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Antiviral activity of medicinal plant Nepeta nuda

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Nepeta nuda subsp. nuda L. is a valuable medicinal plant well-known for its various therapeutic properties. This study assessed the antiviral activity of four extracts derived by methanol and chloroform extractions from in vivo grown and in vitro propagated plants. The cytotoxicity was tested on Madin–Darby bovine kidney (MDBK) cell line. Maximal tolerated concentration (MTC) and cytotoxic concentration (CC50) of both extracts were determined. Antiviral activity on viral replication was evaluated against herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) in MDBK cell line. The 50% inhibitory concentrations (IC50) and selective index (SI) of the extracts against both viruses were calculated. The methanol extract, derived from the native plant exhibited the greatest antiviral activity. The IC50 for both viruses was similar/320 m g/mL against HSV-1 and 510 m g/mL against HSV-2. The SI were 4.94 and 3.1, respectively. Applied in MTC, the extract inhibited viral replication by more than 95% in both HSV-1 and HSV-2. The virucidal effect was determined by the reduction of the infectious virus titer. All four extracts of Nepeta nuda inactivated the extracellular form of HSV-2. The major virucidal activity was demonstrated by the chloroform extract from the native plant – more than 99% viral inactivation. The extracts weakly affected the viral entry into the host cell. The highest inactivation was shown by the chloroform extract form the native plant – more than 90%.

Keywords: medicinal plant; in vitro and in vivo extracts; antiviral activity; HSV-1; HSV-2; Nepeta nuda

Abbreviations

ACV – acyclovir
CC50 – 50% cytotoxicity concentration
CCID50 – cell culture infectious doses
CHR – chloroform extract from laboratory propagated plant
CHV – chloroform extract from native plant
FBS – fetal bovine serum
HSV – herpes simplex virus
IC50 – 50% inhibition concentration
ICP – infectious cell protein
MDBK – Madin–Darby bovine kidney
MER – methanol extract from laboratory propagated plant
MEV – methanol extract from native plant
NCIPD – National Center of Infectious and Parasitic Diseases
MTC – maximal tolerated concentration
MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
P/S – penicillin/streptomycin
PCV – penciclovir
SI – selectivity index – SI = CC50/IC50

Introduction

Herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) are responsible for recurrent labial and genital infections. HSV-2, a sexually transmitted pathogen, infects more than 500 million people worldwide and causes approximately 23 million new infections each year. HSV-1 is even more abundant with an estimated seroprevalence of over 90% in many nations.[1] Transmission occurs by contact with secretions from an infected person with either overt infection or asymptomatic excretion of virus. Antiviral chemotherapy is a standard practice in the management of herpesvirus infections in humans, and currently there are about 11 licensed antiherpetic drugs available over the counter.[2] The standard therapy includes acyclovir (ACV) and penciclovir (PCV) with their respective produgs valacyclovir and famciclovir.[3] ACV, the first selective antiviral agent, is regarded as the drug of choice for more than 20 years.[4] Unfortunately, continuous therapy leads to a selection of resistant strains.[3] Current data indicate the presence of mutant clinical strains with higher prevalence in immunocompromised patients.[5] That is why the search for new therapeutic agents for systemic and local use is an ongoing process.

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Special attention is focused on compounds with natural origin. The advantages of this kind of compounds over the synthetically derived ones are that they have lower cytotoxicity and the occurrence of resistant strains against their action is delayed due to their complex chemical structure. A myriad of natural active compounds have been identified worldwide. Therefore, natural products, including traditional medicinal plants are promising potential sources of new highly effective antiviral drugs.

Genus Nepeta comprises of about 40 species, which possess spasmolytic, antiseptic, diuretic and antiasmatic activity and are widely utilized in various treatments. There are data about the antitherpetic activity of several species from Nepeta genus – Nepeta nepetella L., Nepeta coerulea Aiton and Nepeta tuberosa L. In this work, native Nepeta nuda plants collected from their natural habitats and in vitro propagated under controlled conditions plants were used. Thermostat extractions with methanol and chloroform from both kinds of plants were conducted. This is the first report on the antiviral activity of extracts from this plant.

Materials and methods

Plant materials

Nepeta nuda subsp. nuda was collected from its natural habitat in the Lozen Mountain, Sofia, Bulgaria and multiplied in vitro by using mono-nodal stem segments. The voucher specimen was deposited in the herbarium of Sofia University “St Kliment Ohridski” SO 105807. The aerial parts of in vivo and in vitro cultivated N. nuda were extracted by Soxhlet and used for investigation of their antiviral activity. We have used four extracts – chloroform extract from laboratory propagated plant (CHR), chloroform extract from native plant (CHV), methanol extract from laboratory propagated plant (MER) and methanol extract from native plant (MEV).

Viruses and cells

HSV-1, strain Vic, and HSV-2, strain BA, were supplied by the National Center of Infectious and Parasitic Diseases (NCIPD), Bulgaria. For virus production, monolayers of Madin–Darby Bovine Kidney (MDBK) cells in 75-cm² tissue culture flasks were infected with HSV at a multiplicity of infection of 0.01. After 48 h at 37 °C, the infected cells were harvested with three freeze-and-thaw cycles and the cellular debris were removed with a low-speed centrifugation. The cell line MDBK was provided by the National Bank for Industrial microorganisms and Cell Cultures. The cells were cultured in Dulbecco’s modified eagle medium (DMEM; Gibco® by life technologies) supplemented with 10% fetal bovine serum (FBS) and 1:1000 penicillin/streptomycin (P/S), at 37 °C and 5% CO₂ atmosphere. The cells were passed on and harvested on a regular basis according to a constant scheme.

Cytotoxicity assay

The cytotoxicity was determined by a comparative microscopic examination of the cell morphology in treated and untreated cultures. The maximum concentration, which did not alter the morphology of the cells, was recognized as maximal tolerated concentration (MTC).[14] In a second experiment, the cell viability was determined by the ability of the cells to cleave the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, USA) through the mitochondrial enzyme succinate dehydrogenase, which gives a formazan blue product, following the procedure described earlier. The intensity of the absorbance of the released triazolium crystals from cells treated with serial dilutions of the analysed extracts (250, 500, 750, 1000, 1250, 1500, 1750, 2000 and 2500 µg/mL) was measured on the Elisa reader and consequently standard curve of the cell viability was done. The 50% cytotoxicity concentration (CC₅₀) was calculated based on the curve.

Cytopathogenic effect (CPE) reduction assay

Experiments were performed in multicycle growth conditions. Triplicate confluent cell monolayers distributed in 96-well microplates were infected with 320 cell culture infectious doses (CCID₅₀/0.1 mL) of the virus. After 1 h adsorption at room temperature, the investigated extracts were added to the monolayers in the respective dilutions (75, 100, 150, 250, 500, 750 and 1000 µg/mL). The viral cytopathic effect was determined by the four-cross system when there was full destruction of the cell monolayer in the viral control. The concentration inhibits the viral cytopathic effect by 50% (IC₅₀) with respect to the virus control that was estimated from plots of the data. The selectivity index (SI) was calculated as CC₅₀-to-IC₅₀ ratios.

Effect on the extracellular virus (virucidal effect)

Equal volumes of viral stock containing 10⁵.⁵ CCID₅₀/ml and media with MTC of the appropriate extract were mixed and incubated at different times (5, 10, 15, 30, 60, 120, 250 and 360 min) at 37 °C. The samples were frozen and thawed. Infectious virus titers were calculated at the 48th hour of culturing by the method of Reed and Muench. The virucidal effect was determined by the reduction of the infectious virus titer of each sample as compared to the relevant viral control – equal volumes of viral stock and medium incubated as described above.

Inhibition of attachment and entry assay

Equal volumes of viral stock containing 10⁵.⁵ CCID₅₀/ml and media with MTC of the appropriate extract were mixed and added to MDBK cells at room temperature to allow the attachment and the penetration of the virus. At different time points (15, 30, 45, 60 and 120 min), the mix was taken off and the cells were washed three times with.
fresh medium. After 24 h of incubation, the samples were frozen and thawed. Infectious virus titers were calculated at the 48th hour of culturing by the method of Reed and Muench [17]. The effect was determined by the reduction of the infectious virus titer of each sample as compared to that of the relevant viral control.

Unawares, we found lower antiviral activity of in vitro extract MEV (CC\textsubscript{50} = 1580 μg/mL) compared with the native extract MER (CC\textsubscript{50} = 2250 μg/mL).

**Antiviral activity**

The data showed the dose-dependent manner of the inhibition of the viral replication by all of the investigated extracts. Extracts CHR and MEV applied in MTC inhibited almost completely the replication process of HSV type 1 and type 2 – more than 97% (Figure 2). The values

Figure 1. Cytotoxicity of the extracts against MDBK cell line.

Figure 2. Antiviral activity against (A) HSV-1 and (B) HSV-2 in cell line MDBK.

| Extract | MTC (μg/mL) | CC\textsubscript{50} (μg/mL) | HSV-1 | HSV-2 |
|---------|-------------|-----------------|-------|-------|
|         | IC\textsubscript{50} (μg/mL) | SI   | IC\textsubscript{50} (μg/mL) | SI   |
| CHR     | 750         | 880             | 380   | 2.32  |
| CHV     | 1000        | 2070            | 790   | 2.62  |
| MER     | 1580        | No value*       | No value | 2.1  |
| MEV     | 1000        | 2250            | No value | 2.76 |

Note: *— the inhibition of viral replication was less than 50%.

**Results and discussion**

**Cytotoxic activity**

The extracts of *N. nuda* were applied at concentrations that range from 250 to 2500 μg/mL and both MTC and CC\textsubscript{50} were evaluated simultaneously. The preliminary data suggested that the extracts CHV and MER altered slightly the cell morphology, while the extract CHR caused more significant alterations. The MTC values of extracts were 750 μg/mL for CHR and 1000 μg/mL for the other three extracts (Table 1). The results obtained by the MTT assay confirmed the highest toxicity of the extract CHR. Its CC\textsubscript{50} value was 880 μg/mL, whereas the value of native extract CHV was 2070 μg/mL (Figure 1). The reported difference in the cytotoxicity of the extracts was probably due to the composition of the medium utilized for propagation. In previous publications of this group, we have described higher cytotoxicity of laboratory propagated plants.[8,18]
of IC$_{50}$ for these extracts applied against the HSV-1 replication were similar — 380 and 320 µg/mL, respectively. The effect of CHV on the replication of the same virus was measured twice in a weak and the calculated IC$_{50}$ value was 790 µg/mL. When applied in MTC, the extract decreased viral replication by only 40%. Evaluation of the effect of studied extracts on the replication of HSV-2 showed similar inhibition. The relevant values of IC$_{50}$ were somewhat higher compared to those found for the application on HSV-1 replication (Table 1). The different results for the inhibitory effect obtained from the CPE reduction assay against HSV-1 and HSV-2 may be due to small differences in mechanism of replication — different regulatory proteins infectious cell protein (ICP group) and different activity of some viral enzymes of both viruses.

The demonstrated activity of the extracts CHR and MEV against HSV-1 was higher than the activity of water extracts derived from other members of same genus — N. nepetella, N. coerulea and N. tuberosa.[11]

The values of SI of the three investigated extracts for both viruses were similar (SI = 2.1–4.94). The data manifested the strongest inhibition of the viral replication when the native methanol extract MEV was applied. For HSV-1, the SI value was 4.94 and for HSV-2 SI = 3.1 (Table 1). Unfortunately, these values were much lower than the SI of the reference drug acyclovir (SI = 120).

**Virucidal activity**

The four extracts were tested for the virucidal activity against HSV-2. The strongest effect showed CHV extract with 99% inactivation of the virions (Figure 3). The inactivation was exhibited after 15 min of contact (Figure 3). The antiviral effect increased with the duration of the incubation period and the inactivation reached above 99% ($\Delta$log = 2) at 360 min of contact. The other extracts have weaker activity over an extracellular form of HSV-2.

**Conclusions**

This work revealed that methanol and chloroform extracts derived from N. nuda propagated in vivo and in vitro possess clear antiviral activity. The CHV and CHR, such as the methanol native extract MEV, exerted a strong inhibitory effect against the replication of HSV type 1 and type 2 in MDBK cells. The extract CHV suppressed the first steps of replicative cycle of HSV type 2. The results suggested N. nuda could be an interesting source of natural antiviral substances with potential use in medicine. However, further investigations are needed for the modulation of the synthesis of secondary metabolites in in vitro cultivated plants of N. nuda, for the fractionation of crude extracts and the identification of biological active compounds.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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**Figure 3.** Activity of CHV extract on extracellular HSV-2.

**Figure 4.** Activity of CHV extract on the attachment and entry of HSV-2.
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