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Influence of Flavonoids on the Cytotoxic Activity of Mononuclear Blood Cells in Model Tests

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Abstract

BACKGROUND: The spread of phytocomplex application and justification of its selective effects on tumour cells (mainly due to the presence of flavonoids) require research of its cytotoxic and immunomodulatory activity.

AIM: The goal was to study the direct cytotoxic effect of the phytocomplex and its modulating effect on the cytotoxic activity of the donor's mononuclear blood cells in in vitro experiments.

METHODS: The phytocomplex was a dry extract from marsh cinquefoil, creeping alfalfa and common hop; its main active ingredients were flavonoids. Transplantable monolayer cultures of lung adenocarcinoma, colorectal cancer, erythroblastic leukaemia, and fibroblasts were used as target cells. The cytotoxic activity was assessed using a cytotoxic test based on the selective ability to live cells to reduce MTT (3-[4, 5-dimethyltriazol-2-yl]-2, 5 diphenyltetrazolium bromide) to formazan in mitochondria. Quantitative determination of formazan was performed using spectrophotometry.

RESULTS: A direct cytotoxic effect of the phytocomplex in concentrations of at least 2.5 mg/ml on tumour cells has been established. Its modulating effect on the cytotoxic activity of mononuclear blood cells at a concentration of 0.05 mg/ml was shown. The phytocomplex in doses of 0.25 and 0.5 mg/ml increased the killer activity of the mononuclear cells in a diseased person's blood, but did not affect these blood cells in a healthy donor. Incubation of lymphocytes with a phytocomplex for 24 hours increased the cytotoxic activity of mononuclear cells by 20-25%.

CONCLUSION: The direct cytotoxic effect of the phytocomplex and its modulating effect on the cytotoxic activity of mononuclear blood cells in model experiments in vitro have been established.

Introduction

Natural products in dietary sources including fruits, vegetables, and spices, which consist of biologically active components such as phytosterols, flavonoids, saponins, lycopene, triperpenoids, and many others, are assumed to have anticancer properties [1]. Interest in flavonoids (polyphenols of plant origin) is currently largely due to their anti-inflammatory, antioxidant, antitumor, immunomodulatory, antimicrobial and other types of activity [2], [3], [4] [5].

The most relevant, for purposes of the practical application of flavonoids, are their immunomodulating and antitumor properties [6], [7], [8], [9], [10], [11]. The phytocomplex under study was a dry extract from marsh cinquefoil, creeping alfalfa and common hop. It contained a variety of biologically active substances, including flavonoids, coumestans, polysaccharides, steroids, essential oils, tannins, hydroxycinnamic and phenol carboxylic acids, essential amino acids, vitamins, and mineral components, all of which makes it useful in medicine. The main active ingredients of the phytocomplex were flavonoids [12]. Recent studies have shown the promise and the need for further study of the cytotoxic and immunomodulatory activity of the phytocomplex for use together with physiotherapeutic methods [13].

The goal of this work was to investigate the direct cytotoxic effect of the phytocomplex and its modulating effect on the cytotoxic activity of the donor's mononuclear blood cells (MNCs) in *in vitro* experiments.

Material and Methods

The phytocomplex that was used in the study was a dry extract from leaves and roots of marsh cinquefoil, leaves of creeping alfalfa and multiple fruits (or cones) of common hop (TS 9375-021-00003938-11 “Dry extract of marsh cinquefoil, creeping alfalfa and common hop (phytocomplex)” [14]. The main flavonoids present in the phytocomplex are shown in Figure 1. The flavonoid composition of the raw materials of the used medicinal plants was compatible and balanced in terms of the content of flavones, flavonols and their glycosides, isoflavones, which should have a positive impact on the cytotoxic and immunomodulatory activity of the phytocomplex. The predominant flavonoids were quercetin, rutin and isoflavones: biochanin A, genistein, daidzein.

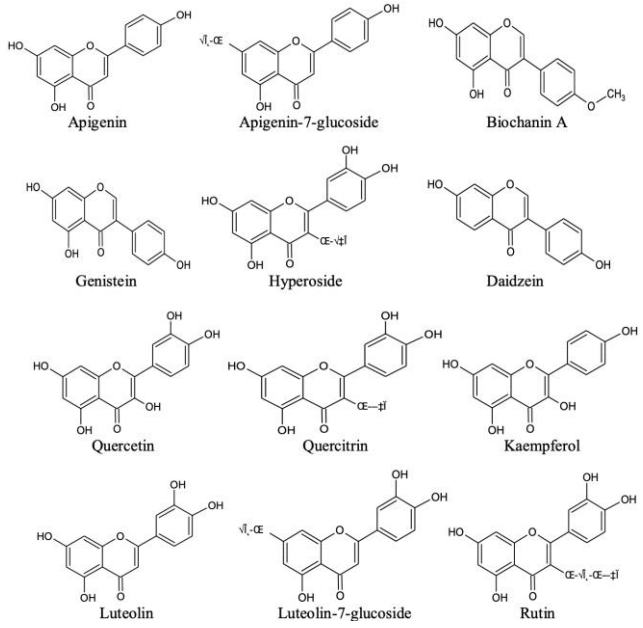

Figure 1: Structural formulas of the main flavonoids of the phytocomplex

A cytotoxic test, based on the selective ability to live cells to reduce MTT (3-[4, 5-dimethyltriazol-2-yl]-2, 5 diphenyltetrazolium bromide, Sigma, USA) to formazan in mitochondria, was used to study the direct cytotoxic effect of the phytocomplex. Quantitative determination of formazan was performed using spectrophotometry. Based on the optical density data, the number of dead target cells (i.e. cytotoxic activity) was calculated.

The following human cell lines were used: lung adenocarcinoma (LAC, A-549) and colorectal cancer (Colo), a model of solid tumours, erythroblastic leukaemia (K-562), a model of hemoblastosis and normal fibroblasts (obtained from the laboratory of tumour cells genetics of the Blokhin Federal Medical Research Center for Oncology) were used as target cells. Target cells were applied in 96-well flat-bottomed microplates in quantity of 2 x 10^6 cells per well in 100 µl of the working culture medium (WCM): RPMI-1640 (No. FSR 2007/00859, Chumakov Institute of Poliomyelitis and Viral Encephalitis, Russia) with 5% fetal calf serum. In the experimental series, working solutions of the phytocomplex were added to the target cells. The final concentrations of the phytocomplex in the wells were 5 mg/ml, 2.5 mg/ml, 0.5 mg/ml, 0.05 mg/ml, 0.005 mg/ml. In the control series, a saline solution was added to the target cells. The total volume of the solution in the wells was 200 µl.

Target cells and effectors were placed in a carbon-oxygen incubator (Binder C 150 CO_2 incubator, Germany) at 37°C for 18 hours. At the end of the experiment, 10 µl of a sterile MTT dye solution was added to each well. Plates protected with aluminium foil were placed in a dark place at 37°C for four hours, then centrifuged for five minutes. The supernatant was removed with a pipette without disturbing the cell pellet. Formazan crystals were dissolved in dimethyl sulfoxide, which was added into the well to bring the volume up to 200 µl. The complete dissolution of the formazan crystals was achieved with stirring on a nutator for 5-15 minutes using an invertoscope to control the process.

The optical density was measured immediately after the complete dissolution of the formazan crystals using a spectrophotometer (Jasco V-730, Japan) at a wavelength of 540 nm.

To study the modulating effect of the phytocomplex on the cytotoxic activity of MNCs, extract concentrations that did not have a direct cytotoxic effect on the target cells were used. MNCs were isolated from the blood samples of six donors, obtained from the blood transfusion department of the Blokhin Federal Medical Research Center for Oncology. Six primary patients with stage II lung cancer (three) and colon cancer (three) without signs of metastasis were included in the study.

Heparinised blood (15 units/ml) was diluted twice with medium 199 (No. ФСР 2011/10969, Chumakov Institute of Poliomyelitis and Viral Encephalitis, Russia), layered on ficoll-verografin (density 1.077 g/cm^3) in the 2:1 ratio and centrifuged for 30 minutes. MNCs were sampled using a Pasteur pipette and washed three times with medium 199 for ten minutes. The concentration of the suspension in WCM was adjusted to 5 x 10^6 cells/ml. In the experiment with donors, MNCs, LAC, Colo, K-562 cells and fibroblasts were used as target cells. For cancer patients, MNCs investigated only NK activity on the NK-sensitive K-562 cells. The ratio of target cells and effectors was 1:5.

Statistical processing of the results was carried out using the SPSS. Statistics. v17. Multilingual-EQUiNOX Software (SPSS Inc.).

Results

The results of the experiment for the study of direct cytotoxic action of the phytocomplex on target cells are presented in Figure 2.

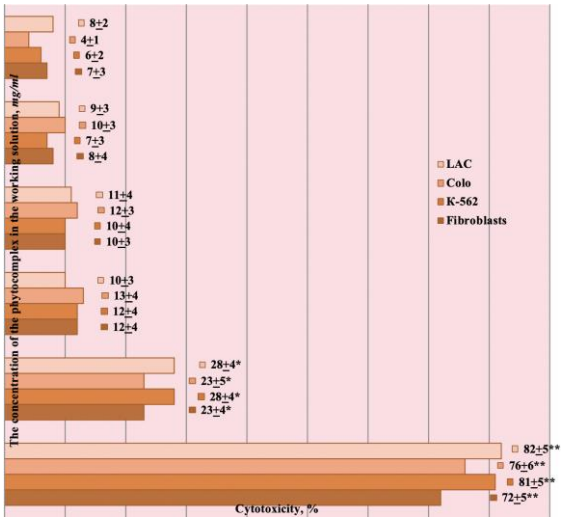

Figure 2: Cytotoxic activity of the phytocomplex (P < 0.05, ** P < 0.01)*

It was found that the addition of working solutions of the phytocomplex at a concentration of 5 mg/ml to the target cells resulted in the death of a significant proportion of tumour cells (72-82%). A two-fold decrease in the effective concentration (2.5 mg/ml) caused lysis of 23-28% of tumour cells. At lower concentrations from 0.5 to 0.005 mg/ml, the cytotoxicity was 4-12%, which was not significantly different from the data for the control series.

Extract concentrations that did not have a direct cytotoxic effect on target cells were used for further studies of the modulating effect of the phytocomplex on the cytotoxic activity of MNCs. The results of the experiment are presented in Figure 3.

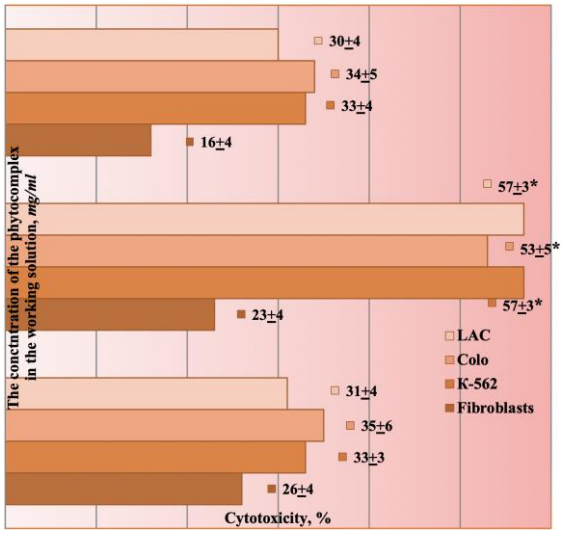

Figure 3: The modulating influence of the phytocomplex on the cytotoxic activity of MNCs (P < 0.05)*

It has been shown that out of the working solutions of the phytocomplex with concentrations ranging from 0.001 to 0.5 mg/ml only the dose of 0.05 mg/ml had a significant stimulating effect on the antitumor cytotoxic activity of MNCs (53-57%, LAC, Colo and K-562 cells were used). Increase of the concentration to 0.5 mg/ml did not lead to an increase in the spontaneous activity of MNCs, and a further increase of the dose could have a direct cytotoxic effect on tumour cells. The obtained data corresponded to the data on the effect of other well-known immunomodulators, such as Interleukin-1 and Interleukin-2, although the latter do not have a dose-dependent effect. No effect of the phytocomplex on the cytotoxic activity of MNCs was shown when using fibroblasts as target cells.

Additionally, the modulating effect of the phytocomplex on the killer activity of MNCs of a healthy donor and a cancer patient was investigated (Figure 4). Cells of K-562 cell culture, which are sensitive to natural killers, were used as target cells. Working solutions with phytocomplex concentrations of 0.5 and 0.25 mg/ml did not have a direct cytotoxic effect on K-562 cells. It was established that the phytocomplex in concentrations of 0.5 and 0.25 mg/ml does not have a significant modulating effect on the cytotoxic activity of MNCs of a healthy donor, but increases the killer activity of MNCs of a cancer patient by 15-18% compared to the control.

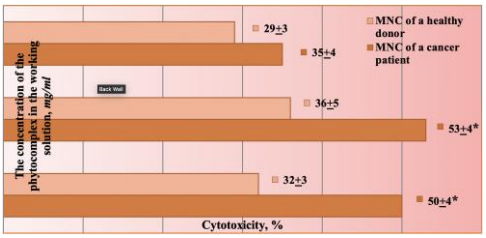

Figure 4: The modulating influence of the phytocomplex on the cytotoxic activity of MNCs of a healthy donor and a diseased person when tested using K-562 cells (P < 0.05)*

It is known that the maximum effect of classical immunomodulators is achieved after 24-48 hours. Therefore, we incubated lymphocytes with the phytocomplex for 24 hours at 37°C (Figure 5).

Experiments have shown that incubation increases the cytotoxic activity of donor MNCs by 20-25% compared with the control.

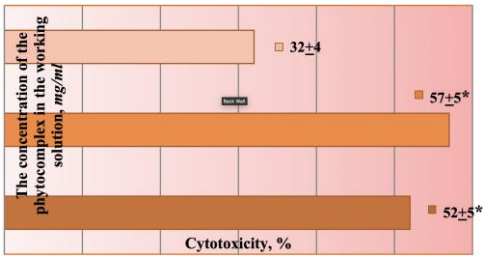

Figure 5: The modulating influence of the phytocomplex on the cytotoxic activity of MNCs of a healthy donor when tested using K-562 cells after incubation for 24 hours (P < 0.05)*

In a special series of experiments, the modulating effect of the phytocomplex on the cytotoxic activity of MNCs was studied using different ratios of target cells and MNCs: 1:1, 1:2 and 1:5 (Figure 6). In the control series, a solution of the phytocomplex was not added.

It was established that the phytocomplex increases the cytotoxic activity of MNCs by 20-29% compared with the control, regardless of the target cells to MNCs ratio.

Figure 6: The modulating effect of the phytocomplex at a concentration of 0.05 mg/ml on the cytotoxic activity of MNCs of a healthy donor when tested using K-562 cells with different MNCs to target cells ratios (P < 0.05)*

Discussion

For the first time, the cytotoxic activity of the phytocomplex was studied using transplantable monolayer cultures of lung adenocarcinoma, colorectal cancer, erythroblastic leukaemia and fibroblasts in *vitro* experiments. It has been established that the phytocomplex has a direct cytotoxic effect on tumour cells in concentrations of 5.0 and 2.5 mg/ml.

The modulating effect of the phytocomplex on the cytotoxic activity of MNCs at a concentration of 0.05 mg/ml (53-57%) was registered when using cell cultures of lung adenocarcinoma, colorectal cancer and erythroblastic leukaemia. An increase in the concentration of the phytocomplex did not lead to an increase in the spontaneous activity of blood mononuclear cells.

It has been established that the phytocomplex in concentrations of 0.25 and 0.5 mg/ml increases the killer activity of the MNCs of a diseased person by 15-18%, but does not affect the cytotoxic activity of the blood mononuclear cells of a healthy donor.

It has been shown that incubation of lymphocytes with phytocomplex for 24 hours increases the cytotoxic activity of mononuclear cells by 20-25%. Variations in the ratio of target cells and blood mononuclear cells did not significantly affect the immunomodulating effect of the phytocomplex.

In conclusion, the direct cytotoxic effect of the phytocomplex and its modulating effect on the cytotoxic activity of mononuclear blood cells in model experiments in vitro have been established.

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