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PHENOLIC COMPOUNDS OF A CULTURE OF *Alhagi kirghisorum* CELLS

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A comparative analysis has been made of a series of phenolic compounds in the initial plant and in a cell culture of Alhagi kirghisorum. *Quercetin, isorhamnetin, isorhamnetin 3-O-neohesperoside, retusin, 8-methylretusin, calycosin and formononetin were isolated from the cultivated cells and identified. Features of the formation of various classes of phenolic compounds in the cultivated cells have been studied by chromatographic methods. It has been shown that the phenolic substances synthesized by cell cultures exhibit an inhibiting influence on the biosynthesis of protein in vitro and show a moderate cytotoxic activity.*

Cells and tissues of higher plants cultivated in vitro are widely used for the study of the capacity of cells for synthesizing various classes of biologically active substances and with the aim of obtaining natural substances by a biotechnological method. On the other hand, the use of cells in vitro permits such processes as growth, cell differentiation, the influence of extreme and harmful actions, and the functional activity of various classes and groups of natural substances to be studied at the tissue and cell levels [1].

We have investigated cultivated cells and tissues of *Alhagi kirghisorum* obtained in the laboratory [2]. This culture has served as a model system for studying the influence of various factors (illumination, temperature, hormonal effectors, fungal elicitors, etc.) on the growth of cells and the biosynthesis of various classes of phenolic compounds in them [3, 4].

The aim of the present investigation was to study features of the formation, isolation, and identification of phenolic compounds synthesized by *Alhagi kirghisorum* cells under in vitro conditions and to reveal the biological activity of these substances.

To study the chemical composition of the phenolic compounds we used callus and suspension cultures at the stages of postexponential and stationary growth, which are characterized by the maximum accumulation of these substances. The investigations performed showed that under in vitro conditions there is a 7- to 12-fold decrease in the level of biosynthesis of soluble phenolic compounds as compared with the epigeal part of the native plant (about 0.8—1.3% in callus cells and 0.3—0.8% in suspension cells). At the same time, the qualitative composition changes considerably. The most stable process, preserved in cell and tissue cultures that have been passaged for a long time, is the biosynthesis of substances that are conjugates of phenolic carboxylic acids [3] and isoflavonoids. In cells that have been passaged for a long time not only is the biosynthesis of isoflavonoids preserved but their relative amount increases 2- to 3-fold, with a considerable decrease in the accumulation of other classes of phenolic substances. Using the HPLC method, four isoflavonoids were isolated from suspension cells. On the basis of physicochemical methods of analysis and comparison with the literature, these compounds have been identified as retusin, 8-methylretusin, calycosin, and formononetin [5—7]. Compounds of this class have not been described for the initial plant. The biosynthesis of compounds of other classes in cells in vitro is less stable. It has been shown that already in the callus cultures of the first passages there is complete inhibition of the biosynthesis of catechins, proanthocyanidins, and their polymeric forms, the amount of which in the initial plant is high and may reach 10—12%.

Of the more than 15 flavonols and their glycosides known for the initial plant, in callus cultures passaged for a short time only three have been detected and these were identified by physicochemical methods of analysis as quercetin, isorhamnetin, and isorhamnetin 3-O-neohesperoside [8]. The authors also observed the synthesis of other flavonols in cultures passaged for

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a long time if the cultivation of the cells was carried out under conditions of stress, especially under fungal attack and with an excess of certain trace elements in the nutrient medium.

We have studied the biological activity of total preparations and of individual phenolic compounds synthesized in cultivated cells. Biological activity was evaluated from the inhibiting action on processes of polypeptide synthesis in a cell-free system from rabbit reticulocytes [9]. Total preparations showed a 20—80% inhibition of the activity of protein biosynthesis initiation factor eIF-2, while individual fractions possessed a stimulating action. Individual phenolic fractions showed a smaller inhibiting effect. The highest activity was possessed by 8-methylretusin (45% suppression) and somewhat smaller ones by calycosin (35%) and retusin (25%). Formononetin, quercetin, and isorhamnetin exerted no influence on the in vitro synthesis of peptides. A test for cytotoxic activity showed that the phenolic compounds of the cultivated cells exerted a moderate cytotoxic effect in tumor strains of mouse myeloma Y-53, Ehrlich carcinoma, Walker carcinosarcoma, and human ovarian carcinoma CaOv. The percentage inhibition of the growth of tumor cells of various strains by the most active fractions amounted to 37—49%.

EXPERIMENTAL

General Observations. The conditions for obtaining and cultivating callus and suspension cells of *Alhagi kirghisorum* have been described in [2]. For the isolation of phenolic compounds we used the freeze-dried biomass of the cultivated cells and tissues. UV spectra were taken on a Hitachi-330 spectrophotometer (in ethanol), and IR spectra on a UR-20 instrument (KBr tablets). Mass spectra were obtained on a Varian-MAT 44 instrument at an energy of the ionizing radiation of 70 eV, and PMR spectra on a Bruker instrument (500 MHz) in CD_3OD or DMSO-d_6 (δ scale, 0 — TMS). Melting points were determined on a Kofler block. Ascending PC was conducted on Filtrak paper in the following solvent systems: 1) butan-1-ol—acetic acid—water (40:12.5:29); 2) 15% acetic acid; 3) butan-1-ol—pyridine—water (6:4:3); 4) benzene—acetic acid—water (6:7:3); and 5) sodium formate—formic acid—water (10:1:200). The phenolic compounds were detected when the chromatograms were viewed in UV light before and after treatment with ammonia or were revealed with diazotized *p*-nitroaniline, a 1% solution of iron ammonium alum $FeNH_4(SO_4)_2 \times 10H_2O$, or a 0.1% solution of vanillin in concentrated HCl. Carbohydrates were detected with *o*-toluidine salicylate or urea. TLC was conducted on Silufol UV-254 plates in the solvent system chloroform—ethanol (95:5). HPLC was performed on Separon SGX C-18, Silasorb C-18, and Partisil 10 ODS columns.

Extraction of the Phenolic Substances. Freeze-dried cells (100 g) were ground in a mortar and were extracted three times with 96% ethanol. The combined extracts were concentrated, diluted with water to 50 ml, and treated with chloroform (6 × 50 ml).

Separation of the Phenolic Substances. a) The chloroform fraction of the total extract was chromatographed on a column of silica gel (Chemapol L, 40—100, 1:20). Quercetin and isorhamnetin were isolated by the elution of the column with chloroform—ethanol (98:2).

b) The aqueous fraction, after being worked up, was chromatographed on a column of polyamide (Woelm, Germany). Isorhamnetin 3-O-neohesperoside was isolated by eluting the column with 10—20% ethanol—water mixtures.

c) **Preparation of a Sample for HPLC.** The dry residue from the initial ethanolic extract was treated with a 1:1 mixture of ethyl acetate and water. The ethyl acetate fraction was washed twice with water (1:1), evaporated to dryness, and redissolved in the minimum amount of methanol. The precipitate of phytosterols that deposited was discarded. The mother solution was evaporated to dryness, and 50 mg of the dry residue was dissolved in 60% aqueous methanol (10 ml). An initial separation of the substances was achieved in an isocratic regime on a column of Silasorb C-18, 5 μm, 15 × 250 mm, eluent 70% aqueous ethanol, space velocity 5 ml/min, column temperature 22°C. The fractions obtained that contained 85—96% of the main component were rechromatographed on a Partisil 10 ODS column, 4.6 × 250 mm, eluent 0 or 60% aqueous ethanol; space velocity 1 ml/min; column temperature 22°C Retusin, 8-methylretusin, calycosin, and formononetin were obtained.

Acid Hydrolysis. A solution of 2 mg of the glycosides in 1 ml of 0.1 N ethanolic HCl was heated in the boiling water bath for 2 h. The hydrolysis products were chromatographed in solvent systems 1—3 with carbohydrate and flavonol markers. Glucose and isorhamnetin were detected in the hydrolysate.

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