The apoptotic activity of flavonoid-containing Gratiola officinalis extract in cell cultures of human kidney cancer

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Abstract: Objective — The discovery of the apoptosis-inducing effects of flavonoid vagonin allowed to make an assumption of existence of similar effect in others flavonoids. This study is devoted to the effects of Gratiola officinalis extract on cell culture of the human kidney cancer.

Methods — Cell cultures of human kidney carcinoma – Caki-1 and SN12c were used in the study. The cells were stained with Hoechst 33258 dye. The number of living cells, cells in a state of apoptosis and mitosis were counted. The Cramer-Welch criterion (T) was used to compare the obtained data.

Results — The activation of apoptosis was noted at all concentrations of the Gratiola officinalis L. extract during the first day of exposure. The apoptotic activity increased with increasing of extract concentration. After 48 hours, this activity was maintained only at a Gratiola officinalis L. extract concentration of 0.9 mg/ml. After 24 hours, the apoptotic activity of the extract was more expressed in the culture of Caki-1. However, after 48 hours the extract induces more pronounced apoptosis in the culture of Sn12c cells. The cytotoxic activity of the extract was not differ after 24 hours in both cultures, after 48 hours it was more pronounced in the culture of Caki-1.

Conclusion — We revealed a pronounced antitumor and apoptotic activity of the Gratiola officinalis L. extract against the cultures of the kidney cancer Caki-1 and Sn12c. Apoptosis of tumor cells can be manifested in the form of pycnosis of the nucleus, the formation of apoptotic bodies and the emergence of cellular debris resulting from complete degradation of tumor cells. The apoptotic activity of the Gratiola officinalis extract depends on their concentration.

Keywords: flavonoids, Gratiola officinalis, kidney cancer, Caki-1, SN12c, apoptosis.


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Introduction

The number of patients with oncological diseases is steadily increasing. Modern methods of treatment are not always sufficiently effective. The used antitumor drugs have some drawbacks: a toxic effect on healthy organs and tissues of the body, as well as the development of tumor resistance to treatment. Therefore, the search for new, more safe and effective medicines is an important challenge today [1, 2].

Medicinal products of plant origin have minimal side effects; therefore, special attention is paid to them today. Plant extracts can be used not only in the form of monotherapy, but also in complex tumor treatment. Flavonoids are able to protect normal cells (including bone marrow stem cells) during the standard course of chemotherapy and radiotherapy [2, 3].

Flavonoids are one of the most promising groups of biological compounds. They have the greatest number of biological effects for the tumor treatment [3]. Some flavonoids are able to activate apoptosis in tumor cells, for example, Vagonin. The apoptotic activity of the flavonoid Vagonin allowed to continue the search for other bioflavonoids with antitumor activity as well as study of mechanisms of their biological activity [3, 4].

Previously, we have developed a method for separation of flavonoid-containing extracts from Gratiola officinalis L. that allows obtaining non-toxic or low toxic extracts even from poisonous plants [5, 6]. It is known that these extract have antioxidant [7, 8], antitumor [9-13], antimicrobial and anti-inflammatory [14, 15] actions.

However, the detail examination of mechanisms of such biological activity of Gratiola officinalis extract was not conducted yet.

The aim of research: to study the morphological changes of cells in human kidney carcinoma Caki-1 and SN12c in experiments in vitro after exposure of Gratiola officinalis extract.
The qualitative reactions with crystalline magnesium and Wagner-Bouchard reagent testify to the presence of bioflavonoids and the absence of alkaloids in obtained *Gratiola officinalis* extract.

### Cell lines

Cultures of human tumor cells of human kidney carcinoma Caki-1 and SN12c were used in the study. Cell cultures were obtained from the Bank of Cell Lines of N.N. Blokhin National Medical Research Centre of Oncology (Moscow, Russia). The lines were cultured on a complete RPMI-1640 culture medium (PanEco, Russia). The nutrient medium contains 10% fetal bovine serum (TPP, HyClone, USA), 2 mM/ml glutamine (PanEco, Russia), 50 mg/ml penicillin-streptomycin (PanEco, Russia). Cells with a 70-80% monolayer were used for experiments. The following parameters were used: a temperature of 37°C and a content of 5% CO₂.

### Immunocytochemical study

Cells were grown up to 70% of the monolayer on the glass. After incubation with the extract during 24 hours, cells were fixed in alcohol and acetone. Then, the cells were stained with Hoechst 33258 dye (1 μg/ml, PanEco, Russia). Hoechst binds to the structures of the cell nucleus and causes their bright glow. We conducted the morphological examination on fluorescence microscope Nikon 80i (Japan) at 435-485 nm. The following indicators were used: the average number of living cells in the field of view (LCVF), the proliferation index – the ratio of LCFV after extract exposure to the LCFV in the control, % of cells in metaphase, anaphase and telophase from the total number of cells, % of cells in apoptosis, % of cells with pyknosis of the nucleus from the total number of cells with signs of apoptosis, percentage of apoptotic bodies from the total number of cells with signs of apoptosis. Cell counting was performed in each group in at least three fields of view with magnification of ×200.

### Statistical analysis

In the statistical processing of data, the normality of the distribution of the indicators was verified in groups using the Shapiro-Wilk test. The Cramer-Welch criterion (T) was used to compare obtained average values, the difference between the arithmetic mean of the two samples (control and experimental) is divided into a natural estimation of the standard deviation of this difference. The mean difference with a probability of 95% is determined with this method at T≥1.96 and p<0.05. All statistical analysis is carried out using STATISTICA 10.0 Interprise software (StatSoft, USA).

### Results

#### Cell culture Caki-1

Low mitotic activity (percentage of cells in metaphase, anaphase and telophase phases of mitosis 1.2±0.3%) and weak apoptotic activity were observed after 24 hours in control cell culture of Caki-1 without exposure (*Figure 1*). Signs of apoptosis and mitosis were not observed in the cells after 48 h (*Figure 1B*), LCFV increased by 8%.
compared to the control group at Caki-1 (Fig. 2). At concentration of 0.9 mg/ml, a decrease in LCFV at 24 hours was observed (94.6%) (Fig. 2B). 0.035; 0.18 and 0.9 mg/ml. An increase in the number of apoptotic cells by 22.9% (50% in the stage of pyknosis and 50% apoptosis bodies) and cellular debris as result of cell degradation were noted at an extract concentration of 0.9 mg/ml.

Cell culture Sn12c

The absence of cells in metaphase, anaphase and telophase phases of mitosis, and a low percentage of cells in apoptosis (1.8±0.1%) were observed after 24 hours in the control group of Sn12c culture (without exposure). After 48 h, a 10% decrease in LCFV, a low percentage of cells in a mitosis state, and a low percentage of cells in apoptosis were observed in the control group of Sn12c culture (Fig. 3, Table 2).

24 hours after exposure to concentrations of 0.035, 0.18 and 0.9 mg/ml of Gratiola officinalis extract, an increase in apoptotic cells was observed in Sn12c cell culture of 7.9%, 11.2%, and 17.6%, respectively, mainly in the pyknosis stage (more than 80%) (Fig. 4A). At concentration of 0.9 mg/ml, a decrease in LCFV at 63.3%, a low proliferation index of 0.36 and a lack of cells on stages of metaphase, anaphase and telophase mitosis were observed (Table 2).

The increase of ANCL up to 14% was observed 48 hours after exposure to 0.035 mg/ml of Gratiola officinalis extract. An increase in LCFV up to 19.5% was noted at extract concentration of 0.18 mg/ml, the proliferation index was 1.19. An increase in apoptotic cells (up to 92.9% at the stage of pyknosis) was noted only at 0.9 mg/ml (Fig. 4B) (Table 2).

Decrease in LCFV to 94.6% was observed, and the proliferation index was 0.06 at extract concentration of 0.9 mg/ml.

Table 1. Morphometric parameters of Caki-1 cells after exposure of Gratiola officinalis extract

<table>
<thead>
<tr>
<th>Groups (mg/ml extract concentration)</th>
<th>LCFV</th>
<th>The index of proliferation</th>
<th>% of cells in metaphase, anaphase and telophase</th>
<th>% apoptosis’s cells in field of view</th>
<th>% cells in apoptosis</th>
<th>Pyknosis</th>
<th>Apoptotic bodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>172.7±10.8</td>
<td>1</td>
<td>1.2±0.3</td>
<td>2.1±0.3</td>
<td>49.2±11.5</td>
<td>50.8±11.5</td>
<td></td>
</tr>
<tr>
<td>Extract 0.035 mg/ml</td>
<td>168.0±7.5</td>
<td>0.97</td>
<td>2.5±0.3</td>
<td>10.0±0.6*</td>
<td>80.7±1*</td>
<td>19.3±1.0*</td>
<td></td>
</tr>
<tr>
<td>Extract 0.18 mg/ml</td>
<td>154.6±14.7</td>
<td>0.89</td>
<td>1.2±0.4</td>
<td>13.3±1.6*</td>
<td>86.2±2.9*</td>
<td>13.8±2.9*</td>
<td></td>
</tr>
<tr>
<td>Extract 0.9 mg/ml</td>
<td>63.3±6.7*</td>
<td>0.37</td>
<td>0.0±0</td>
<td>19.7±2.5*</td>
<td>96.7±2.1*</td>
<td>3.3±1.2*</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>187.0±8.7</td>
<td>1</td>
<td>0.0±0</td>
<td>0.0±0</td>
<td>0.0±0</td>
<td>0.0±0</td>
<td></td>
</tr>
<tr>
<td>Extract 0.035 mg/ml</td>
<td>2.0±0.1*</td>
<td>0.01</td>
<td>0.0±0</td>
<td>0.0±0</td>
<td>0.0±0</td>
<td>0.0±0</td>
<td></td>
</tr>
<tr>
<td>Extract 0.18 mg/ml</td>
<td>1.0±0.4*</td>
<td>0.01</td>
<td>0.0±0</td>
<td>0.0±0</td>
<td>0.0±0</td>
<td>0.0±0</td>
<td></td>
</tr>
<tr>
<td>Extract 0.9 mg/ml</td>
<td>5.5±1.0</td>
<td>0.03</td>
<td>0.0±0</td>
<td>22.9±6*</td>
<td>50.0±28.9*</td>
<td>50.0±28.9*</td>
<td></td>
</tr>
</tbody>
</table>

Data presented as mean with standard deviation - M±SD. LCFV, the average number of living cells in the field of view. * – The differences are significant compared to the control group at p<0.05 and T≥1.96.

Figure 2. Cell line Caki-1: A) 24 h after exposure to the Gratiola officinalis extract at a concentration of 0.9 mg/ml (arrows denotes apoptotic bodies); B) 48 hours after exposure to the Gratiola officinalis extract (arrows labeled cellular debris), the mode of fluorescence at 435-485nm, Hoechst 33258 staining. x200.

Gratiola officinalis extract, which was due to an increase in cells at the stage of pyknosis (80.7%).

A decrease of 63.3% in LCFV was observed at the concentration of 0.9 mg/ml of Gratiola officinalis extract (Fig. 2). Also, an increase in the number of apoptotic cells by 17.6% was observed at concentration of 0.9 mg/ml of Gratiola officinalis extract (Fig. 2), due to increasing of cells at the stage of pyknosis (47.5% more).

The proliferation index was 0.36. Cells in stages of metaphase, anaphase and telophase of mitosis were absent after exposure to Gratiola officinalis extract at a concentration of 0.9 mg/ml (Fig. 2).

The decrease in LCFV in 97%, 99%, and 99%, respectively, was observed 48 hours after exposure of Gratiola officinalis extract in cell culture of Caki-1 (Fig. 2B): 0.035; 0.18 and 0.9 mg/ml. An increase in the number of apoptotic cells by 22.9% (50% in the stage of pyknosis and 50% apoptosis bodies) and cellular debris as result of cell degradation were noted at an extract concentration of 0.9 mg/ml.

24 hours after extract exposure, LCFV did not change at 0.035 mg/ml of Gratiola officinalis extract in the cell culture of Caki-1 (Table 1). The number of apoptotic cells at the stage of pyknosis increased (by 31.5% more), the proliferation index was 0.97, the cells in mitosis increased by 1.3%. The number of cells in apoptosis increased by 11.2% when exposed to 0.18 mg/ml of Gratiola officinalis extract, which was due to an increase in cells at the stage of pyknosis (80.7%).

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24 hours after exposure to concentrations of 0.035, 0.18 and 0.9 mg/ml of Gratiola officinalis extract, an increase in apoptotic cells was observed in Sn12c cell culture of 7.9%, 11.2%, and 17.6%, respectively, mainly in the pyknosis stage (more than 80%) (Fig. 4A). At concentration of 0.9 mg/ml, a decrease in LCFV at 63.3%, a low proliferation index of 0.36 and a lack of cells on stages of metaphase, anaphase and telophase mitosis were observed (Table 2).

The increase of ANCL up to 14% was observed 48 hours after exposure to 0.035 mg/ml of Gratiola officinalis extract. An increase in LCFV up to 19.5% was noted at extract concentration of 0.18 mg/ml, the proliferation index was 1.19. An increase in apoptotic cells (up to 92.9% at the stage of pyknosis) was noted only at 0.9 mg/ml (Fig. 4B) (Table 2).

Decrease in LCFV to 94.6% was observed, and the proliferation index was 0.06 at extract concentration of 0.9 mg/ml.
Table 2. Morphometric parameters of Sn12c cells after exposure of Gratiola officinalis extract

<table>
<thead>
<tr>
<th>Groups (mg/ml extract concentration)</th>
<th>LCFV</th>
<th>The index of proliferation</th>
<th>% of cells in metaphase, anaphase and telophase</th>
<th>% apoptosis's cells in field of view</th>
<th>% cells in apoptosis Pyknosis</th>
<th>Apoptotic bodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>261.3±16.04</td>
<td>1.00</td>
<td>0±0</td>
<td>1.8±0.1</td>
<td>71.7±3.8</td>
<td>28.3±3.8</td>
</tr>
<tr>
<td>Extract 0.035 mg/ml</td>
<td>269.0±22.0</td>
<td>1.03</td>
<td>0±0</td>
<td>3.4±0.6*</td>
<td>91.9±2.6*</td>
<td>8.1±2.6*</td>
</tr>
<tr>
<td>Extract 0.18 mg/ml</td>
<td>291.6±9.2</td>
<td>1.11</td>
<td>0±0</td>
<td>3.6±0.9*</td>
<td>55.7±11.3</td>
<td>44.3±11.3</td>
</tr>
<tr>
<td>Extract 0.9 mg/ml</td>
<td>16.7±5.5</td>
<td>0.06</td>
<td>0±0</td>
<td>84.2±10*</td>
<td>100.0±0*</td>
<td>0±0*</td>
</tr>
<tr>
<td>After 48 hours</td>
<td>Control</td>
<td>235.6±8.0</td>
<td>1</td>
<td>0.4±0.1</td>
<td>48.8±15.4</td>
<td>51.2±15.4</td>
</tr>
<tr>
<td>Extract 0.035 mg/ml</td>
<td>268.0±3.5*</td>
<td>1.14</td>
<td>0.6±0.1</td>
<td>1.7±0.1</td>
<td>77.5±1.4</td>
<td>22.5±1.4</td>
</tr>
<tr>
<td>Extract 0.18 mg/ml</td>
<td>281.8±5.5*</td>
<td>1.19</td>
<td>0±0*</td>
<td>3.3±1.3</td>
<td>50.4±11.2</td>
<td>44.6±5.7</td>
</tr>
<tr>
<td>Extract 0.9 mg/ml</td>
<td>12.5±1.1*</td>
<td>0.05</td>
<td>0±0*</td>
<td>95.0±2.9*</td>
<td>100.0±0*</td>
<td>0±0*</td>
</tr>
</tbody>
</table>

Data presented as mean with standard deviation - M±SD. LCFV, the average number of living cells in the field of view.

* – The differences are significant compared to the control group at p<0.05 and T≥1.96.

Table 3. Concentration of the Gratiola officinalis L. extract cause a pronounced apoptotic and cytotoxic activity after 24 h and 48 h

<table>
<thead>
<tr>
<th>Apoptotic activity</th>
<th>Cytostatic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.18 - 0.9 mg/ml</td>
<td>0.9 mg/ml</td>
</tr>
<tr>
<td>0.9 mg/ml</td>
<td>0.9 mg/ml</td>
</tr>
<tr>
<td>0.035 - 0.9 mg/ml</td>
<td>0.9 mg/ml</td>
</tr>
<tr>
<td>0.9 mg/ml</td>
<td>0.9 mg/ml</td>
</tr>
</tbody>
</table>

Figure 3. Cell line Sn12c without exposure: A) After 24 h, B) After 48 h, fluorescence at 435-485 nm, Hoechst 33258 staining. x200.

Figure 4. Cell line Sn12c after exposure of Gratiola officinalis extract at concentration of 0.9 mg/ml: A) after 24 h; B) after 48 h, fluorescence at 435-485 nm, Hoechst 33258 staining. x200.
Discussion

In the experiments in vitro, activation of apoptosis was noted at all concentrations of *Gratiola officinalis* L. extract during the first day of exposure. The dependence was direct and linear, with increasing concentration, apoptotic activity increased. After 48 hours, this activity was maintained only at *Gratiola officinalis* L. extract concentration of 0.9 mg/ml. The apoptotic activity of the extract will manifest more in the culture of CaKi-1 after 24 hours. However, the extract induces apoptosis more after 48 hours for the culture of Sn12c cells. The result obtained indicates a greater sensitivity of the CaKi-1 culture. The cytotoxic activity of the extract does not differ after 24 hours in the cultures studied. After 48 hours, it is pronounced more in the culture of CaKi-1 (Table 3).

The antitumor properties of flavonoids are discussed in the literature [21-23]. The data obtained by us are consistent with the results of other authors demonstrating that flavonoids activate apoptosis in tumor cells [24, 25], while not having toxicity. Thus, it is known that the flavonoid Vogonin as part of the popular Chinese herbal remedy Huang-Qin (based on grass skullcap Baikal) induces apoptosis in a wide range of human tumor cells in vitro and inhibits tumor growth in vivo in various models of mouse tumors [3, 29-34]. It is important that at doses lethal to tumor cells, vogonin showed absence of cytotoxicity for normal cells and had also no obvious toxicity with regard to animals [30-35].

Some authors suggest that apoptosis, induced in tumor cells by flavonoids, is not realized by caspase [26]. However, most authors think that flavonoids activate caspase 3 [4, 27, 28]. Other authors consider that flavonoids activate caspase 3 [4, 27, 28]. Previously, we found that flavonoid-containing extract of *Gratiola officinalis* L. is able to activate p53 in rat kidney cancer cells [13]. p53 can transcriptionally activate a number of target genes involved in cell death signaling including proapoptotic Bax and Bak, which regulate the release of cytochrome c from the mitochondria, and result in cell apoptosis by activating the cleaving of caspase-3 and caspase-9 [36]. Thus, our data suggests that the extract may trigger the caspase pathway of apoptosis. Further studies of the mechanisms of antitumor action of *Gratiola officinalis* extract are necessary for possible clinical application.

Conclusion

As a result of our study, we established a pronounced antitumor and apoptotic activity of *Gratiola officinalis* extract against the cell cultures of human kidney cancer CaKi-1 and Sn12c. The most sensitive culture of kidney cancer to the action of extract was CaKi-1. Apoptosis of tumor cells can be manifested in the form of nucleus pyknosis, the formation of apoptotic bodies and the emergence of cellular debris resulting from complete degradation of tumor cells. The apoptotic activity of the *Gratiola officinalis* extract depends on the concentration and occurs at a concentration of 0.18-0.90 mg/ml.

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Conflict of interest: none declared.

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