of NOS and NO, no significant changes were observed, in addition, the plant does not have a pronounced antiangiogenic effect. At the same time, quantitative changes in MDA showed the influence of the plant on the activity of enzymes of the antioxidant system, promoting an increase in the amount of MDA, which, in turn, indicates the occurrence of oxidative stress. Furthermore, the high level of ROS and low level of polyamines, develop apoptosis and show antiproliferative effect, preventing the process of cancer development (Fig. 3).

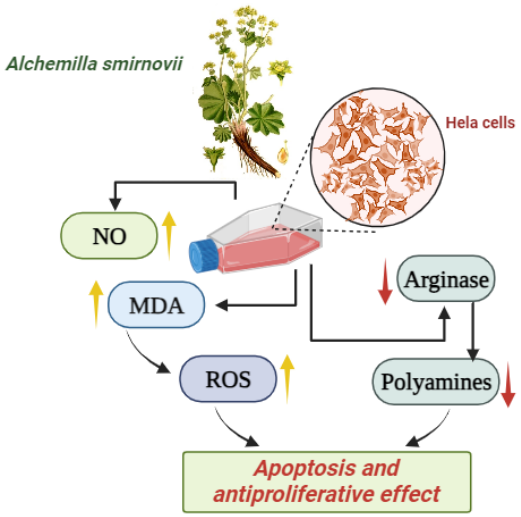

Fig. 3. Anticancer and antiproliferative mechanism of *A. smirnovii* ethanol extract on HeLa cancer cells.

Conclusion. *A. smirnovii* ethanol extract acts synergically increasing the growth-inhibiting properties of 5-fluorouracil when used in combination. *A. smirnovii* extract brought to decrease in the activity of the arginase enzyme and an increase in the amount of MDA in the HeLa cell medium treated with plant extracts, which suggests that *A. smirnovii* induce oxidative stress in cells and thereby can inhibit the progression of cancer via inducing apoptosis. These promising results will serve as a basis for further, more comprehensive research, including identifying active phytochemicals contained in the extract which can be responsible for their bioactive properties and elucidate the molecular mechanisms of their joint anticancer effects.

This work was supported by the Science Committee of MESCS RA, in the frames of Basic support to the Research Institute of Biology, YSU, of the research project № 21T -1F283.

Received 15.11.2023
Reviewed 29.11.2023
Accepted 15.12.2023

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Ն. Վ. ԱՎԹԱՆԴԻԼՅԱՆ

ALCHEMILLA SMIROVII JUZ. ԲՈՒՅՍԻ ԼՈՒԾԱՄՋՎԱԾՔԻ ԲՋՋԱՏՈՔՍԻԿ ԱԶԴԵՑՈՒԹՅԱՆ ԵՎ ՀԱԿԱՔԱՂՑԿԵՂԱՅԻՆ ՀԱՏԿՈՒԹՅՈՒՆՆԵՐԻ ԳՆԱՀԱՏՈՒՄԸ HELA ՔԱՂՑԿԵՂԱՅԻՆ ԲՋՋԱՅԻՆ ԳԾՈՒՄ ԱՐԳԻՆԱԶԻ ԳՈՐԾՈՒՆԵՈՒԹՅԱՆ ԿԱՐԳԱՎՈՐՄԱՆ ՄԻՋՈՑՈՎ

Alchemilla ցեղի բույսերը հայտնի են իրենց դեղաբանական ազդեցությամբ: *Alchemilla smirnovii* Juz.-ը, որը նաև հայտնի է «կանացի թիկնոց» անվամբ, համաձայն ավելի վաղ զեկույցների՝ ունի քաղցկեղի բջիջների աճը արգելակող բարձր հատկություններ: Աշխատանքի նպատակն է ուսումնասիրել *A. smirnovii* էթանոլային լուծամզվածքի հակաքաղցկեղային մոդուլացնող ազդեցությունը 5-ֆլուորոուրացիլի նկատմամբ և պարզաբանել բջջատոքսիկ ազդեցության հնարավոր մեխանիզմը HeLa մարդու արգանդի վզիկի քաղցկեղի բջիջների մոդելում: Բույսի լուծամզվածքի սիներգետիկ փոխազդեցությունը քիմիաթերապևտիկ միացության հետ գնահատվել է 3-(4,5-դիմեթիլթիազոլիլ)-2,5-դիֆենիլտետրազոլիում բրոմիդ թեստի միջոցով: Արդյունքները ցույց են տվել, որ *A. smirnovii*-ի էթանոլային լուծամզվածքը ցուցաբերում է սիներգետիկ ազդեցություն 5-ֆտորուրացիլի հետ՝ ուժեղացնելով վերջինիս քաղցկեղային բջիջների աճը արգելակող հատկությունները: Բացի այդ, լուծամզվածքը արգելակում է արգինազ ֆերմենտի ակտիվությունը և բարձրացնում ՄԴԱ (մալոնիլդիալդեհիդ) մակարդակը HeLa բջիջների միջավայրում: Սա ենթադրում է, որ *A. smirnovii*-ն առաջացնում է օքսիդային սթրես բջիջներում՝ արգելակելով քաղցկեղի զարգացումը:

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ЦИТОТОКСИЧЕСКИЕ СВОЙСТВА ЭКСТРАКТА *ALCHEMILLA SMIRNOVII* НА РАКОВЫЕ КЛЕТКИ HELA, ПРОЯВЛЯЕМЫЕ ЗА СЧЕТ СНИЖЕНИЯ АКТИВНОСТИ АРГИНАЗЫ

Растении рода Alchemilla хорошо известны своим фармакологическим действием. Согласно более ранним сообщениям, *Alchemilla smirnovii* Juz., также известная как дамская манжетка, обладет высокими ингибирующими свойствами на рост раковых клеток. В данной работе мы изучили противораковое модулирующее действие экстракта *A. smirnovii* по отношению к 5-флуороурацилу и возможные механизмы его цитотоксического действия на раковые клетки HeLa. Синергическое взаимодействие растительного экстракта с химиотерапевтическим средством оценивали с помощью МТТ-анализа. На основании полученных данных этаноловый экстракт *A. smirnovii* проявляет синергический эффект, усиливая ростингибирующие свойства 5-флуороурацила при совместном применении. Кроме того, экстракт приводит к снижению активности фермента аргиназы и повышению уровня MDA в клеточной среде HeLa. *A. smirnovii* может вызывать окислительный стресс в клетках, потенциально подавляя прогрессирование рака.