Antiviral Research 97 (2013) 376–380

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Evaluation of antiherpetic activity of crude extract and fractions of *Avicenna marina*, *in vitro*

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ARTICLE INFO

Article history:
Received 31 October 2012
Revised 3 January 2013
Accepted 7 January 2013
Available online 17 January 2013

Keywords:
Avicenna marina
HSV-2
Antiviral activity
Flavonoid
Real time PCR

ABSTRACT

Aim: This study was carried out to check antiherpetic substances of crude methanol leaf extract of *Avicenna marina* and its column chromatographic fractions.
Background: Herpes simplex virus 2 (HSV-2) is a harmful pathogen especially in highly susceptible individuals.
Materials and methods: The antiherpetic activity of crude methanol extract and sub-fractions was performed in different concentrations (20, 2, 0.2, and 0.02 μg/ml) by use of plaque-forming unit (PFU) assay and real time polymerase chain reaction (PCR) assay.
Results: The most active fraction analyzed by NMR contained luteolin 7-O-methylether 3′-O-beta-D-glucoside (LMEG). The other active fraction was detected by HPLC as luteolin. The apparent effective concentrations for 50% plaque reduction (EC50) of crude methanol extract, LMEG, luteolin and ACV were 10, 5, 16.6 and 2.97 μg/ml, respectively. The three extracts showed no cytotoxic effect on Vero cell line at concentrations of 32 μg/ml or below. According to the consequences of time-of-addition studies, antiherpetic compound LMEG exerted an inhibitory effect on the early stage of HSV-2 infection during which it was added.
Conclusions: In conclusion, LMEG isolated from *A. marina* could probably inhibit HSV attachment to the cell membrane and its entry into the cell.

1. Introduction

Avicenna marina (commonly known as grey mangrove or white mangrove), is one of the mangrove species trees native to Southern Africa (Duke, 1991). It has been used to treat some diseases such as ulcers, rheumatism and small pox (Bandaranayake, 1995). Through extracting natural compounds from *A. marina* leaf in recent decades, a number of iridoid glucosides, fatty acids, sterols and hydrocarbons have been isolated (König and Rimpler, 1985; Hogg and Gillan, 1984). Since these compounds may have the potential to inhibit viral infection, here the anti HSV activity of this plant has been studied. HSV-related opportunistic infections cause a variety of malignancies. As resistance of virus to conventional antiviral drugs has been reported, it is necessary to find new alternative antiviral compounds (Cheng et al., 2004; Saijo et al., 2005). Currently, the only aspect of the herpes simplex life cycle for which antiviral therapy has been developed is the process of DNA replication, which is targeted by a small group of nucleoside analogues including acyclovir (ACV), Valaciclovir, Penciclovir and Famciclovir. ACV is useful during primary HSV infection but induces development of drug-resistant species. Valacyclovir and Famciclovir are approved for recurrent infections and foscarnet used for ACV-resistant cases. However, despite their high efficacy, they are expensive and cause severe side effects. Resistance is also observed clinically when foscarnet is used in patients with AIDS (Piret and Boivin, 2011). Therefore, *in vitro* assays to study the anti HSV activities of a variety of herbal medicines used by world's folk medical systems had been performed and revealed that their mechanism of function is either through inhibiting viral replication or by viral genome synthesis (Cheng et al., 2004; Schnitzler et al., 2007; Saddi et al., 2007; Chattopadhyay and Khan, 2008). The antiviral activity of *A. marina* has been investigated against poliovirus and HSV-1 (Zandi et al., 2009), but the antiviral properties of crude methanol extract and sub-fractions against HSV-2 have not been investigated, yet. Therefore, we designed a study to examine anti-HSV-2 activity of *A. marina* grown in Iran agricultural conditions.

2. Materials and methods

2.1. Plant material

Two kilograms of the aerial parts of *A. marina* were collected from Agriculture and Natural Resources Research Canter of

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http://dx.doi.org/10.1016/j.antiviral.2013.01.001

Gheshm, Iran, in December 2009. Voucher specimens were deposited in the herbarium of the University of Isfahan. Two kilograms of plant leaves were carefully dried in a well-ventilated dark room and then finely powdered. Finally, 0.7 kg of the dried leaf powder was obtained.

2.2. Extraction and isolation of compounds

Methanol extract (98%) of dried and powdered *A. marina* leaf (1000 g) was prepared. The extraction was done thrice at 40 °C. Then, the resulting liquid was collected, filtered and concentrated using a rotary evaporator (Stroglass, Italy) at 40 °C and dried using a Freeze dryer (Zirbus, Germany). Silica-gel column chromatography was carried out with the dried methanol extract (5 g) of *A. marina* eluted with Chloroform: ethyl acetate (9:1–1:9, v/v) and 100% methanol. Fractions 1–12 (0.51, 0.41, 0.45, 0.41, 0.45, 0.39, 0.35, 0.32, 0.35, 0.20, 0.39, 0.40 g) were obtained. Fraction 9 was found to have anti herpetic activity and rechromatographed on silica gel column eluted with Aceton:MeOH (30:1, 20:1, 10:1, 5:1, 3:1, 2:1, 1:1, v/v) to yield fractions 9a, 9b, 9c and 9d (0.12, 0.16, 0.15, 11 mg). Fractions 9b and 9c were detected as anti herpetic active compounds. Fraction 9b was the most active fraction and was analyzed by NMR as 7-O-methylether 3′-O-beta-D-glucoside (LMEG). Fraction 9c was impure and was analyzed by HPLC as luteolin when compared to standard peak.

2.3. HPLC analysis

HPLC screening of methanol extract, fraction 9b and fraction 9c was carried out. HPLC was performed on a HITACHI Series HPLC system equipped with L-7100 pump and an L-7100 UV–vis detector. Peaks were separated on a RPC18 column using the mobile phase [methanol/acetone/water (70:20:10 v/v)]. The flow rate of the mobile phase was 1.5 ml min^{-1}. The absorption of analytes was detected at 450 nm. Samples were injected to the HPLC bed manually with injection volume as 5 μl. T2000 software was used for peak integration and calculation.

2.4. NMR analysis

NMR screening was used to detect trace compounds in fraction 9b. ^{1}H NMR spectra were recorded on Bruker 500 MHz spectrometer by use of DMSO (δH = 7.26) as residual solvent with chemical shifts expressed in parts per million (ppm).

2.5. Cells and viruses

African green monkey kidney (Vero) cells (ATCC No. CCL-81) were purchased from the Cell Bank of Pasteur Institute in Tehran, Iran. Vero cells were cultured in RPMI, supplemented with 10% (v/v) Fetal Calf Serum (FCS), 100 U ml^{-1} penicillin and 100 mg ml^{-1} streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate. All reagents were purchased from Gibco Company, Germany. A provided virus stock of HSV-2 from University of Isfahan, Iran was propagated in Vero cells as follows: Vero cells were infected with a low multiplicity of virus and incubated for 4 days. Then, supernatant of viral stock was harvested every day over a period of 4 days post-infection. Virus titers were determined by plaque assay in Vero cells and expressed as plaque forming units per ml (PFU/ml). The viruses were stored at −70 °C until use.

2.6. Cytotoxicity assay

Cellular toxicity of *A. marina* extract and sub-fractions was tested *in vitro* to find their non-cytotoxic concentrations for the antiviral test experiments by dissolving the extracts in sterilized double distilled water or dimethyl sulfoxide (DMSO). DMSO was added to the medium at sub-toxic concentration. To avoid toxicity or interference by the solvent, the maximum concentration of DMSO in the test medium was 0.019%. To evaluate the proliferative effect of methanol extract and fractions of *A. marina* on uninfected Vero cells, dilutions at concentrations of 32, 60, 120, 250 and 500 μg/ml were added to Vero monolayers in the maintenance medium by use of 96-multiwell microplate with 4.0×10^4 cells per well. After 72 h of incubation at 37 °C, cytotoxicity was determined through a MTT (Roche, Germany) proliferation assay (Twentyman et al., 1987). Then 50% cell cytotoxic concentration (CC50) of *A. marina* was calculated. All assays were carried out in triplicate.

2.7. Antiviral activity

Anti HSV-2 activity of methanol extract and different fractions of *A. marina* was investigated via plaque reducing assay in a final non-toxic concentration <32 μg/ml with 24 h old monolayer of Vero cells grown in microtitre tissue culture plates. The cell monolayers were infected with 25 PFU of HSV-2 and incubated at 37 °C for 2 h. Then they were washed and overlaid by medium supplemented with 2.5% methylcellulose and different concentrations of extract (20, 10, 5 and 2.5 μg/ml). 0.1% DMSO and different concentrations of ACV (20, 10, 5 and 2.5 μg/ml) were used as negative and positive controls. After 3–4 days of incubation, the overlay medium was removed. The cell monolayer was fixed with 3.7% formalin for 5 min. Then visible plaques were counted after staining with 1% crystal violet. The antiviral activity was determined by following formula:

$$\text{Percentage of inhibition} = \left[1 - \frac{(\text{number of plaque})_{\text{tested}}}{(\text{number of plaque})_{\text{control}}}\right] \times 100$$

The required minimal concentrations of extracts to suppress the formation of virus plaque number by 50% (EC50) were calculated by regression analysis of the dose–response curves generated from data according to Cheng et al. (2002).

2.8. Time of addition study

The time of addition effect was examined for most active fraction" LMEG" as described by Yang et al. (2005). Vero cells, 2×10^5 per well, were seeded into 24-well culture plates (Nunc; Nalge Nunc International, Rochester, NY, USA) and incubated for 24 h. Then, confluent monolayer was treated by 5 μg/ml of LMEG (added into the wells) either concurrent with HSV-2 (0 h) or at intervals of 2, 4, and 6 h pre-infection and also post-infection. The procedure was similar to "plaque forming assay" section, except that cells were washed thrice by PBS to eliminate extract prior to inoculation of virus for "pre-infection" (−6, −4 and −2 h) group. The extracts were added at different times during HSV-2 (0 h) and post-infection (2, 4 and 6 h) group. After 2 h of infection, cells were washed and overlaid by medium supplemented with 5% FBS. After incubation at 37 °C for 72 h, the inhibition percentage was calculated. The reduction in the virus titer was obtained by real time polymerase chain reaction (real time PCR) assay and infection cultures containing the extracts were compared with the control cultures. ACV was used as positive control for antiviral assay during HSV-2 infection.

2.9. Quantitative real time PCR assay for HSV-2

For real time PCR, 200 ml of supernatant of each treated-infected, or untreated-infected (virus control) wells were collected. DNA was purified from 200 μl of each specimen by QIAamp DNA Mini Kit (Qiagen) according to the standard protocol. Purified