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Effect of Phlojodicarpus sibiricus on the proliferative activity of the HepG2 cell line

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ABSTRACT

The climate in the Republic of Sakha (Yakutia) is sharply continental, characterized by long winters and short summers. It is known that under extreme climatic conditions in plants, the total content and structural diversity of biologically active substances of regulatory and protective action increases. In the experimental study, the plant Phlojodicarpus sibiricus was used, which has been used in ethnomedicine among the peoples of Siberia and Yakutia since ancient times.The leaf extract of the bloater contains phytosterols, which are considered in the work as the active substance under study, belong to the group of steroid alcohols naturally present in plants. The aim of this study was to evaluate the cytotoxic effect on the proliferative activity of cultured HepG2 cancer cells. The measurement of antitumor activity was based on the MTT-assay method, which is an indicator of mitochondrial function in viable cells, based on tetrazolium reduction.o perform this test, HepG2 cancer cells were cultivated at 37°C with 5% CO2 in DMEM medium with the addition of 10% fetal bovine serum until a monolayer was formed. To obtain a cell suspension, the formed monolayer of cells was trypsinized, followed by inactivation of trypsin with the addition of a nutrient medium. A 96-well plate was used to carry out the test. From 2500 to 2800 cells were added to each well in 200 ?l of culture medium. Next, the plates with cells were incubated for 24 hours in a CO2 incubator for cell adhesion at a temperature of 37°C. The reference substance for comparing the antitumor activity of Ph. Sibiricus was the drug Doxorubicin. As a result of the three stages of the experimental study, the most effective concentration of the antitumor activity of the Ph. Sibiricus lyophilisate suspension against cultured HepG2 cancer cells was established, equal to the range from 0.025 g/ml (1:4) to 0.017 g/ml (1:6). Thus, this plant growing in the cryolithozone can be considered as a potential promising medicinal plant material with antitumor activity, further more in-depth study is necessary.

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INTRODUCTION

The Republic of Sakha (Yakutia) is a constituent entity of the Russian Federation, located in the northeast Asian part, occupying one fifth of the territory of Russia. The distance between the extreme points from north to south is about 2000 kilometers,from west to east about 2300 kilometers. Most of the protected mountains and plateaus. The climate, depending on Sakha (Yakutia), is sharply continental, with long winter and long summer periods.

On the territory of the Republic of Yakutia, in Oymyakon, there is a cold pole of the northern hemisphere of the planet, where a temperature of -71.2 °C is recorded. The temperature difference between the coldest month - January and the warmest - July is 70-75°C. In terms ofthe absolute value of significant temperature and the duration of the period with a negative temperature (from 6.5 to 9 months a year), the republic does not matter in the Northern Hemisphere. The absolute minimum temperature almost everywhere in the country is below - 50 degrees.

KEYWORDS:
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One of the most important mechanisms of adaptation of northern plants to extreme growing conditions at the biochemical level is not just an increase in the content of physiologically active substances of regulatory and protective action by 1.5–2.5 times, but alsoan increase in their structural diversity by 3–5 times (isomers, homologues, derivatives according to the degree of oxidation, etc.), compared with similar species growing in temperateclimates [1].

Phlojodicarpus sibiricus (Steph. ex Spreng.) K.-Po1. Celery family - *Apiaceae* (umbrella - Umbelliferae) - is a valuable medicinal species of the Siberian-Mongolian type. Listed in the "Red Book of the Republic of Sakha (Yakutia)" in the 2b category of rarity: a species whose populations are declining as a result of their excessive use by humans and can be stabilized by special conservation measures. Herbaceous tap-rooted perennial up to 60 (70)cm in height, stems branched, glabrous, dense, basal leaves are bluish, matte, thrice pinnate, umbels with 6-16 (28) rays, an involucre of 5-7 bare leaflets, fruits with narrow dorsal and widepterygoid flat white marginal ribs, glabrous or sparsely pubescent. It grows on the edges of steppe forests and thickets of shrubs, on gravelly slopes, less often on sands, in stony steppes. Blooms in July and August [2].

Among the peoples of Siberia and Yakutia, *Phlojodicarpus sibiricus* (*P. sibiricus*), which is used as a food and medicinal plant, is among the most valuable plants. In ethnomedicine of the peoples of Yakutia, it was used for obesity, pulmonary tuberculosis, diseases of the thyroid gland, heart, stomach and esophagus, including cancerous tumors, and rheumatism [3]. At present, Siberian bloater is a promising, but poorly studied medicinal plant, growing on permafrost, it can be a source of biologically active compounds with oncoprotective and antitumor activity.

Chemical components *P. sibiricus* belong to the group of chemical compounds of coumarins: pterixin, umbelliferone, 7-hydroxycoumarin, isoimperatorin, dihydrosamidin, visnadin. The therapeutic effect of the underground parts of this plant is m-anticholinergic, a- adrenergic blocking, hypotensive, vasodilating, anthelmintic, antispasmodic [4, 5, 6]. In folk medicine, rhizomes are used, a decoction is prepared from it and used for vascular diseases, sleep disorders and chronic intestinal diseases. Collection of rhizomes of *P. sibiricus* isproduced in early spring or autumn [7].

It is known from the literature that polyphenols of natural origin have a protective effecton the development of tumors of the oral cavity, stomach, duodenum, liver, lungs, skin, ovaries, cervix, mammary and prostate glands [8, 9, 10, 11].

In the leaf extract of *P. sibiricus* contained phytosterols (phytosterols; also plantsterols/sterols), which are considered in the work as the active substance under study, are contained in the leaf extract of the bloater, belong to the group of steroid alcohols naturally present in plants. They look like a non-solid white powder with a characteristic odor, insolublein water and soluble in alcohol. It is known that phytosterols have high immunomodulatory, hypocholesterolemic and oncoprotective activity and are widely used in medicine. Also, the composition of the leaves of *P. sibiricus* includes phenylpropanoids [12,13]. The presence of caffeoylquinic acids in the leaf extract can cause hypoglycemic, hypocholesterolemic, hepatoprotective, antitumor effects on the human body [14, 15].

Thus, given the fact that the considered species of medicinal plant *P. sibiricus*, being a source of flavonoids, essential oils growing in the permafrost, can be considered for medical purposes as a potential promising medicinal plant material with antitumor activity. Cancer cell lines are undoubtedly excellent models for studying the biological mechanisms responsible forthe occurrence of oncology. They allow the use of cell models for the development and testingof anticancer drugs, as well as for the development of new treatments.

The aim of this study was to evaluate the cytotoxic effect of *Phlojodicarpus sibiricus* on the proliferative activity of cultured HepG2 cancer cells.

MATERIALS AND METHODS

Since *P. sibiricus* is listed in the Red Book of the Republic of Sakha (Yakutia) in the study, not rhizomes were used, but only leaves that were collected in an ecologically clean area of the Botanical Garden of Yakutsk in 2018 and stored as a lyophilisate in a sterile darkened glass vessel at a temperature of 4- 5°C. This method of collection does not pose a threat to the reduction of natural populations. Biologically active substances were obtained by extraction according to the protocol of the scientific laboratory of the Institute of Biological Problems of the Cryolithozone of the Siberian Branch of the RussianAcademy of Sciences.

The studies were carried out under sterile conditions *in vitro*. The efficacy of *P. sibiricus* on commercial HepG2 cell lines was evaluated.

HepG2 is a human hepatocellular carcinoma cell line derived from the liver tissues of a15-year-old white Caucasian human with well-differentiated hepatocellular carcinoma [16]. The measurement of antitumor activity was based on the Standard MTT Test Compendium. For this procedure, HepG2 cancer cells are cultivated at 37 °C with the presence of 5% CO2 inDMEM medium with the addition of 10% fetal bovine serum until a monolayer is formed. To obtain a cell suspension, the formation of a monocellular layer was trypsinized with rapid inactivation of trypsin with the addition of a nutrient medium. For implementation, a 96-well plate was used. 2500 to 2800 HepG2 cells were dropped into each well in 200 µl of culture medium. Next, the plates with cells were incubated for 24 hours in a CO2 incubator for cell adhesion to the bottom of the wells at a temperature of 37°C. Further, after a day of incubation, the prepared suspension of Ph lyophilisate was added. sibiricus, 22 µl per well, except for control wells. After isolation of the substance, the cells were incubated for 72 hours, removing the culture medium with emissions using a vacuum aspirator. Separately, a solution of 9 ml wasprepared in a bath for a multichannel dispenser. culture medium + 1 ml. MTT reagent (5 mg/ml in Hanks' solution). The prepared solution was added in 100 µl to each well of the plate and incubated in a CO2 incubator for 3.5 hours. According to the approximate incubation time, thesupernatant was carefully removed and then 100 µl of DMSO was added to each well and further discubed for 10 min. After the last incubation, the wells show a bright purple color. Violet stain detection performed on a plate reader (Bio-Rad iMark) at 650 spectrum spectra.

The choice of MTT-test is due to the fact that MTT has a positive charge and easily penetrates into living eukaryotic cells. 3-[4,5-dimethylthiazolyl-2-el]-2,5-iphenyltetrazolium bromide - MTT reagent under the action of mitochondrial dehydrogenases of viable cells turns into water-insoluble formazan, which has a purple color. During cell lysis, crystals formed in living cells easily pass into a DMSO (dimethyl sulfoxide) solution. The optical density of the formazan solution was used to determine the activity of mitochondrial

dehydrogenases and, accordingly, cell viability.

Doxorubicin was chosen as a drug sample with proven antitumor activity. Doxorubicin, described in US Pat. No. 3,590,028, has a wide range of uses in the treatment ofcancer and is used in the treatment of leukemias, lymphomas and solid tumors. It inhibits the synthesis of DNA and RNA: it intercalates into the DNA double helix between pairs of nitrogenous bases (the matrix is broken and the spatial structure changes) and causes DNA cleavage due to the formation of free radicals. In addition, the antitumor effect may be due toa change in cellular functions as a result of binding to cell membrane lipids and interaction with topoisomerase II. The drug "Doxorubicin", which refers to antitumor antibiotics of the anthracycline series, isolated from the culture of Streptomyces peucetius var. Caesius and hasantimitotic and antiproliferative effects. The concentration of the tablet form of the drug wasadjusted to 0.5 mg/ml, taking into account its therapeutic dose.

All obtained quantitative results were subjected to statistical processing using the IBM SPSS Statistics, 23 package. The Kolmogorov-Smirnov test was used to determine whether the data corresponded to the normal distribution law. The equality of the sample means was tested using the parametric Student's t-test (in the case of a normal distribution) and the non- parametric Mann-Whitney U-test for independent samples (in case of deviation from the normaldistribution). The value of $p<0.05$ was taken as the threshold level of significance. Continuousvalues were presented as median (Me) and 25th and 75th percentiles.

RESULTS

This pilot study was carried out in 2022 and consisted of three stages. At the first stage of the study (May 2022), experiments were carried out with different concentrations of the test substance - a suspension of lyophilisate *P. sibiricus:* 0.01 g/ml (1:10);0.001 g/ml (1:100); 0.0001 g/ml (1:1000) and 0.00001 g/ml (1:10000) based on a starting concentration of 0.1 g/ml (1:1). To obtain the initial concentration, we took into account the doses used in traditional medicine. For each concentration, we evaluated 16 wells of the plate, i.e. 16 x 6 = 96 holes. Comparative analysis revealed statistically significant differences, indicating the most effective concentration of cytotoxicity P. sibiricus in relation to HepG2 cancer cells equal to 0.01 g/ml (1:10) (Fig. 1`).

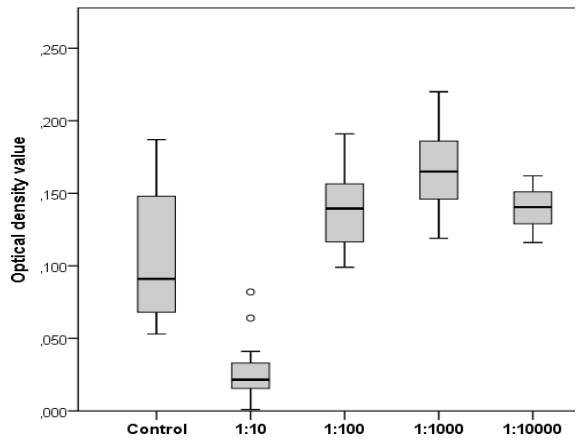

Fig 1: Cytotoxic effect of various concentrations of *P. sibiricus* versus control

Differences in optical density values of this effective concentration of 0.01 g/ml (1:10) compared with other concentrations and the control group (no lyophilisate, only cancer cells) were statistically significant $p \leq 0.05$. Thus, at the first stage, we found a more effective concentration of *P. sibiricus* with a maximum cytotoxicity value for HepG2 cancer cells equalto 0.01 g/ml (1:10).

The second stage of our study (October 2022) was that, in addition to the control group(only cancer cells without the addition of *P. sibiricus)*, a group with the drug Doxorubicia was added to the experimental groups. Taking into account the previous results obtained, indicatinga pronounced degree of cytotoxicity at a concentration of 0.05 g / ml (1:10), the following high concentrations of Ph were determined at this stage. sibiricus: 0.02 g/ml (1:5), 0.01 g/ml (1:10)and 0.006 g/ml (1:15). Each comparison group, as in the preliminary experiment, was evaluatedin 16 wells. The results of statistical analysis indicated that *P. sibiricus* concentration of 0.02 g/ml (1:5) had a pronounced cytotoxic effect, which turned out to be very close to that of the drug (Fig. 2).

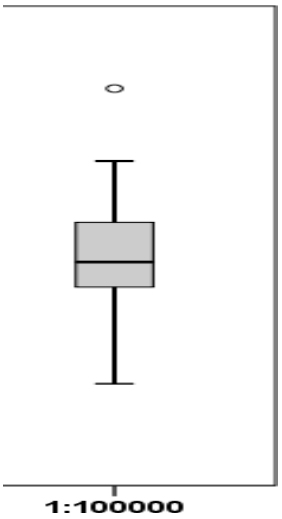

Fig 2: Cytotoxic effect of various concentrations of *Ph. sibiricus* compared with controland doxorubicin.

Comparative analysis did not reveal statistically significant differences in optical density between the values of the suspension with *Ph. sibiricus* with a concentration of 0.02 g

/ ml (1:5) (0.008 (0.005; 0.012)) and doxorubicin (0.007 (0.003; 0.0107)). Compared with the control, statistically significant differences in the values of optical densities are the value of thedrug and the concentration of 0.02 g/ml (1:5) and 0.01 g/ml (1:10) *Ph. sibiricus.*

DISCUSSION

In our study, we obtained results indicating that *P. sibiricus,* being sources of phytosterols, phenylpropanoids, can be considered as a potential promising medicinal raw material with antitumor activity. The antitumor activity of the substances was evaluated in vitro. This study was carried out in a biotechnological laboratory on a model tumorcell line HepG2. At the same time, a standard drug screening protocol was used, and the directeffect of the extract on directly cultured cells

was studied. Cytotoxicity was investigated by MTT - test. The MTT test is an indicator of mitochondrial function in viable cells based on tetrazolium reduction. The most effective concentration of lyophilized P. sibiricus extract was of 0.02 g/ml (1:5)

As it turned out, the aerial part of the medicinal plant of the Siberian bloater (lat. *Phlojodicarpus sibiricus*) is of great value for medicine, as it has an antitumor effect. This is due to the presence in them of effective biologically active substances that can be isolated andmodified as a drug. The lack of raw materials can be compensated by plantation cultivation ofthe plant.

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