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*

Mandana Behbahani *, Mehrnaz Shanehsaz Zadeh, Hassan Mohabatkar

Department of Biotechnology, Faculty of Advanced Sciences and Technologies, University of Isfahan, Isfahan 81746-73441, Islamic Republic of Iran

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.
© 2013 Elsevier B.V. All rights reserved.

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

* Corresponding author. Tel.: +98 311 7934327; fax: +98 311 7932342.
E-mail addresses: Ma_behbahani@yahoo.com, Ma.behbahani@ust.ui.ac.ir (M. Behbahani).

0166-3542/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
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

DNA was resuspended in 200 μl of elution buffer and 5 μl aliquots were used as PCR templates. As previously described forward primer, HSV-2 pol F (5′ GCTCGAGTGCGAAAAAACGTTC 3′) and the reverse primer, HSV-2 pol A (5′ CGG GGC GCT CGG CTA AC 3′) were used to amplify a 391 bp product (Espy et al., 2000). One of the HSV-2 probes is 3′ labeled with FAM and the other 5′ labeled with Cy5 (supplied by Biosearch Technologies, USA). To prevent extension of the Cy5 probe, an additional phosphate is attached to the 3′-end of this probe. The sequences of the label probes (5′ GTA CAT CGG CGT CAT CTG CGG GGG CAA G-FAM) and (5′-CY5 TGC TCA TCA AGG GCG TGG ATC TGG TGC-phosphate-3′) were used for HSV-2 detection. The real time PCR master mix contained the following: Taq DNA polymerase (Molecular Diagnostics), 2.5 mM dNTP mix (containing dUTP instead of dTTP), 2.5 mM $MgCl_2$, 1.3 μM of forward primer, 12 μM reverse primer, 10 μM FAM-labeled probe and 10 μM Cy5 labeled probe. Cycling conditions were as follows: initial denaturation/Fast Start Taq DNA polymerase activation at 95 °C/5 min, 40 cycles of denaturation at 95 °C/15 s, annealing at 58 °C/20 s and extension at 72 °C/10 s.

2.10. Statistical analysis

Data from three independent experiments were presented as mean ± SD. The EC50 and CC50 values were calculated by Microsoft Excel 2000. A selectivity index (SI) was calculated for each viral strain by the ratio of CC50 to EC50 value. Student's unpaired *t*-test was used to assess significance of the test sample and solvent control. *P* value <0.05 was considered to be statistically significant.

3. Results

3.1. HPLC analysis

Fig. 1A–C shows the HPLC chromatograms of the methanol extract, LMEG and fraction 9c of *A. marina*. Several peaks were observed in the HPLC spectrum of methanol extract (A). After purification only one peak, 9b, was observed at retention time of 12.2 min which was similar to one of the peaks in methanol extracts. This peak was observed with high intensity and was later identified as LMEG (B). Fraction 9c was impure and three peaks (one major peak and two minor peaks) were detected (C). The major peak of fraction 9c was observed at a retention time of 13.5 min which was similar to luteolin standard peak (D). Two small peaks were also observed at retention times between 12.5 and 13.5 min.

3.2. NMR analysis

Fraction 9b which was the most active fraction was analyzed using ^{1}H NMR (DMSO, 400 MHz): δ 7.70 (s, 2H), 6.95 (d, 1H, J = 9.0 Hz), 6.90 (s, 1H), 6.80 (d, 1H, J = 2.0 Hz), 6.40 (d, 1H, J = 2.0 Hz), 5.10 (d, 1H, J = 7.0 Hz), 3.90 (s, 3H), 3.20–3.70 (m, sugar proton). The ^{1}H NMR spectrum suggested that fraction 9b was luteolin7-O-methylether 3′-O-β-D-glucoside.

3.3. Cytotoxic effects on viability of Vero cells

The concentrations of methanol extract from *A. marina* did not affect the viability of Vero cells by MTT assay. Results showed that methanol extract, LMEG and Fraction 9c had no cytotoxic effect up to 32 μg/ml (Fig. 2). The CC50 values of methanol extract, LMEG and Fraction 9c were 250 ± 3.2, 125 ± 3.0 and 88.2 ± 2.8 μg/ml, respectively. Therefore, the anti HSV-2 activity of these three extracts was assayed at concentrations of 20.0 μg/ml or lower.

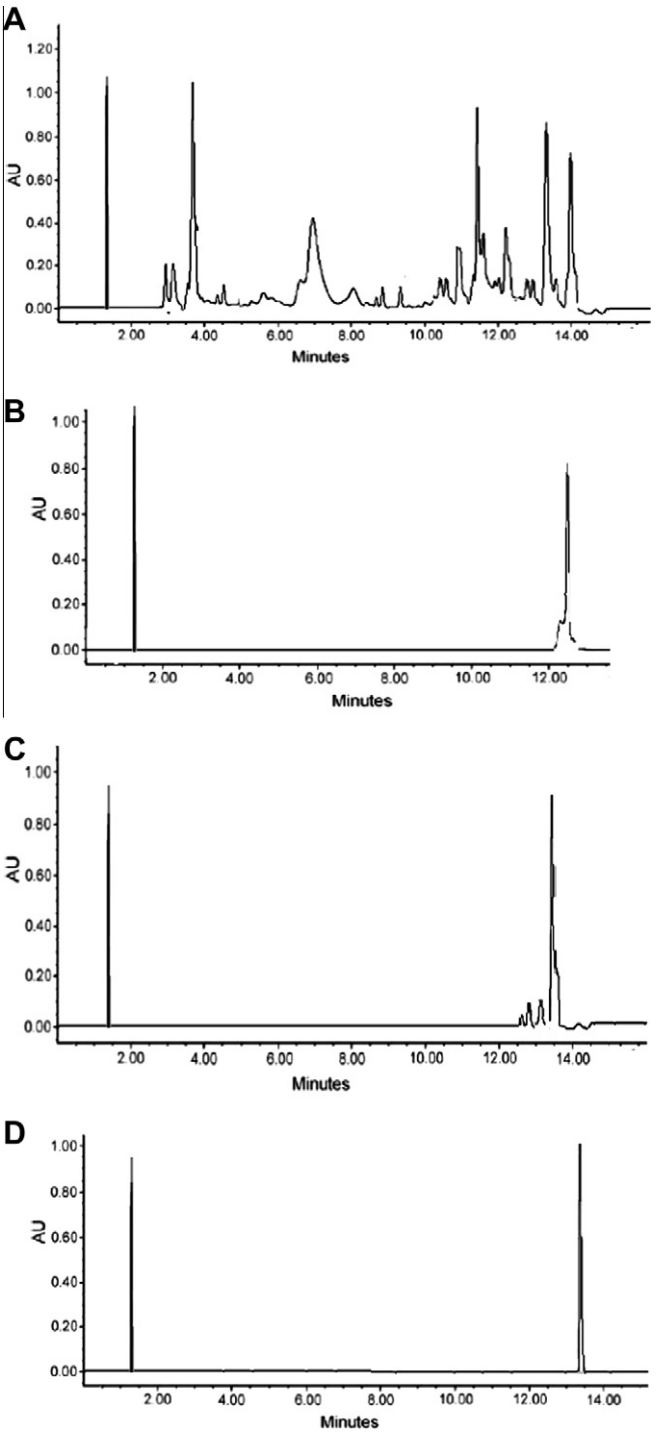

Fig. 1. HPLC analysis of the methanol extract (A), LMEG (B), luteolin (C) and standard luteolin (D) isolated from *A. marina*. Chromatographic conditions: RPC18 column, mobile phase: methanol/aceton/water = 70/20/10 (v/v/v) at 1.5 ml min^{-1}.

3.4. Anti HSV-2 activity

Antiviral activity of 20 μg/ml of methanol extract and twelve fractions of *A. marina* were evaluated by plaque reduction assay. Results showed that methanol extract, fractions 9b and 9c inhibited HSV-2 multiplication with an inhibition rate of 80 ± 1.6%, 100 ± 2.9% and 65 ± 1.3%, respectively. Other fractions had inhibition rate of lower than 20% (data not shown). The antiviral activity of these three extracts was further examined by different concen-

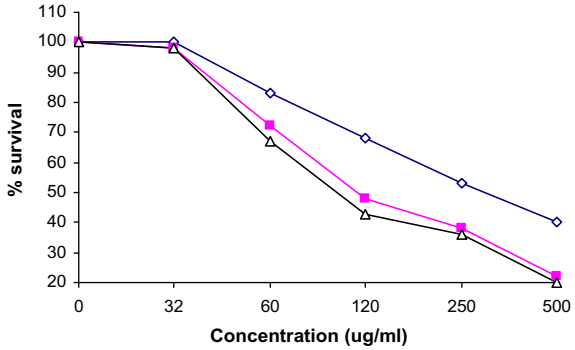

Fig. 2. Cytotoxic activity of crude methanol extract (◇), LMEG (■) and fraction 9c (Δ) from *A. marina* leaf.

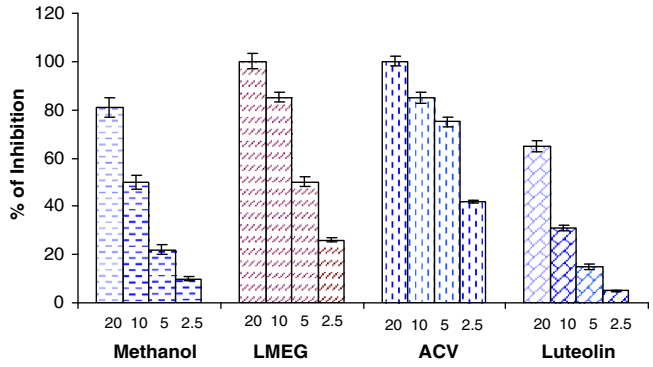

Fig. 3. Effect of methanol extract, LMEG, ACV and luteoin on HSV-2 replication in Vero cells. The 50% inhibitory concentration (EC50) of each extract was calculated using regression line. Each bar represents the mean ± SD of three independent experiments.

trations of 20, 10, 5, 2.5 μg/ml (Fig. 3). The antiviral activity of fraction 9c was weaker than 9b. EC50 values of methanol extract, LMEG, fraction 9c and ACV were 10, 5, 16.6 and 2.97 μg/ml. Also the calculated selective index (SI) for methanol extract, LMEG and luteolin were 25, 25, and 5.3, respectively.

3.5. Mechanism of action on HSV-2 replication

A time of addition experiment of the most active fraction "LMEG" was performed by measuring the viral DNA yields in infected culture supernatants, by means of real time PCR assay. The viral DNA concentrations in cultures treated with 20 μg/ml of the LMEG before (−6, −4 and −2 h), during (0 h), and after (2, 4 and 6 h) period of HSV-2 infection and in untreated virus control were calculated. The curves generated during PCR reactions showed a prominent drop in HSV-2 DNA amounts in treated cultures compared to those of virus controls (Fig. 4). The strongest HSV-2 proliferation inhibition was achieved when the extract was added during the initial stages of infection and 2 h after infection. The result showed that the amount of viral DNA during and 2 h after infection was 10^2-fold lower compared to untreated virus control. No viral DNA was detected in non-infected cells.

4. Discussion

Medicinal plants have been shown to treat a variety of human viral infections. Some of them appear to pose the broad-spectrum antiviral activity without extensive toxic effects as ideal candidates in antiviral therapy (Mukhtar et al., 2008). Flavonoids isolated from

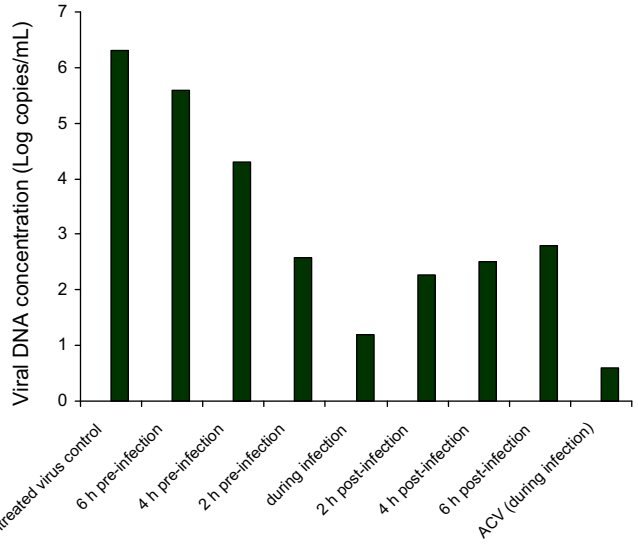

Fig. 4. Time of addition effect of LMEG obtained from *A. marina* on HSV-2 replication in Vero cells. Twenty μg/ml of LMEG was added either before (−2, −4 and −6 h), during (0) and after (2, 4 and 6 h) virus infection. The extracts added before virus infection was rinsed off prior to the virus exposure. Each value is the result of mean ± SD of three independent experiments.

medicinal plants have been reported to have HSV-1 and HSV-2 antiviral properties (Amaral et al., 1990).

Three prepared extracts from *A. marina* were investigated for their herpes virus inhibitory effect by two separate assays. The tested crude methanol extract, sub-fractions 9b and 9c showed moderate and excellent antiherpetic activity, respectively. Among the tested fractions, sub-fraction 9b was the most active compound reducing the viral titer of HSV-2 DNA. Fraction 9b has been characterized as a LMEG. The antiviral activity of 9c was weaker and was analyzed by HPLC as luteolin. The naturally occurring flavonoids are believed to possess the ideal chemical structure for scavenging free radicals (Choi et al., 2002).

Some flavonoids have anti herpetic effects and the direct inactivation of HSV by some flavonoids such as quercetin, procyanidin, pelargonidin, catechin and hesperitin has been verified (Amoros et al., 1992). Many *in vitro* and *in vivo* studies have been published on the antiviral activity of flavonoids. Debiaggi et al. (1990) have investigated propolis-derived flavonoids and shown high concentrations of quercetin can reduce HSV replication. Similarly, it was reported that some flavonoids such as hyperbrasilol B, amentoflavone, hyperoside, guaijaverine and luteoforol isolated from *Hypericum connatum* inhibit HSV replication (Fritz et al., 2007). Previous studies examined anti herpetic activities of luteolin and luteolin-7-glucoside *in vitro* and *in vivo* (Amaral et al., 1990; Chiang and Chiang, 2002).

Our results showed that isolated LMEG and luteolin from methanol extract of *A. marina* inhibit HSV-2 infection significantly. The calculated selective index (SI) for methanol extract, LMEG and luteolin were 25, 25 and 5.3, respectively. This important criterion determines that SI $\geqslant$ 4 should be considered suitable for an antiviral agent (Tsuchiya et al., 1985). The time of addition study revealed that LMEG was most effective when added concurrently with HSV-2 infection. The antiviral activities in LMEG-treated Vero cells were almost similar during and 2 h after HSV-2 infection. Preincubation of the Vero cells with extracts of *A. marina* and then washing the extracts out did not protect the cells from HSV-2 infection (Fig. 4). Furthermore, the extracts of *A. marina* could further inhibit HSV-2 infection when it was added during and after 2 h virus inoculation period. These observations indicated that LMEG inhibited the first stage of HSV-1 infection which included

attachment of the virus to the cell membrane, diffusion of the virus through the cell membrane and transfer of viral DNA into the cell nucleus. Nevertheless, further studies are needed to verify the mechanism of isolated compounds from *A. marina* extract.

5. Conclusion

Based on the findings of the study, it can be concluded that *A. marina* leaves have an inhibitory effect on HSV-2 activity. The results indicate that LMEG constituted most active sub-fraction in the crude methanol extract from *A. marina* and exhibits an effective inhibitory mechanism on the early stage of HSV-2 infection.

Acknowledgments

The authors would like to acknowledge the University of Isfahan for the financial support of this study. We also thank Faculty staff of the University of Isfahan for their research efforts on the study design and Agriculture and Natural Resources Research Center of Gheshm, Iran for collection of *A. marina* seeds.

References

Amaral, A.C.F., Kuster, R.M., Gonçalves, J.L.S., et al., 1990. Antiviral investigation on the flavonoids of *Chamaesyce thymifolia*. Fitoterapia 70, 239–295.

Amoros, M., Simes, C.M., Girre, L., et al., 1992. Synergistic effect of flavones and flavonols against *herpes simplex* virus type 1 in cell culture. Comparison with the antiviral activity of propolis. J. Nat. Prod. 55, 1732–1740.

Bandaranayake, W.M., 1995. Survey of mangrove plants from Northern Australia for phytochemical constituents and UV-absorbing compounds. Curr. Top. Phytochem. 14, 69–78.

Chattopadhyay, D., Khan, M.T., 2008. Ethnomedicines and ethnomedicinal phytophores against herpesviruses. Biotechnol. Annu. Rev. 14, 297–348.

Cheng, H.Y., Lin, C.C., Lin, T.C., 2002. Antiherpes simplex virus type 2 activity of casuarinin from the bark of *Terminalia arjuna* Linn. Antiviral Res. 55, 447–455.

Cheng, H.Y., Lin, T.C., Yang, C.M., et al., 2004. Putranjivain A from *Euphorbia jolkini* inhibits both virus entry and late stage replication of herpes simplex virus type 2 *in vitro*. J. Antimicrob. Chemother. 53, 577–583.

Chiang, L.C., Chiang, W., Chang, M.Y., et al., 2002. Antiviral activity of Plantago major extracts and related compounds *in vitro*. Antiviral Res. 55, 53–62.

Choi, C.W., Kim, S.C., Hwang, S.S., et al., 2002. Antioxidant activity and free radical scavenging capacity between Korean medicinal plants and flavonoids by assay-guided comparison. Plant Sci. 163, 1161–1168.

Debiaggi, M., Tateo, F., Pagani, L., et al., 1990. Effects of propolis flavonoids on virus infectivity and replication. Microbiologica 13, 207–213.

Duke, N.C., 1991. A systematic revision of the mangrove genus *Avicennia* (Avicenniaceae) in Australasia. Aust. Syst. Bot. 4, 299–324.

Espy, M.J., Ross, T.K., Teo, R., et al., 2000. Evaluation of lightcycler PCR for implementation of laboratory diagnosis of herpes simplex virus infections. J. Clin. Microbiol. 38, 3116–3118.

Fritz, D., Venturi, C.R., Cargnin, S., et al., 2007. Herpes virus inhibitory substances from *Hypericum connatum* Lam., a plant used in southern Brazil to treat oral lesions. J. Ethnopharmacol. 113, 517–520.

Hogg, R.W., Gillan, F.T., 1984. Fatty acids, sterols and hydrocarbons in the leaves from eleven species of mangrove. Phytochemistry 23, 93–97.

König, G., Rimpler, H., 1985. Iridoid glucosides. Avecinnia Marina 24, 1245–1248.

Mukhtar, M., Arshad, M., Ahmad, M., et al., 2008. Antiviral potentials of medicinal plants. Virus Res. 131, 111–120.

Piret, H., Boivin, G., 2011. Resistance of herpes simplex viruses to nucleoside analogues: mechanisms, prevalence, and management. Antimicrob. Agents Chemother. 55, 459–472.

Saddi, M., Sanna, A., Cottiglia, F., et al., 2007. Antiherpesvirus activity of *Artemisia arborescens* essential oil and inhibition of lateral diffusion in Vero cells. Ann. Clin. Microbiol. Antimicrob. 6, 10.

Saijo, M., Suzutani, T., Morikawa, S., et al., 2005. Genotypic characterization of the DNA polymerase and sensitivity to antiviral compounds of foscarnet-resistant herpes simplex virus type 1 (HSV-1) derived from a foscarnet-sensitive HSV-1 strain. Antimicrob. Agents Chemother. 49, 606–611.

Schnitzler, P., Koch, C., Reichling, J., 2007. Susceptibility of drug-resistant clinical herpes simplex virus type 1 strains to essential oils of ginger, thyme, hyssop, and sandalwood. Antimicrob. Agents Chemother. 51, 1859–1862.

Tsuchiya, Y., Shimizu, M., Hiyama, Y., et al., 1985. Antiviral activity of natural occurring flavonoids *in vitro*. Chem. Pharm. Bull. 33, 3881–3886.

Twentyman, P.R., Luscombe, M., 1987. A study of some variables in tetrazolium dye (MTT) based assay for cell growth and chemosensitivity. Brit. J. Cancer 56, 279–285.

Yang, C.M., Cheng, H.Y., Lin, T.C., et al., 2005. Acetone, ethanol and methanol extracts of *Phyllanthus urinaria* inhibit HSV-2 infection *in vitro*. Antiviral Res. 67, 24–30.

Zandi, K., Taherzadeh, M., Yaghoubi, R., et al., 2009. Antiviral activity of *Avicenna marina* against herpes simplex virus type 1 and vaccine strain of poliovirus. J. Med. Plant Res. 3, 771–775.