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Inhibitory Effect of a Standard Extract of *Zhumeria majdae* Rech.f. and Wendelbo. against *Herpes simplex-1* Virus

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Rosmarinic Acid (RA) is an interesting phenolic acid with a variety of biological activities such as antioxidant, anti inflammatory and anti viral effects. In the present study, we aimed to study the inhibitory effect of methanolic extract of *Zhumeria majdae* against *Herpes simplex* type 1 (HSV-1). This extract has been standardized on the basis of RA content. Aerial parts of *Zhumeria majdae* was extracted with methanol 80% by maceration method. Calibration curve of RA was prepared and content of plant extract was measured on the basis of this curve by spectrophotometry method. Neutral red method was used for determining of maximum non toxic concentration (MNTC) of the plant extract. A serial dilution of plant extract up to MNTC were examined *in vitro* on vero cells for their anti HSV-1 effect using a plaque reduction assay. Acyclovir was used as positive control. For studying of time-dependent antiviral effect of *Z. majdae*, plant extract was added to HSV-1 infected vero cells at different stages of infection. The percentage of RA was determined as 1.3% in *Z. majdae*. This plant could inhibit plaque formation at a period of 3 h after cell infection at all used concentrations (5, 10, 20 and 50 $\mu\text{g mL}^{-1}$). This extract revealed both a time and concentration inhibition against HSV-1. Considering the antiviral effect of *Z. majdae* against HSV-1, can conclude that this plant is a good candidate for further studies and rosmarinic acid would be an important factor for this activity.

Key words: *Herpes simplex*, *Zhumeria majdae*, rosmarinic acid, neutral red

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INTRODUCTION

Herpes simplex virus type 1 (HSV-1) is a common human pathogen which causes a variety of diseases with different degrees of severity (Marques and Straus, 2000). This virus causes the vast majority of oral herpes (cold sores or fever blisters) (Whitley and Roizman, 2001). The infection with this virus is recurrent and its removal might persist for many times beyond treatment. Patients who use current drugs licensed for the HSV infections, often experience severe side effects and/or prevalence resistance (Schuhmacher *et al.*, 2003). However, resistance to the current drugs has emerged due to the increase in the use of the drug and more attention has been paid to medicinal plants as a source of therapeutic active agents. There are some reports in the literature which have shown anti HSV-1 effect of medicinal plants and different phytochemicals such as phenolic compounds, essential oils and flavonoids (De Logu *et al.*, 2000; Reichling, 1999).

Rosmarinic Acid (RA) is an important phenolic compound which occurs in several families and is responsible for anti herpes effect of some medicinal plants such as lemon balm (Toth *et al.*, 2003). Antibacterial, antioxidant, antiallergic and anticarcinogenic effect of RA has been reported previously (Lee *et al.*, 2007; Osakabe *et al.*, 2004; Sanbongi *et al.*, 2004; Swarup *et al.*, 2007). This compound is found in sub family of Nepetoideae from Lamiaceae family. In continuing for finding medicinal plants with anti HSV-1 effect, here, we have studied inhibitory effect of a standard extract of *Zhumeria majdae* Rech.f. and Wendelbo (Lamiaceae family) against HSV-1 virus. This plant is a unique endemic plant to Iran known in Persian as "Mohrkhosh". Different biological activities has been reported for the plant such as anticonvulsant, antioxidant, antibacterial and antileishmanial effect (Mandegary *et al.*, 2012; Moein *et al.*, 2008; Mohaddese and Nastaran, 2009; Sharififar *et al.*, 2007). *Z. majdae* has been suggested for colic, dysmenorrheal and antispasmodic in Iranian traditional medicine (Aynechi, 1992). So in the present work we aimed to study the inhibitory effect of this plant against HSV-1 infection using plaque assay.

MATERIALS AND METHODS

Plant materials: The plant was collected from Hormozgan province at June, 2011. A voucher specimen of the plant was deposited at the Herbarium center of Faculty of Pharmacy, Kerman, Iran. Aerial parts of the plant were extracted with methanol 80% by maceration method and concentrated in vacuum and finally dried in oven at 40°C. Dried extract storage at -20°C until experiment.

Chemicals: Standard rosmarinic acid was prepared from Fluka, Dulbecco's modified Eagle's medium (DMEM), Fetal Calf Serum (FCS), penicillin, streptomycin were prepared from Sigma, Neutral red and the other compounds were prepared from Merck. Acyclovir was prepared from Farabi Co., Iran.

Determination of RA content of *Z. majdae* by spectrophotometric method

Calibration curve of RA: An amount of 10 mg of standard RA was weighed accurately and dissolved in methanol (80%) in a 100 mL calibration flask to give a 100 µg mL⁻¹ stock solution. Serial dilutions were prepared from stock solution and the absorbance spectra of the RA were recorded in wavelength range between 200 to 400 nm with a UV-visible spectrophotometer (Lambda 25, Perkin Elmer, USA). At the λ max of 328 nm absorbance of different dilutions (2, 5, 10, 15 and 20 µg mL⁻¹) of RA was read. The calibration curve of standard solutions was constructed by plotting RA concentration versus absorbance at 328 nm. The experiment was repeated three times on different days and the mean of the absorbance was used to draw a suitable standard curve. The percent of relative standard deviation (%RSD) and error (%) were calculated as a measure of precision and accuracy of the method, respectively. In addition, a third derivative spectrophotometric (Δλ = 5 nm) method using the amplitude of the standard solutions at λ = 349.9 nm was used to construct a calibration curve to determine RA amount in the extract. This method could help to avoid interferences of accompanying constituents present in the extract.

Preparation of plant sample for RA content determination

A quantity of 100 mg of dried extract was dissolved in 100 mL methanol and filtered using filter paper (stock solution). A volume of 10 mL of the sample diluted to 100 mL with methanol, UV spectrum was recorded and absorbance was read at 349.5 nm and Δλ = 5 nm. By putting the related absorbance in the calibration curve, RA content of the plant was determined. Each experiment was done in triplicate and the results were reported as mean±SD.

Anti HSV-1 experiments

Cell culture: A vero cell from African monkey kidney cells was purchased from the National Cell Bank of Pasteur Institute of Iran (Tehran, Iran). Cells were maintained in DMEM medium supplemented with 5% (v/v) FCS (fetal bovine serum), 100 units mL⁻¹ penicillin and 100 µg mL⁻¹ streptomycin, at 37°C in a CO₂ incubator (5% CO₂ and 95% relative humidity) (Reichling *et al.*, 2005).

Viruses: *Herpes simplex* virus type 1 strain KOS was prepared from Research Center of Virology (Tehran University of Medical Sciences) which has been isolated from infected cells and stored at -80°C . Infectivity titers were determined by monoclonal antibodies. Determination of TCID_{50} (the concentration of virus suspension which infects 50% of cells) was used for virus titration.

Cytotoxicity assay: Neutral red method was used for evaluation of cytotoxicity of the extract. Neutral red is a dye for staining the living cells. Viable cells will take up the dye and incorporate the dye into the lysosomes. Uptaking and accumulation of the neutral red has linear correlation with the number of viable cells (Soderberg *et al.*, 1996). The cells were seeded into 24-well plates. The medium was removed after 24 h incubation at 37°C and then 100 mL of fresh DMEM containing different dilutions of the sterile extract (5, 10, 20, 50 $\mu\text{g mL}^{-1}$) was added and incubated again. After 48 h, the extract was aspirated and 0.2 mL of the neutral red solution (40 $\mu\text{g mL}^{-1}$) was added to wells and incubated for 1 h at 37°C . After removing the neutral red, rinsing with 0.5 mL acetic acid buffer and shaking for 15 min, the absorbance was measured at 550 nm (Koch *et al.*, 2008). Control wells contained extract free medium. The mean absorbance of the cell control wells was assigned as 100% viability. The maximum non toxic concentration (MNTC) of the plant was determined as the concentration of a plant which had no toxicity on viable cell number (100% viability).

Plaque inhibition assay: The potency of tested plants for anti HSV-1 effect was evaluated by plaque inhibition assay. Briefly, the cell monolayers infected with HSV-1 (2×10^5 pfu cell $^{-1}$) and incubated at room temperature. Plant extract was added to wells in different dilutions (at least 5 dilutions less than MNTC). After 48 h incubation at 37°C , the medium was aspirated and rinsed with sterile phosphate buffer saline (PBS). Then 500 μL of methanol was added to each well and after 15 min, re-aspirated and rinsed with PBS and fixed with formalin (10% v/v). Microwells were stained with 200 μL of crystal violet (1%) and after 30-45 min. re-rinsed with PBS. Subsequently, the plates were considered under microscope for plaque counting (Nolkemper *et al.*, 2006). In all experiments untreated virus infected cells were used as control. The percentage of plaque reduction was calculated relative to the amount of plaque formation in the absence of the tested extract (extract was dissolved in medium).

Time-dependent antiviral effect: In order to study a possible time-dependent antiviral effect, the culture media in different wells containing cells monolayers was

replaced with plant extract just after viral infection (h_0), one hour (h_1), two hours (h_2) and three hours after viral infection (h_3). After 72 h of incubation the monolayer at 37°C and fixation by formalin (10% v/v), the cells were stained with crystal violet (1%) and the plaques were counted (Nolkemper *et al.*, 2006). Acyclovir was used as positive control. These experiments were repeated three times on various days and untreated virus infected cells as well as medium treated viruses were used as control.

Data analysis: In plaque assay, by counting the plaques, the percentage of plaque inhibition was calculated. Each experiment was repeated for three times and the results were reported as Mean \pm SEM. Differences between test and control group were analysed using analysis of variance (ANOVA).

RESULTS

The results of extraction and RA content of the plant has given in Table 1. The yield of extraction of *Z. majdae* was 25.22% (g/100 g dried plant). Calibration curve of RA in third derivation in different dilutions has given in Fig. 1. This plant contained 1.3% RA.

Cytotoxicity of plant extract: Plant extract was dissolved in medium and added to wells in different dilutions. In neutral red method, MNTC was determined for the plant as the maximum concentration which has absorbance equal to control. As shown in Table 1, the MNTC of the plant was determined as 50 $\mu\text{g mL}^{-1}$ in comparison to acyclovir (MNTC = 500 $\mu\text{g mL}^{-1}$).

Inhibition of plaque formation: The viruses were treated with plant extract in various concentrations ranges up to MNTC. The results show that *Z. majdae* could inhibit plaque formation by HSV-1. The results were presented as a percentage of plaque inhibition which were the mean values from three independent experiments. The extract of *Z. majdae* inhibited plaque formation of HSV-1 in a concentration-dependent manner. At concentrations of 50 $\mu\text{g mL}^{-1}$ (MNTC), 94.5 \pm 3.6% plaque formation was inhibited by this plant extract at the time of 0 h after incubation. This plant reduced plaque formation by 91.7 \pm 3.1 and 89.4 \pm 3.5%, respectively at concentrations of 20 and 10 $\mu\text{g mL}^{-1}$. Acyclovir exhibited 100% plaque inhibition at concentrations of 500 (MNTC), 250 and 100 $\mu\text{g mL}^{-1}$ and 96.7% plaque inhibition at 50 $\mu\text{g mL}^{-1}$.

Time dependent antiviral effect: The extract of *Z. majdae* could inhibit 100% the plaque formation at all concentrations up MNTC at a period of 3 h after cell

Table 1: Results of extraction and determination of rosmarinic acid content of *Zhumeria majdae* Rech.f. and Wendelbo

Plant	Herbarium number	Part used	Percentage of extraction (g/100 g dried plant)	Rosmarinic acid (g/100 g dried extract)	MNTC ($\mu\text{g mL}^{-1}$)
<i>Zhumeria majdae</i> Rech.f. and Wendelbo	KF1387	Aerial parts	25.22	1.3	50

MNTC: Maximum non toxic concentration

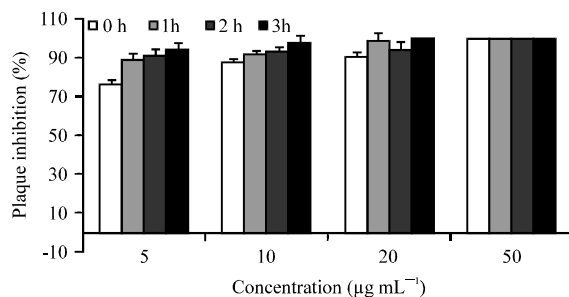


Fig. 1: Anti herpes effect of different concentrations of *Zhumeria majdae* Rech.f. and Wendelbo. Each experiment was repeated three times and results were shown as mean \pm SE

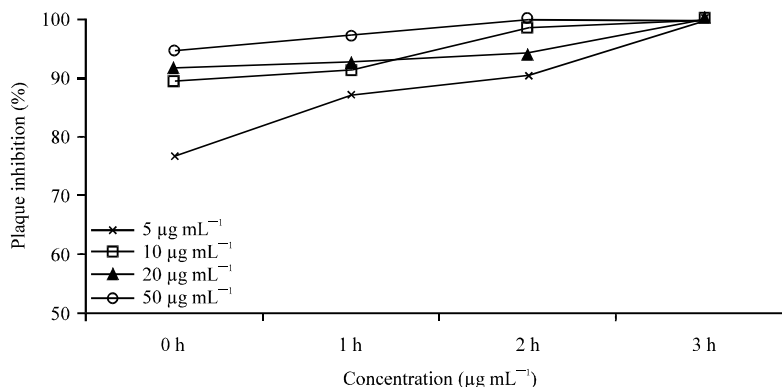


Fig. 2: Time-dependent anti HSV-1 effect of different concentrations of *Zhumeria majdae* Rech. f and Welbo. Each experiment was repeated three times and the results were demonstrated as mean \pm SE

infection. This plant also revealed 100% inhibition after 2 h at MNTC, while 94.1 \pm 4.1, 98.5 \pm 4.1 and 90.2 \pm 3.4% inhibition at concentrations of 20, 10 and 5 $\mu\text{g mL}^{-1}$ at a time of 2 h after incubation. *Z. majdae* extract also demonstrated 97.1 \pm 4.4% and 92.6 \pm 3.55 inhibition of plaque at a time of 1 h after incubation at concentrations of 50 and 20 $\mu\text{g mL}^{-1}$.

DISCUSSION

In the present study, methanolic extract of *Z. majdae* was standardized on the basis of the rosmarinic acid content. RA is an interesting phenolic compound with anti viral effect (Swarup *et al.*, 2007). The amount of RA content of the plant was determined as 1.3%. When the extract of *Z. majdae* was added during the time of adsorption, viral amplification was decreased significantly clearly in a concentration-dependent manner. A maximum

94.5 \pm 3.6% plaque inhibition was occurred at concentrations of 50 $\mu\text{g mL}^{-1}$ (MNTC) at the time of 0 while 91.7 \pm 3.1 and 89.4 \pm 3.5% inhibition was induced at concentrations of 20 and 10 $\mu\text{g mL}^{-1}$, respectively at the same time. For elucidation of time-dependent effect of plant extract, different concentrations up to MNTC of *Z. majdae* was incubated with HSV-1 for different time periods prior and after to cell infection. The results showed that this plant extract exhibited the highest plaque inhibition at a time of 3 h after incubation. This extract reduced plaque formation 100% at a period of 3 h after cell infection in all used concentrations (5, 10, 20, and 50 $\mu\text{g mL}^{-1}$) (Fig. 2). *Z. majdae* extract exhibited plaque inhibition more than 94.5 \pm 3.6% inhibition at concentration of 50 $\mu\text{g mL}^{-1}$ at all time of after cell infection (Fig. 2). Acyclovir showed to be effective in both phase of adsorption and replication. In all, we can conclude that the RA content would be an effective and

determining factor for antiviral effects of this plant. The observed antiviral activity might be due to the other known plant constituents. Flavonoids, different derivatives of caffeic acid and tannins can block the viral surface ligands or host cell receptors and inactivate the *Herpes simplex* virus (Cohen *et al.*, 1964; Jassim and Naji, 2003; Kucera and Herrmann, 1967; May and Willuhn, 1978; Reichling, 1999).

As we know, it is for the first time that *Z. majdae* has been studied for antiviral effect. This plant exhibited inhibitory effect against HSV-1 at MNTC in a time and concentration-dependent route. Further studies for identifying the accurate mechanism are being carried out.

CONCLUSION

The obtained results indicated that *Z. majdae* has shown high anti-HSV-1 effect in low concentrations. The plant exhibited the greatest antitherpetic effect at a time of 3 h after incubation. This activity was both time and concentration-dependent. Further studies are needed for finding the active components and accurate mechanism of the plant.

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