Research Article

Metabolic cell disorders under the influence of lectin-like proteins of *Cuscuta europaea*

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Abstract

Lectins are one of the functionally significant proteins involved in the plant protective strategy and possess a wide range of action, which in many ways stimulates researchers to search for new sources of their isolation. **Objective:** The work was initiated to study cytotoxic activities and membrane –active properties of lectin-like glycoproteins isolated from the dodder (*Cuscuta europaea*). **Methodology:** LLP were isolated from the seeds of the dodder by extraction with phosphate buffer solution with subsequent gradient precipitation with 20%, 50% and 80% ammonium sulfate and designated as $LLP_{20,}$ LLP_{50} and LLP_{80}, respectively. Cytotoxic activity of LLP was assessed biochemically in HeLa (cervical carcinoma) and B-16 (skin cancer) cell cultures by MTT, LDH assay. The membrane-active properties of the total fraction of dodder containing LLP were studied, as well as its effect on the conductivity of the lipid bilayer. **Results:** The results showed that the B-16 cell line was more sensitive than HeLa to lectin-like proteins of *Cuscuta europaea*. Also, it was shown that the HeLa cell line was more resistant than B-16 to cisplatin. All fractions contribute to the manifestation of high activity of extracellular LDH. **Conclusion:** Thus, we isolated and characterized lectin-like proteins form *Cuscuta europaea*, as well as demonstrated cytotoxic effect of $LLP_{20,}$ $LLP_{50}$and LLP_{80} on HeLa and B-16 cells. The membrane-active properties of LLP, as well as heir influence on the conductibility of the lipid bilayer and the degree of cell membrane damage, are shown.

***Keywords*:** Lectin-like proteins, *Cuscuta europaea,* cytotoxicity, membrane –active properties, cisplatin

Introduction

At present, the attention of scientists has been attracted by a large group of parasitic plants (*Haustorya*), which includes various species of mistletoe (*Viscus alba* L) and dodder (*Cuscuta* L), from which various cytotoxins have been isolated (Ya Chee et al., 2016). One of the little-studied object is lectin-like glycoproteins of parasitic plants. Lectins and lectin-like proteins have a wide range of activities, including the control malignant tumors, which largely stimulates researchers to search for new sources of their isolation and study of biological effects (Akev et al., 2020).

The most studied protein cytotoxins are viscotoxins and purotoxins isolated from various types of mistletoe, which actively suppress the cancer cell growth in tissue culture (Marvibaigi et al., 2014).

Preparations obtained from the parasite plant of mistletoe (*Viscum album L.*) in the form of extracts as well as their individual components (lectin, agglutinin) and recently synthesized recombinant form of the drug are widely used in Europe in the treatment of cancer patients (Garcia et al., 2021). Thus, in Western Europe, the patented drug Iskador is popular, which is a fermented water extract prepared using a special technology from white mistletoe leaves, standardized for ML-I lectin.

One of the little-studied objects are lectin-like proteins of the *Cuscuta L* family, which is an obligate flower parasite that mainly affects dicotyledonous plants. The seeds of some species of dodder contain the poisonous substance saponin.

Among chemical compounds with biological activity, coumarins, flavonoids, phenolcarboxylic acids and their derivatives, polysaccharides, saponins, alkaloids, etc. have

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been isolated from seeds and vegetative parts of different dodder species (Noureen et al., 2019). An ethanol extract of dodder showed antioxidant and antiproliferative activity on cancer cells (Bulut et al., 2021).

An aqueous extract of *Cuscuta chinensis* inhibited the proliferation of cancer cells (CCRF-CEM-human leukemia cells) and normal cells (JM-normal lymphocytes) at various concentrations (Zeraati et al., 2010).

An aqueous extract from the seeds of *Cuscuta chinensis* delayed the appearance and development of papillomas and the spread of carcinomas induced in mice by oral administration of 7,12-dimethylbenzaanthracene (Noureen et al., 2019). The antitumor activity of *Cuscuta kotschyana* on the MCF7 cell line (breast cancer) was studied (Sepehr et al., 2010).

A glycoprotein with molecular weight of 27-28 kDa that binds the C3 component of the complement system was isolated and characterized from the seeds of *C. europaea*. This glycoprotein exhibited a strong immunostimulating effect both in vivo and in vitro experiments. The main target of C3-binding glycoprotein is macrophages. This protein induces the formation of interleukin-6, subsequently γ-interferon and, to a very small extent, interleukin-1α and interleukin-10 (Stanilova and Carpenter, 1994).

Despite intensive studies of the components of the parasitic plant *Cuscuta europaea*, the protein–peptide components, in particular, the lectin-like proteins of the seeds of *Cuscuta europaea*, and their effect on the cell remain the least studied.

In the future, our interest in dodder is due to the need for a highly active cytotoxic substance effective against cancer cells, including skin cancers and psoriasis (Atyabi et al., 2018) for further development of a tumor-targeted therapeutic agent.

Materials and methods

Isolation of lectin-like proteins

We used the seeds of dodder (*Cuscuta europaea*) parasitizing on *Alhagi* L. Lectin-like proteins (LLP) were isolated from the seeds according to (Kakhorova et al., 2018). For that air-dried raw material was ground up and extracted with ten parts (m/v) of phosphate-buffer (pH 7.7) containing 0.1 M NaCl PMSF (SIGMA, USA) for 2 hours, and aliquot was taken to use it as total fraction (Σ). The extract was centrifuged for 20 minutes at 5,000 rpm. After centrifugation the supernatants (total proteins) were subjected to gradient precipitation with ammonium sulfate; one part was precipitated by ammonium sulfate to the final concentration of 20%, the second part to the final concentration of 50%, the third part to the final concentration of 80%. After centrifugation the LLP_{20}, LLP_{50} and LLP_{80} precipitations were dialyzed against the distilled water. All the protein fractions were lyophilized. Concentrations of protein were measured by Lowry method; total sugars were determined by anthrone-sulfuric method.

Cells and reagents

Two cancer cell lines cervical carcinoma cells (HeLa) and skin cancer cells (B-16) were used for cytotoxicity screening of the lectin-like proteins. The verified cell lines were cultivated in the Bank of Cell Cultures in the Institute of Bioorganic Chemistry, Academy of Science of the republic of Uzbekistan. Cell lines were cultured in RPMI-1640 (SIGMA, USA) supplemented with 10% inactivated FBS (HiMedia, India) and 2 mM L-glutamine (HiMedia, India) and 1% antibioticantimycotic solution (HiMedia, India), and grown at 37°C in a humidified atmosphere of 5% CO_2 in air. To sub culture the cells were 2 detached with Versen solution.

MTT assay

To assess cytotoxic effect of the proteins cervical carcinoma cells (HeLa) and skin cancer cells (B-16) were disseminated in 96-well plates in amounts of 20,000-30,000 of cells/ml in 100 µl of RPMI-1640 with 10% fetal bovine serum to be cultivated at 37°C in CO_2 incubator. In 24 hours the proteins in the doses of 100, 10 and 1 µg/ml in 100 µl of the medium were added to cells to be cultivated for 24 hours, to be subsequently mixed with 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Hi Media, India) for viable cells to be found. After one-hour incubation the medium was decanted with care to add DMSO and incubated for 20 minutes; optical density of the solution was measured at 620 nm (En Spire Multimedia Reader 2300, Singapore).

LDH assay

To assess the degree of damage to cell membranes, a method based on measuring the release of lactate dehydrogenase (LDH) into the culture medium was used. To do this, 24 hours after the addition of proteins, 5 µl of the culture fluid was taken from each well of growing cells and the contents were transferred in parallel to each well of a flat-bottomed 96-well plate containing substrates (135 µl of 0.1 M phosphate buffer, pH 7.4, 5 µl 5 mM NADH, 5 µl 23 mM sodium pyruvate) to measure LDH activity. The contents were quickly mixed and the increase in optical density at 340 nm was measured on the microplate reader (En Spire Multimedia Reader 2300, Singapore) every minute for 1–3 min (Freyer and Christoph, 2017).

Statistical analysis

Statistical data processing was carried out using standard "Origin" and "Microsoft Excel" software packages. Differences were considered as statistically significant at p

<0.05 and p <0.01. Experimental results are presented as the average of data obtained from at least 3 experiments.

Results

LLP were isolated from the seeds of the dodder by extraction with phosphate buffer solution with subsequent gradient precipitation with 20%, 50% and 80% ammonium sulfate and designated as $LLP_{20,}$ LLP_{50} and LLP_{80}, respectively.

Cytotoxic effects of LLPs

We studied cytotoxic activity of a glycoprotein (LLP) isolated from the dodder in various cell systems, to name, human cervical carcinoma (HeLa) and skin (B-16) cancers. Cultured cells that mimic the human body in vitro can replicate indefinitely. HeLa cells are of epithelial origin. On their surface, they carry a reasonably universal set of receptors, which allows them to be used to study the action of various biologically active substances.

HeLa and B-16 cells were cultured in RPMI-1640 growth medium containing an antimycotic antibiotic, L-glutamine, and 10% fetal calf serum and adapted to our culture conditions.

Cytotoxic activity was assessed biochemically using the MTT test. MTT is which can be used to determine mitochondrial dysfunction, i.e. the degree of damage to mitochondria and, as a result of inhibition intensity of cellular respiration.

To determine the cytotoxic effect, the cells were seeded into 96-well plates in 100 µl of the RPMI 1640 growth medium and cultured at 37^0C in a CO_2 incubator. A day later, proteins were added at different concentrations for cultivation with cells for 24 h, and then MTT was injected into cell culture. After a 3-hour incubation, the medium was carefully poured off, DMSO was added and incubated for 20 minutes, then the optical density of the solution was measured at the wavelength of 620 nm **(Table 1).**

Table 1. *Cytotoxic activity of* Lectin-like Protein from *Cuscuta europaea*

Samples	MTT assay, IC50 (mg/mL)	
	HeLa	B-16
∑ fraction	9.76±0.3	9.98±0.06
LLP_{20}	12.54±0.5	9.37±0.95
LLP_{50}	10.0±0.4	9.6±0.72
LLP_{80}	12.8±0.5	9.2±0.5
Cisplatin	8.4±0.8	8.0±0.6

Results are expressed as means ± SD of three independent MTT assay performed in triplicate. Cisplatine was tested as positive control.

Cells without exposure to substances served as control, where the level of MTT incorporation into cells was 100% (100% of live cells). Cisplatin was used as a positive control indicating the sensitivity of cells to the effects of drugs.

The result showed that the lectin-like proteins significantly ($P < 0.05$) inhibited the cell growth of HeLa and B-16 cell lines. As shown in Table 1, the IC50 values (The inhibitory concentrations that could reduce 50% of HeLa and B-16 cells) were 9,76 mg/mL (∑), 12,54 mg/mL (LLP_{20}), 10 mg/mL (LLP_{50}), 12,8mg/mL (LLP_{80}) and 9,98 mg/mL (∑), 9,37 mg/mL (LLP_{20}), 9,6 mg/mL (LLP_{50}), 9,2 mg/mL (LLP_{80}) for fractions and 8,4 and 8 mg/mL for cisplatin, respectively. The results showed that the B-16 cell line was more sensitive than HeLa to lectin-like proteins of *Cuscuta europaea*. Also, it was shown that the HeLa cell line was more resistant than B-16 to cisplatin (**Table 1**).

It can be assumed that the cytotoxic effect on cells violates the integrity of not only the cell membrane, but also the mitochondria; in general, cell metabolism is disturbed.

One of the fundamental properties of the cell is the ability to actively maintain the constancy of the volume under conditions of changing osmolality of the medium.

We have previously studied the effect of dodder lectin-like proteins on the regulation of thymocyte volume under hypoosmotic stress. We have shown that lectin-like proteins from the seeds of the dodder *Cuscuta evropaea* have an overwhelming effect on the regulation of thymocyte volume under conditions of hypoosmotic stress. The mechanism of action of the studied substances on RVD (regulatory volume decrease), is possibly associated with their effect on volume-activated potassium channels (Kakhorova et al., 2015).

Membrane –active properties of LLPs

In order to study the membrane-active properties of LLP, we carried out experiments on flat bilayer membranes formed on the opening of the Teflon septum of the sample cell used in the method of measuring conductivity at the fixed voltage between two compartments of the cell. Egg phosphatidylethanolamine (PE) was used as the phospholipid. Control experiments showed that in the absence of membrane-active molecules, the formed membrane washed with a buffer solution (Tris-HCI, pH 7.5) has a lifetime of several hours, i.e. the membrane did not disintegrate for a long time. The measurements were carried out at various values of voltage, fixation, and at the same time the membrane retained its integrity. We chose K^+ and Na^+ ions (salts of KCI and NaCI) as conduction ions.

The influence of the total LLP fraction on the conductivity

of the bilayer membrane was carried out as follows. A membrane was formed, then within about 0.5 hour if the membrane retained its integrity, LLP was introduced into the cis-compartment of the cell and the conductance curve was recorded. It turned out that LLP at the concentration of 1 ng/ml is not able to form conduction ion channels in bilayer membranes in the presence of NaCI in solutions washing the membrane (Tris-HCI-25mM, pH 7.4, clamping voltage +30mV).

However, single channels were found in the presence of KCI. No more than 10 single openings of potassium channels were experimentally recorded, after which the membrane ruptured. Similar results were also obtained on bilayer membranes formed from PE + 5% phosphatidylserine isolated from bovine brain. Only the lifetime of the membrane differed. The rupture of integrity under the action of the protein occurred in a shorter period. The data obtained indicate that under the action of the total LLP fraction of *Cuscuta europaea*, a destruction of the integrity of bilayer membranes was observed and that the membrane activity of dodder proteins depends on the lipid composition of bilayer membranes.

Effect of LLPs *Cuscuta europaea* on LDH activity

The measurement of lactate dehydrogenase activity is also used as the basis for various cytotoxicity assays. This enzyme is normally present in the cytoplasm of living cells and is released into the cell culture medium by leakage through the membranes of dead or dying cells that have been damaged by the toxic agent. Small amounts of the culture medium can be removed at various time intervals after cell chemical treatment to measure LDH activity and assess the degree of damage to cell membranes ((Freyer and Christoph, 2017). 24 hours after the addition of various LLP fractions to HeLa cells, 50 µl of culture fluid was taken from each well of growing cells to measure LDH activity and added to each well of a flat-bottomed 96-well plate containing substrates for measuring LDH, as described in materials and methods. The contents were rapidly mixed and the increase in absorbance at 340 nm was measured on the microplate reader (En Spire Multimedia Reader 2300, Singapore) every minute for 1-3 minutes. The data can be seen in **figure 1.**

Table 2. Effect of LLPs *Cuscuta europaea* on LDH activity

Sample	LDH assay IC50 mg/ml
∑-fraction	11.4 ± 0.03
LLP_{20}	12.6± 0.1
LLP_{50}	11.5± 0.04
LLP_{80}	11.4± 0.04
Cisplatin	7.7± 2.0

LDH levels in HeLa cells treated with different concentrations (1-100 µg/L) of lectine-like proteins for 24 h. Results are expressed as means ± SD of three independent LDH assay performed in triplicate. Cisplatin was tested as positive control.

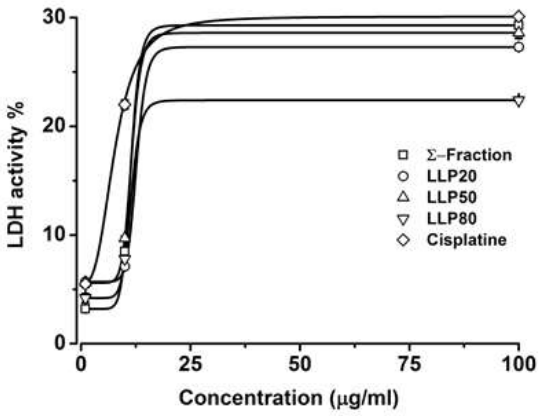

Figure 1. Effect of LLP *Cuscuta europaea* on LDH activity.

As shown in Figure 1, the IC50 values (The inhibitory concentrations that could reduce 50% of HeLa cells) were 11,4 mg/mL (∑), 12,6 mg/mL (LLP_{20}), 11,5 mg/mL (LLP_{50}), 11,4 mg/mL (LLP_{80}) for fractions and 7,7 mg/mL for cisplatin, respectively (**Table 2)**. In the present study, MTT and LDH cytotoxicity assays on HeLa cells gave similar results (**Table 2**).

As follows from **Figure 1**, all LLP fractions contribute to the manifestation of activity of extracellular LDH, which correlates with our data on the cytotoxic activity of these fractions.

Discussion

The study of the mechanism of action of biologically active substances on target cells requires an integrated approach, consisting of various tests using several indicators of toxicity at the cellular level: a set of cell cultures, the study of interrelated processes leading to damage to the integrity of biological membranes, impaired synthesis and secretion of the most important molecules occurring inside the cell.

We have studied the effect of *Cuscuta europaea* lectin-like proteins on various types of continuous cell cultures, as well as studied the membrane-active properties of the protein and the degree of damage to cell membranes.

Seeds of *Cuscuta europaea* growing on Alhagi were used as plant raw materials.

Lectin-like proteins (LLPs) were isolated by saline extraction (∑- total fraction) followed by 20, 50 and 80% salting out with ammonium sulfate and were designated by us as LLP_{20}, LLP_{50} and LLP_{80}, respectively, and as described previously (Khashimova et al., 2022). The protein content

was determined by the Lowry method. The content of total sugars was determined by the anthrone-sulfuric acid method, and it was found that proteins are glycoproteins (Kakhorova et al., 2018).

In the frame of this work, the effect of *Cuscuta europaea* lectin-like proteins on continuous HeLa (human cervical carcinoma) and B-16 (skin cancer) cell culture lines was studied. We also studied the comparative effect of different fractions of *Cuscuta europea* lectin-like proteins on two types of transplanted cancer cells HeLa and melanoma B-16. From the comparative analysis it follows that the B-16 cell line was more sensitive than HeLa to lectin-like proteins of *Cuscuta europaea*.

One of the fundamental properties of the cell is the ability to actively maintain the constancy of the volume under conditions of changing osmolality of the medium.

We have previously studied the effect of dodder lectin-like proteins on the regulation of thymocyte volume under hypoosmotic stress. We have shown that lectin-like proteins from the seeds of the dodder *Cuscuta evropaea* have an overwhelming effect on the regulation of thymocyte volume under conditions of hypoosmotic stress. The mechanism of action of the studied substances on RVD (regulatory volume decrease), is possibly associated with their effect on volume-activated potassium channels (Okten et al., 2014). The membrane-active properties of the total fraction of dodder containing LLP were studied, as well as its effect on the conductivity of the lipid bilayer (Ionov et al., 2009). It can be assumed, that it is the channel-forming properties of dodder proteins that may be the cause of its antitumor activity and may be determined by the lipid composition of the cancer cell, which differs from that of healthy cells.

One of the approaches to determine the integrity of cell membranes is the activity of intracellular lactate dehydrogenase (LDH). Lactate dehydrogenase is one of the key metabolic enzymes and has a significant effect on the redox potential of the cell. The under the action of lectin-like proteins, the activity of lactate dehydrogenase increases and a more pronounced character is observed in the case of LLB_{50}. These data indicate that, probably, the integrity of the cell membrane is disrupted.

Conclusion

Thus, we found that the lectin-like proteins of *Cuscuta europaea* have a dose-dependent cytotoxic effect on the transplanted cell culture. Cytotoxic activity is probably due to damage to the cell membrane. In future, this herbal source can be used for developing anti-tumor drugs.

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Conflict of interests

The authors declare no conflict of interests.

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