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Original article

Examining apoptotic activity of *Gratiola officinalis* L. (*Scrophulariaceae*) extract on cultured human tumor cell lines

Natalya N. Polukonova [1], Nikita A. Navolokin [1], Maria A. Baryshnikova [2], Galina N. Maslyakova [1], Alla B. Bucharskaya [1], Anna V. Polukonova [1]

[1] V.I. Razumovsky Saratov State Medical University, Saratov, Russia
[2] N.N. Blokhin Research Center for Oncology, Moscow, Russia

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Abstract: *Objective* — To investigate the apoptotic activity of *Gratiola officinalis* L. extract on human tumor cell lines by flow cytofluorometry.
Material and Methods — The extract of *Gratiola officinalis* L. was manufactured via our original methodology. Studies were performed on human tumor cell lines: HeLa – cervical carcinoma, Jurkat – T-cell lymphoblastic leukemia, MCF-7 – breast adenocarcinoma, A549 – lung carcinoma, PC-3 – prostate carcinoma, HCT-116 – colon carcinoma, A498 – renal carcinoma, and SK-BR-3 – human breast carcinoma. Induction of apoptosis was studied after incubating cell lines with *Gratiola officinalis* L. extract at a concentration of 0.9 mg/mL using the Annexin V-FITC Apoptosis Kit. Caspase-dependent apoptosis was examined on a flow cytometer using anti-caspase-3-FITC (BD) kit on the Jurkat cell line. Morphological studies of HeLa cervical carcinoma cells in the *alive and dead* test were performed using two stains, acridine orange and propidium iodide, at different concentrations of the extract. The statistical data processing was performed using Microsoft Office Excel software.
Results — One day after their exposure to *Gratiola officinalis* L. extract at a concentration of 0.9 mg/mL, tumor cells were mostly in late apoptosis stage. Cytotoxic activity of *Gratiola officinalis* L. extract was established for all investigated tumor cell cultures but their sensitivities to the extract were different. Mechanisms of antitumor action of *Gratiola officinalis* L. extract were identified: we established that the extract induced caspase-dependent apoptosis in tumor cells.
Conclusion — The identified mechanisms of apoptotic activity of *Gratiola officinalis* L. extract confirmed the prospects of bioflavonoids as new-generation antitumor agents.

Keywords: *Gratiola officinalis* L. extract, flavonoids, human tumor cell lines, flow cytofluorometry.

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Correspondence to Alla B. Bucharskaya. Address: 112 Bolshaya Kazachya St., Saratov 410012, Russia. Phone: +79053850895. E-mail: allaalla_72@mail.ru.

Introduction

Despite the large number of available antitumor medicines used in the treatment of cancer patients, many unresolved problems remain, including pronounced toxicity of multi-course chemotherapy [1] and formation of multiple drug resistance [2].

Bioflavonoids with a verified broad spectrum of action can become potential new-generation antitumor agents. Their ability to enhance the effectiveness of cytostatic therapy by weakening its toxic effects on healthy cells and activation of apoptosis in tumor cells was demonstrated [3]. Previously, in *in vitro* experiments on renal cancer cell cultures (Caki-1 and SN12c) [4] and in *in vivo* experiments on transplanted tumors of laboratory animals [5], we discovered that the extract of *Gratiola officinalis* L., containing flavonoids in its composition [6], has antitumor and apoptotic effects. The study was conducted only on two cell cultures of human kidney cancer; therefore, the study of apoptosis in tumors of other histogenesis is relevant. In addition, the mechanisms of the *Gratiola officinalis* L. extract antitumor activity were not fully investigated.

Objective: To explore the apoptotic activity of *Gratiola officinalis* L. extract on cultured human tumor cell lines (HeLa – cervical carcinoma, Jurkat – T-cell lymphoblastic leukemia, MCF-7 – breast adenocarcinoma, A549 – lung carcinoma, PC-3 – prostate carcinoma, HCT-116 – colon carcinoma, A498 – renal carcinoma, SK-BR-3 – human breast carcinoma) and identify possible mechanisms of its development.

Material and Methods

The extract of *Gratiola officinalis* L. was manufactured using our original methodology [6]. The chemical composition of *Gratiola officinalis* L. extract was examined via high-performance liquid chromatography and tandem mass spectrometry (HPLC-MS/MS), and luteolin-7,3'-di-O-glycoside, apigenin-7-O-glucoside, along with trace amounts of eupatin and pentacyclic triterpenoid, soyasapogenol B 3-(1-2)-glucoside, were detected [7].

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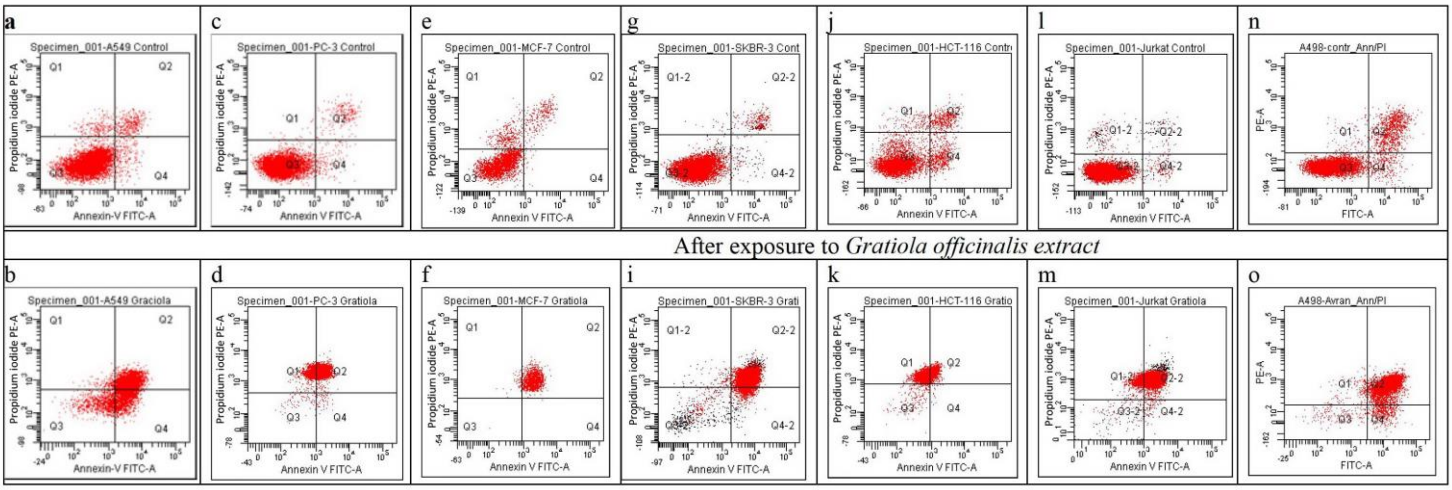

Figure 1. The results of flow cytofluorometry after the exposure to *Gratiola officinalis* L. extract at a concentration of 0.9 mg/mL on human tumor cells. A549 – lung carcinoma (a – no exposure; b – with extract); PC-3 – prostate carcinoma (c – no exposure; d – with extract); MCF-7 – breast adenocarcinoma (e – no exposure; f – with extract); SK-BR-3 – human breast carcinoma (g – no exposure; h – with extract); HCT-116 – colorectal carcinoma (i – no exposure; j – with extract); Jurkat – T-cell lymphoblastic leukemia (l – no exposure; m – with extract); A498 – renal carcinoma (n – no exposure; o – with extract).

Table 1. Distribution of tumor cells after exposure to *Gratiola officinalis* L. extract according to flow cytofluorometry

Cell lines	*Group*	*Square Q3 Living cells (AnV^-/PI^-), %*	*Square Q4 Early apoptotic cells (AnV^+/PI^-), %*	*Square Q2 Late apoptotic cells (AnV^+/PI^+), %*	*Square Q1 Necrotic cells (AnV^-/PI^+), %*
A549	Control	90.8±3.1	2.9±1.5	4.1±1.2	2.3±0.2
	Extract	15.2±5.3*	26.9±3.4*	55.4±7.1*	2.5±0.5
PC-3	Control	95.7±2.7	1.6±1.1	2.4±0.8	0.2±0.1
	Extract	1.2±0.4*	0.9±0.4	68.6±3.7*	29.3±4.8*
HCT-116	Control	79.4±2.4	9.9±2.8	7.6±1.8	3.0±1.2
	Extract	4.4±1.1*	0.4±0.8*	27.8±4.5*	67.4±7.1*
MCF-7	Control	81.5±4.9	0.7±0.5	8.0±2.2	9.9±2.4
	Extract	0.1±0.1*	0.1±0.1	96.8±5.5*	3.1±1.2*
SK-BR-3	Control	96.7±2.8	0.9±0.4	2.3±1.2	0.2±0.1
	Extract	1.3±0.2*	1.5±0.5	96.3±2.7*	0.9±0.2
Jurkat	Control	97.2±1.5	1.4±0.7	0.4±0.2	1.0±0.4
	Extract	1.0±0.2*	0.9±0.3	79.7±3.8*	18.4±3.2*
A498	Control	82.9±3.1	3.8±3.1	12.2±3.1	1.1±0.2
	Extract	2.1±0.6*	6.4±1.8	89.5±3.3*	2.0±0.4

* statistical significance of differences at $p<0.05$ and $T>1.96$ between experimental and control groups was determined via Cramér-Welch's test. AnV, Annexin V-FITC staining solution; PI, propidium iodide staining solution.

We investigated apoptotic activity on human tumor cell lines: HeLa – cervical carcinoma, Jurkat – T-cell lymphoblastic leukemia, MCF-7 – breast adenocarcinoma, A549 – lung carcinoma, PC-3 – prostate carcinoma, HCT-116 – colon carcinoma, A498 – renal carcinoma, and SK-BR-3 – human breast carcinoma, obtained from the Cell Line Bank of N.N. Blokhin Research Center for Oncology of the Russian Federation Ministry of Healthcare. Cell lines were cultured on complete nutrient medium RPMI-1640 (PanEco, Russia) containing 10% fetal calf serum (TES, HyClone, USA); 2 mM/mL glutamine (PanEco, Russia), 50 mg/mL penicillin-streptomycin (PanEco, Russia) at 37^{0}C in an atmosphere containing 5% CO_2. Cells at 70-80% of monolayer surface were used for experiments. The cell lines were cultivated in the growth medium RPMI-1640 (PanEco, Russia) containing 10% fetal calf serum (TES, HyClone, USA); 2 mM/mL of glutamine (PanEco, Russia), 50 mg/mL of penicillin-streptomycin (PanEco, Russia) at 37 °C in an atmosphere containing 5% of CO_2. For experiments, cells were used on 70-80% of the monolayer surface.

Morphological studies of HeLa cervical carcinoma cells in the *alive and dead* test were performed using two dyes, acridine orange and propidium iodide according to [8] at different concentrations of the extract: 0.4; 0.9; 1.5; 3.0; 6.0; 12.0; and 24.0 (mg/mL). The experiment was carried out in three repetitions for each concentration, the values were evaluated after 24 hours in at least five fields of view for each repetition. Leica DMI microscope (Leica Microsystems, Germany) was used to visualize cells. Images were captured and analyzed using Leica

DFC420 C digital video camera and Leica Application Suite V 3.1 software (Leica Microsystems, Germany). ImageJ software (USA) was used for cell counting.

Induction of apoptosis was examined on a FACS CantoII flow cytometer (Beckton Dickenson, USA). Tumor cells were incubated with *Gratiola officinalis* L. extract at a concentration of 0.9 mg/mL for 24 h using propidium iodide and Annexin V-FITC Apoptosis Kit (Invitrogen, Life Technologies, USA). Annexin V binds to phosphatidylserine, which reaches the surface of the cell membrane in the early stage of apoptosis. Propidium iodide (PI), binding to DNA of destroyed cells, is a marker of the late-stage apoptosis or necrosis.

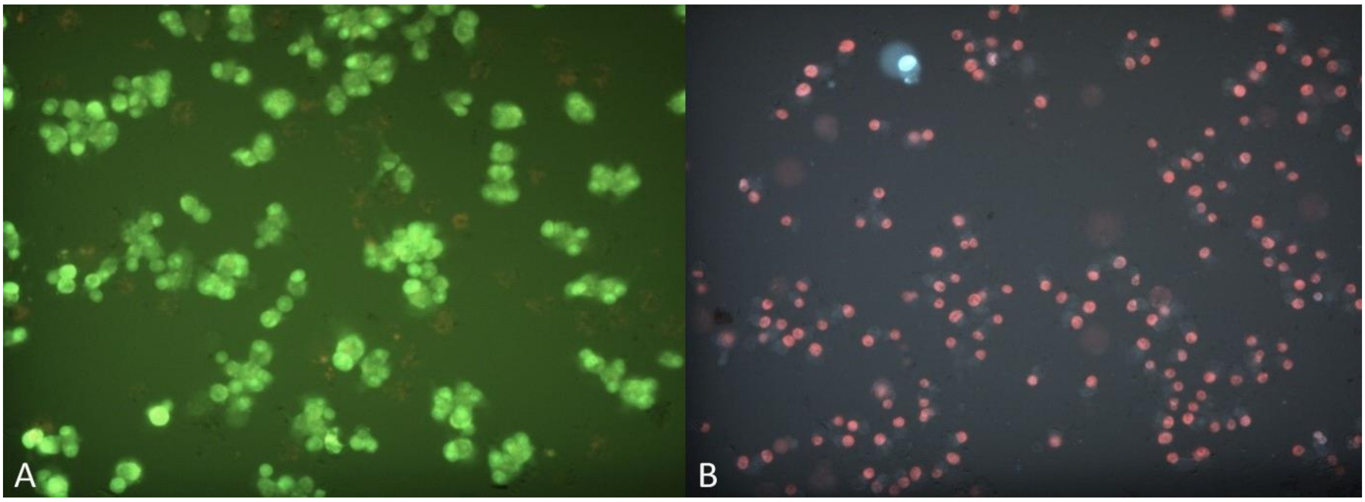

Figure 2. HeLa cultured cell line after exposure to *Gratiola officinalis* L. extract at 0.90 mg/mL (A) or 3.0 mg/mL (B). Fluorescence staining with acridine orange (green) and propidium iodide (red). Magnification ×200.

Table 2. Morphological parameters of HeLa cultured cell line after exposure to *Gratiola officinalis* L. extract

Group	*Control*	*0.4 mg/mL*	*0.9 mg/mL*	*1.5 mg/mL*	*3.0 mg/mL*	*6.0 mg/mL*	*12 .0 mg/mL*	*24.0 mg/mL*
MNC	510±12.61	161±13*	133.3±10*	123.0±9*	132±11*	95.4±12*	66±7*	142±10*
Proportion (%) of dead cells	0	8±1*	13.2±1.5*	41±5*	97.6±8*	93.6±10*	99.5±12*	100±8*
Proportion (%) of cells with karyopyknosis	0	4.4±0.3*	6.2±0.5*	19.1±1*	0	0	0	0

* statistical significance of differences at p<0.05 and T>1.96 between experimental and control groups was determined via Cramér-Welch's test. MNC, Mean number of cells per field of view.

Caspase-dependent apoptosis (activation of caspase-3) was investigated via a flow cytometer using anti-caspase-3-FITC (BD) on the Jurkat lymphoblastic leukemia cell line. To carry out the reaction, cells were removed, washed in phosphate-buffered saline, and resuspended in Annexin V binding buffer at a concentration of 1 million cells/mL. Then 100 μL of cells were transferred into tubes containing 5 μL of Annexin V-FITC and 5 μL of PI, and incubated at room temperature in the dark for 15 minutes. Next, 400 μL of Annexin V binding buffer was added, and cells were counted on a FACS CantoII flow cytometer.

The statistical data processing was performed using Microsoft Office Excel software. Normality of distribution in in the studied variables was tested using the Shapiro-Wilk criterion. To compare the indicators obtained in the study with their parametric distribution, but without equality of variances, the Cramér-Welch (T) criterion was used, in which the difference between the arithmetic means of two samples (control and experimental) is divided by the natural estimate of the mean squared deviation of this difference. With this method, differences between the group means with a probability of over 95% ($p<0.05$) are determined at $T\geq1.96$.

Results

The analysis of apoptotic activity of *Gratiola officinalis* L. extract demonstrated that in all studied human tumor cell lines, there was an increase in the number of cells in a state of apoptosis under the action of *Gratiola officinalis* L. extract at a concentration of 0.9 mg/mL vs. the control (*Figure* 1, *Table* 1).

After 24 hours of exposure to *Gratiola officinalis* L. extract at a concentration of 0.9 mg/mL, we discovered that larger proportions of tumor cells were in advanced apoptosis stage (from 27.8% in colon carcinoma line HCT-116 to 96.8% in breast adenocarcinoma line MCF-7) (*Figure* 1, *Table* 1).

The lung cancer line A549 had the highest share of cells in early apoptosis (26.9%), and late apoptosis was observed in 55.4% of cells in this line. In the PC-3 prostatic carcinoma line, 68.6% of the cells were in late apoptosis stage, while 29.3% of the cells had necrotic changes after their exposure to the extract. In the colorectal cancer line HCT-116, the smallest share of cells in the apoptosis stage under the influence of the extract was noted (27.8% in the stage of late apoptosis), along with the largest proportion in the necrosis stage (67.4%). The largest number of cells in the late apoptosis stage was found in the MCF-7 breast adenocarcinoma line (96.8%) and the SK-BR-3 breast carcinoma line (96.3%) after exposure to the extract. Slightly lower numbers of cells in the stage of late apoptosis after exposure to the extract were exhibited by cultured renal cell carcinoma line (89.5%) and Jurkat T-cell lymphoblastic leukemia line (79.7%). These results implied that all studied cultures were sensitive to the action of the *Gratiola officinalis* L. extract, but the sensitivity of tumor cells to the extract was different.

Morphological changes in cervical cancer (HeLa) tumor cells in the alive and dead test

In the control group, the cells were arranged in an even monolayer, tightly adhering to each other. The cells were spindle-shaped and polygonal, their cytoplasm was homogeneous, and their nucleus was clearly shaped. The mean number of cells (MNC) per field of view was 510±12.61. Dead cells were not detected. After exposure to *Gratiola officinalis* L. extract, we noticed that with increasing concentration of the extract, MNC values became smaller, while the number of dead cells increased (*Figure* 2, *Table* 2).

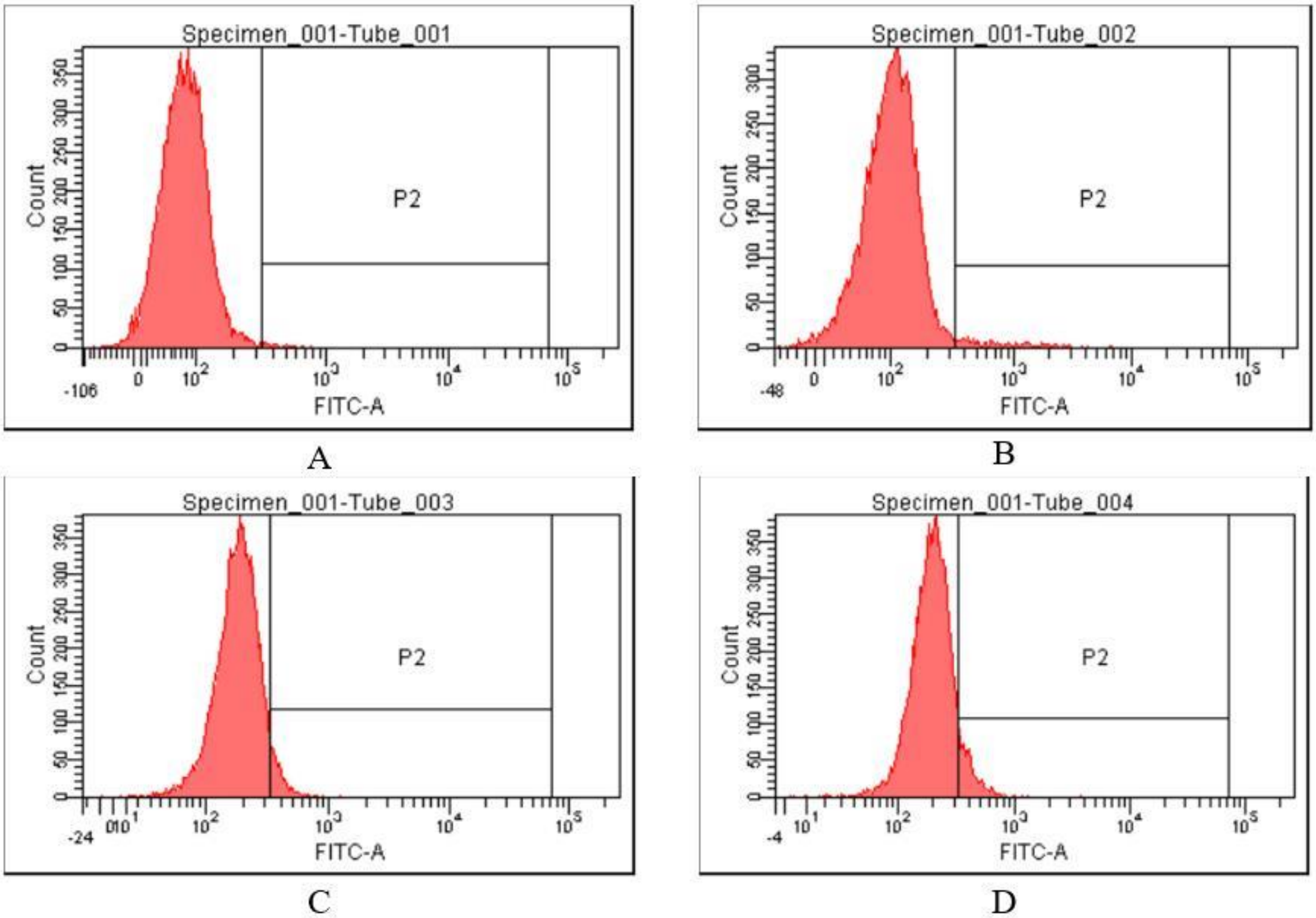

Figure 3. The results of flow cytofluorometry in Jurkat cell line. A, control: cells without exposure – 0.5%; B, control + anti-caspase-3-FITC (BD) – 2.3%; C, control: cells with *Gratiola officinalis* L. extract (extract fluorescence check) – 4.6%; D, cells + *Gratiola officinalis* L. extract + anti-caspase-3-FITC (BD) – 7.2%.

Reduction in MNC was observed already at the concentration of 0.4 mg/mL, i.e., from this concentration on, the extract started exhibiting its cytotoxic effect towards tumor cells. At the concentrations of 0.4-1.5 mg/mL, we observed the signs of karyopyknosis and karyorrhexis, while further increase in concentration these conditions were not observed (*Table* 2).

The study of caspase-dependent apoptosis pathway in T cells of lymphoblastic leukemia

In Jurkat cell line, the caspase-dependent apoptosis (caspase-3) was investigated on a flow cytometer after the exposure to *Gratiola officinalis* L. extract: 24 hours after exposure, 7.2% of positive cells were detected in Jurkat cell line with anti-caspase-3-FITC (BD) (*Figure* 3).

The weak signal, apparently, was associated with the sensitivity of the cell line to the action of this extract, as indicated by the rapid transition of Jurkat cells to the zone of late apoptosis and necrosis; therefore, caspase-dependent early apoptosis was poorly detected after 24 hours. However, despite the weak signal, it could be concluded that caspase-dependent apoptosis was induced in the Jurkat tumor line under the action of *Gratiola officinalis* L. extract.

Discussion

Previously, both in *in vitro* experiments (on cultures of kidney cancer, Caki-1 and SN12c) and *in vivo* experiments (on laboratory rats with transplanted liver cancer and sarcoma), we discovered that the extract of *Gratiola officinalis* L. had antitumor effect [4,5]. In a study on Caki-1 and SN12c kidney cancer cultures, we detected apoptotic activity of *Gratiola officinalis* L. extract was; the activity depended on the concentration of the extract and was observed at concentrations of 0.18-0.90 mg/mL [4]. The present study demonstrated that apoptotic activity depended not only on the extract concentration, but also on the cell culture type. Among published sources, we found no data from other authors on the antitumor activity and mechanisms of action of *Gratiola officinalis* L. extract, but there was information on such effects of individual bioflavonoids contained in the extract of *Gratiola officinalis* L. E.g., in a study by Ho H.-Y. et al. (2021), it was found that luteolin in the form of luteolin-7-O-β-d-glucoside significantly reduced the proliferation of nasopharyngeal carcinoma cells through cell cycle arrest, chromatin condensation and apoptosis activation [9]. A study by Kim et al. (2020) discovered that apigetrin reduced the proliferation of gastric cancer AGS cells and stimulated apoptosis by cleavage of caspase-3 and poly (ADP-ribose) polymerase (PARP), increased the expression of extrinsic apoptotic pathway proteins and mRNA, and promoted autophagic cell death via the

PI3K/AKT/mTOR pathway, leading to inhibition of gastric cancer development [10]. The cytotoxic potential of apigetrin was shown in a study by Smiljkovic et al. (2017) on HCT116 colorectal cancer cells by measuring cell viability, apoptosis rate, and expression of genes associated with apoptosis and colorectal cancer [11]. Wang et al. (2016) demonstrated that eupatin promotes apoptosis of glioma cells depending on the concentration [12]. Kim et al. (2005) showed that eupatin can induce apoptosis in AGS gastric cancer [13]. All these data are consistent with our results on the antitumor effect of *Gratiola officinalis* L. extract due to the activation of apoptosis in tumor cells, and this process depends on the concentration of the extract.

Limitations

We used eight cell cultures of different histogenesis to study the apoptotic effect of flavonoid-containing extract of *Gratiola officinalis* L. The experiment was carried out in three repetitions for each concentration of the extract, the number of repetitions provides a sufficient control sample and allows to reliably extrapolating the results of the study. The advantage of using cell cultures in antitumor activity studies is that they are relatively cheap and easy to maintain, and yield results faster than *in vivo* studies. A limitation of our study is that the effective concentration values of the extract obtained in this study cannot always be easily extrapolated for use in the human body.

Conclusion

In our *in vitro* experiments, we confirmed that *Gratiola officinalis* L. extract had antitumor activity. Apoptotic activity of the extract was established against all examined human tumor cells (HeLa – cervical carcinoma, Jurkat – T-cell lymphoblastic leukemia, MCF-7 – breast adenocarcinoma, A549 – lung carcinoma, PC-3 – prostate carcinoma, HCT-116 – colon carcinoma, A498 – renal carcinoma, SK-BR-3 – human breast carcinoma), but their sensitivities to the extract were different. At the tested extract concentration of 0.9 mg/mL, the MCF-7 line of breast adenocarcinoma was the most sensitive to its action (late apoptosis was noted in 96.8% of cells), as well as the lung cancer line A549 (late apoptosis was observed in 55.4% of cells). In our study, Jurkat T-cell lymphoblastic leukemia tumor cells were shown to induce caspase-dependent apoptosis in tumor cells under the effect of *Gratiola officinalis* L. extract.

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Conflict of interest

None declared.

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Authors:
Natalya V. Polukonova – DSc, Associate Professor, Department of General Biology, Pharmacognosy and Botany; Head of Laboratory of Cell Technologies, Collective Use Center for Experimental Oncology, V.I. Razumovsky Saratov State Medical University, Russia. http://orcid.org/0000-0001-9228-6808.
Nikita A. Navolokin – PhD, Assistant Professor, Department of Anatomic Pathology; Head of Experimental Department, Collective Use Center for

Experimental Oncology, V.I. Razumovsky Saratov State Medical University, Russia. https://orcid.org/0000-0001-7876-9758.
Maria A. Baryshnikova – PhD, Head of the Laboratory of Experimental Diagnostics and Biotherapy of Tumors, N.N. Blokhin Research Center for Oncology, Moscow, Russia. http://orcid.org/0000-0002-6688-8423.
Galina N. Maslyakova – MD, DSc, Professor, Chair of the Department of Anatomic Pathology, V.I. Razumovsky Saratov State Medical University, Saratov, Russia. https://orcid.org/0000-0001-8834-1536.
Alla B. Bucharskaya – DSc, Head of the Collective Use Center for Experimental Oncology, V.I. Razumovsky Saratov State Medical University, Russia. https://orcid.org/0000-0003-0503-6486.
Anna V. Polukonova – Junior Researcher, Laboratory of Cell Technologies, Collective Use Center for Experimental Oncology, V.I. Razumovsky Saratov State Medical University, Russia. http://orcid.org/0000-0001-7198-1254.