



International Journal of Molecular and Clinical Microbiology



Comparison of Antiviral Effects of *Chelidonium majus L.* Hexane and Aqueous Extracts against Herpes Simplex Virus Type 1

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ARTICLE INFO

Article history:

Received 30 September 2018

Accepted 30 November 2018

Available online 1 December 2018

Keywords:

HSV-1, *Chelidonium majus* Hexane and Aqueous Extracts, Real-time PCR

ABSTRACT

Herpes simplex virus type 1 (HSV-1) leads to various clinical manifestations in humans. Due to drug resistance, the use of herbs extracts for treatment has been considered as a significant solution. One of these compounds is *Chelidonium majus L.* herb extracts which is known as Mamiran in Iran. In this study, two aqueous and hexane extracts of *Chelidonium majus L.* were used to investigate the antiviral effects against HSV-1. The toxicity of hexane and aqueous extracts on HeLa cells was determined by MTT assay. Anti-viral effects of the extracts were evaluated directly, at different concentrations and times. The effect of the aforementioned extracts on reducing the virus replication was evaluated by quantitative Real-time PCR based on the glycoprotein D (gD) gene expression level of the herpes simplex virus. We observed that a 3500 µg/ml concentration of aqueous extract of *C.majus* and 600 µg/ml concentration of hexane extract were not toxic to HeLa cells. None of the extracts had a direct anti-viral effect on the virus. The aqueous extract at the above concentrations after 1, 2, 4, and 8 h of adsorption significantly reduced on virus replication. In contrast, the hexanic extract of this plant had no viral and inhibitory effect on HSV-1 replication. The aqueous extract of *C.majus* has an antiviral effect on HSV-1 compared to the hexane extract.

1. Introduction

The herpes simplex virus is the member of the herpes virus family, i.e., *Herpesviridae*, and the *Alphaherpesvirinae* subfamily. This species has two common types. Type 1 leads to various clinical manifestations of simple cold sore to acute encephalitis in humans. Type 2 virus causes genital herpes infection by transmitting through sexual intercourse; however, type 1 can

also cause genital infection in some cases (LeGoff et al., 2014; Zolini et al., 2014; Cunningham et al., 2006). Infections caused by this virus may be early or recurrent. Primary infections occur in people without antibodies and most clinically asymptomatic people produce antibodies and latent infections in nerve ganglia (McAllister and Schleiss, 2014).

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In recent years, many studies have been done on the effects of natural substances inhibition on microorganisms. These materials are the most appropriate choice for not having side effects and limiting their use during pregnancy and breastfeeding compared with chemicals and drugs. Today, various drugs such as acyclovir, cytarabine, and vidarabine are used to treat viral herpes infections. In Iran, due to plant diversity, many studies have been conducted on the effects of medicinal plants on a number of microorganisms, including herpes viruses. One of these herbs is *Chelidonium majus L.* (Hook et al., 1992; Fahad and Stepher, 1996).

Chelidonium majus L., which is known as Mamiran in Iran as a significant plant in western herbal medicine and traditional Chinese medicine is widely distributed in Europe and Western Asia. Raw extracts of various parts of the plant, such as roots, stems, and leaves, contain large amounts of secondary metabolites of isoquinoline alkaloids including Sanguinarine, Chelidonine, Chelerythrine, Berberin, Coptisine, which have anti-bacterial, anti-viral, and anti-inflammatory properties. Each of the extracts and alkaloids can be extracted from the plant by a series of organic solvents (Gilca et al., 2010; Ghorbanli et al., 2009).

There are certain compounds in each of the hexane and aqueous extracts of the *Chelidonium majus*. The aim of this study is to evaluate and compare the antiviral effects of these extracts on the herpes simplex virus type 1. Because of its high sensitivity and efficiency, the real-time PCR is a common tool for finding and evaluating the expression of selected gene expressions in biological samples, especially viral specimens (Karsai et al., 2002). One of the most practical uses of real-time PCR is the evaluation of genes expression using relative quantitative measurements. Currently, relative quantification is the most accurate method for examining changes in gene expression (Valasek and Repa, 2005; Ginzinger, 2002).

There are various methods for preparing the extract from medicinal herbs. Each extract has specific substance depends on solvent used in extraction. In this study we evaluated aqueous and hexane extracts of *C.majus* on Herpes simplex virus type 1. Since each of the above extracts contains specific compounds lack of any study in Iran on the effect of *C.majus* on Herpes

simplex virus type 1 by Real-time PCR, the present study was conducted to investigate the effect of hexane and aqueous extracts of *C.majus* on HSV-1 in HeLa cell line and to compare the antiviral effects of different concentrations of these extracts on viruses using Real-time PCR.

2. Materials and Methods

2.1. Cell line and virus

In this study, HeLa cell line was used and purchased from the Pasteur Institute of Iran. Herpes simplex virus type 1 (KOS strain) was donated by the Department of Virology of Tarbiat Modares university. The titration of the virus was determined by 50% Tissue Culture Infective Dose (TCID₅₀) method and kept at -70°C for use at different stages of research.

2.2. *C.majus* Extracts preparation

To prepare the extracts of *C.majus*, the plant was collected from Gorgan city by experts of the Faculty of Pharmacy, the University of Tehran, dried in shade, and then grinded. After sieving by a 500- μ m sieve, 140 g of plant powder was weighed and poured inside the decanter. Hexane solvent and distilled water were separately added to this amount. The aqueous extract was boiled and after 72 h the extract was passed through a paper filter and was dispensed with a volume of 100 ml. Then, 10 ml of these extracts were separately placed at 37°C to evaporate the solvent. About 0.2 g of a powdered extract of the plant was dissolved with hexane and water solvents separately in 10 ml of the Dulbecco's Modified Essential Medium (DMEM). After 24 h, the solvents were passed through a 0.22- μ m filter and kept inside the sterilized tube at +4°C.

2.3. MTT Assay for cellular cytotoxicity

MTT method was used to determine the threshold of toxicity of plant extracts on cells. In this method, a 96-well culture plate was considered and in each well, 15,000 cells per 200 μ l of DMEM medium were cultured with 10% of the FBS. After 24 h of incubation and formation of the monolayer in each well, different concentrations of extract with 2% FBS were added so that for each concentration, 3 wells were considered as cell control. After 72 h of incubation, 96-well plate cells was washed

with Phosphate Buffered Saline (PBS) and 20 μ l of MTT solution and 80 μ l of DMEM medium were added to each well. MTT solution was prepared by adding 0.005 g of MTT powder to 1 ml sterile PBS. After 4 h of incubation at 37°C and formation of formazan crystals, the supernatant was removed slowly. To dissolve crystals, 100 μ l of DMSO was added to each well. The optical density of each well was measured at 540 nm wavelength. The average OD of 3 wells of each concentration and 3 other control wells was calculated. To 3 wells without cells, 100 μ l of DMEM medium was added. After removing it, 100 μ l of DMSO was added and their average OD was considered as blank. The following formulas were used to calculate the viability of the cells using the MTT method:

$$\text{Cytotoxicity\%} = 1 - \frac{\text{Mean OD}_{\text{test}} - \text{Mean OD}_{\text{blank}}}{\text{Mean OD}_{\text{control}} - \text{Mean OD}_{\text{blank}}} \times 100$$

$$\text{Viability\%} = 100 - \text{Cytotoxicity\%}$$

The 50% cytotoxic concentration (CC50) was assessed from dose-response curve. The concentration of the extract, which led to the survival of 90% of the cells, was considered as a non-toxic concentration.

2.4. Virucidal assay

To evaluate the direct effect of the extract on the HSV-1, the HeLa cells were cultured in a 24-well culture plate. Next, 100TCID50 of the virus was prepared in non-toxic extracts and the extract-free medium (as control) separately and the viral suspension was added to the wells at 0, 1, 2, 3, and 4 h after preparation. After 48 h, the supernatant of the wells was sampled and the amount of virus was evaluated in comparison with the control by Real-time PCR.

2.5. Effects of different concentrations of plant extracts on HSV-1

In order to evaluate the inhibitory effect of extracts in different concentrations on virus replication, after preparation of monolayers in a 24-well plate, the cells of each well were infected with 100TCID50 virus. After 1 h of viral adsorption, different concentrations of plant extract comprise nontoxic concentrations and lower concentrations were added to wells containing 2% FBS and medium. After 48 h, the supernatant of each well was sampled and the virus was evaluated in comparison with the control by Real-time PCR.

2.6. Effects of plant extract on HSV-1 replication at different times

To assess the effect of the extract on the virus at different times of its replication, after preparing the 24-well culture plate, which contained monolayer cell, and evaluating the extract effect immediately after preparation of 100TCID50 virus in the nontoxic extract concentration, 200 μ l of the solution was added to wells of the washed cells.

After 1 h of adsorption, the supernatant was removed and the cells were washed. Afterward, 1000 μ l of the medium containing 2% FBS was added to the well. In the next step, 7 wells containing cell monolayers were washed and 100TCID50 was added to each of them. All wells were washed again, after 1 h of adsorption. Next, 1, 2, 4, 8, 12, and 24 h after adsorption, the supernatant was removed from the rest of the wells and was replaced with the extract containing nontoxic concentrations and 2% FBS. To control the virus, 1 h after viral adsorption, the medium containing 2% FBS without extracts was added in an isolated well. Then, 48 h after the initial infection of the cells with the virus, the supernatant of all wells was removed and the amount of virus was evaluated in comparison with the control by quantitative Real-time PCR. All of the above treatments were performed separately for both aqueous and hexane extracts.

2.7. Quantitative PCR

Total viral DNA was isolated using the QIAamp MinElute Virus Spin kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The glycoprotein D (gD) gene from the HSV-1 was compared with the Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene as a standard gene. Once all the samples obtained from the steps of evaluating the virucidal effects of hexane and aqueous extracts on the virus, the inhibitory effect of these two extracts in different concentrations on virus replication and the determination of the effect of hexane and aqueous extracts on the virus at different times were carried out using the relative Quantification method. In other words, gD gene C_T (cycle threshold) was compared at each step with standard gene C_{TS} GAPDH. The result of this quantitative analysis was then obtained by the following formula:

$$\text{Fold Difference} = 2^{-(B-A)}$$

A: C_T standard gene (GAPDH) control sample - C_T gene gD control sample

B: C_T Sample standard gene (GAPDH) containing extract - C_T Sample gD gene containing extract

Information on the primers designed for the gD gene for HSV-1 and human GAPDH is presented in Table 1:

Table 1. Characterization of primers for gD of HSV-1 and Human GAPDH genes

Primer name	5'-Primer sequence-3'	Melting Temperature (T _m)
gD qRTF	GAA TGC TCC TAC AAC AAG TC	54.0 °C
gD qRTR	GTA ATC TCC GTC CAG TCG	54.2 °C
GAPDH qRTF	CTC TTG CTA CTC TGC TCT G	54.7 °C
GAPDH qRTR	GCC TGC CTG GTG ATA ATC	55.1 °C

Table 1: gD primers reproduce the 182 base pair (bp) of gD gene of HSV-1. The length of the PCR product for human GAPDH gene primers is 179 bp.

2.8. Statistical analysis

Data are presented as the mean \pm standard deviation (SD). All data were analyzed using t-test and SPSS software. Significant changes were identified where $p < 0.05$.

3. Results

3.1. Cellular cytotoxicity

Cytotoxicity for hexane and aqueous extracts concentrations of *C. majus* was obtained by MTT assay (Table 2). The CC50 values of extracts were assessed at 7150 $\mu\text{g/ml}$ for aqueous extract and at 1550 $\mu\text{g/ml}$ for hexane extract. After 72 h, more than 90% of cells survived at 3500 $\mu\text{g/ml}$ concentration of aqueous extract and 600 $\mu\text{g/ml}$ concentration of hexane extract in comparison to control.

3.2. Virucidal assay

There was no significant difference between controls and tests at 0, 1, 2, 3, and 4 h in the direct investigation of the virucidal effect of aqueous and hexane extracts on the virus in the extracellular environment. Consequently, 3500 $\mu\text{g/ml}$ concentration of aqueous extract and 600

$\mu\text{g/ml}$ concentration of hexane extract had no significant effect on the virus ($P = 0.63$).

3.3. Effects of different concentrations of plant extracts on HSV-1

In order to assess the effect of various concentrations of non-toxic aqueous and hexane extracts on HSV-1, all concentrations of aqueous extracts compared to control showed a significant decrease in inhibitory viral replication ($P < 0.001$; fig. 1).

Among the above concentrations, 3500 $\mu\text{g/ml}$ of aqueous extract was the most significant. Thus, it can be concluded that 3500 $\mu\text{g/ml}$ concentration of aqueous extract has the greatest effect on inhibiting virus replication compared with lower concentrations. In comparison, according to figure 2 and observing a very low difference between the control and higher non-toxic levels of hexane extracts and lower concentrations, it can be concluded that the concentration of 600 $\mu\text{g/ml}$ and lower concentrations of this extract had no significant inhibitory effect on HSV-1 virus replication ($P = 0.17$).

Table 2. Toxicity of Hexane and aqueous extracts on HeLa cells after 72 hours by MTT assay

Concentration of Aqueous extract ($\mu\text{g/ml}$)	Viability% after 72 hours (MTT)	Concentration of Hexane extract ($\mu\text{g/ml}$)	Viability% after 72 hours (MTT)
8000	44.26 \pm 1.06	1600	43.91 \pm 1.12
7000	52.14 \pm 1.29	1400	61.87 \pm 1.84
6000	68.89 \pm 1.25	1200	66.66 \pm 1.93
5000	72.26 \pm 1.25	1000	76.62 \pm 1.86
4000	78.31 \pm 1.07	800	82.33 \pm 2.17
3500	93.19 \pm 1.71	600	90.13 \pm 1.2
3000	93.28 \pm 2.3	400	91.54 \pm 2.09
2500	92.51 \pm 2.36	200	91.85 \pm 1.31
Control	-	Control	-

Table 2: HeLa cells were treated with different concentrations of aqueous and hexane extracts. The cells viability after 72 h was determined by the use of MTT assay. The tests were performed in triplicate and the standard deviation (\pm) was obtained.

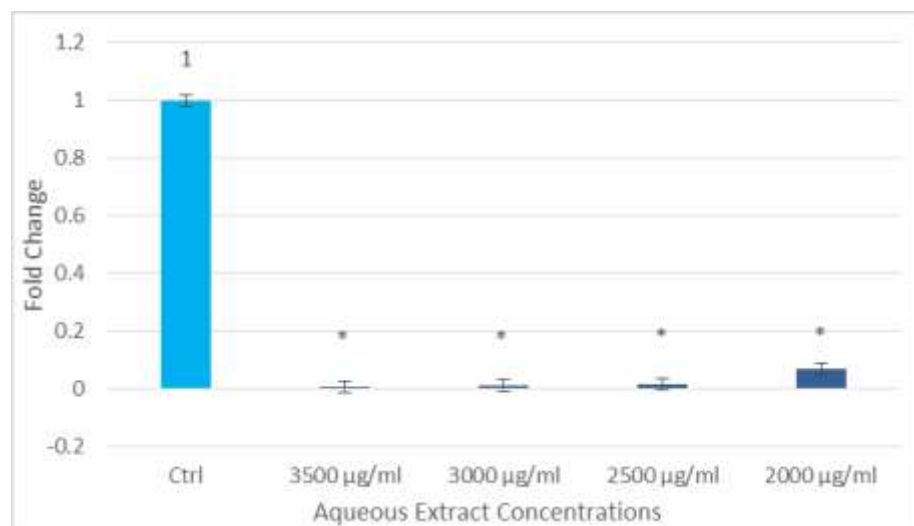


Figure 1. The effect of various concentrations of *C.majus* aqueous extract on HSV-1 infected HeLa cells. Adding the different concentrations of aqueous extract to virus-infected cells significantly reduced the replication of the virus ($P < 0.001^*$). The average virus titer is shown based on three independent replicates for each test. Error bars represent the standard deviation.

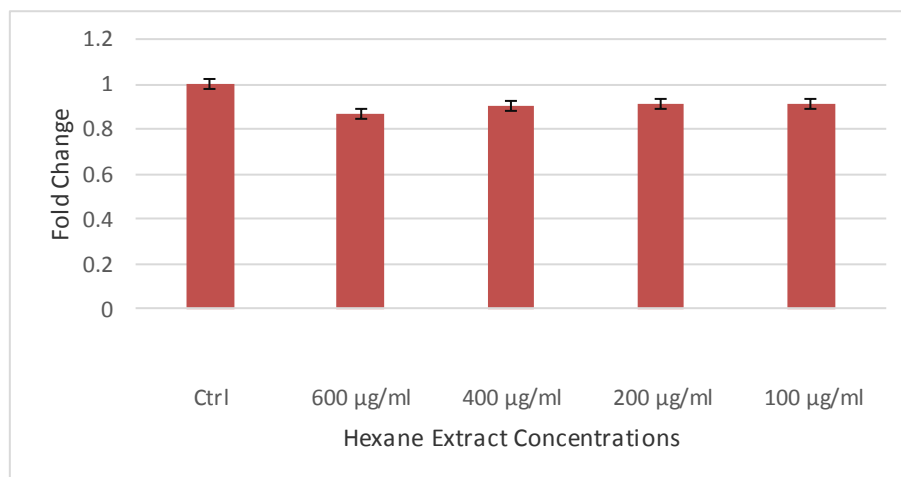


Figure 2: Effect of Different Concentrations of *C.majus* Hexane Extract on HSV-1 Infected HeLa Cells; the average virus level is shown based on three independent replicates for each test. Error bars represent the standard deviation. None of hexane extract concentrations had a significant effect on virus replication than control.

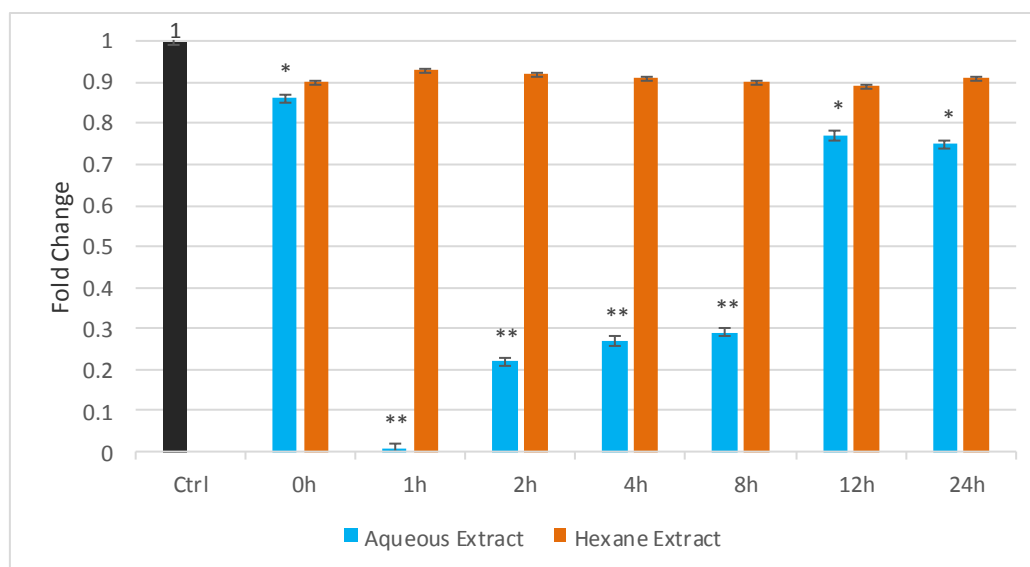


Figure 3. The effect of aqueous and hexane extracts on the replication of HSV-1 in different times of cell infection. The average virus level is shown based on three independent replicates for each test. Error bars represent the standard deviation. Adding the aqueous extract to virus infected cells after 1, 2, 4, and 8 h of adsorption ($P=0.0001^{**}$) and at 12 and 24 h ($P=0.001^{*}$) significantly reduced the replication of the virus. Hexane extract did not significantly decrease the replication of virus ($P=0.054$).

3.4. Effects of plant extract on HSV-1 replication at different times

Studying the effect of aqueous and hexane extracts on HSV-1 replication in different times of cell infections with the virus and according to the results (Fig. 3), it was found that 3500 µg/ml concentration of aqueous extract compared to control significantly reduced the amount of the

virus 1, 2, 4, and 8 h after adsorption. In order to investigate the effect of aqueous and hexane extracts on HSV-1 replication in different times of cell infection with the virus, according to the results obtained (Fig. 3), it was found that 3500 µg/ml concentration of aqueous extract compared to control 1, 2, 4, and 8 h after adsorption significantly reduced the amount of the virus ($P<0.001$). At 12 h and 24 h, there was

a significant effect on virus replication ($P=0.01$), however, this effect was less than the 1, 2, 4, and 8 h after adsorption. During adsorption, there was no significant effect on virus propagation ($P=0.28$). In contrast, the non-toxicity concentration of hexane extract compared to control during adsorption and post-adsorption times did not significantly decrease the replication of virus ($P=0.054$).

4. Discussion

Recently, due to drug resistance and their side effects, the use of herbal medicine for the treatment of infectious diseases has increased. As the results of this study showed (fig3), the aqueous extract of the plant has the most effect on virus replication, compare to hexane extract. There are many studies on the antiviral effects of herbal extracts that can result in the development of new drugs.

El-Mekkawy screened 59 indigenous Egyptian herbs such as *C.majus*, which had inhibitory effects on essential HIV-1 enzymes; to this end; the MT-4 cell line was used. MT-4 cells were infected for 1 h with HIV-1 at TCID₅₀ of 0.001 per cell. Afterward, 96-well culture plate (contained RPMI-1640 medium) was treated with various concentrations of extracts, including *C.majus* extract, separately for 5 days. The results showed that Chelidonium Majus aqueous and ethanolic extracts had the highest inhibitory effect on HIV-1 protease on MT-4 cells. The minimum concentration required for complete inhibition of HIV-1 induced CPE on Mt4-cells was found to be 3.9 µg/ml (El-Mekkawy et al., 2009).

Monavari assessed the antiviral effects of Chelidonium majus against HSV-1 in Vero cell line. In this study, Vero cells were treated with the extract of Chelidonium majus (up to 1000 µg/ml concentration), before, simultaneous with, and after infection with HSV-1. Neutral red staining for cytotoxicity at different times and direct immunofluorescence technique was used to monitor the synthesis of viral proteins. The results showed that anti-viral effects of extract, simultaneously and after infection, are more significant compared with before infection effects. They showed that the extract did not have any effect on the cell to a concentration of 1000 µg/ml. The strongest inhibitory effect of

the extract was detected 1 h after Infection, suggesting that the extract can inhibit the synthesis of α gene proteins or possibly the action of the VP16 protein (Monavari et al., 2011).

Monavari examined the antiviral effects of 25 different Iranian herbs, including *C. majus*. Different parts of the plants were extracted with aqueous solvents to obtain crude extracts. Plants extracts were screened for their cytotoxicity against Vero, BSC-1, Hep-II and RD cell lines by micro-culture neutral red dye absorption and microscopically follow up for CPE. Antiviral properties of the plant extracts were determined by cytopathic effect inhibition assay and plaque reduction assay. *C. majus* extracts showed great anti-viral activity against HSV-1 such that the concentration of 500 µg / ml of extract completely prevents virus replication (Monavari et al., 2007).

Gerenčar reviewed the anti-retroviral activity of *C.majus* aqueous extract in vitro and in vivo. The results showed that higher concentrations than 100 µg / ml *C.majus* fraction pool2 (ChM-P2) has cytotoxic effects on H9 and AA2 cells. 25 µg / ml ChM-P2 reduces the activity of the reverse transcriptase of the enzyme. However, the inhibitory effect is reduced by 10 µg / ml, and no effect is seen at 2.5 µg / ml (Gerenčar et al., 2006).

In this study, the highest non-toxic concentration of aqueous extract of *C.majus* was determined at 3500 µg/ml and this concentration had an antiviral effect on HSV-1. The difference between the highest non-toxic concentration in this study and other studies can be attributed to the difference in cell type and the treatment time of the extract with the cell as well as the amount of powder obtained from the dried leaves of the plant used for the extraction. In general, by comparing this study with other ones, it can be concluded that the aqueous extract of *C.majus* may show its effect on the expression of genes or interactions with viral proteins. However, how this is related to the effect of the extract of *C.majus* on Herpes simplex virus requires further investigation.

Conclusion

The hexane extract did not have any effect on virus replication compared with aqueous extract. The concentration of 3500 µg/ml of aqueous

extract, as the highest non-toxic concentration for HeLa cells, has the strongest effect on virus replication compared to lower concentrations. This concentration of aqueous extract after 1, 2, 4, and 8 h of adsorption significantly reduced on virus replication.

Acknowledgments

This research is a graduate thesis on microbial biotechnology in Department of Biotechnology, Faculty of Advanced Sciences and Technology, Tehran Medical Sciences, Islamic Azad University that was conducted at the Medical Genomic Research Center in Tehran Medical Sciences, Islamic Azad University. We would like to thank Medical Genomics Research Center, Tehran Medical Sciences Branch, Islamic Azad University for the equipment of molecular and cell culture laboratories.

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