Salvadora persica as natural anti-viral agent

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Abstract

The antiviral and cytotoxic activity of etanolic extract of *Salvadora persica(L.)* roots was investigated on bovine herpes-1 (BHV-1) infection in vitro. An ethanolic extract of *Salvadora persica* was used in two concentrations. MDBK cells grown on Minimum Essential Medium (MEM) were used for virus propagation and titration. The effects of two concentrations of *Salvadora persica* on viral growth in MDBK cells as well as cytolytic activity of BHV-1 were evaluated at different times pretreatment ,simultaneouslly and after adsorption. The results demonstrated that the safest concentration (non-cytotoxic) of the plant extract under study on MDBK cells was 0.1 mg/ml.The plan extract showed a significant antiviral effect against BHV-1 at simultaneous treatment experiment. The virus titeration assay showed a marked decrease from 10^5 TCID50/ml to 10^3 TCID50/ml in adsorption step simultaneouslly incubated with *Salvadora persica* extract . In contrast, the plan extract didn't show any activity against BHV-1 neither in pre- nor post- treatment experiments. Indeed, the present study confirms that ethanolic extract of *Salvadora persica* roots had an antiviral effect against BHV-1.

Key wards*:* *Salvadora persica (L.)*, antiviral , BHV-1, MDBK cells.

INTRODUCTION

Salvadora persica (Miswak – siwak) is a medical plant whose roots, twigs or stems have been used for centuries as oral hygiene tool in many parts of the world **(Al–Sadhan and Almas, 1999)**. It is available as chewing gum, toothpaste and tooth brushes. Prophet Mohammed,peace be upon him, advised Muslims to use Miswak before every praying saying "The Miswak cleanse the mouth and pleasing to Allah**" (Khoory, 1983)**. Several studies have shown that extract of Salvadora Persica possess many biological properties including antibacterial **(Al–Lafi and Ababneh, 1995)**, antifungal **(Al-Bagieh, et al., 1994)**, anticaries **(Fadulu, 1975 and Al–Lafi & Ababneh, 1995)**, anti–inflammatory **(Monforte et al., 2001)**, antiplaque effect **(Homes, 1992)**, and reduces gingivitis and gingival bleeding **(Al–Otaibi, 2004 and Khalessi et al., 2004)**. Recently Salvadora persica extract was evaluated as root canal irrigant **(Al–Salman et al., 2005 and Al–Sabawi et al., 2007)** A number of antiviral drugs have been formally licensed and are widely used for the chemotherapy of specific viral infections **(Nakamichi et al., 2000)**. These drugs are primarily classified as three classes: anti-herpesvirus, anti-retrovirus, and to lesser extent, anti-rhinovirus compounds **(De Clercq et al., 1980; De Clercq, 1993)**. The major antiviral agents that act against (BHV-1) are called nucleosides and nucleotide analogues, through blocking the viral replication. They include acyclovir, valacyclovir, famciclovir and cidofovir **(De Clercq, 2002; Qiu et al., 2004)**, As few laboratory studies documented the effect of Salvadora persica on (BHV-1), the present study aimed to investigate the

antiviral effects of ethanolic extract of Salvadora persica (Siwak) against (BHV-1).

MATERIAL AND METHODS

1- Virus and cells:

Bovine herpesvirus-1 (Colorado strain) was kindly provided by Prof. Dr. Hussien Ali Hussien, Prof. and Head of Virology Department, Faculty of Vet. Medicine, Cairo University. It was propagated on MDBK cells (provided from VACSERA). Virus was harvested by freezing and thawing of the infected cell cultures and stored at -80°C until used. Bovine herpesvirus-1 (BHV-1) propagation and titration on MDBK cells according to **(Reed and Muench, 1938)**.

2- Plant extract:

Dried roots (1 kg) of Salvadora persica (L.) were collected from medical local market in Giza (Egypt) then they were identified and authenticated from National Research center (NRC) using the live specimens and photographs .(515 g) of dried roots of *Salvadora persica* (L.) were weighed then sliced into small pieces then grinding and extracted with ethanol 99%(Provided from SIGMA) at 70ºC, by maceration for several times (3 times) using rotavapor.The resulting extract was concentrated by lyophilization, leaving residue weighing about 11 g. Starting material about 250 mg of the residue was reconstituted with sterile MEM to 1 mg/ml. Then it was stored at refrigerator until use. Preparation of ethanolic extract of Salvadora persica (L.):according to Egyptian pharmacopeia.

3- Cytotoxicity assay:

as previously descriped in **(El-Hoseiny 2010 and Shosha 2011).**

The plant extract was used at concentration (10 mg/ml) in M-MEM maintainamce media (2% FBS), Confluent monolayer (80-90%) of MDBK cells was prepared in 96- well microtiter plates. The 96-well microtiter plates were incubated at 37ºC with 5% CO_2 for 24 hours. After 24 hours, the growth medium was replaced by 100 µl/well ten fold serial dilutions of plant extract (10 mg/ml) in M-MEM (2% FBS). Plant extract dilutions (100 µl/well) was inoculated in each column of each 96-well microtiter plates. The 96-well microtiter plates were incubated at 37ºC with 5% CO_2 for 72 hours. The safest concentration of non cytotoxic effect and the 50% cytotoxic concentration (CC50) were determined using ANOVA system analysis.

4- In vitro antiviral activity of *Salvadora persica* (L.) on BHV-1: according to **(El-Hoseiny 2010 and Shosha 2011).**

Ethanolic extract was used at 2 concentrations (0.1 and 0.01 mg/ml).

1- Before treatment:

Confluent monolayer (80-90%) of MDBK cells was prepared in 96-well microtiter plate. The cell monolayers were pretreated with (100µl/well) of the plant extract at the previously mentioned concentrations and the plate was incubated at 37°C for one hour. After incubation, the plant extract was aspirated off and equal volumes of 100 TCID50/ml of BHV-1 (100µl/well) were added to the cell monolayer and the plate was incubated at 37°C for 2 hours for virus adsorption. After adsorption, the virus suspension was aspirated off and replaced with maintenance medium (100µl/well) and the plate was incubated at 37°C with 5% CO2 for 2-3 days. The appearance of cytopathic effect (CPE) after 2-3 days was monitored and the virus titer was calculated.

2- Simultaneously:

Confluent monolayer (80-90%) of

MDBK cells was prepared in 96-well microtiter plate. Equal volumes (100 µl) of the plant extract at the previously mentioned concentrations and 100 TCID50/ml of BHV-1 (100 µl) were added to the cell monolayer at the same time and the plate was incubated at 37°C with 5% CO_2 for 2-3 days. The appearance of cytopathic effect (CPE) after 2-3 days was monitored and the virus titer was calculated.

3- After adsorption:

Confluent monolayer (80-90%) of MDBK cells was prepared in 96-well microtiter plate. The MDBK cells were infected with 100 TCID50/ml of BHV-1 (100 µl/well) and incubated at 37°C for 2 hours for virus adsorption . After virus adsorption, the virus suspension was aspirated off and the maintenance medium containing plant extract at the previously mentioned concentrations were added to the cells (100µl/well). The plate was incubated at 37°C with 5% CO_2 for 2-3 days. The appearance of cytopathic effect (CPE) after 2-3 days was monitored and the virus titer was calculated.

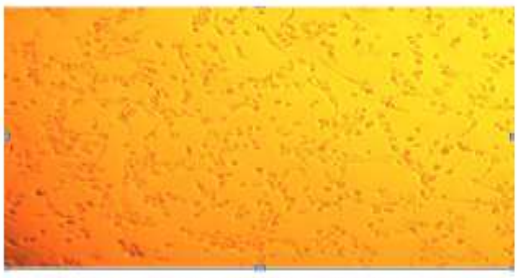
Statistical analysis: The data were evaluated by a one-way analysis of variance (ANOVA) using the SPSS® 6.1.3 software package (SAS Institute, Cary, NC, USA) and the difference between the mean values were assessed using the test of least significant difference (LSD). Statistical significance was at $P \leq 0.05$.

RESULTS

Propagation and titration of BHV-1:

BHV-1 was propagated and titrated on MDBK cells. Its characteristic cytopathic effect (CPE) was developed within 1-2 day post infection (d.p.i.). The CPE of infected cells was appeared as cell rounding with an increase of granularity and progress rapidly to form a characteristic bunches of grapes. The virus titer was determined as 10^5 $TCID_{50}$ /ml.

Microscopic pictures for normal uninfected MDBK control cells



CPE developed by BHV-1. Granulation and rounding of infected MDBK cells with a characteristic grape like appearance were observed

Cytotoxicity testing of ethanolic extract of *Salvadora persica (L.)* on MDBK cells:

The results demonstrated that the safest (non cytotoxic) dilution of the plant extract under study on MDBK cells was 0.1 mg/ml.

In vitro antiviral activity of ethanolic extract of *Salvadora persica (L.)* on BHV-1:

The antiviral effect of *Salvadora persica (L.)* on BHV-1 when inoculated simultaneously:

An equal volume of 100 TCID50/ml of BHV-1 and etanolic extract of Salvadora persica (L.) at concentrations of 0.1 mg/ml and 0.01 mg/ml were added to the monolayers of the MDBK cells at the same time without removal of the mixtures. The 96-well microtiter plate was incubated at 37°C with 5% CO2 for 3-4 days. The results revealed that the ethanolic extract have significant effect on BHV-1 during adsorption (figure 1). As the virus titer was 10^3 TCID50 /ml at 0.1mg/ml while it was 105 TCID50/ml at 0.01mg/ml which was equal to the virus titer of non treated control cells. These results denote to that the virus titer was decreased 2 log at 0.1 mg/ml of the plant extract only comparable to non treated control cells.

The antiviral effect of Salvadora persica (L.) on BHV-1 using pretreated and posttreated MDBK cells:

Ethanolic extract of *Salvadora persica (L.)* didn't show a notable activity against BHV-1 in pretreatment and post treatment experiments.

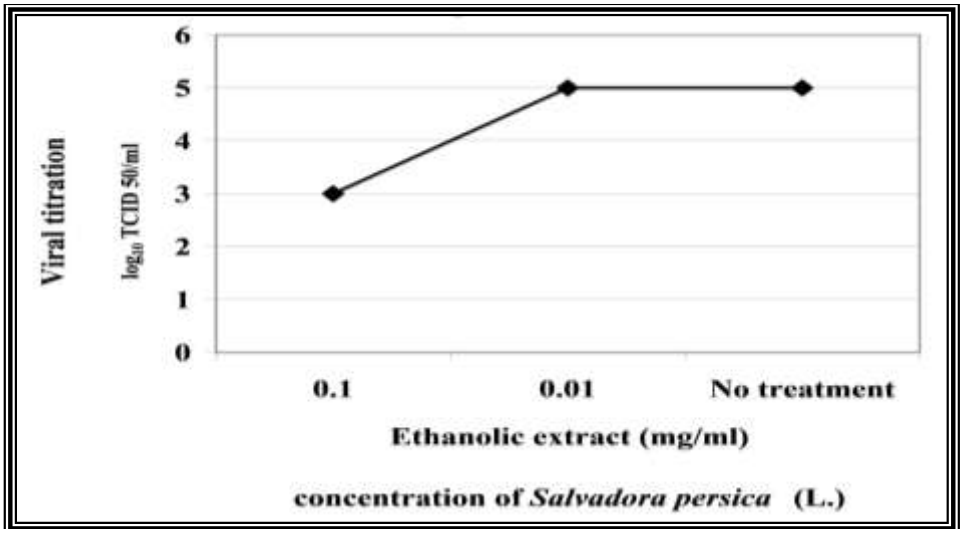
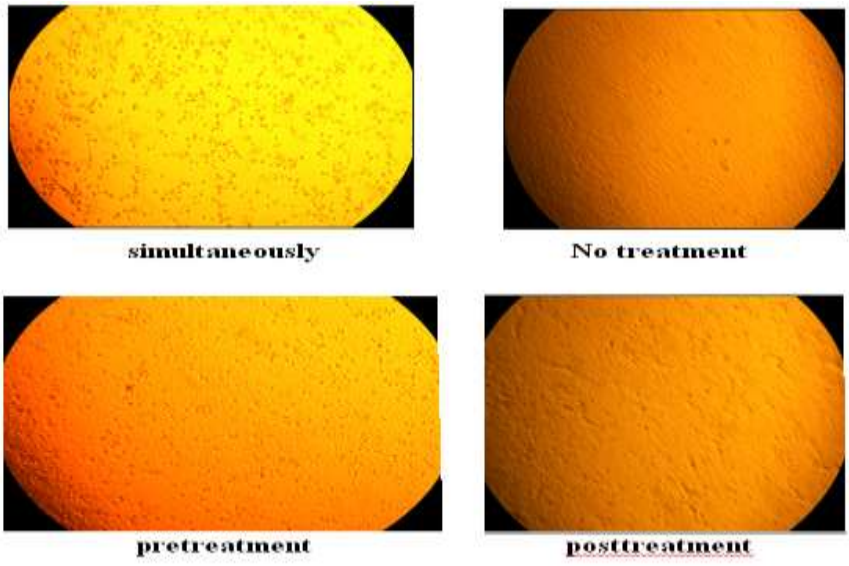
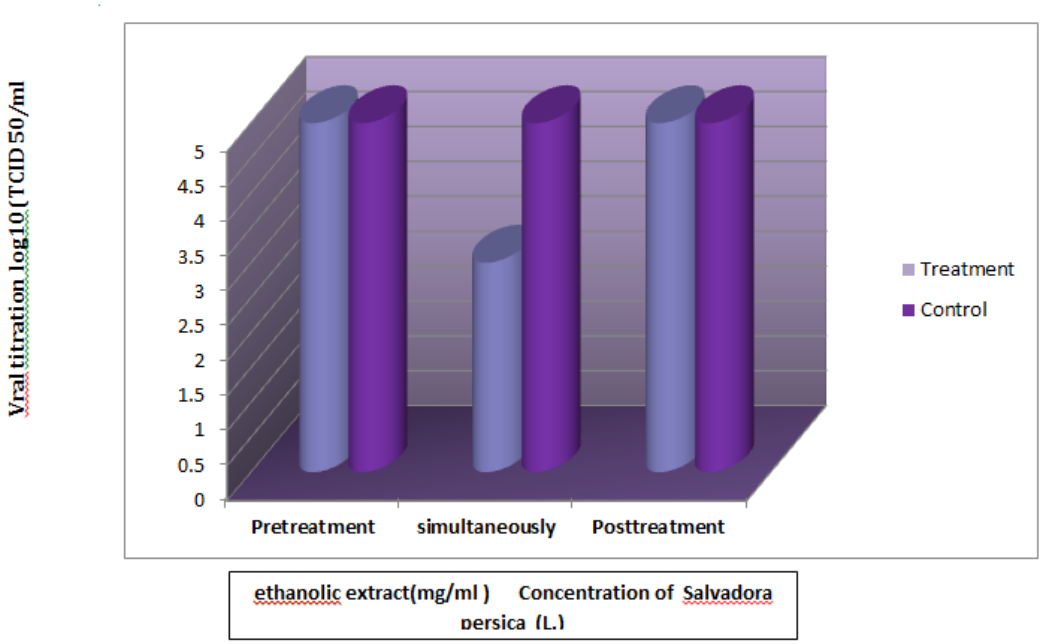


Fig. (1): The antiviral effect of *Salvadora persica (L.)* on BHV-1 when inoculated simultaneously



In vitro antiviral activity of ethanolic extract of Salvadora persica (L.) on BHV-1



In vitro antiviral activity of ethanolic extract of *Salvadora persica (L.)* on BHV-1

Virus	Cell line	Plant extract	Dilution of the plant under study	Virus titer (TCID $_{50}$/ml)				
				Virucidal activity of the plant extract on BHV-1	Pretreatment of MDBK cells with the plant extract	Antiviral effect of *Salvadora persica (L.)* on BHV-1 when inoculated simultaneously	Antiviral effect on BHV-1 after adsorption	No treatment

BHV-1	MDBK cells	Ethanolic extract of *Salvadora persica*	0.1	10^5	10^5	10^3 (decrease 2 log)	10^5	10^5
			0.01	10^5	10^5	10^5	10^5	

DISCUSSION

Salvadora persica has many synonyms such as Arak, Galenia asiatica, Meswak, Peelu, Pilu, Mustard tree, Salvadora indica or Natural toothbrush tree***(Akhilanand et al.)***. ‘‘Miswak’’ (synonyms in different Arabic dialects and countries include ‘‘miswaak,’’ ‘‘misswak,’’ ‘‘miswaki,’’ ‘‘meswak,’’ ‘‘mswaki,’’ ‘‘sewak,’’ ‘‘siwak,’’ and ‘‘siwaki’’) is an arabic word meaning tooth-cleaning stick **(Hattab, 1997)**. The fresh leaves are eaten as salad and are used for cough, asthma, scurvy, rheumatism and piles. The flowers are small, fragrant and used as stimulant and purgative. The berries are small and eaten both fresh and dried ***(Akhilanand et al.)***. *S. persica* may be recommended for regular use, given its favorable effects on oral health, low cost, ready availability, and simplicity of use, Administered in prevention or treatment of infectious pathologies like bacteria, fungi, protozoa and viruses in animals and humans, have been led to many applications for health. This enabled to use *S. persica (L.)* in a large number of fields. It is used to prevent smoking in adults and thumb sucking in children. It can be used in the development of dentition during eruption**(Attar ,1979),** It improves appetite and regulates peristaltic movements of the gastro-intestinal tract **(Akhtar,1981)**. The main use of *S. persica* is as a tool for teeth, tongue and gum cleaning and has also been used to treat toothache. This plant has large spectrum of biological activities including: antifungal, antibacterial, anti-inflammatory and hypoglycemic activities besides astringent and detergent effects. Regarding its different parts, *S. persica* has many uses in traditional medicine. **Ahmad et *al.* (2011)** investigated the various pharmacological activities of *Salvadora persica* family Salvadoracea and that includes anti inflammatory, analgesic, CNS, bleeding and clotting time activity and its analgesic activity was compared with aspirin. **Almas et al.,(1997)** found no difference in antibacterial action of fresh and one-month old miswak extracts.In the present study, ethanolic extract of *S. persica* (L) was used like others who **(Al-Bagieh et al., 1994)** concluded that alcoholic extract is more effective than aqueous extract for antibacterial activity. Our aim was to investigate the antiviral effects of ethanolic extract of Salvadora persica (Siwak) against (BHV-1**)**. The results in the present study showed the effective antiviral activity against BHV-1which in accordance with the conclusion **(Taha, 2008)**.The active componant in S. Persica extract was found to be benzylisothiocyanate (BIT) **(Farooqi et al., 1968)**. **Al- Bagieh(1992)** found the virucidal activity of benzylisothiocyanate against herpes simplex virus type 1 and the results of the plaque reduction assay indicated that BIT has a virucidal activity against HSV-1 at a concentration of 133.3

g/ml. A typical viral infection process consists of two parts: the early events, including adsorption, penetration and uncoating; and the later events, including transcription, translation, virion assembly and release **(Delgado *et al.*, 1998; Hosmane, 2003)**. Based on this process, the possible targets for antiviral chemotherapy include the attachment of virion to cell receptor, (*e.g*., *S. persist*), at concentrations higher than 0.1%, exerted a very significant cytotoxic effect on all the cell lines (P < or = 0.01), at a concentration of 0.001%,and persica are toxic to macrophage, epithelial, fibroblast, and osteoblast cells in a concentration – dependent manner **(Rajabalian et *al.*,2009)**. The best concentration of Salvadora Persica which showed a significant effect was 5%.The previous studies on Salvadora persica (L.)have showen that Salvadora Persica inhibited the replication of HSV–1 in BHK cells as well as the cytolytic activity of cell free virus **(Taha, 2008)**. In the present study, antiviral activity of S. persica was evidance. The results demonstrated that the safest dilution of the plant extract under study on MDBK cells was 0.1 mg/ml (non cytotoxic). The effective concentration on BHV-1 was 0.1 mg/ml as showed a marked decrease in virus titer from 10^5 TCID50/ml to 10^3 TCID50/ml in the adsorption step. In contrast, the plan extract didn't show any antiviral activity against BHV-1 neither in pre- nor post-treatment experiments. These results are similar to those evaluated the effect of ethanolic extract of *S. persica* (L.) herpesvirus on BHK when plant extract was added during adsorption, the mean inhibition of viral replication was 95 % **(Taha, 2008).** These results suggested that the active constituents of the roots ethanolic extract need to be metabolized in side the mouth to exhibit its antiviral activity. Although other reports demonstrated the safe use of Salvadora Persica in concentration 10% in experimental injection of laboratory animals, results presented in this study confirmed that ethanolic extract of *S. persica* (L.) exerts its effect during the early phases of the BHV-1 multiplication cycle, since the inhibitory effect was highest when ethanolic extract of *S. persica* (L.) was added during the viral attachment step only, suggesting that plant extract competes also in MDBK cell system with BHV-1 for heparan sulphate receptor on cell surface **(Van der Strate *et al.*, 2001; Marchetti *et al.*, 2004; Susana *et al.*, 2009)**. Further studies are recommended on the molecular level of BHV-1 and cells to clarify the mode of action of this plant extract on BHV-1 in vivo. The use of *S. persica* (L.) in combination with other milk components or drugs will be an increasing consideration. *S. persica* (L.) and could be investigated for topical application of external lesions induced by BHV-1 or for topical application as local anathesia.

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