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Anti *Herpes simplex-1* Activity of A Standard Extract of *Zataria multiflora* Boiss

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Abstract: Rosmarinic Acid (RA) is a phenolic acid which has many biological activities such as antioxidant, anti-inflammatory and anti-viral effects. In the present study, we have studied the anti *Herpes simplex* type 1 (HSV-1) effect of methanolic extract of *Zataria multiflora* which has been standardized on the basis of RA content. Methanolic extract of *Zataria multiflora* was prepared by maceration method. RA content of plant extract was measured by spectrophotometry method using the calibration curve of RA. Maximum non-toxic concentration (MNTC) of the plant was determined by neutral red method. MNTC and lower serial dilutions of extract were examined *in vitro* on vero cells for their effect against HSV-1 using a plaque reduction assay. Acyclovir was used as positive control. Time-dependent antiviral effect of *Z. multiflora* was studied by adding the extract to HSV-1 infected vero cells at different stages of infection. The percentage of RA was determined as 2.2% in *Z. multiflora*. This plant was effective in all used concentrations and significantly reduced plaque formation up to 100% at concentrations of 800 and 1000 $\mu\text{g mL}^{-1}$. Clearly *Z. multiflora* revealed both a time and concentration inhibition. It seems that the presence of rosmarinic acid would be a determining factor for anti HSV activity of *Z. multiflora*.

Key words: Rosmarinic acid, *Herpes simplex*, *Zataria multiflora*, neutral red

INTRODUCTION

Herpes simplex Virus (HSV) is a common pathogen from Herpesviridae family which causes a broad range of infections in human. The HSV type 1 (HSV-1) is involved in a variety of lesions of oral cavity, epidermal, mucous membrane, eyes and pharynx problems especially in immunodeficiency patient (Schnitzler *et al.*, 2008). The HSV-1 is a DNA virus which is distinguished by serological and molecular methods. 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). So, the tendency for accessing the new therapeutic agents with different mechanisms is increasing. Previous studies have exhibited the antiherpetic effect of several medicinal plants and their secondary metabolites such as flavonoids,

anthraquinones, essential oil, phenolics (Logu *et al.*, 2000; Reichling, 1999). Rosmarinic Acid (RA) is a phenolic acid with different properties such as anti-bacterial, anti-allergic, antioxidant, anticarcinogenic and antiviral including anti-HIV-1 effect (Lee *et al.*, 2007; Osakabe *et al.*, 2004; Sanbongi *et al.*, 2004; Swarup *et al.*, 2007). This compound widely is distributed in Lamiaceae family especially in Nepetoideae plants. Here, we have studied anti-herpetic effect of a standard extract of *Zataria multiflora* which belongs to Nepetoideae subfamily in comparison to acyclovir. This plant is an Iranian endemic plant known in Persian as Avishane-shirazi. This plant has different biological activities such as antioxidant, antibacterial, anticholinesterase and regulation of *mdm2* and *atm* genes expression (Sharififar *et al.*, 2011a, b; Sharififar *et al.*, 2007; Gohar *et al.*, 2010). In traditional medicine, *Z. multiflora* has been used for common cold, respiratory and gastric disorders and as antitussive (Zargari, 1990). In the present study, we have studied

the anti *Herpes simplex* type 1 (HSV-1) effect of methanolic extract of *Zataria multiflora* which has been standardized on the basis of RA content.

MATERIALS AND METHODS

Plant materials: The plant was collected from Kerman Province at July, 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 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. multiflora* by spectrophotometric method

Calibration curve of RA: Ten 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: One hundred milligram of each completely dried extract was dissolved in 100 mL methanol and filtered using filter paper (stock solution). Ten milliliter of each 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 TCID₅₀ (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 extracts. Neutral red is a dye for staining the living cells. Viable cells will take up the dye and incorporate the dye into the lysosomes. Uptake and accumulation of the neutral red has linear correlation with the number of viable cells (Derberg *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 µL of fresh DMEM containing different dilutions of the sterile extracts (5, 10, 50, 100, 250, 500, 1000 and 1250 µg mL⁻¹) was added and incubated again. After 48 h, the extracts were aspirated and 0.2 mL of the neutral red solution (40 µg 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) 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 $500 \mu\text{L}$ 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 $200 \mu\text{L}$ 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 percent 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.

RESULTS

The results of extraction and RA content of the plant has given in Table 1. The yield of extraction of *Z. multiflora* was 28.77% (g/100 g) dried plant. Calibration

curve of RA in third derivation in different dilutions has given in Fig. 1. This plant content contained 2.2% RA.

Cytotoxicity of plant extracts: 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 $1000 \mu\text{g mL}^{-1}$ in comparison to acyclovir (MNTC = $500 \mu\text{g mL}^{-1}$).

Inhibition of plaque formation: The viruses were treated with plant extract in various ranges up to MNTC. The results show that *Z. multiflora* could inhibit plaque formation by HSV-1. The results presented as a percentage of plaque inhibition which was the mean values from three independent experiments. The extract of *Z. multiflora* inhibited plaque formation of HSV-1 in a dose-dependent manner. At concentrations of 800 and $1000 \mu\text{g mL}^{-1}$ (MNTC), 100% plaque formation was inhibited by this plant extract. This plant reduced plaque formation by 93.2 and 86.2% respectively at concentrations of 500 and $250 \mu\text{g mL}^{-1}$. Acyclovir exhibited 100% plaque inhibition at concentrations of 500 (MNTC), 250 and $100 \mu\text{g mL}^{-1}$ and 96.7% plaque inhibition at $50 \mu\text{g}$.

Time dependent antiviral effect: The extract of *Z. multiflora* could inhibit 100% the plaque formation at concentrations of 500 and $1000 \mu\text{g mL}^{-1}$ (MNTC) after a period of 1, 2 and 3 h cell infection. This plant also revealed 86.1% inhibition after 0 h and 100% plaque inhibition after 1, 2 and 3 h cell infection at $250 \mu\text{g mL}^{-1}$.

Table 1: Results of extraction and determination of rosmarinic acid content of *Zataria multiflora* Boiss

Plant	Herbarium number	Part used	Percent of extraction (g/100 g dried plant)	Rosmarinic acid (g/100 g dried extract)	Maximum non toxic concentration ($\mu\text{g mL}^{-1}$)
<i>Zataria multiflora</i>	KF1241	Aerial parts	28.77	2.2	1000

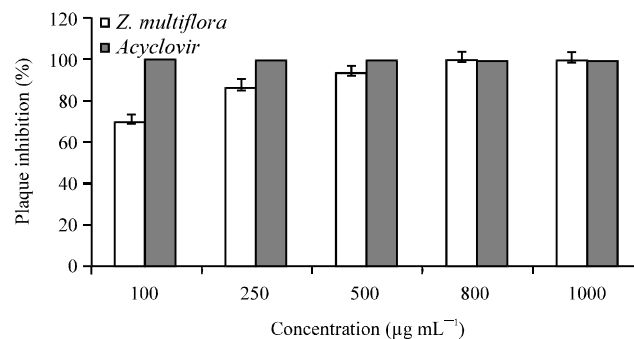


Fig. 1: Anti herpes effect of different concentrations of *Zataria multiflora* Boiss. Each experiment was repeated three times and results were shown as Mean \pm SE

DISCUSSION

In the present study, methanolic extract of *Z. multiflora* was standardized on the basis of the rosmarinic acid content. RA is the ester of caffeic acid with a diversity of biological activities for example anti viral effect (Swarup *et al.*, 2007). The amount of RA content of the plant was determined as 2.2%). Previous study detected no RA in this plant (Shekarchi *et al.*, 2012). This difference might be due to several factors including soil and climatic conditions, plant ontogenesis phases, harvest and plant storage which affect the RA content (Adzet *et al.*, 1992a, b; Hose *et al.*, 1997; Mrlianova *et al.*, 2002).

When the extract of *Z. multiflora* was added during the time of adsorption, viral amplification was decreased significantly clearly in a concentration-dependent manner. A 100% plaque inhibition was occurred at concentrations of 800 and 1000 $\mu\text{g mL}^{-1}$ while 93.3 and 86.7% inhibition was induced at concentrations of 500 and 250 $\mu\text{g mL}^{-1}$, respectively. For elucidation of time-dependent effect of plant extract, different concentrations up to MNTC of *Z. multiflora* was incubated with HSV-1 for different time periods prior and after to cell infection. The results showed that *Z. multiflora* reduced plaque formation 100% at a period of 1, 2 and 3 after cell infection in all used concentrations (250, 500, 800 and 1000 $\mu\text{g mL}^{-1}$) (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 the only factor affecting the antiviral effects of medicinal plants, however its effect should not be ignored. The observed antiviral activity might be due to the other known plant constituents. Flavonoids, different derivatives of caffeic acid and tannin 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

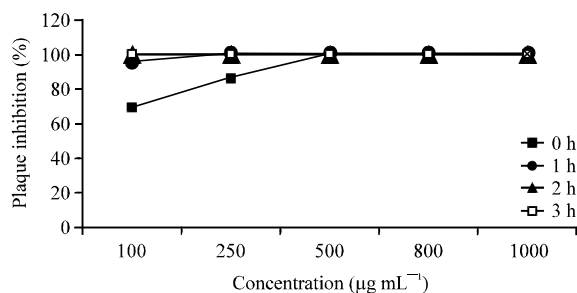


Fig. 2: Time-dependent inhibitory effect of different concentrations of *Zataria multiflora* Boiss. against *Herpes simplex*-1

Herrmann, 1967; May and Willuhn, 1978; Reichling, 1999). As we know, it is for the first time that *Z. multiflora* 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.

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