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Full Length Research Paper

Anti-viral activity of the methanolic leaf extract of an Iranian medicinal plant *"Hyssopus officinalis"* against herpes simplex virus

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Herpes simplex virus (HSV) infection is a major opportunistic infection in immunosuppressed persons. The development of resistant strains of HSV to the available drugs for infection management, as is evident in the first drug of choice acyclovir, has further compounded this situation. There is therefore an urgent need to identify and develop new alternative agents for management of HSV infections, more so, for those due to resistant strains. We report here on methanolic extract preparation from the leaves of *Hyssopus officinalis*, a medicinal plant locally growing in Iran that has exhibited remarkable anti-HSV activity *in vitro* and *in vivo* for both wild type and resistant strains of HSV. The extract significantly inhibited formation of plaques in Vero E6 cells infected with 100 PFU of wild type strains of HSV (7401H HSV-1 and Ito-1262 HSV-2) or resistant strains of HSV (TK$^-$7401H HSV-1 and AP^r 7401H HSV-1) by 100% at 25 µg/ml *in vitro* with minimal cell cytotoxicity (CC_{50} = 960 µg/ml). When the extract was examined for *in vivo* efficacy in a murine model using Balb/C mice cutaneously infected with wild type or resistant strains of HSV, the extract at an oral dose of 125 mg/kg significantly delayed the onset of HSV infections by over 50%. It also increased the mean survival time of treated infected mice by between 55 and 65% relative to the infected untreated mice ($p < 0.05$ versus control by Student's *t*-test). The mortality rate for mice treated with extract was also significantly reduced by 90% as compared with the infected untreated mice that exhibited 100% mortality. No acute toxicity was observed in mice at the oral therapeutic dose of 125 mg/kg. These results suggest that this herbal extract has potent anti-viral agents against herpes simplex viruses that can be exploited for development of an alternative remedy for HSV infections.

Keywords: Herpes simplex virus, *Hyssopus officinalis,* anti-viral activity.

INTRODUCTION

The use of herbal medicines is widely practiced in Iran. Reasons for this use range from the general acceptance of herbal remedies as alternative medicines for management of disease, the ready availability of these medicines at local village markets and the high cost of modern pharmaceuticals which is prohibitive to most of the general population. While the benefits of these herbal remedies are appreciated within communities, most of them lack documented evidence of therapeutic effectiveness.

Infections due to herpes simplex virus (HSV) have been reported worldwide (Shi et al., 2007). This virus can produce latent infection in the host for life and is reactivated by stimulus to cause recurrent infections and lesions (Fatahzadeh and Schwartz, 2007). On the other hand, the severe side effects and the development of some resistant mutants of this virus especially during long term medication with antiviral drugs were reported (Miserocchi et al., 2007). Severe forms of disseminated HSV infection are often seen in patients with reduced cell mediated immunity as in bone marrow transplant reci-

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Abbreviations: HSV, Herpes simplex virus; **HIV-1,** human immunodeficiency virus type 1; **AIDS,** acquired immunodeficiency syndrome; **ACV,** acyclovir; **STD,** sexually transmitted disease; **DMSO,** dimethyl sulfoxide; **FBS,** fetal bovine serum; **MC,** methyl cellulose; **MEM,** minimum essential medium; **PFU,** plaque forming units; **EC_{50},** half maximal effective concentration; **CC_{50},** 50% cytotoxic concentration.

pients or patients with acquired immunodeficiency syndrome (AIDS) (Wolfgang et al., 1985).

Immunosuppression in patients with AIDS due to human immunodeficiency virus type 1 (HIV-1) infection has been closely associated with increased cases of HSV infection in regions with high HIV-1 seroprevalence. In a study aimed to examine the *in vivo* relationships between HSV-2 seroprevalence (and shedding) and HIV-1 seropositivity in African women in Bangui, Central African Republic, findings indicated a significant seroprevalence of HSV-2 in HIV-1 seropositive than in HIV-1 seronegative women (Mbopi-Keou et al., 2000). These results indicate that infections due to HSV are therefore a major health problem in regions with high HIV-1 seroprevalence. Herpes simplex virus infections are usually managed with anti-viral drugs such as the nucleoside analogue acyclovir (ACV), the drug of choice (Kleymann, 2003). However, resistance to ACV has been reported mainly among the immunocompromised patients (Morfin and Thouvenot, 2003). In a study on persons attending sexually transmitted disease (STD) and human immunodeficiency virus (HIV) clinics in the United States, 226 patients were reported as being HIV-positive. Out of this HIV positive group, 5.3% yielded resistant HSV isolates to oral and topical acyclovir (Reyes et al., 2003). Since HSV infection and recurrence is still expected to increase in frequency and severity in areas with high HIV prevalence (e.g. in sub-Saharan Africa) because of immunosuppression (Safrin et al., 1991; Mbopi-Keou et al., 2000), the level of resistance to the available drugs is also expected to increase. There is therefore a need to develop new therapeutic agents for the management of HSV infections. We here report on a methanolic extract from the leaves of *Hyssopus officinalis* (Lamiaceae) that has shown significant anti-HSV activity *in vitro* and *in vivo*. The plant *H. officinalis* is reputed in Traditional Medicine as a good source of medicine for treatment of ear infections, bronchitis and stomach problems. The leaves can be used as compresses on wounds and bruises (Kazazi et al., 2007).

MATERIALS AND METHODS

Collection and preparation of the medicinal plant extract

The leaves of *H. officinalis* were taken from a tree in Herbal Unit, at Isfahan University, (Central of Iran) in December 2006. The dried plant sample (60 g) was placed in a stopped conical flask and macerated with 500 ml of 98% (v/v) methanol (Merck, Germany) at room temperature (25 – 28°C) for 3 days with occasional stirring. Each experiment was performed in triplicate (n = 3). The solvent was then filtered and evaporated in a vacuum rotary evaporator (Somatco, Germany) at 45°C. The residue was placed in oven (Napco, USA) at 40ºC until dry. The crude extract was stored in a well-closed container, protected from light and kept in a refrigerator at –4°C.

100 mg of the sample extract were dissolved in 10 ml 100% (v/v) of Dimethyl sulfoxide (DMSO) and mixed by sonicating with a sonicator. For *in vitro* experiments, a working solution of 1 mg/ml was aseptically prepared by diluting 10 µl stock with 0.99 ml (990 µl) Eagle's Minimum Essential Medium (MEM) (Merck, Germany) supplemented with 2% heat inactivated fetal bovine serum (FBS) (Merck, Germany). For the initial *in vitro* screening, the test concentrations of 50 and 100 µg/ml was achieved by adding 25 and 50 µl working solution to 975 and 950 µl of MEM containing 2% FBS and 0.8% nutrient methyl cellulose (MC) (Merck, Germany), respectively, for treatment of infected cells.

For the *in vivo* experiments, a working solution of 20 mg/ml was aseptically prepared from the stock solution by diluting 200 µl stock solution in 0.8 ml (800 µl) sterile double distilled water. A 0.125 ml of the working solution was then orally administered to a 20 g body weight mouse (equivalent to 125 mg/kg).

Preparation of acyclovir (ACV)

Acyclovir was used as the reference drug and was purchased as tablets (Zovirax 200, Wellcome) from Nippon Wellcome K.K. Japan. A stock suspension of 10 mg/ml active drug was prepared by carefully powdering a tablet (one tablet contained 200 mg of the active drug) in a clean mortar using a pestle and directly adding 20 ml of sterile double distilled water in the mortar, under aseptic conditions. The milky suspension was transferred into a 50 ml vial and vortexed for 1 min. The suspension was kept at 4°C and used within a week.

For *in vitro* assays, a working concentration of 1 mg/ml was achieved by diluting 100 µl stock solution with 0.9 ml (900 µl) MEM supplemented with 2% FBS. 5 µl of the working solution was added in 995 µl MEM containing 2% FBS and 0.8% MC for a standard test concentration of 5 µg/ml for treatment of HSV infected Vero E6 cells.

For *in vivo* assays, a working concentration of 0.4 mg/ml was prepared by diluting 40 µl of stock solution with 0.96 ml (960 µl) of sterile double distilled water and 0.25 ml of the working suspension administered to a 20 g body weight mouse to give 100 µg/20 g (5 mg/kg) (Kurokawa et al., 1993).

Viruses and cells

The Vero E6 cells were donated by the Virology Department, Medical University of Isfahan, Iran and are duly acknowledged. The cells were grown under 5% CO_2 at 37°C in MEM supplemented with 5% FBS for cell growth and 2% FBS for cell maintenance.

The herpes simplex virus (HSV) strains used in the study were wild-type 7401H HSV-1 (Kurokawa et al., 1993), wild type HSV-2 (Ito-1262) (Kurokawa et al., 1995), thymidine kinase deficient (TK^-) B2006 HSV-1 (Dubbs and Kit, 1964) and acyclovir resistant (AP^r) 7401H HSV-1 (Kurokawa et al., 1995). These viruses were donated by the Virology Department, Medical University of Isfahan, Iran and is duly acknowledged. The virus stocks were prepared from infected Vero E6 cells. The infected cultures were frozen and thawed three times to lyse the cells and centrifuged at 3000 rpm for 15 min. Their supernatants, containing HSV, were harvested and stored at –80°C until use (Kurokawa et al., 1993).

Plaque inhibition assay

The herbal extract was examined for extent of inhibition of plaque formation on HSV infected tissue culture as an indication of antiviral activity *in vitro* as described by Kurokawa et al. (1993, 2001). Briefly, Vero E6 cells in 60 mm Petri dishes were cultured to a confluent monolayer in MEM supplemented with 5% FBS in 5% CO_2 at 37°C for 3 days. The cells were infected with 100 plaque forming units (PFU) of HSV and left to adsorb for 1 h at room temperature on a tray shaker. The infected cells were then overlaid with MEM supplemented with 2% FBS and 0.8% MC and various

concentrations of herbal extract or acyclovir and further incubated at 37°C in 5% CO_2 for 2 days. The treated infected cells were fixed by adding 5% formalin (Sigma, USA) and washed with running tap water followed by staining with 0.03% methylene blue solution (Merck, Germany). The plaques, appearing as transparent dots, were counted using a dissecting microscope and the percent plaque inhibition calculated.

The effective concentration inhibiting formation of plaques by 50% (EC_{50}) was determined by the plaque inhibition assay for the extract at various concentrations (5, 10, 20, 40, 60, 80 and 100 µg/ml). The EC_{50} value was obtained from a graph of concentration versus % plaque inhibition. The EC_{50} determination was independently done three times from which the mean and standard deviation (S.D.) was calculated.

Cell cytotoxicity assay

The cytotoxic concentration causing 50% cell lysis and death (CC_{50}) was determined for the extract by a method described by Kurokawa et al. (2001). Vero E6 cells were seeded at a concentration of 2.5×10^4 cells/well in 24-well plates and grown at 37°C for 2 days. The culture medium was replaced by fresh medium containing extract at various concentrations and cells further grown for 24 h. The cells were treated with trypsin and the number of viable cells determined by the trypan blue exclusion test. The concentration of herbal extract reducing cell viability by 50% (CC_{50}) was determined from a curve relating percent cell viability to the concentration of extract.

Virus yield reduction assay

The leaves of *H. officinalis* were compared with acyclovir for its anti-viral activity on the growth of the wild type strains HSV-1 and HSV-2 and resistant strains, AP^r HSV-1 and TK^- HSV-1, in the virus yield reduction assay as described by Kurokawa et al. (1998). Confluent monolayers of Vero E6 cells in 60 mm diameter dishes were infected with HSV at multiplicity of infection of 5 (5 M.O.I). Separate dishes each containing infected cells was then treated with extract at various concentrations (25, 50 and 100 µg/ml). The infected treated cells were incubated in 5% CO_2 at 37°C for 24 h. The treated cell cultures were then frozen at –80°C and thawed at 37°C for 1 h. This procedure was repeated three times. The lysed cultures were collected in 15 ml tubes and centrifuged at 3000 rpm for 10 min and supernatant harvested. A serial dilution of each supernatant was prepared in MEM and the virus titre determined by the plaque inhibition assay.

Determination of therapeutic effect of *H. officinalis* extract in Balb/C mice cutaneously infected with HSV

Balb/C mice purchased from the Virology Department, Medical University of Isfahan, Iran, were used for determination of *in vivo* efficacy of the extract. The animals were handled according to guidelines laid down by the animal care and maintenance committee of the Isfahan Medical Research Institute that follows internationally acceptable standards on animal care and use in laboratory experimentation. The determination of the therapeutic effect of the *H. officinalis* extract was carried out as described by Kurokawa et al. (2001) with minor changes. Female Balb/C mice 7 weeks old weighing about 20 g were housed for a week to acclimatize. The animals were fed with food (Mice cubes, Unga feeds, Iran) and water *ad libitum*. The mid flank of each mouse was shaved using an electric hair trimmer (Wahl super taper, England) and hair completely removed by applying a chemical hair remover (Shiseido, Co., Ltd., Japan) on the shaved area. The mice were randomly divided into four groups each comprising of 5 mice. The first group, the control-1 group, were not infected and received no treatment. The second group, control-2 group, were infected but received no treatment. The third group, the test group, were infected and received the plant extract treatment. The final fourth group was the reference drug group. In this group, mice were infected and received ACV treatment. An initial oral dose of the test extract or ACV was administered to the mice in the test and reference groups accordingly before infection. The two control groups were administered with distilled water. Four hours after the oral administrations, the shaved mid flank of each mouse was scratched using a bundle of G27 needles and the sacrificed area infected with 1×10^6 PFU HSV apart from the control-1 group. Four hours after infection, the second oral administration was given followed with the final administration for the day, 12 h after infection. Oral administration of the test sample and reference drug was given three times a day at 8 h intervals for seven consecutive days. The control mice were administered with distilled water at the same time. The development of skin lesions and mortality were continuously monitored every 8 h daily and scored as follows: 0, no lesion; 2, vesicles in local region; 4, erosion and/or ulceration in the local region; 6, mild zosteriform; 8, moderate zosteriform; 10, severe zosteriform and death (Kurokawa et al., 1993). The mice were fed and observed for 30 days to determine their mortality.

Determination of acute toxicity

The determination of *in vivo* acute toxicity was performed as described by Kurokawa et al. (1993, 2001). Seven weeks old 20 female and 20 male Balb/C mice were used. 250 mg per kilogram extract (the concentration found to exhibit therapeutic effect) was orally administered to the uninfected mice three times daily for 7 days following the same schedule as that under the determination of therapeutic effect. The mice were weighed every day in the morning for 21 days to determine any change in weight. The mortality of the uninfected mice was calculated on the 30th day. The weights and mortality were used for the determination of acute toxicity.

Data analysis

In the *in vitro* assays, the number of plaques formed in infected cells were used to calculate the percentage inhibition of plaques and graphical determination of EC_{50}. In the cytotoxicity experiments, the number of viable cells was used to calculate the percentage toxicity at each tested concentration that was then used for the graphical determination of the CC_{50}. In the *in vivo* experiments, the Student's t-test was used to evaluate the significance of differences between mean survival times and the mean times at which skin lesion were initially scored 2 (vesicles in local region) or 6 (mild zosteriform lesion) after infection. The significance of differences in mean weights between control and extract treated mice in the toxicity experiment was also evaluated by the Student's t-test. Analysis of variance (ANOVA) was used to analyse the interaction between *H. officinalis* leave extract or acyclovir, with methanol (control), in mean skin lesion score for 3 – 10 days after infection. A p-value of less than 0.05 was statistically defined as significant. The Stat View computer soft software by SAS Institute Inc. USA was used for the statistical analysis.

RESULTS

Effects of the extract in inhibiting plaque formation

Plaque inhibition assay was carried out using methanol extract of *H. officinalis*. Preliminary screening concentra-

Table 1. EC_{50} and CC_{50} determinations for the medicinal plant extract.

[b] (µg/ml)	[b] (µg/ml)				[b] (µg/ml)
[b] (µg/ml)	[b] (µg/ml)	[b] (µg/ml)	HSV-1 AP^r	HSV-1 TK$^-$	
[b] (µg/ml)	[b] (µg/ml)	[b] (µg/ml)	4.1 ± 0.40	5.1 ± 0.40	960
[b] (µg/ml)	[b] (µg/ml)	[b] (µg/ml)	> 5.0	> 5.0	100

[a]Effective concentration for 50% plaque reduction
[b]Cytotoxic concentration causing 50% cell lysis and death. The results are a mean of three independent experiments.

Table 2. *In vitro* activity of *H. officinalis* extract on the growth of wild type and resistant strains of HSV in vero E6 cells.

Virus	Virus titre (PFU/ml) [a]	Treatment (µg/ml)			%[b]
	Control	25	50	100	
HSV-1	(6.0 ± 0.10)X10^6	(0.41 ± 0.02)X10$^{6\,c}$	(1.8 ± 0.14)X10^{5c}	0 [c]	100
HSV-2	(6.0 ± 0.11)X10^6	(0.96 ± 0.03)X10$^{5\,c}$	(2.9 ± 0.14)X10^{4c}	0 [c]	100
AP^r HSV-1	(6.0 ± 0.10)X10^6	(0.2 ± 0.01)X10$^{5\,c}$	(2.0 ± 0.14)X10^{4c}	0 [c]	100
TK$^-$HSV-1	(6.0 ± 0.11)X10^6	(0.1 ± 0.08)X10$^{5\,c}$	(1.60 ± 0.14)X10^c	0 [c]	100

[a] Values are mean ± S.D. of two independent experiments.
[b] Values are % virus yield reduction at 100 µg/ml of extract.
[c] $p < 0.05$ vs. control, by Student's t-test.

Table 3. *In vitro* activity of acyclovir extract on the growth of wild type and resistant strains of HSV in vero E6 cells.

Virus	Virus titre (PFU/ml) [a]	Treatment (µg/ml)			%b
	Control	0.5	1.0	5	
HSV-1	(6.0 ± 0.14)X10^6	(3. 0 ± 0.30)X10^{5c}	(7.0 ± 0.11)X10^{4c}	0 [c]	100
HSV-2	(6.01 ± 0.11)X10^6	(3. 0 ± 0.10)X10^{5c}	(7.0 ± 0.12)X10^{2c}	0 [c]	100
AP^r HSV-1	(6.61 ± 0.14)X10^6	(6.0 ± 0.10)X10^{5c}	(6.32 ± 0.21)X10^6	(3.12 ± 0.07)X10^6	1.4
TK$^-$HSV-1	(6.72 ± 0.11)X10^6	(6.16± 0.01)X10^{5c}	(6.41 ± 0.32)X10^6	(4.15 ± 0.07)X10^6	3.2

[a] Values are mean ± S.D. of two independent experiments.
[b]Cytotoxic concentration causing 50% cell lysis and death. The results are a mean of three independent experiments.
P < 0.05 vs.control, by student' t-test.

tions of 25 and 50 µg/ml extract provided complete inhibition of plaques of non-resistant HSV. The effects of the extract at concentrations lower than 25 µg/ml are demonstrated by the EC_{50} values presented in Table 1. The results show that the resistant strains of the virus were more susceptible to the extract than the wild type strains. The CC_{50} of the extract was several magnitudes higher than the EC_{50} values, an indication of a wide therapeutic index (TI =CC_{50}/EC_{50}). The non-sensitivity of the resistant strains to ACV at 5 µg/ml confirmed that they were indeed resistant to ACV at that concentration.

Effects of the extract on reduction of virus yield

The activity of the *H. officinalis* extract on the growth of wild type and resistant strains of HSV was compared to that of acyclovir in the virus yield reduction assay. Table 2 shows the anti-HSV activity of the extract at 25, 50 and 100 µg/ml. At 100 µg/ml, the extract significantly reduced the virus yields of AP^r HSV-1, HSV-2, HSV-1 and TK– HSV-1 by 100% ($p < 0.05$ versus control by Student's t test).

Table 3 shows that Acyclovir at 5 µg/ml reduced the virus yields by 100% for both HSV-1 and HSV-2 but ≤ 3% virus yield reduction on the resistant strains (AP^r HSV-1 and TK– HSV-1) at the same concentration.

Therapeutic effect of the extract in female Balb/C mice infected with wild type strain *7401H HSV-1*

In order to follow interpretation of results in the mouse model, it is necessary to first explain that the scores 2

Table 4. *Therapeutic* effect of *H.I officinalis* extract on cutaneous wild strain 7401H HSV-1 infection in female Balb/C mice.

Animals	Treatment (µg/ml)	Meantime(days ± S.D)			Mortality [a] %
		Score 2[b]	Score 6[b]	Survival [c]	
Control-1	0 mg/ml	SNO	SNO	NC	0
Control-2	0 mg/kg	3.0 ± 0.18	5.0 ± 0.12	5.0 ± 0.98	100
ACV	5 mg/kgd	5.80 ± 0.51e	6.13 ± 0.18	7.00f	8
H. officinalis	125 mg/kgd	5.00 ± 0.70e	6.30 ± 0.15	12.13 ± 0.66	10

SNO: score not observed; NC: not calculated because all the mice survived; control-1: uninfected untreated mice; control-2: infected untreated mice. The table shows
the activity of the extract in mice infected with 1×106 PFU/ml/10µl HSV-1 and given an oral treatment with extract at 125 mg/kg orally.
[a] Number of dead nice/number of mice tested per %. Mortality was calculated on day 30.
[b] Mean times at which score 2 or 6 was first observed.
[c] Surviving mice were not included for the calculation of mean survival times.
[d] $p < 0.0005$ vs. control by repeated measures ANOVA (Bonferroni/Dunn).
[e] $p < 0.005$ vs. control by Student's t-test.
[f] Less than 2 mice died so S.D. could not be calculated.

and 6 represented the critical stages of the HSV infection. The score 2 represented the onset of infection by the appearance of vesicles at the site of infection. On the other hand, score 6 indicated the development of mild zosteriform lesions at the infection site. The settling of the mild zosteriform lesion was an indication of a lethal stage of infection in which the experimental animal could not recover from even with continued treatment. The mean survival time represented the mean time, in days, the animals of a group survived before they succumbed to death as a result of infection. Mortality in the tables was a representation of the total number of animals percent that died in a group at the close of the experiment 30 days post infection.

Table 4 shows the results of the determination of therapeutic effect of the *H. officinalis* extract in female Balb/C mice cutaneously infected with 1×10^6 PFU/10 µl/mouse HSV-1 after being given an oral dose of 125 mg/kg. The extract delayed the onset of HSV-1 infection by 2 days (after onset of infection in the control-2 group). This observation was comparable to the reference drug ACV which also showed a similar effect. The same table shows that the group of mice treated with the extract developed infection earlier than the ACV treated group (the 5.0th and 5.8th days, respectively). However, the control-2 group experienced onset of infection much earlier (the 3rd day) than mice treated with either *H. officinalis* or ACV. The extract and ACV treated mice also had a longer mean survival time (>6 days as opposed to 5 days for control-2) and lower mortality rate than the untreated infected mice (control-2).

Therapeutic effect of the extract in Balb/C mice infected with acyclovir resistant strain (AP^r) HSV-1

Table 5 shows the therapeutic effect of the *H. officinalis* extract at 125 mg/kg on cutaneous AP^r HSV-1 infection in female Balb/C mice. The onset of infection in mice treated with extract was significantly delayed (score 2 observed on the 6th day as opposed to 3rd day in control-2). The non-responsiveness to ACV treatment in infected mice was observed in which infection appeared the same day as untreated control. No mild zosteriform lesions (score 6) were observed in mice treated with the extract or ACV. There was 67% mortality for the infected untreated mice and no mortality for the extract or ACV treated mice.

Therapeutic effect of the extract in Balb/C mice infected with wild type strain HSV-2

Table 6 shows the therapeutic effect of the *H. officinalis* extract on cutaneous HSV-2 infection in female Balb/C mice following treatment with an oral dose of 125 mg/kg extract. The extract delayed the onset of HSV-2 infection in the infected treated mice by 2 days when compared with untreated infected control (score 2 appearing on 3rd day control-2 and 6th day for the infected extract treated mice). ACV treatment significantly delayed the onset of HSV-2 infection by 3.5 days in mice compared to control-2. No mild zosteriform lesion was observed in the ACV treated mice. The mean survival time for mice treated with the extract or ACV was the same (9 days). The reduced mortality rate of mice treated with the extract or ACV was 12 and 37%, respectively, as opposed to 100% mortality for the infected untreated mice (control-2).

The acute toxicity of the extract in uninfected mice

Table 7 shows the evaluation of acute toxicity for the *H. officinalis* extract at the therapeutic oral dose of 125 mg/kg in uninfected male and female Balb/C mice. The results indicate that there was no significant difference in

Table 5. Effecacy of *H. officinalis* extract on cutaneous acyclovir resistant (AP^r) in female Balb/C mice.

Animals	Treatment (µg/ml)	Mean time (days±S.D.)			Mortality %
		Score 2[b]	Score 6[b]	Survival [c]	
Control-1	0 mg/ml (not infected)	SNO	SNO	NC	0
Control-2	0 mg/kg (infected)	3.1 ± 0.14	8.00 ± 0.13	10.60 ± 0.30	67
ACV	5 mg/kg	3.3 ± 0.11	SNO	NC	0
H. officinalis	125 mg/kg	6.0 ± 0.11[d]	SNO	NC	0

SNO: score not observed; NC: not calculated because all the mice survived; control-1: uninfected untreated mice; control-2: infected untreated mice. The table shows
the activity of the extract in mice infected with 1×10^6 PFU/ml/10µl HSV-1 and given an oral treatment with extract at 125 mg/kg orally.
[a] Number of dead mice/number of mice tested per %. Mortality was calculated on day 30.
[b] Mean times at which score 2 or 6 was first observed.
[c] Surviving mice were not included for the calculation of mean survival times.
[d] $p < 0.005$ vs. control by Student's t-test.

Table 6. Effecacy of *Hyssopus officinalis* extract on wild type strain HSV-2 infection in female Balb/C mice.

Animals (µg/ml)	Treatment	Meantime (days ± S.D.)			Mortality %
		Score 2[b]	Score 6[b]	Survival [c]	
Control-1	0	SNO	SNO	NC	0
Control-2	0	3.0 ± 0.0	6.40 ± 0.69	7.67 ± 0.33	100
ACV	5 [d]	6.50 ± 0.21 [c]	SNO	9.0 ± 0.0	37
H. officinalis	250 [d]	6 ± 0.0	6.0 ± 0.0	9.0 ± ND	12

SNO: score not observed; NC: not calculated because all the mice survived; control-1: uninfected untreated mice; control-2: infected untreated mice. The table shows
the activity of the extract in mice infected with 1×10^6 PFU/ml/10µl HSV-1 and given an oral treatment with extract at 125 mg/kg orally.
[a] Number of dead mice/number of mice tested per %. Mortality was calculated on day 30.
[b] Mean times at which score 2 or 6 was first observed.
[c] Surviving mice were not included for the calculation of mean survival times.
[d] $p < 0.0005$ vs. control by repeated measures ANOVA (Bonferroni/Dunn).
[e] $p < 0.005$ vs. control by Student's t-test.

Table 7. Acute toxicity of *H. officinalis* in uninfected Balb/C mice following an oral treatment with 125 mg/kg extract.

Animals	Treatment (µg/ml)	Mean weight ±S.D. on[a]				Mortality [b] %
		Day 1	Day 7	Day 14	Day 21	
Female Balb/C mice						
Control	0	19.10 ± 0.8	20.0 ± 0.7	20 ± 0.45	20.2 ± 0.15	0
Test: *H. officinalis*	125	19.50 ± 0.12	20.15 ± 0.13	20 ± 0.35	20.12 ± 0.25	0
Male Balb/C mice						
Control	0	19.10 ± 0.9	20.70 ± 1.7	20 ± 0.95	21.2 ± 0.15	0
Test: *H. officinalis*	125	20.10 ± 0.99	21.60 ± 2.7	21 ± 0.45	21.2 ± 0.15	0

the mean weights of mice given an oral dose of extract at 125 mg/kg and the control that were given no drug in both sexes of mice.

DISCUSSION

The Lamiaceae family of plants consist of members of whom some are known potent poisons, for example the *Origanum vulgare* used traditionally as an arrow poison by some communities. However, not all genera in the family are poisonous. *H. officinalis* has been used in the Iran Traditional Medicine over the years for treatment of various ailments (Kazazi et al., 2007). *H. officinalis* is an indigenous plant naturally growing at different geogra-

phical localities in Iran with a wide spread use in traditional medicine practice. The most utilized part of the plant are its leaves which are usually boiled and given or used as compresses on wounds and bruises. There are no reports indicating that this Iranian variety has anti-viral activity. The methanol extract from the leaves of *H. officinalis* was evaluated for *in vitro* and *in vivo* anti-HSV activity. The results have shown that both the wild and resistant strains of HSV were sensitive to the extract. The resistant strains were even more sensitive to the extract than the wild strains, this being very interesting and therefore calling for further work. The extract appeared to have synergistic effects on the resistant strains and this perhaps may explain why the viruses were not responsive to acyclovir, a pure compound. The cell cytotoxic concentration (CC_{50}) of this extract was several magnitudes higher than the effective concentrations inhibiting plaque formation by 50% (EC_{50}), indicating the high safety margin of the extract. The virus yield reduction assay which simulates an *in vivo* environment of infection, demonstrated that the extract had a virocidal activity, further supporting the potency of the extract. The virus yield reduction assay also confirmed the observations of the plaque inhibition assay on anti-HSV activity. Another theory that can be advanced from the activity of this extract to the resistant strains is the possibility of a difference in the mechanism of anti-HSV action to that of acyclovir. The results shows that the extract at 100 µg/ml reduced the virus yields of the resistant HSV strains by 100% and that acyclovir at 5 µg/ml could not reduce the virus yields of the resistant strains above 3% can be used to support this view. Acyclovir is phosphorylated to its active form by the viral encoded thymidine kinase where it acts as a chain terminator of the viral DNA polymerase thereby stopping any further viral replication (De Clercq, 2001). The two resistant strains used in the study were either thymidine kinase deficient (TK–7401H HSV-1) implying that this resistant strain had no thymidine kinase to phosphorylate acyclovir to its active form, or a plaque purified in the presence of high concentrations of acyclovir from strain 7401H HSV-1 that could replicate in high concentrations of acyclovir (AP^r HSV-1) (Kurokawa et al., 1995). Since the mechanisms of action of acyclovir are well understood, it would therefore be logical to assume that the only means by which this extract was acting against these resistant viral strains was through a different path of anti-viral action to that of acyclovir. This angle of thought needs investigations. In the animal experiments using Balb/C mice cutaneously infected with wild type strains of HSV-1 or HSV-2 at 1×10^6 PFU/mouse, all the infected untreated mice ultimately died. Oral treatment with the extract however provided some protection. For instance, there was a delay in the onset of infection in the treated mice when compared to untreated infected control (Tables 4 – 6). And when the infection eventually picked up in the treated mice, the progression was slow and less lethal, with an increased mean survival time and a significantly reduced mortality rate (Tables 4 and 6). All these were indicative of the therapeutic effect of the extract. The therapeutic effect of the extract in mice receiving extract treatment after infection with the resistant strain (AP^r HSV-1) was also evident (Table 5). The inability of acyclovir to delay the onset of infection in the AP^r HSV-1 infected mice was seen, while the delay in the extract treated mice group was evident (Table 5); this suggests the possibility of a synergistic effect of the extract observed in the *in vitro* experiments. Similarly, the possibility of a difference in mechanisms of anti-viral action to that of acyclovir was again observed, where activity of extract against the resistant strain was observed. However, the zero percent mortality noted in mice infected with the resistant strain and treated with extract could not be fully attributed to treatment since 50% of the infected untreated mice also survived. An explanation for the 50% survival of the infected untreated control mice could lie in the attenuation and thereby losing virulence of the resistant strain virus achieved during plaque purification *in vitro* (Field and Darby, 1980; Kurokawa et al., 1995). It is worth noting that in all the animal experiments, the normal control mice (contro-1 in the experiments) neither showed infection nor mortality. This confirmed that the observed deaths in the infected untreated mice to be all due to HSV infection. The therapeutic concentration of 125 mg/kg administered exhibited no lethal toxicity.

In conclusion, the *H. officinalis* methanolic leaf extract contains potential agents with activity against HSV. These agents need to be isolated and identified and screened for possible anti-viral activity. Such agents could be developed as anti-HSV agents or provide a template for the synthesis of a new anti-HSV agents.

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