

Full Length Research Paper

Antiherpetic activity of some endemic *Hypericum* species in Turkey

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This study was designed to investigate the antiherpetic activities of the crude methanolic extracts of the aerial parts of three species of *Hypericum* growing in Turkey (*Hypericum neurocalycinum* Boiss. & Heldr., *Hypericum salsugineum* Robson & Hub.-Mor. and *Hypericum kotschyianum* Boiss.). For this purpose, firstly, the cytotoxicity potential of the extracts against Vero cells were assessed using MTT [3-(4,5-dimethylthiazol-2-yl) - 2,5-diphenyltetrazolium bromide] colorimetric assay. The 50% cytotoxic dose (CD₅₀) corresponded to the concentration required to kill 50% of the Vero cells for each extract was calculated by nonlinear regression analysis using GraphPad Prism software. Additionally, the maximum non-cytotoxic concentrations (MNCCs) were determined as the maximal concentration of the extracts that did not exert a toxic effect. Later, the values of the maximum non-toxic concentration obtained (50.00, 100.00 and 25.00 µg/ml for the methanolic extracts of *H. neurocalycinum*, *H. salsugineum* and *H. kotschyianum*, respectively) were used in antiherpetic activity determination of the extracts using MTT assay. Nevertheless, it was determined that none of the extracts have antiherpetic activity in tested maximum non-toxic concentrations.

Key words: *Hypericum* species, antiherpetic activity, colorimetric MTT assay.

INTRODUCTION

Herpes simplex viruses [Herpes simplex virus (HSV) type 1 and type 2] are DNA viruses belonging to the family Herpesviridae and responsible for a variety of mild to severe diseases, which are sometimes life threatening, especially in immunocompromised patients (Snoeck, 2000). Drugs with clinically relevant activity against HSV infections include interferons (IFNs), acyclovir (ACV), vidarabine (ara-A), gancyclovir (DHPG) and phosphonoformic acid (foscarnet, PFA). However, undesirable complications and the emergence of drug-resistant viruses urge the development of new antiherpetic agents (Fritz et al., 2007).

Hypericum L., a member of the Hypericaceae (Guttiferae) family, is a genus with a number of species growing in Europe, West Asia, North Africa and North

America (Axarlis et al., 1998). Turkey is an important centre for *Hypericum* species. The *Hypericum* genus is represented in Turkey by 89 species of which 43 are endemic (Dönmez, 2000). "Members of the Guttiferae family, for example, have been used in traditional medicine to treat wounds, lymphatitis, parotitis, hepatitis, gastrointestinal disorders and tumours, which could be related to viral agents. Many compounds have been isolated from plants of this family and have had their antiviral activity studied. Hypericin and pseudohypericin, isolated from plants of the genus *Hypericum* also received attention due to the antiviral action on lipid enveloped and non-enveloped DNA and RNA viruses" (Fritz et al., 2007). Considering the presence of antiviral compounds in the Guttiferae family and the long traditional reputation of many species of *Hypericum* genus as medicinal plants for the treatment of a variety of conditions, commonly related to viral infections, the extracts and isolated compounds from one of these species, *Hypericum neurocalycinum*, *Hypericum salsugineum* and *Hypericum kotschyianum*, were tested

Abbreviations: MTT, 3-(4,5-Dimethylthiazol-2-yl) - 2,5-diphenyltetrazolium bromide; MNCCs, maximum non-cytotoxic concentrations.

for antiherpetic activity.

MATERIALS AND METHODS

Plant materials and preparation of the extracts

Endemic *Hypericum* species (*H. neurocalycinum* Boiss. & Heldr., *H. salsugineum* Robson & Hub.-Mor. and *H. kotschyianum* Boiss.) were collected from Konya province, Turkey. The taxonomic identification of plant materials was confirmed by Osman Tugay in Department of Biology, Selçuk University, Konya, Turkey. The specimens are kept in the Herbarium, Department Biology of Selçuk University.

Plant materials (50 g) were dried in an oven at a temperature of 40°C and ground in a manual mill. The dried and powdered plant materials were extracted with methanol (MeOH) in a Soxhlet extraction apparatus for 8 h. The extracts obtained were filtered and the filtrate centrifuged at 10,000 rpm. The supernatants were lyophilised for further use and were stored at a temperature of 4°C protected from the light, until they were used in the cytotoxicity and antiviral assays, no longer than two months (Betancur-Galvis et al., 1999).

Cell and virus

African green monkey kidney (Vero) cell line as well as the test virus Herpes simplex virus type 1 (HSV-1) used in this study were provided by the Department of Virology, Faculty of Veterinary Science, Selçuk University (Turkey). Vero cells grown in Dulbecco's Modified Eagle Medium (D-MEM, Gibco Limited, P. O. Box. 35, Paisley, Scotland) containing 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 100 µg/ml streptomycin and 5 µg/ml amphotericin B were used for both cytotoxic and antiviral studies. HSV-1 was propagated in Vero cells and the titre of propagated viral stock was determined as TCID₅₀ (50% tissue culture infective dose) / 0.1 ml by using Kaerber method (Kaerber, 1964). The viral stock after titration was dispensed in some sterile tubes which were stored at -70°C. The infective titre of the viral stock was 10⁻² TCID₅₀ / 0.1 ml.

Cytotoxicity assay (colorimetric MTT assay)

100 mg of each lyophilised extract was separately dissolved in 0.5 ml of distilled dimethyl sulphoxide (DMSO) and volume was made up to 10 ml with maintenance medium (DMEM with 2% FBS) to obtain a stock solution of 10 mg/ml concentration, sterilized by filtration. The stock solutions were diluted with Dulbecco's Modified Eagle's Medium (DMEM) to desired concentrations ranging from 6.25 to 200 µg/ml. The final concentration of DMSO in each sample did not exceed 0.1% v/v. The cytotoxic activity of the extracts were tested in Vero cell line by using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) method (Andrighetti-Fröhner et al., 2003).

Briefly, Vero cells were seeded in 96 well-plates (80 µl/well at a density of 5 × 10⁵ cells/ml). After a 24 h period of incubation at 37°C in a humidified 5% CO₂ atmosphere cell monolayers were confluent, the medium was removed from each well and replenished with 200 µl of extract dilutions per well (1:2) ranging from 200 to 6.25 µg/ml prepared in DMEM. Additionally, 200 µl of DMEM without extract and with 0.1% DMSO were used as cell control and solvent control, respectively. After 4 days, at 37°C in humidified 5% CO₂ atmosphere, medium was removed by suction from all wells and 50 µl of MTT (Sigma, 1 mg/ml) solution prepared in DMEM was added to each well and the plates were incubated for 4 h at 37°C. The MTT solution was removed without disturbing the Formosan crystals. After gently shaking the plates for 5 min,

whereby crystals were completely dissolved, the absorbance was read on a multiwell ELISA reader (Multiskan EX, Labsystems) at 540 nm. The percentage of cytotoxicity was calculated as $[(A - B) / A \times 100]$, where A and B are the absorbance of cell control and treated cells, respectively. The 50% cytotoxic dose (CD₅₀) corresponded to the concentration required to kill 50% of the Vero cells for each extract was calculated by nonlinear regression analysis using GraphPad Prism software. Additionally the maximum non-cytotoxic concentrations (MNCCs) were determined as the maximal concentration of the extracts that did not exert a toxic effect in comparison with cell controls. Later, these values of the maximum non-toxic concentration determined were used in antiherpetic activity determination of the extracts using MTT assay.

Antiviral assay by the MTT method

The colorimetric MTT assay (Müller et al., 2007) was used to determine the antiviral activities of samples against HSV-1. Vero cell cultures (100 µl/well at a density of 4 × 10⁵ cells/ml) were prepared in 96-well tissue culture plates (Corning, USA). The culture medium was removed from the monolayer cells after culturing for 24 h at 37°C in a 5% CO₂ incubator. To confluent monolayers of cells in 96-well tissue culture plates, 100 µl of virus suspension containing 100 TCID₅₀ and 100 µl of maintenance medium containing appropriate serially diluted concentrations of the samples were added. The MNCC of the test sample was used as the highest concentration from which a serial twofold dilution was made with the culture medium. Acyclovir (ACV) [9-(2-hydroxyethoxymethyl) guanosine, Sigma, 10 µg/ml] was used as positive control for HSV-1 inhibition. The maximum concentration of DMSO (0.1%) was used as negative control. To act as a virus control and cell control, the virus suspension and maintenance medium without samples were added, respectively. The plates were incubated at 37°C in a 5% CO₂ incubator for 4 days until the cells in the virus control wells showed a complete virus-induced cytopathic effect (CPE) as observed under the light microscope. The same method used to evaluate cell viability with MTT as described previously was followed. The percentages of protection were calculated spectrophotometrically as $[(A - B) / (C - B) \times 100]$, where A, B and C indicate the absorbance of samples, virus and cell controls, respectively.

RESULTS

In this study, the methanolic extracts obtained from three plant species belonging to the family *Clusiaceae* (*H. neurocalycinum*, *H. salsugineum* and *H. kotschyianum*) were evaluated for their *in vitro* antiviral activities against herpes simplex virus type 1 (HSV-1) using the colorimetric MTT assay in Vero cells. As prerequisite for antiviral tests, the cytotoxicity of the extracts against virus-host cells was investigated by the colorimetric cell viability test. The CD₅₀ values of methanol extracts obtained from *H. neurocalycinum*, *H. salsugineum* and *H. kotschyianum* were determined as 361.92, 208.53 and 93.70 µg/ml, respectively. Additionally, their maximum non-cytotoxic concentrations (MNCCs) used in determining of the antiherpetic activities of the extracts of the above-mentioned *Hypericum* species were determined as 50.00 µg/ml, 100.00 µg/ml and 25.00 µg/ml, respectively. These results are shown graphically in Figures 1 to 3. After determining the cytotoxicity of

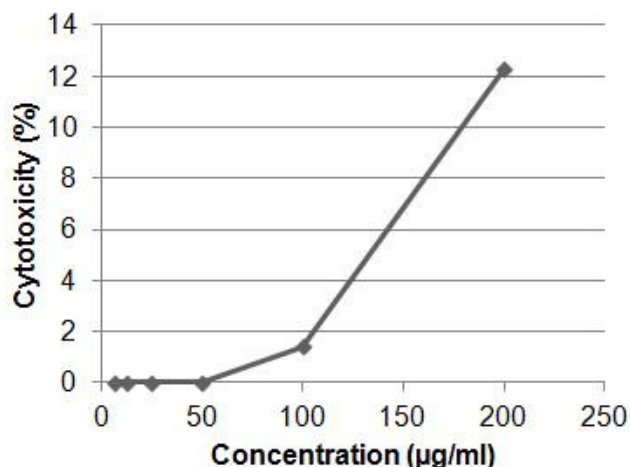


Figure 1. Cytotoxic activity of *H. neurocalycinum* methanolic extract against Vero cells after 96 h incubation (MNCC: 50.00 µg/ml).

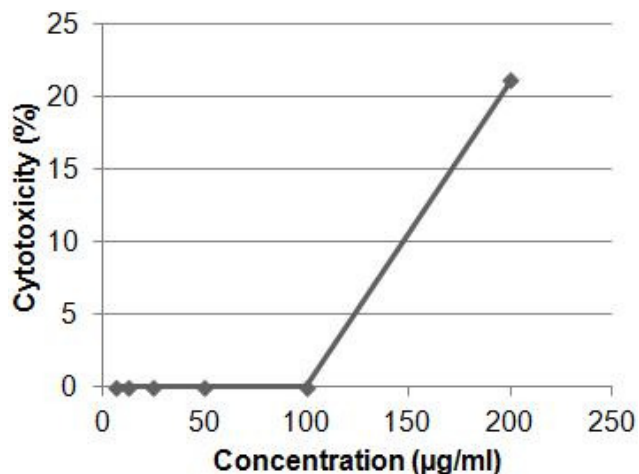


Figure 2. Cytotoxic activity of *H. salsugineum* methanolic extract against Vero cells after 96 h incubation (MNCC: 100.00 µg/ml).

extracts, their antiviral activity against HSV-1 was examined using the colorimetric MTT assay. As a result, it was determined that none of the extracts have not antiherpetic activity in tested maximum non-cytotoxic concentrations. The results are summarized in Table 1.

DISCUSSION

Evidence of antiviral effects on the genus *Hypericum* has especially been shown for *Hypericum perforatum* (Lavie et al., 1995; Barnes et al., 2001; Bombardelli and Morazzoni, 1995). Plant preparations with different chemical composition have been assayed against various viruses and both, hypericin and pseudohypericin, were found to be particularly effective as virucidal agents. The

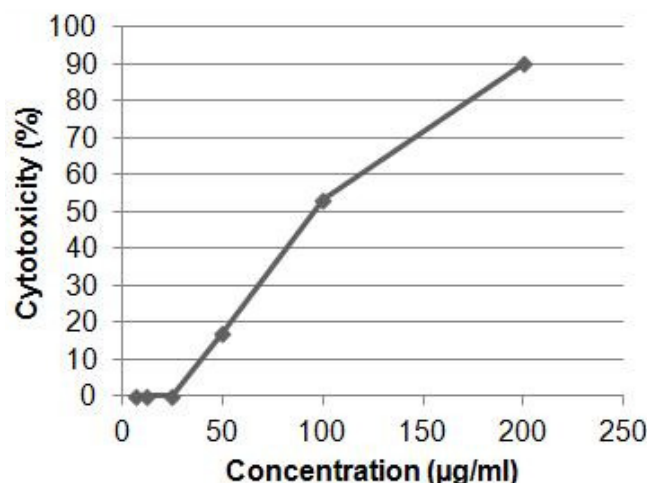


Figure 3. Cytotoxic activity of *H. kotschyianum* methanolic extract against Vero cells after 96 h incubation (MNCC: 25.00 µg/ml).

two compounds have been shown to be active against a broad range of viruses such as HSV-1 and 2, vesicular stomatitis and influenza viruses, cytomegalovirus and human immunodeficiency virus 1 (HIV-1) (Lavie et al., 1995; Barnes et al., 2001; Bombardelli and Morazzoni, 1995; Wood et al., 1990; Meruelo et al., 1988). Studies on the mechanism of action suggested that hypericin antiviral effect is directed towards enveloped viruses, while non enveloped viruses, such as adenovirus and poliovirus, are unaffected (Chatterjee et al., 1998). The high biological value of *H. perforatum* has worldwide led to an increased interest for the study of the antiviral, antimicrobial, chemical and various biological properties of other related species. 18 plants with ethnomedical backgrounds from different families have been screened for antiviral activity against HSV-1, and three extracts, from *Hypericum mysorense*, *Hypericum hookerianum* and *Usnea complanta*, have exhibited significant anti-HSV-1 activity at concentrations without toxic effects on cells *in vitro* (Vijayan et al., 2004). Sassi et al. (2008) studied on the anti-HSV-1 activities for 50 plant species belonging to 10 families in Tunisia and they found that methanol extract of *Hypericum crispum* had moderate antiviral activity (inhibition < 50%) against HSV-1. Traditionally, plants used to treat viruses caused diseases in Nepal and were tested for the antiviral activities against three mammal's virus (HSV, Sindbis virus and poliovirus) (Taylor et al., 1996), and it was determined that the methanol extracts of *Hypericum cordifolium* and *Hypericum uralum* had significant antiviral activity against HSV-1 while the extract of *Hypericum elodeoides* showed any antiviral effect against HSV-1. An investigation was made for the antibacterial, antifungal and antiviral activities on 45 plant extracts belonging to 37 different plant species out of 21 families in the district of Butare in Rwanda (Cos et al., 2002),

Table 1. Cytotoxic and antiviral effects of methanol extracts from *Hypericum* species determined by MTT assay in Vero cells after 96 h incubation^(a)

<i>Hypericum</i> species	MNCC*(µg/ml)	Antiviral activity against HSV-1
<i>H. neurocalycinum</i>	50.00	-
<i>H. salsugineum</i>	100.00	-
<i>H. kotschyannum</i>	25.00	-

^(a) The values are the mean of three experiments; *, maximum concentration of the extract that does not exert a toxic effect; -, no measurable effect.

Hypericum revolutum extracts having high antiviral activities against HSV-1 has been determined. The crude methanol extract obtained from *Hypericum connatum*, the extract function and compounds derived from these extracts, is tested for antiviral activity against HSV-1 (Fritz et al., 2007). All of the tested samples were to be effective in varying degrees against HSV-1, but the most effective compound was found to be luteoforol. The plants extracts obtained some Turkish medicinal plants including *H. capitatum* and *H. scabrum* and their extracts obtained from cell cultures were investigated for the cytotoxic properties and antiviral activities against herpes simplex viruses (HSV-1 and 2) and HIV-1 (Vlietinck, 2002). As a result of the research that none of the tested extracts showed a significant activity against HSV-1 and 2, it was reported that an extract derived from cell cultures of *H. capitatum* was detected as the weak antiretroviral activity against HIV-1.

Accordingly, in order to determine the antiherpetic activity of *Hypericum* species in all these studies, the antiviral activity of some *Hypericum* species that have and do not have this activity was determined in the current study. These differences for the anti-HSV activities between *Hypericum* species may be due to the compounds of the species responsible for the activity of antiviral, in particular whether hypericin do not contain pseudohypericin, the amount of these compounds and the time of collection of plant samples. Hypericin and pseudohypericin being responsible for the activity of antiviral compounds can be absent in some *Hypericum* species (for example, in *Hypericum connatum*) (Sökmen, 2001), if these compounds are present, the highest rate in the species including the period of flowering time can vary for collection time of the plants (Schmitt et al., 2001; Southwell and Campbell, 1991; Southwell and Bourke, 2001). In this research, although the presence of hypericin and pseudohypericin were not determined from plant samples, plant materials collected during the flowering period reported the highest proportion of containing these compounds. Unfortunately, anti-HSV-1 activity was not detected in any of the plant extracts.

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