Nitraria schoberi L. hairy root culture as a source of compounds with antiviral activity against influenza virus subtypes A(H5N1) and A(H3N2)

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Abstract

The hairy root culture of halophyte *Nitraria schoberi* was obtained by transforming seedlings' primary leaves with a wild strain of *Agrobacterium rhizogenes* 15834 SWISS (transformation frequency was 78.7%). The roots' transgenic status was confirmed by PCR analysis using *rolB*-, *rolC*-, *virC*-, *virD1*-specific primers. There was a significant increase in the biosynthesis of flavonoids, hydroxycinnamic acids, pectins, protopectins and saponins in hairy roots as compared to intact plant roots. The catechin content in ethanol extracts of transformed roots' exceeded this factor 3.8 times in plant roots, but the compound composition of catechin nature in the latter was more diverse. The high antiviral activity of *N. schoberi* hairy root ethanol extracts against influenza virus subtypes A(H5N1), A(H3N2) was revealed, which manifested itself in the effective inhibition of infectivity both in vitro—in MDCK cell culture—and in vivo increasing survival of mice infected with them.

Keywords *Nitraria schoberi* · Hairy roots · Catechins · Influenza virus · Antiviral activity

Introduction

Nitraria schoberi L. is a halophytic shrub of the family *Nitrariaceae*, a typical representative of deserts and semi-deserts, grown in saline gypsum soils of foothills, lowlands, and salt lakes shores. The species has a disjunctive distribution (Caspian, Turanian lowlands, China (Dzungaria), Afghanistan, Northern Iran, Syria, Transcaucasia, Altai Region, southeast of West Siberia, Kazakhstan, Crimea, spurs of southern Carpathians). Due to their high tolerance to arid growth conditions the genus *Nitraria* species are in demand in phytoremediation to reduce salinity as well as powerful stabilizers of sand soils (Zhao et al. 2002). *Nitraria* species are highly valued for their nutritional qualities, while *N. schoberi* is one of the few fruit-berry plants able to grow on solonchaks.

The most important mechanism of stress resistance to severe conditions in nitre bushes is their ability to synthesize antioxidant substances, which determine a wide range of medicinal uses. *Nitraria* contains flavonoids, hydroxycinnamic acids, anthocyanins, pectins and carbohydrates (Vysochina et al. 2011). In addition, species of the genus *Nitraria* accumulate alkaloids that are structurally unusual and promising in terms of biological activity (Mohamed et al. 2014). The cytotoxic effect of leaf extracts from *N. retusa* on melanoma cells has recently been established (Boubaker et al. 2015). Moreover, this species is characterized by antiviral activity against Rotavirus Wa, Adenovirus type 7 and Coxsackievirus B4 (Mohamed et al. 2015). Extracts of fruits, leaves and roots of *N. schoberi* have powerful antioxidant properties preventing free radical formation (Sharifi-Rad et al. 2014). Further studies have shown that extracts from the fruits of *N. schoberi* fruits are characterized by not only antioxidant but antimicrobial, fungicidal and anti-inflammatory activities (Sharifi-Rad et al. 2015).

The interest in drugs originating from plants has significantly increased in recent years due to their availability, lower toxicity and broad spectrum of action compared to synthetic drugs (Vahabpour-Roudsari et al. 2007; Salehi et al. 2017; Sharifi-Rad et al. 2017a, b, c). Influenza

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infections remain a major health problem worldwide. Among the existing influenza virus genera, A viruses are the most virulent pathogen periodically causing epidemics and epizootics. The reason for such regularity is the emergence of new virus strains due to the high variability of their genome. Based on the antigen specificity of surface glycoproteins of hemagglutinin (H) and neuraminidase (N) nowadays known as 18 H and 11 N subtypes of influenza A virus. Viruses containing three subtypes H (H1, H2, H3) and two subtypes of N (N1, N2) are of epidemic importance for human population. Seasonal morbidity is caused by strains of the influenza A virus of two H1N1 and H3N2 subtypes (Tong et al. 2012). A/H5N1 avian influenza has the highest pandemic potential among known subtypes (James and Whitley 2017). It is supposed that its genetic reassortment with a seasonal human influenza virus could lead to the emergence of a novel and potentially highly pathogenic pandemic influenza virus strain (Octaviani et al. 2011). Up to the present, two classes of drugs have been used for antiviral therapy: inhibitors of the adamantane group (amantadine and rimantadine), which depress viral proliferation, and neuraminidase inhibitors (oseltamivir, zanamivir), which play an important role in viral release. However, the high rate of mutations leads to the emergence of drug-resistant strains. Thus, based on virologic surveillance data, a significant proportion of influenza A (H3N2) viruses are resistant to the adamantane drugs but susceptible to neuraminidase inhibitors (Harper et al. 2009). Although, to date, the neuraminidase inhibitors (oseltamivir, zanamivir) are both therapeutic and prophylactic in value for population treatment (James and Whitley 2017), the emergence of drug-resistant virus strains highlights the need to develop novel antiviral drugs that effectively target other viral proteins or cellular factors involved in the influenza virus life cycle (Liu et al. 2011; Kim et al. 2013). Available data on the biological activity of *N. schoberi* are the basis for studying the possible antiviral activity of extracts obtained from this species plants.

However, raw material gathering for the preparation of medicines from *Nitraria* is difficult to implement and can cause serious damage to natural populations. The introduction of these plants is complicated by their biological peculiarities. The solution to the problem of raw material shortage is possible using alternative biotechnological approaches, one of which is developing techniques for in vitro hairy roots cultivation. Hairy root cultures are induced through the genetic transformation of plant cells with *Agrobacterium rhizogenes*, when a part of plasmid DNA bacterium (T-DNA Ri-plasmid) is inserted into the plant cell genome. These cultures are considered to be the most promising in vitro systems in comparison with callus or suspension cultures because of fast growth without hormones, biochemical and genetic stability (Bourgaud et al. 1999). Moreover, the level of valuable secondary metabolite production is comparable

and in some cases exceeds the level of their biosynthesis in intact plants (Sevon and Oksman-Caldentey 2002).

Applying hairy root cultures as an alternative technology in vitro has advantages in comparison with traditional gathering of raw medicinal plants because it allows not only for year-round production of valuable metabolites under controlled conditions but also makes the process cheaper and standardized. Furthermore, there are several approaches from the elicitation of cultures to stressors or chemicals, to the utilization of genetic engineering manipulation with hairy root cultures that can significantly increase the desired metabolite production (Ludwig-Muller et al. 2014).

The aims of this study were to establish *N. schoberi* hairy root culture, to carry out the phytochemical analysis and to evaluate the antiviral activity of ethanol extracts obtained from hairy root culture against influenza A virus subtypes H3N2 and H5N1 subtypes.

Materials and methods

Plant material

Seeds of *N. schoberi* were collected in the surroundings of Kulunda Lake, Altai Region (coordinates: 52°52'94"N, 79°71'60"E). The botanical identity of *N. schoberi* was confirmed by Dr. Sc. E. V. Banaev. The voucher specimen NSK3000987 was deposited at the M.G. Popov Herbarium (NSK, USU_440537) of the Central Siberian Botanical garden SB RAS, Novosibirsk, Russia. Seeds of *N. schoberi* were germinated in vitro using the embryo culture procedure (Zhelezniichenko et al. 2016) on the hormone-free nutrient medium of Murashige and Skoog (MS) (Murashige and Skoog 1962). The roots derived from approximately 5-year-old-plants of *N. schoberi* gathered in the same natural populations were used as intact roots in phytochemical analysis.

Bacterial strains and conditions of cultivation

A wild (unmodified) agropin strain of *A. rhizogenes* 15834 SWISS kindly provided by I. N. Kuzovkina (K.A. Timiryazev Institute of Plant Physiology of the Russian Academy of Sciences, Moscow) was used for transformation. Agrobacteria were grown for 2 days on YEB solid medium (Vervliet et al. 1975) in the dark at $25 \pm 2^\circ\text{C}$, then the culture was collected with a glass spatula and resuspended in MS liquid medium supplemented with 30 g/L sucrose. Cell density in suspension was adjusted to an $\text{OD}_{600} = 0.2$ with MS medium.

Establishment of hairy root cultures

Hairy root cultures of *N. schoberi* were obtained from the primary leaves of 2-week sterile shoots. The leaves were

cut with a scalpel along the middle vein and inoculated overnight in a liquid medium of MS containing agrobacterium suspension and then washed with sterile water. After transformation, the explants were transferred to the solid hormone-free Dunsten and Short (BDS) (Dunsten and Short 1977) medium supplemented with 30 g/L sucrose and 500 mg/L Ceftriaxone (Rafarma, Russia) to eliminate the residual bacteria. Cultivation was carried out under conditions of reduced illumination ($10 \mu\text{mol} \times \text{m}^{-2} \text{s}^{-1}$) for 20 days at $22 \pm 1^\circ\text{C}$. The transformed roots were then separated from explants and cultivated with a reduced content of Ceftriaxone in medium (250 mg/l) for two passages in the dark (passage time 25 days). Then the culture was maintained on a hormone-free nutrient medium of the same composition for two passages. Thereafter, the apical parts of the roots (3–4 cm) were transferred to antibiotic-free BDS liquid medium. Cultivation was carried out on a shaker (Elmi, S-3-02 L, Latvia) at 100 rpm and $22 \pm 1^\circ\text{C}$ in the dark (passage time 35 days). Transformation frequency was calculated as a ratio of transformed explants to their total number. The growth index (GI) was calculated as follows: [(final fresh weight – initial fresh weight)/initial fresh weight] (Vinterhalter et al. 2015). YEB medium before autoclaving was adjusted to pH 7 and the remaining media to pH 5.8. The media were autoclaved at 121°C for 20 min.

PCR confirmation of transformation

To confirm the transgenic status of the hairy root culture, PCR analysis was performed for *rolB* (797 bp) and *rolC* (543 bp) genes. To exclude the possible contamination of cultures with agrobacterial DNA, the preparations were tested by amplifying the sequence of *virC* (730 bp) and *virD1* (441 bp) gene.

The total genomic DNA was identified by CTAB procedure (Suman et al. 1999) with some modifications. Non-transformed *N. schoberi* roots DNA were used as a negative control. Ri-plasmid (pRi) DNA *A. rhizogenes* 15834 SWISS isolated with Plasmid Miniprep set (Evrogen, Russia) was applied as a positive control.

The following pairs were used as primers for detecting: for *rolB* (5'-CAATGGATCCCAAATTGCTATTCC-3' and 5'-CGGCTTTAGGCTTCTTTCTTGAGG-3'); for *rolC* (5'-ATGGCTGAAGACGACCTGTGT-3' and 5'-TAGCCGATTGCAAACTTGAC-3'); for *virC* (5'-ATCATTTGTAGCGACT-3' and 5'-AGCTCAAACCTGCTTC-3'); for *virD1* (5'-ATGTCGCAAGGACGTAAGCCCA-3' and 5'-GGA GTCTTTCAGCATGGAGCAA-3').

PCR was carried out in a thermal cycler C-1000 (Bio-Rad, USA). The final volume of the reaction mixture was 25 μL : 10 ng of DNA, 0.4 μM of each primer (Evrogen, Russia), 0.25 mM of each dNTP (Evrogen, Russia), 1 unit, HS Taq DNA polymerase (Evrogen, Russia), 2.5 mM MgCl_2

(Evrogen, Russia), and 1 $\times$ Taq buffer (Evrogen, Russia). Amplification was performed according to the following programme: initial denaturation at 94°C —3 min; 35 cycles (denaturation 94°C —20 s, primers annealing at 60°C for *rolB*, *virC* and at 55°C for *rolC*, *virD1*—40 s, elongation at 72°C —60 s); final elongation—7 min.

The amplification products were stained with 3 $\times$ SYBR-Green (Medigen, Russia) and separated in 1.7% agarose gel in 1 $\times$ TAE-buffer at voltage 2.7 V/ cm^2 . Gels were visualized and recorded by the GelDoc-It imaging system (Bio-Rad Laboratories Pty Ltd.). The amplified fragment sizes were determined using 100 bp DNA marker (Eurogen, Russia).

Extraction

Antiviral activity has been studied in an ethanol extract of the hairy root culture of the 4th passage. Extracts were prepared from the powdered and homogenized dry material (5 g) of the hairy root culture, which was suspended in 100 mL of 70% ethanol aqueous solution triply for 8 h of the total extraction time at 60°C . Then combined cooled extracts were filtered through glass filters, and evaporated in a rotary evaporator and dried at 60°C in a drying oven.

Phytochemical composition of the hairy root culture of the 4th passage and intact roots was studied in ethanol extracts obtained from air-dried and ground to fine powder materials. To determine the content of flavonoids, hydroxycinnamic acids and catechins, the dry material (5 g) was extracted three times with a 70% ethanol aqueous solution for 30 min in water bath at 72°C . Methods of preparing extracts to define the content of saponins and pectic substances are described in detail below.

Phytochemical analysis

The total flavonoid content of hairy roots and intact roots of *N. schoberi* was determined spectrophotometrically according to the method, based on the formation of a complex flavonoid–aluminum (Brighente et al. 2007). In brief, the extract (0.2 mL) with 0.1 mL of 10% (w/v) aluminum chloride solution, 0.1 mL of 1 M potassium acetate solution, and distilled water (4.6 mL) were mixed. After incubation at room temperature for 15 min, the absorbance of the reaction mixture was measured at 415 nm using SF-26 spectrometer (LOMO, Russia). Rutin (Sigma-Aldrich, Germany) was used to make the calibration curve. The total flavonoid content was calculated as milligram of rutin equivalents per gram dry weight of sample (mg RE/g).

To analyze the total hydroxycinnamic acid content, Arnov's reagent was used (Matkowski et al. 2008). The extract studied (1 mL) was added to 5 mL of water. This mixture was supplemented with HCl (1 mL, 0.1 M), Arnov reagent (1 mL of 10% w/v of sodium molybdate and 10%

w/v of sodium nitrite), NaOH (1 mL, 1 M), and filled up to 10 mL. Thereafter, absorbance was measured at 490 nm using SF-26 spectrometer (LOMO, Russia). The results were expressed as milligram of caffeic acid equivalents per gram dry weight of sample (mg CAE/g).

The saponin content in samples was determined by the double-extraction gravimetric method described by Harbone (1973). A measured weight (5 g) of the powdered sample was mixed with 50 mL of 20% aqueous ethanol solution, then heated in a water bath for 90 min at 55 °C and filtered. The residue was extracted with 50 mL of 20% ethanol. Both extracts were combined and reduced to about 40 mL at 90 °C and transferred to a separating funnel, where 40 mL of diethyl ether was added and shaken hard. Re-extraction through partitioning was done until the aqueous layer had become clear. The saponins were extracted with 60 mL of normal butanol. The combined extracts were washed with 5% aqueous sodium chloride (NaCl) solution and evaporated to dryness in a pre-weighed evaporation dish. The precipitate was weighed and the saponin content in the sample was calculated in milligram per gram dry weight of material (mg/g).

The pectic substance amount was evaluated by a carbazole method based on obtaining a specific violet-pink staining of uronic acids with carbazole in a sulfuric medium (McComb and McCready 1952). A ground sample of dry material was extracted three times with ethanol in boiling water bath to extract sugars, filtered through a paper filter into a volumetric flask. The residue, together with the filter, was placed in a flask and the water-soluble pectin was extracted in a water bath at 45 °C for an hour. The liquid was filtered into a 100-mL volumetric flask. To recover protopectin, after extraction with water the residue was hydrolyzed with 50 mL of 0.3 N HCl for half an hour in a water bath with a reflux condenser; filtered into a 200-mL volumetric flask and washed 2–3 times with hot water. The filter, along with the residue, was put back into the same extraction flask, adding 50 mL of a 1% solution of ammonium citrate, and placed in water bath for half an hour; filtered into a volumetric flask containing the filtrate of the hydrochloric acid extract, washed with hot water, after cooling it was adjusted to mark with water. Before carrying out the reaction with carbazole, demethoxylation was carried out. Then the reaction with carbosol was fulfilled, and the solution optical density was measured at 480 nm on an Agilent 8453 spectrophotometer. Its content was found on a calibration curve for a galacturonic acid. Water-soluble pectin or protopectin content was determined as milligram of galacturonic acid equivalents per gram dry weight of sample (mg GAE/g).

As the inhibitory effect of green tea catechins on the replication of A/H3N2 influenza virus was recently discovered (Kim et al. 2013), it was interesting to study in detail the catechins of *N. schoberi* hairy roots and intact plant roots. An analytical HPLC system consisting of a chromatograph

Agilent 1200 with a diode array detector and a ChemStation system were used to analyze the catechin's quantitative and qualitative content. Separation was performed at a Zorbax SB-C18 column, 4.6 × 150 mm in size, with 5 µm particle diameter, using a gradient elution mode. The mobile phases were 0.1% the methanol (solvent A) and 0.1% the aqueous solution of orthophosphoric acid (solvent B). The methanol content in the aqueous solution of orthophosphoric acid (0.1%) varied from 19 to 70% for 30 min, from 70 to 100% in 30th–32nd min, and from 100 to 22% since 32–36 min. The injected sample volume was 5 µL. The eluent flow rate was 1 mL/min. The column temperature was 26 °C. The detecting was executed at a wavelength $\lambda = 270, 280$, and 290 nm. The individual compound quantitative estimations in the eluates were carried out according to an external standard method (Van Beek 2002). To make standard samples, preparations of Sigma-Aldrich company (Germany) were used. Standard solutions were prepared at 10 µg/mL concentration in ethyl alcohol. UV spectra of unidentified components were specific for catechins: the spectral range was 246–325 nm with $\lambda_{\max}$ 270–280 nm. The amounts of catechins was expressed as milligram of $\pm$ catechin equivalents per gram dry weight of sample in milligram-per gram of dry weight (mg CE/g).

Determination of cytotoxicity

The preparation cytotoxicity and antiviral activity were estimated in a cell monolayer of a transplantable Madin–Darby canine kidney cell culture (MDCK) obtained from the Cell Culture Collection of the State Research Center of Virology and Biotechnology “VECTOR” in 96-well plates using RPMI-1640 medium (Biolot, Russia) and 5% fetal bovine serum (HyClone, USA). To determine the preparation cytotoxicity 10 mg of the dry extract was dissolved in 1 mL of RPMI-1640 medium without serum, then diluted 5, 10, 20, 40, 100, 1000 times in the same medium, then 100 µL in plate wells (four wells for each dilution) and incubated at 37 °C, 5% CO₂, and 100% humidity. After 2 days, an inverted microscope was used to evaluate toxic effects on the cells and determine the maximum nontoxic concentration at which 100% of cells were retained in the monolayer.

Antiviral assay

The preparation antiviral activity was assessed using a highly pathogenic avian influenza virus A/chicken/Kurgan/05/2005 (H5N1) and A/Aichi/2/68 (H3N2) one adapted to laboratory mice. Influenza virus strains of subtypes important for seasonal diseases and possible influenza pandemics have been used in this study. These strains were obtained from the State Collection of Viral Infections and Rickettsioses Agents of SRC VB “VECTOR” (Koltsovo, Novosibirsk Region) and

grown in 10-day embryonated chicken eggs (CE). The virus concentration in samples was determined by their titration in MDCK cell culture calculated by the Spearman–Kerber algorithm, expressed in decimal logarithms of 50% tissue culture infectious doses in millilitre ($\log_{10}$ TCID₅₀/mL) and represented as $M \pm I_{95}$ (where I_{95} is 95% confidence level) (Sachs 1976). The influenza virus A/H5N1 and A/H3N2 titers in the infectious allantoic fluid (IAF) were 7.50 ± 0.82 and $6.50 \pm 1.24 \log_{10}$ TCID₅₀/mL, respectively.

The antiviral activity of *N. schoberi* hairy root extract with respect to the influenza virus was investigated in MDCK cell culture using its maximum nontoxic concentration and tenfold dilutions of the influenza virus in RPMI-1640 medium without serum containing trypsin (2 µg/mL) TPCK (Sigma, USA). For this purpose, 50 µL of an extract solution in RPMI-1640 medium without serum containing 2 µg/mL of trypsin and 50 µL of tenfold dilutions of IAF prepared in the same medium were added to the wells with the cells. After 2 days of incubation at 37 °C and 5% CO₂ in each well, the virus was detected by a hemagglutination reaction with a 1% suspension of chicken erythrocytes, the virus titer was expressed in $\log_{10}$ TCID₅₀/mL. As a reference, Oseltamivir known by its brand name “Tamiflu” (Hoffmann-La Roche Ltd., Basel, Switzerland) was used (Smith 2010).

The preparation concentration in the culture liquid was 0.01 mg/mL. A nutrient medium was used as a control in 50 µL volume. The antiviral effect was evaluated by lowering the virus titer in the experiment compared to the control one, and the virus neutralization index (NI) under the preparation effect was calculated: NI = virus titer in the control – virus titer in the experiment ($\log_{10}$).

In addition, the preparation antiviral activity was evaluated in experiments with ICR outbred mice weighing 14–16 g obtained from the SRC VB VEKTOR nursery. Research and manipulations on animals were conducted in accordance with the veterinary legislation and requirements for humane animal care and use in experimental studies (National Research Council 2011).

In preliminary experiments, the values ($M \pm I_{95}$) of 50% lethal doses (LD₅₀) of influenza virus strains were estimated with intranasal infection of mice, which were 0.10 ± 0.33 and $2.30 \pm 0.33 \log_{10}$ TCID₅₀/head for A/chicken/Kurgan/05/2005 (H5N1) and A/Aichi/2/68 (H3N2), respectively. When assessing the antiviral activity of extract studied, influenza viruses were used at a dose of 10 LD₅₀ for A/chicken/Kurgan/05/2005 (H5N1), and 25 LD₅₀ for A/Aichi/2/68 (H3N2) causing death of 100% of animals in the respective control groups.

The extract solution and Tamiflu were administered to mice orally two times a day in a volume of 0.2 mL with a daily dose of 25 µg/g of mouse weight from an hour before virus inoculation until 5 days after infection. Infected animals in the control group did not receive a cure. There were

10 mice in each group. The animals were being observed for 16 days, the share of deaths and average life expectancy (ALE) were calculated. 16 days were accepted as the maximum life expectancy of animals, i.e. the guaranteed time of termination of death of mice infected with the influenza virus.

Statistical analysis of data

The results' statistical analysis was carried out by a standard procedure (Sachs 1976), using software package “Statistica 7.0” (StatSoft Inc. USA), estimating the reliability of differences with error probability $p \leq 0.05$. To compare virus titers in vitro in the control and in the presence of preparations, Student's *t* test was applied. ALE values are represented as $M \pm SD$, where *M* is the arithmetic mean, and *SD* is the standard deviation. The comparison of mice ALE was performed according to Mann–Whitney *U* test. In addition, differences in animal survival rates were assessed using Kaplan–Meier curves by the log-rank test (Rebrova 2006).

Results and discussion

Hairy root induction and maintenance

Under-transforming primary leaves of *N. schoberi* aseptic seedlings with strain *A. rhizogenes* 15834 SWISS, callus and short root formation were observed on the wound sites of explants after 2 weeks of incubation (Fig. 1a). After 20 days of cultivation, the roots elongated (Fig. 1b), and had plagiotropic growth, which was a morphological characteristic of a successful genetic modification.

The frequency of primary leaves transformation with strain *A. rhizogenes* 15834 SWISS was 78.7%. After several passages on agar-solidified BDS medium with ceftriaxone, formed roots were transferred to solid medium without antibiotics and cultured in the dark at 17 °C to maintain the stock culture. Using selection, a well-growing line of the hairy root culture of *N. schoberi* was revealed, transferred in a liquid nutrient medium (Fig. 1c), and cultured for several passages. In submerged cultivation, the culture GI at the first passage amounted 10.1, then gradually increased, and at the fourth passage achieved 20.4. As the culture reached the required biomass only at the fourth passage, the roots of this passage were applied for molecular, phytochemical and virological studies.

PCR confirmation of transgenic hairy roots

It is known that agrobacterial Ri-plasmids contain a mobile element, T-DNA, which is able to be integrated into a plant cell genome. Herewith, the presence of four *rol*-genes: *rolA*,

Fig. 1 Establishment of *N. schoberi* hairy root cultures: **a** emergence of roots in primary leaves after 2 weeks of inoculation with strain *A. rhizogenes* 15834 SWISS; **b** hairy roots subcultured on agar-solidified BDS medium supplemented with 30 g/L sucrose and 500 mg/L ceftriaxone at 20 days of cultivation; **c** hairy root culture growing in BDS liquid medium after 35 days of cultivation. Scale: 1 cm

rolB, *rolC* and *rolD* is critical for the formation of the hairy root culture, but *rolB* plays a key role in rooting (Altamura 2004). In addition to the mobile element (T-DNA), the Ri-plasmid also contains a *vir*-region comprising approximately 35 genes of virulence grouped into at least 8 operons (*virA*, *virB*, *virC*, *virD*, *virE*, *virG*, *virF*, *virH*), which is not transferred to a plant cell, but provides T-DNA transfer into the plant cell genome (Pacurar et al. 2011).

The transgenic nature of obtained hairy roots was confirmed by the PCR method with primers to agrobacterial oncogenes *rolB* and *rolC*, whereas the absence of agrobacterium contamination was examined with primers for *virC* and *virD1* genes. Product formation of the expected length (Fig. 2) indicated a successful result of agro-transformation. PCR analysis using primers for the *virC* and *virD1* genes provided no bands corresponding to the size of *virC* and *virD1* from the hairy root lines, thus eliminating the

possibility of *A. rhizogenes* contamination; the absence of hybridization with *vir* probes indicated that samples were not contaminated with agrobacterium.

Phytochemical analysis of hairy roots and intact plant roots

The comparison of metabolites' content and the composition of ethanol extracts from *N. schoberi* intact roots and cultures of genetically modified roots has shown that the content of all the studied compounds in the hairy root culture was significantly higher than their amount of the same components in native roots (Table 1). Thus, the content of flavonoids in the hairy root cultures was more than 41.14 times higher than their level in the intact plant roots. Flavonoids accumulate mainly in plant leaves, which is due to the biosynthesis induction of these secondary metabolites by ultraviolet light

Fig. 2 PCR detection of *rolB* (a), *rolC* (b), *virC* (c), *virD1* (d) genes. 1: Plasmid DNA of *A. rhizogenes* 15834 SWISS—positive control; 2: total DNA of the hairy root culture of *N. schoberi* obtained by trans-

formation of primary leaves with strain *A. rhizogenes* 15834 SWISS; 3: total DNA of *N. schoberi* non-transformed roots—negative control; M molecular weight marker 100 bp

Table 1 Metabolites comparative content in the roots of intact plants and in the hairy root culture of *N. schoberi*

Compound composition	Roots of intact plants	Hairy roots
Flavonoids (mg RE/g)	0.35 ± 0.01	14.4 ± 0.30
Hydroxycinnamic acids (mg CAE/g)	7.09 ± 0.02	96.0 ± 2.50
Pectins (mg GAE/g)	6.6 ± 0.11	14.4 ± 0.28
Protopectins (mg GAE/g)	29.0 ± 0.43	131.2 ± 4.20
Saponins (mg/g)	31.4 ± 0.54	208.6 ± 8.30

Values are mean of ± SE of three replicates

Table 2 Qualitative and quantitative composition of catechins in roots of intact plants and the hairy root culture of *N. schoberi* according to HPLC

No. of compounds	Retention time (min)	Content (mg CE/g)	
		Intact plant roots	Hairy roots
1—gallic acid	2.7	0.7	0.5
2—± catechin	5.4	0	1.2
4—L-epicatechin	8.6	0	1.4
3, 5–17—unidentified compounds	7.0; 10.3–31.1	2.2	7.9
Σ catechins		2.9	11.0

Values are mean of ± SE of three replicates

The relative standard deviation of repeatability in determining phenolic compounds was $\sigma_r = 0.11$

(Koyama et al. 2012); these results are evidence of changing flavonoid metabolism in the hairy root culture compared to the intact plants. The content of hydroxycinnamic acids in genetically modified roots also increased significantly, 13.54 times higher than the level of these phenolic acids in the intact roots. A significant increase of saponin biosynthesis in hairy roots was noted (Table 2). Pectins were accumulated to the least extent, but their content in the transformed roots was 2.18 times higher than in the intact plant roots. The content of protopectins in hairy root cultures was 4.53 times higher than in the intact roots. Furthermore, a significant increase of saponin content in genetically modified roots was observed: it was 6.64 times higher than in intact roots.

These results are consistent with research performed on the hairy root cultures of *Fagopyrum tataricum* Gaertn., which has shown increased biosynthesis of phenolic compounds (Kim et al. 2009). Studying the hairy root cultures chemical composition of *Rubia cordifolia* has reported that anthraquinone accumulation in them is also significantly higher compared to roots of intact plants (Bulgakov et al. 2002). Probably, the activation of secondary metabolism in genetically modified roots is explained by expression of

rol-genes (Bonhomme et al. 2000), since it is known that besides the rooting, *rolB* and *rolC* are potent inducers of secondary metabolism in transgenic plants and roots (Shkryl et al. 2008). Thus, the hairy root culture of *N. schoberi* has noted a significant accumulation of metabolites with a broad spectrum of biological activity. Furthermore, some of them possess antiviral activity at various stages of infection and replication (Buzzini et al. 2008).

Since it has been established that catechins included in the flavonoid composition have high antitumor, antioxidant, and antiviral activity against influenza A (Kim et al. 2013), the subsequent detailed analysis was focused on studying these compounds. Using the HPLC technique, it was shown that the qualitative composition of catechins in genetically transformed and of intact plant roots was different (Table 2; Fig. 3). So, in the roots of intact plants, there were 13 compounds of catechin nature, whereas in genetically modified roots only 10 ones were observed. However, only 6 compounds were found in both samples. This might be due to the capability of the hairy root culture to produce not only valuable metabolites peculiar to original plants (Zarate 1999), but also substances being their precursors or derivatives (Fukui et al. 1999).

Comparing chromatograms of analyzed samples, the retention times of substance signals with the retention times of standard sample signals and spectra made it possible to identify gallic acid ($t_R = 2.7$ min), ± catechin (5.4 min.), and L-epicatechin (8.6 min). The total content of catechins in the hairy root culture sample has exceeded 3.8 times this indicator in the intact roots and reached 11 mg/g of the dry weight.

According to some reports, the antiviral activity of catechins is determined by the presence of a gallic group in 3-OH position of a catechin structure (Kim et al. 2013). It has been shown that catechins are potent inhibitors of the development of influenza A virus subtypes H1N1 and H3N2 as well as the influenza B virus. The antiviral effect of catechins on the influenza viruses is mediated by interfering with haemagglutination, inhibits the action of neuraminidase, and influences the synthesis of viral RNA at its high concentration (Song et al. 2005).

Anti-influenza activities

A preliminary assessment of solutions cytotoxicity of the studied plant extract has revealed that the toxic effect of the extract on the cell culture of MDCK was not observed at a concentration of ≤ 0.01 mg/mL; therefore, in the analysis of the antiviral effect of the preparations in vitro, the extract of the hairy root culture of *N. schoberi* and Tamiflu was used at a concentration equal 0.01 mg/mL. The inhibition results of the infectivity (titers) of influenza virus strains A/chicken/Kurgan/ 05/2005 (H5N1) and A/Aichi/2/68 (H3N2) under the influence of extract and Tamiflu in MDCK cell culture

Fig. 3 HPLC analysis of catechin production: **a** standard gallic acid; **b** standard $\pm$ catechin; **c** standard L-epicatechin; **d** *N. schoberi* extracts of intact roots; **e** *N. schoberi* hairy root culture (4th passage). 1—Gallic acid, 2— $\pm$ catechin, 4—L-epicatechin, 3, 5–17—unidentified compounds

has shown a significant antiviral effect related to influenza virus strains used in the experiment (Table 3).

Further experiments with laboratory mice have demonstrated that upon administration of the extract and Tamiflu to mice infected with the influenza A/chicken/Kurgan/05/2005 virus (H5N1) ALE reliable ($p \leq 0.004$) increase (up to 15.2 ± 1.7 and 13.2 ± 3.6 days, respectively) was observed as compared with the value in the control group (8.0 ± 1.3 days). In addition, while these drugs were administered to mice infected with A/Aichi/2/68 (H3N2) virus,

ALE also increased significantly ($p \leq 0.004$) (up to 13.3 ± 3.9 and 11.4 ± 3.4 days, respectively) relative to the value of this indicator in the control (6.1 ± 0.9 days).

The analysis of Kaplan–Meier survival curves presented in Fig. 4 has revealed considerable differences according to the log-rank test between groups of mice infected with influenza strains A/chicken/Kurgan/05/2005 (H5N1) and A/Aichi/2/68 (H3N2), and received the extract or Tamiflu, and the corresponding control groups. Thus, the survival rate of mice infected with strain A/chicken/Kurgan/05/2005 (H5N1)

Table 3 Infectiousness (titers) of influenza virus strains in MDCK monolayer of cells in control (virus only) and in the preparation present in a culture medium

Preparation ^a	Infectiousness rates of the influenza virus strains			
	A/Aichi/2/68 (H3N2)		A/chicken/Kurgan/05/2005 (H5N1)	
	Virus titre, log ₁₀ TCID ₅₀ /mL (± I ₉₅)	NI, log ₁₀	Virus titre, log ₁₀ TCID ₅₀ /mL (± I ₉₅)	IN, log ₁₀
<i>Nitraria schoberi</i> extract	1.83 ± 0.41*	2.67	3.00 ± 0.44**	4.50
Tamiflu (Oseltamivir)	2.33 ± 0.33*	2.17	5.00 ± 0.44*	2.50
Virus control	4.50 ± 0.00	–	7.50 ± 0.00	–

*Difference from the corresponding control according to Student's *t* criterion for $p \leq 0.002$; **difference from the corresponding control according to Student's *t* test for $p = 0.00003$; number of experiment replicates was 3 in all cases

^aThe concentration of drugs in the culture liquid was 0.01 mg/mL; *NI* neutralization index (virus titer in the control – virus titre in the experiment)

Fig. 4 Kaplan–Meier survival curves of mice infected with influenza virus strains A/chicken/Kurgan/05/2005 (H5N1) (a) and A/Aichi/2/68 (H3N2) (b) in control groups (dotted line: control) and groups treated with drugs (dashed line: Tamiflu, solid line: *Nitraria schoberi* extract). +: the end of the observation period, i.e. empirically established, guaranteed time of termination of mice death post infection with influenza virus

has significantly increased up to 80% ($p = 0.00009$) under the extract administration, and up to 60% ($p = 0.002$) under Tamiflu administration relative to the value (0%) in the control group (Fig. 4). When the extract and Tamiflu were administered into mice infected with strain A/Aichi/2/68 (H3N2), a considerable increase in survival rates to 60% ($p = 0.002$) in both groups has been observed, while in the control group survival was 0% (Fig. 4).

Thus, for the first time it has been shown that ethanol extracts from the hairy root culture of *N. schoberi* possess high antiviral activity against the influenza virus subtypes A(H5N1) and A(H3N2), which manifests itself in the effective inhibition of infectivity not only in vitro—in MDCK cell culture—but also in vivo, increasing the survival and life expectancy of infected mice. In this case, the anti-influenza efficacy parameters of the extract and Tamiflu are comparable.

The obtained results open up prospects for research aimed at developing a new effective antiviral drug for influenza prevention and treatment. The primary phytochemical analysis has demonstrated the biosynthesis enhancement of a number of natural compounds in the hairy root culture of *N. schoberi* in comparison with intact roots. The subject of further study is the action mechanism of an extract capable to inhibit the influenza A virus replication.

It is interesting to find out if the activity of catechins was present in *Nitraria* hairy roots, some of which effect haemagglutination, neuraminidase activity, and the viral RNA synthesis, as shown by Song et al. (2005) with catechins in green tea, or the synergistic action of the extract components.

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Compliance with ethical standards

Conflict of interest The authors declare no financial or other conflicts of interest.

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