



## Inhibition of herpes simplex virus type 1 infection by *Sambucus ebulus* extract *in vitro*

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### Abstract

**Background:** The emergence of drug-resistant strains of herpes simplex virus type 1 (HSV-1) has been increasingly reported. Therefore, attempts to discover new antiviral agents in particular from natural compounds are required. In this study, we evaluated the possible inhibitory effects of hydroalcoholic extract of *Sambucus ebulus* (*S. ebulus*) against HSV-1.

**Methods:** *S. ebulus* extract was produced by maceration method. MTT assay was used to evaluate the cytotoxicity effects of the *S. ebulus* extract; also, antiviral effects were measured both by test TCID<sub>50</sub> and quantitative real-time PCR methods. To study the inhibitory impact of *S. ebulus* extract on the expression of HSV-1 antigens, indirect immunofluorescence assay (IFA) was also performed. All analyses were performed using the GraphPad Prism software v. 7.0.

**Results:** In the postexposure assay of HSV-1 with *S. ebulus* extract at the highest nontoxic concentration (75 µg/mL), *S. ebulus* extract led to 2.6 log<sub>10</sub> TCID<sub>50</sub> reduction in infectious virus titer. At the highest nontoxic concentration, the *S. ebulus* extract led to inhibition rates of 91.2%, based on the quantitative real-time PCR assay results ( $p < 0.001$ ). Also, in the immunofluorescence assay, a significant reduction was observed in fluorescence emission intensity in HSV-1-infected cell treated with *S. ebulus* extract compared to the control group.

**Conclusion:** *S. ebulus* extract is a novel and effective natural compound in reducing HSV-1 titer and future studies should be conducted to discover the complete mechanism of antiviral effect of this natural compound.

**Keywords:** Herpes simplex virus, Antiviral activity, Natural product, *Sambucus ebulus*

**Conflicts of Interest:** None declared

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### Introduction

Herpes simplex virus type 1 (HSV-1) belongs to the *herpesviridae* family, which causes severe diseases, including oral-labial or oral-facial herpes, corneal blindness, encephalitis, and peripheral nervous system disorders. Also, HSV-1 causes difficult infections such as genital herpes in the genital or anal area. HSV-1 is a highly contagious infection, and most infected people suffer from

recurrent HSV infection several times a year (1, 2). Antiviral drugs can help to reduce the severity and frequency of symptoms, but as a result of the side effects of existing antiviral drugs and the increasing emergence of drug-resistant organisms during treatment, searching for an alternative antiviral agent is crucially necessary (3-5). Natural compounds have been considered as important

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#### ↑What is “already known” in this topic:

Due to some limitations of the present approved antiviral drugs against HSV-1, interest in discovering antiviral activity of natural compounds has recently been the subject of intense scientific investigation.

#### →What this article adds:

*S. ebulus* extract is a novel and effective natural compound in reducing HSV-1 titer and can be considered as a potent inhibitor of HSV-1 infection.

sources of potential treatments for cancer and infectious diseases due to their cost-effectiveness and potential therapeutic properties (6). Considering the undesirable side effects and drug-resistant organisms of antiviral agents, using an effective, economical, and accessible treatment is essential. *Sambucus ebulus* (*S. ebulus*), Iranian native botany, is one of the best natural compounds in the world that is a good source of polyphenols, anthocyanin, quercetin and vitamin C, which may be related to its favorable properties such as antioxidant and antimicrobial activity (7, 8). Essential oils and monoterpene compounds from *S. ebulus* contains  $\alpha$ -terpinene,  $\gamma$ -terpinene,  $\alpha$ -pinene,  $p$ -cymene, terpinen-4-ol,  $\alpha$ -terpineol, thymol, citral and 1,8-cineole are responsible for the biological properties of medicinal plants, including anti-inflammatory, antiviral, antitumor, cytotoxic, and antimicrobial activities (9, 10). A wide range of medicinal applications of *S. ebulus*, including improvement of lipid profile, antiparasitic, antiviral, antibacterial, antiulcerogenic, anti-inflammatory applications, radical scavenging activities, and wound healing activities, has been reported (11-13). *Sambucus ebulus* (*S. ebulus*) grows in many temperate regions and does not require special conditions for maintenance and has amazing effects on humans. However, its subsistence may be surmised (14). Therefore, in this study, we evaluated the antiviral activity of *S. ebulus* hydroalcoholic extract on HSV-1 replication in cell culture system.

## Methods

### Plant collection, extraction, and analyses of essential oils

*S. ebulus* plant (Fruits) was collected from different parts of northern Iran and herbarium E1-38-131 was registered in the Faculty of Pharmacy, Mazandaran University of Medical Sciences and its identity was confirmed by Mohamad Ali Ebrahimzadeh. In the next step, the fruits of the plant were dried at room temperature and pulverized in the grinder. Then, the *S. ebulus* extract was prepared by maceration method at room temperature for 3 days and the macerated solution was sterilized by 0.22  $\mu$ m microbiological filters. Also, the macerated solution was concentrated in a rotary evaporator until all the solvents were cleared. After preparation of the *S. ebulus* hydroalcoholic extract, quantitative and qualitative data for all the essential oils were determined by Shimadzu GC-14A gas chromatograph equipped with a Supelcowax 10 (Supelco, Bellefonte, PA) capillary column (60 m - 0.25 mm i.d.). Essential compounds were identified by comparing the mass spectra and their relative gaps with valid samples (the Wiley Registry of Mass Spectral data and literature citations) (15). Then, serial concentrations of *S. ebulus* extract were prepared to estimate its toxicity threshold and antiviral activity.

### Cell and virus culture

In this study African green monkey kidney cells (Vero), taken from the Razi Vaccine and Serum Research Institute (Karaj, Iran), were used to evaluate the antiviral activity of the *S. ebulus* extract. Vero cells were grown under the following conditions: DMEM plus 10% fetal bovine serum

(FBS; Gibco, USA); 2mM L-glutamine (Merck, Germany); 1mM sodium pyruvate (Merck, Germany); 100 IU/mL penicillin; and 100  $\mu$ g/mL streptomycin (Sigma- Aldrich, USA) at 37°C in a humidified incubator in an atmosphere of 5% CO<sub>2</sub>. The HSV-1 primary stock (strain KOS), a gift from the Virology Department of Tarbiat Modares University (Tehran, Iran) that was cultured in the Vero cells, and 50% tissue culture infectious dose (TCID50) assay were used to determine the viral infectious titer.

### Cytotoxicity assay

The cytotoxicity of *S. ebulus* extract on Vero cells was determined by methylthiazolyltetrazolium (MTT) method. First, Vero cells were grown in DMEM media with 10% FBS, and then harvested using trypsin and 100  $\mu$ L of trypan blue-treated cell suspension counted using hemocytometer. Then, Vero cells at a density of  $1 \times 10^5$  cells/mL were cultured on flat-bottomed 96-well microtiter plate (SPL Life Science, South Korea) in triplicate and incubated at 37°C in 5% CO<sub>2</sub> environment for 24 hours. Different concentrations from 25 to 225  $\mu$ g/mL of *S. ebulus* extract were added to the plate and incubated for 48 hours. After treatment time, the *S. ebulus* extract was removed and replaced with 10  $\mu$ L of MTT reagent and 100  $\mu$ L RPMI (Bio-Idea, Iran). After 4 hours, medium was removed and 50  $\mu$ L of DMSO solution was added to the plate and the plate was gently shaken for 15 minutes. Finally, the plate was read at 550 nm using a microplate reader (Hiperion MPR 4+, Roedermark, Germany).

### Evaluation of antiviral activity

The confluent monolayer of Vero cells in a flat-bottomed 96-well microtiter plate were preincubated with 100  $\mu$ L of 100 TCID50/mL HSV-1 in triplicate for 1 hour at 37°C in 5% CO<sub>2</sub> atmosphere. After attachment time, the media containing HSV-1 were discarded from the wells, and the cells were washed 3 times with PBS (PBS; Bio-Idea, Iran). Then, the infected cells were incubated with 100  $\mu$ L of different noncytotoxic concentrations of *S. ebulus* extract for 48 hours at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Also, the virus and cell controls were kept as described above. This assay was also performed for acyclovir. At the indicated time of all above experiments (at 48 hours), plates of cells were subjected to a single freezing–thawing cycle to release the cell-associated virus particles. Finally, the lysates were collected from the wells and used for TCID50 and quantitative real-time PCR methods to determine the amount of total progeny virus.

### Quantitative real-time PCR assay

To determine HSV-1 viral load, HSV-1 DNA was extracted from the harvested lysates using Genomic DNA Extraction Mini kit (Favorgen, Taiwan), based on manufacturer's protocol. Then, DNA extracted was subjected to quantitative real-time PCR assay using HSV-1 specific primers for the US3 region, as described in previous study (1). This assay was performed using the Rotor-Gene Q instrument (Qiagen, Germany).

### Indirect immunofluorescence assay (IFA)

Vero cells were seeded on sterile glass coverslips (Nunc, Denmark) in a 24-well plate (SPL Life Science, South Korea) and grown to 80%-90% confluence. The media were discarded and the cells were washed with PBS and incubated with 200  $\mu$ L of 100 TCID<sub>50</sub>/mL HSV-1 virus suspensions for 1 hour at 37°C in a humidified 5% CO<sub>2</sub> incubator. After discarding the virus inocula, the cells were washed 3 times with PBS. The maximum noncytotoxic concentration of *S. ebulus* extract was suspended in DMEM and was added to the wells and the plate was incubated at 37°C in 5% CO<sub>2</sub> atmosphere. The virus and cell controls were also included in this experiment. After 15 hours, the cells were fixed with cold acetone (4°C) for 20 minutes, and the fixed cells were overlaid with HSV-1 specific human antibody, followed by incubation at 37°C for 30 minutes. In the next step, the cells were washed 3 times with PBS and were then overlaid with fluorescein isothiocyanate (FITC) (Dako, Germany), followed by incubation at 37°C for 30 minutes. Afterwards, the cells were washed 3 times with PBS and coverslips were mounted by glycerol buffer on slides. Finally, Olympus BH2-RFCA fluorescence microscope (Tokyo, Japan) was used to visualize the cells.

#### Statistical analysis

The mentioned values were obtained by the average of 3 independent experiments. The results of the experiments were tabulated and one-way analysis of variance (ANOVA) was used to analyze the difference between the means and Tukey's multiple comparison test was also performed. GraphPad Prism software, version 7.0 (GraphPad software, USA) was used for all analyses and p values less than 0.05 were considered as statistically significant.

## Results

### Gas chromatography (GC) and mass spectrophotometry (MS)

A total of 28 compounds were identified in *S. ebulus* extract, and the main components of *S. ebulus* extract were  $\alpha$ -Terpinene,  $\beta$ -Pinene,  $\alpha$ -Terpineol, Trans carveol, Cis carveol, Chavicol, Terpinen-4-ol, and Iso borneol (Table 1). In this study all experiments were performed with the same lot of essential oil to ensure reproducibility due to the inherent variability observed from lot to lot.

### Cytotoxicity of extract

MTT assay was used to determine the cytotoxic effect of different concentrations of *S. ebulus* extract on Vero cells. As shown in Figure 1, acyclovir did not show significant effects on Vero cells in the cytotoxicity test. In cytotoxicity experiments, acyclovir showed a significant reduction compared to the *S. ebulus* extract because by increasing the concentration of essential oils of hydroethanolic extracts, cell viability is significantly reduced. Thus, the viability was determined greater than 90% up to the concentration of 75  $\mu$ g/mL of *S. ebulus* extract and these concentrations were used for subsequent antiviral assays.

### Antiviral activity of the extract

In this study to evaluate *S. ebulus* extract with antiviral properties, we investigated the anti-HSV-1 activity (in vitro) of *S. ebulus* extract, prepared from *S. ebulus* collected in northern Iran. The antiviral activity was evaluated by 100 tissue culture infecting doses (TCID<sub>50</sub>) and qPCR real-time assays. The results of TCID<sub>50</sub> assay showed the preexposure and coexposure of Vero cells to *S. ebulus* extract did not lead to any reduction of the HSV-1 titer.

Table 1. Gas chromatography and mass spectrophotometry of *S. ebulus* fruit extract

No	Compounds	KI = Kovats Index on HP-5 column	Percent (%)
1	Pino carvone	1156	0.31
2	Iso pulegol	1209	0.79
3	$\alpha$ -Terpinene	1125	1.28
4	Safrol	1273	0.19
5	$\beta$ -Pinene	976	0.76
6	Iso estragol	1254	1.39
7	Linalool oxide	1121	0.74
8	Camphor	1185	0.76
9	$\alpha$ -Terpineol	1211	2.76
10	Myrtenol	1189	0.64
11	Verbenone	1206	1.06
12	Fragranol	1264	0.23
13	Trans carveol	1195	0.65
14	$\alpha$ -Thujone	1121	0.89
15	Geranial	1165	0.95
16	Cis carveol	1187	3.86
17	Geraniol	1257	1.63
18	$\beta$ -Thujone	1095	0.98
19	Chavicol	1253	2.43
20	Carvone	1284	0.57
21	Myrtenal	1124	0.42
22	Pulegone	1263	1.18
23	Ocimenone	1275	0.47
24	Ocimene oxide	1071	1.16
25	Terpinen-4-ol	1189	3.39
26	Borneol	1205	1.05
27	Pulegol	1189	0.17
28	Iso borneol	1094	0.92

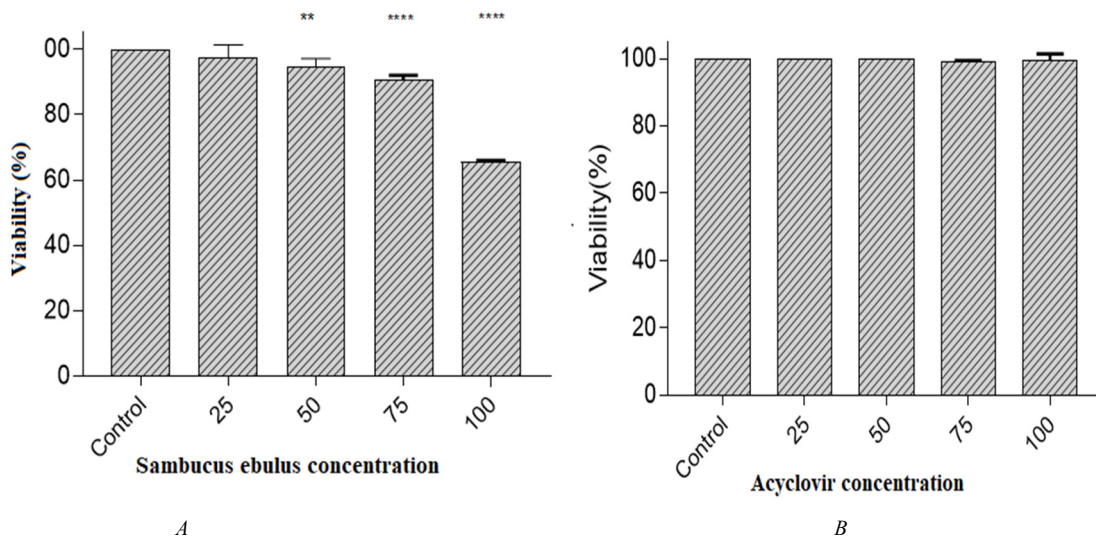


Fig. 1. Evaluation of cytotoxicity of *S. ebulus* (A), acyclovir as a positive control (B) on Vero cells

Meanwhile, virucidal activity was not observed at any of the studied concentrations of *S. ebulus* extract, indicating that *S. ebulus* extract could not directly inactivate the HSV-1 particle and could lead to the inactivation of viral infection. The significant finding of our study is that *S. ebulus* extract exerts its antiviral effects only when added 1 hour after absorption of HSV-1 to the Vero cells, which could result in a striking decrease in viral titer. Postexposure of HSV-1 with *S. ebulus* extract at the concentrations of 25, 50, and 75 µg/mL showed that it could lead to 0.5, 1.2, and 2.6 log<sub>10</sub> TCID<sub>50</sub> reduction in virus titer when compared to the virus control, respectively ( $p < 0.0001$ ) (Fig. 2). In our study acyclovir was used as a positive control to compare the anti- HSV-1 activities of the test compounds. Z test was used to confirm the results. In the next step, qPCR real-time assay was used to confirm the antiviral activities of *S. ebulus* extract against HSV-1 and

the antiviral activity followed a dose-dependent pattern. Thus, the *S. ebulus* extract at the concentration of 25, 50, and 75 µg/mL led to inhibition rates of 4.2%, 48.2%, and 91.2%, respectively. The most antiviral effect of *S. ebulus* extract was observed at the concentration of 75 µg/mL, which could lead to a 91.2% inhibition rate. The production of HSV-1 was completely inhibited by acyclovir at the concentration of 75 µg/mL (Fig. 3).

**Indirect immunofluorescence assay**

An indirect immunofluorescence assay (IFA) was used to evaluate the inhibitory activity of *S. ebulus* extract on the expression of HSV-1 antigens on the Vero cells surface. In this step, the highest nontoxic concentration of *S. ebulus* extract, which showed the highest inhibitory effect against the HSV-1, was used. Equally, both positive and negative controls were also included. Figure 4 shows a significant reduction in fluorescence emission intensity in HSV-1-infected cell treated with *S. ebulus* extract at the

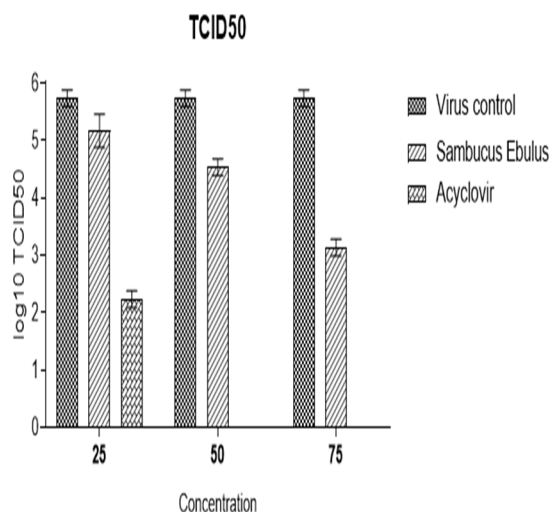


Fig. 2. Assessment of antiviral activity of *S. ebulus* on the infectious titer of HSV-1 by TCID<sub>50</sub> assay, compared with acyclovir.

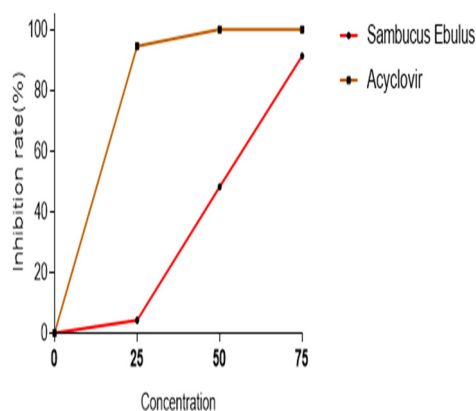


Fig. 3. Comparison of the inhibitory rates determined by real-time PCR assay in the postexposure of HSV-1 with *S. ebulus* and acyclovir

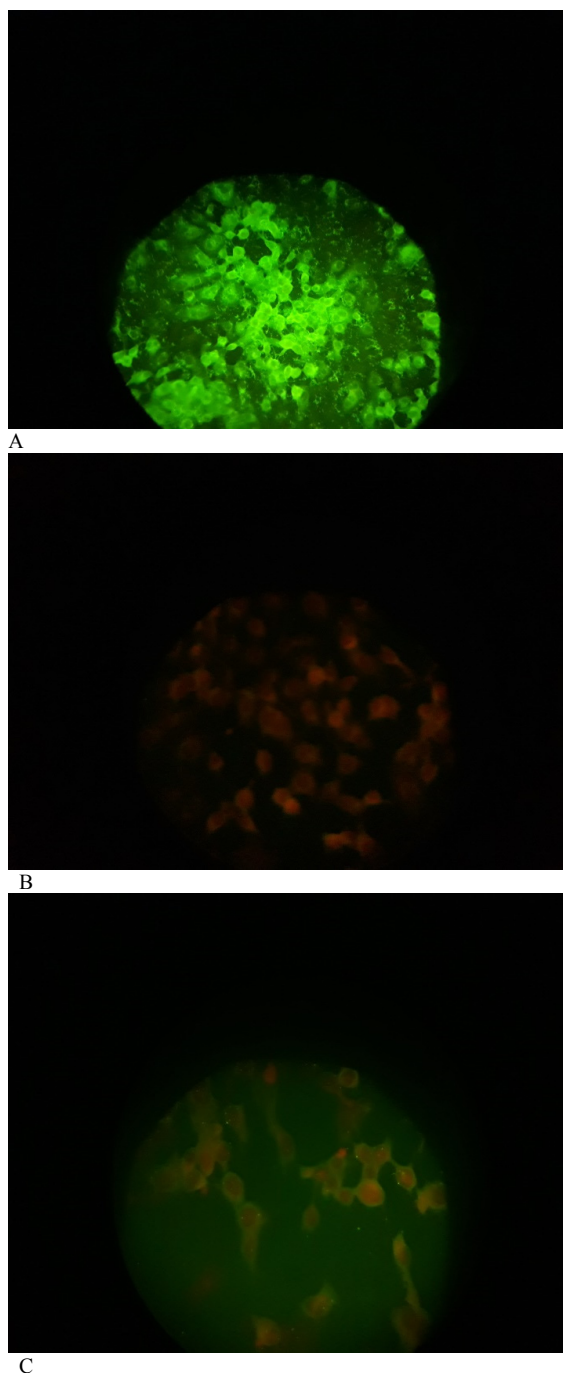


Fig. 4. Indirect immunofluorescence assay for detection of HSV-1 antigens in the Vero cells. (a) Virus control, (b) Cell control, (c) Infected cells treated with *S. ebulus* extract (75 µg/ml) at 15 h post infection.

concentration of 75 µg/mL compared to the positive control.

### Discussion

The HSV-1 infection is a significant global public health problem. Antiviral drugs used to treat HSV-1 infection are weakened due to major side effects and the emergence of drug-resistant strains during treatment. Hence, the research and development of effective HSV-1 drugs especially from the natural product seems interesting and necessary (16, 17). Compared to the current antiviral drugs, there are

several important advantages to use natural product for therapeutics, such as effectiveness in low concentrations, potent antiviral activity against drug-resistant strains, and cost effective preparation (18, 19). In this study we used HSV-1 (strain KOS) as the virus model and evaluated the antiviral properties of *S. ebulus* extract on HSV-1. Therefore, a series of laboratory experiments were designed and performed based on the cell culture system. The plant *S. ebulus* (Caprifoliaceae L.) as a native and well-known Iranian herb is abundant in northern Iran and has been noted to exhibit exceptional antimicrobial and antiparasitic functions (20-23). Also, it consists of anticancer substances, such as ebulin (RIP-II, ribosome inactivated protein-II), flavonoids, steroid substances, cardiac glycosides, tannins, caffeic acid derivatives, and other isolated substances (24). To date, several studies have reported the inhibitory capacity of the Caprifoliaceae family on viral infections.

Essential oils from *S. ebulus* are complex natural mixtures and their main terpenes, such as  $\alpha$ -Terpinene,  $\beta$ -Pinene,  $\alpha$ -Terpineol, Trans carveol, Cis carveol, Terpinen-4-ol, Iso borneol, and phenylpropanoids, are responsible for their biological properties and their major monoterpene compounds have been scientifically proven to possess antiviral activity. These compounds interact with the viruses and could alter the intracellular events of virus replication. Previous studies have reported a potent antiviral activity of terpenes such as Isoborneol that specifically inhibit glycosylation of viral polypeptides (15, 25). In the study by Christian Krawitz et al, the inhibitory activity of *S. nigra* L. against influenza A and B viruses was investigated in vitro (26). Their results have shown that the aqueous extract displays an inhibitory effect on the propagation of human pathogenic influenza viruses. They suggested that additional investigations are required to elucidate the mechanism of action. In another study by Christie Chen et al, antiviral properties of *S. nigra* extracts were tested for anti-infectious bronchitis virus activity on Vero cells and a dose-dependent antiviral activity were found in their investigation (27). The results of their study suggest that *S. nigra* extract may inhibit IBV at the onset of infection by presenting a noninfectious virus. In our study, we aimed to assess the antiviral activity of *S. ebulus* extract at different time of the virus replication cycle, so that the *S. ebulus* extract was added before and after virus adsorption. Our results have shown in preexposure and coexposure of cells to *S. ebulus* extract did not result in any decrease in the titer of HSV-1 and exerted its own antiviral effects only when added 1 hour after infection of the cells, which can significantly inhibit HSV-1 infection in the Vero cell culture system. The inhibitory of *S. ebulus* extract was confirmed by several laboratory techniques, containing TCID<sub>50</sub>, real-time PCR, IFA, and CPE inhibition assays. Previous studies have shown that after HSV-1 entry into a host cell, it transports its nucleocapsid through the nuclear pores from the cytosol to the cell nucleus. HSV-1 then completes viral gene expression, replication, capsid formation, and viral DNA packaging in the nucleus of infected cells. Our experiments showed that the most antiviral potency is observed after viral adsorption. *S. ebulus*

extract may be targeted and interfere with some stages in the life cycle of the HSV-1, such as DNA, RNA, and protein synthesis, which occurs after viral adsorption and internalization by the cells, leading to inhibition of intracellular replication (28, 29). The comparison between our results with those of Gavanji et al in 2015 shows that *S. ebulus* extract is more effective than *Z. multiflora* and *E. caesia*; and anti-HSV-1 activity of these oils showed that by increasing the concentration of oils, virus infection is inhibited. Thus, it can be concluded that the antiviral effect of different plant oils is related to their active components and natural phenolic compounds of oils have ideal antimicrobial activity (30). Additional investigations are required to explore the exact antiviral mechanism of the *S. ebulus* extract, such as transmission electron microscope (TEM), and to examine the pattern of HSV-1 gene expression in the presence of the *S. ebulus* extract.

### Conclusion

In this study the inhibitory activity of SE-HAE on HSV-1 was investigated for the first time. The results showed *S. ebulus* extract have a high anti- HSV-1 activity with low cytotoxicity, suggesting that the *S. ebulus* extract can be effective in enhancement of antiviral effects against HSV-1. The results of in vitro experiments in the present study also showed that the *S. ebulus* extract have inhibitory effects against HSV-1 only after the virus entered the host cells.

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### Conflict of Interests

The authors declare that they have no competing interests.

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