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Evaluation of Antiviral Effect of *Rutagraveolens* Extract on Herpes Simplex Virus Type 1 in Human Cell Line

Seyed Milad Moosavi Jazaeri[1], MasoudParsania[2]*, Paria Sadat Lavasani[3] Elahe Ebrahimi[4]

[1]Microbiology Department, Science and Research Branch of Guilan, Islamic Azad University, Guilan, Iran
[2]Assistant Professor, Microbiology and Medical Sciences Department, Science and Research Branch of Tehran, Islamic Azad University, Tehran, Iran
[3]Molecular Medicine Department, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran
[4]Microbiology Department, Islamic Azad University of Pharmaceutical sciences Branch, Tehran, Iran

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ABSTRACT

Herpes simplex virus type 1 (HSV-1) is a human specific pathogen causes several disorders. Acyclovir, Famiciclovir and Vidarabine are common antiviral synthetic drug used to treat infectious patients. However, drug resistance has been increased recently, a large abundant researches are being done to find more effective replacement therapy with these drugs. Nowadays, herbal medicine is being regarded because of its less side effects and complications. *Rutagraveolens* is a medicinal herb with anti-spasmodic, anti-inflammatory and anti-bacterial effects and can be used for treating hypertension and eyestrain. This study evaluates antiviral effect of *Rutagraveolens* extract on HSV-1 proliferation in human cell line (Hela cell). In this study virus was proliferated on Hela cell. Extract of *Rutagraveolens* was prepared. Then toxicity threshold of extract on Hela cell was determined with MTT and trypan blue methods. Maximum non-toxic concentration of extract on cells was measured. Extract anti-proliferative effect on virus was investigated at different time. The measure of infectious virus titer was determined by TCID50 method, respectively. The results show that maximum non-toxic concentration of extract on Hela cell was 0.125 µg/ml and with this concentration, extract had no effect on virus titer. In addition, the most effective exposure time to prevent virus proliferation was 2 hours after virus adsorption to the cells. According to the results, *Rutagraveolens* hydro alcoholic extract affects virus replication potentlyat early time of infection in vitro, so it can be recommended as a natural drug candidate to cure patients suffer from HSV-1 infection.

KEYWORDS: *Rutagraveolens*, HSV-1, Hela cell,anti-proliferative effect

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) is a human specific pathogen causes several disorders such as meningitis, blister, encephalitis and eye infection [1, 2]. Acyclovir, Famiciclovir and Vidarabine are common antiviral synthetic drug used to treat infectious patients but worldwide usage of these drugs cause drug resistance incidence among population around the world [3, 4]. Recently, increase in drug resistance is observed. So, researchers want to replace another effective antiviral drugs against infections. Among them, herbal medicines have been concerned because of their low side effects and complications [5-7]. *Rutagraveolens*, belongs to Rutaceae family, is one of the traditional medicinal herb used for treating diseases [8, 9]. It is being regarded for its anti-spasmodic, anti-inflammatory and antibacterial effects and can be used for treating hypertension and eyestrain [10-14]. This study evaluates antiviral effect of *Rutagraveolens* extract on HSV-1 proliferation in human cell line (Hela cell) at different time of exposure to propose this extract as a candidate drug.

MATERIAL & METHODS

Virus and cell culture:

KOS strain of HSV-1 was obtained from virology department of Tarbiat Modares University. Hela cell was cultured in Dulbecco Modified Eagle Medium (DMEM, Gibco, Germany) containing 10% fetal bovine serum (FBS, Biosera, Germany), 100 µg/ml penicillin and 100 µg/ml streptomycin. Then cultured medium was incubated at 37°C and 5% CO2 for about 24-48 hours. After viruses proliferation, titer of viruses was measured with tissue culture infective dose$_{50}$ standard protocol (TCID$_{50}$).Cells that show apoptosis signs were discarded either before extract treatment or after facing to viruses.

*** Corresponding Author:** Masoud Parsania, Assistant Professor, Microbiology and Medical Sciences Department, Science and Research Branch of Tehran, Islamic Azad University, Tehran, Iran.
E-mail: masoud_parsania@yahoo.com Phone: (98) (21) 22006660 Mobile: (98) (912) 4055246

Extract Preparation:

After detection and confirmation of herb's leaves by research institute of forest and rangelands experts, the leaves were dried and ground. To obtain hydro alcoholic extract, 30 gr of grinded herb was added to 96%methanol and was filtered through membrane. For the next step, 20 ml of extract solution was dried at 60°C temperature. The powder of extract was added to DMEM medium with the 1:4 ratio, and filtered through0.22 µ membrane, respectively. Different extract concentrations were prepared for following experiments.

Cell viability assessment against *Rutagraveolens* extract:

In this study**,** in order to detect extract cytotoxicity effects on living cells, MTT assay and trypan blue method were applied.

Trypan blue method:

Hela cell was grown in the 24 wells micro titer plate to reach monolayer confluence. Then 1000 µl of DMEM medium with 1% FBS containing 0.062, 0.125, 0.25, 0.5, 0.75 extract concentrations was added to each well. As a negative control, three wells were treated only with DMEM medium with 1% FBS. After incubation period (24, 48, 72 hour) in condition referred before, cells were tripsinized and trypan blue reagent was added to cell suspension. The ratio of the living cells were measured according to the formula:

Percentage viability= living cell/ living cell + death cell × 100

MTT assay:

2 × 10 4 cells per 200 µl DMEM containing 1% FBS were cultured in 96 well micro titer plate and incubated in condition as mentioned before. After 48 hours incubation, extract dilution with 0.062, 0.125, 0.5 and 0.7 concentration were added to each well of grown cells. This experiment was done without herb extract as a negative control. After 72 hours of incubation, supernatants were removed and each well was washed with Phosphate buffered saline (PBS). Then 20 µl of ready MTT reagent (0.005 gr/ml) was added to each well containing 80 µl of DMEM medium. Three wells filled with only DMEM medium were used as a blank. Then plates were incubated for 4 hours at 37°C. Supernatants were removed, 100 µl of DMSO (Merck) was added to each well and wells were shaken for 15 seconds. At last, OD of each well was measured by ELISA reader in 540 nm. According to the formula, living cells were calculated.

% cytotoxicity = a-b/d-b × 100

a= mean OD of test wells

b= mean OD of test wells

d= mean OD of control wells

Virucidal activity test:

In this research, the extract effect on HSV-1 was found according to TCID50 protocol. For this purpose, first, 100 $TCID_{50}$ of viruses were prepared and added to extract with non-toxic concentration and medium deprived of extract. Secondly, at 4 ° C, these solution were introduced to each well containing cell line at different times (0, 1, 2, 3 and 4 hours). Then the measurement of infectious virus titer was determined by $TCID_{50}$ method after 48 hours.

Extract anti-proliferative effect on HSV-1 at different time of exposure:

Cell treatment with extract without virus adjacency:

For determining extract effect on cells, briefly, cultured cells in 2 wells of micro titer plates were treated with maximum non-toxic concentration of extract. Then virus seeds were inoculated to each well and after passing 1 hour of virus adsorption, wells were washed with PBS buffer. Thus, 1000 µl of DMEM medium containing extract and 2% FBS was added to each well. Virus titer was determined by $TCID_{50}$ standard protocol after 48 hours incubation and removing supernatant.

Infectious cell treatment with extract through and after virus adsorption:

To investigate extract effect through virus adsorption to cells, 100 virus doses according to TCID50methodalong with DMEM containing 2% FBS and non-toxic concentration of extract were added to a well. Thisprocedure was performed to assets extract effect after 1, 2, 4, 8, 12 and 24 hours during virus adsorption tocells. As a control, one well received only DMEM medium containing 2% FBS after virus adsorption. Then virus titer was calculated with $TCID_{50}$ method after 48 hours.

RESULTS

Results from trypan blue assay show that maximum non-toxic concentration of *Rutagraveolens* extract on cell line was 0.125 µg/ml and this concentration was used for the next steps (table1).

To confirm results obtaining from trypan blue assay, MTT method was conducted with three different concentrations of extract and living cells reported respectively. After 72 hours duration. It can observe that in 0.125 µg/ml concentration More than 90% of treated cells significantly remained alive comparison with other concentrations. Results are listed in table 2.

Results from virucidal activity test were summarized in chart 1, concluded that extract had no influence on HSV-1 located outside of the cells.

Inhibitory effect of extract on virus proliferation was investigated at different time and results demonstrated that the extract has the ability to inhibit viruses propagation. Although inhibitory effect depends on the time viruses infected cell culture. The most effective exposure time was seen 2-4 hours after HSV-1 adsorption to Hela cells, while extract had no effect on proliferation before and through infection. These results are illustrate in chart 2.

Table 1. Detection of extract cytotoxicity which measured by trypan blue method

	Percentage of living cell		
Extract Concentration (mg/ml)	**after 24 hours**	**after 48 hours**	**after 72 hours**
0 (control)	97%	95%	95%
0.75	81%	81%	62%
0.5	90%	87%	80%
0.25	92%	90%	85%
0. 125	93%	92%	92%
0.062	94%	94%	93%

Table 2. Detection of extract cytotoxicity which measured by MTT method

	Percentage of living cell		
Extract Concentration (ug/ml)	**after 24 hour**	**after 48 hours**	**after 72 hours**
0.5	88%	80%	73%
0.25	93%	90%	88%
0. 125	94%	93%	93%
0.1	96%	95%	93%
0.062	97%	96%	96%

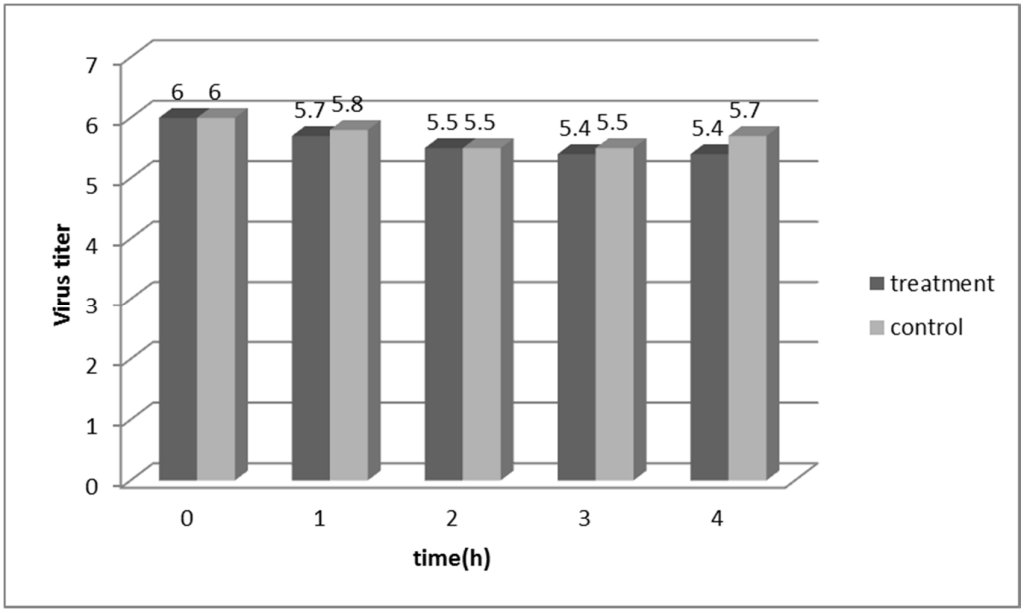

Chart 1. Virus titer before and after treatment with 0.125 µg/ml Rutagraveolens extract

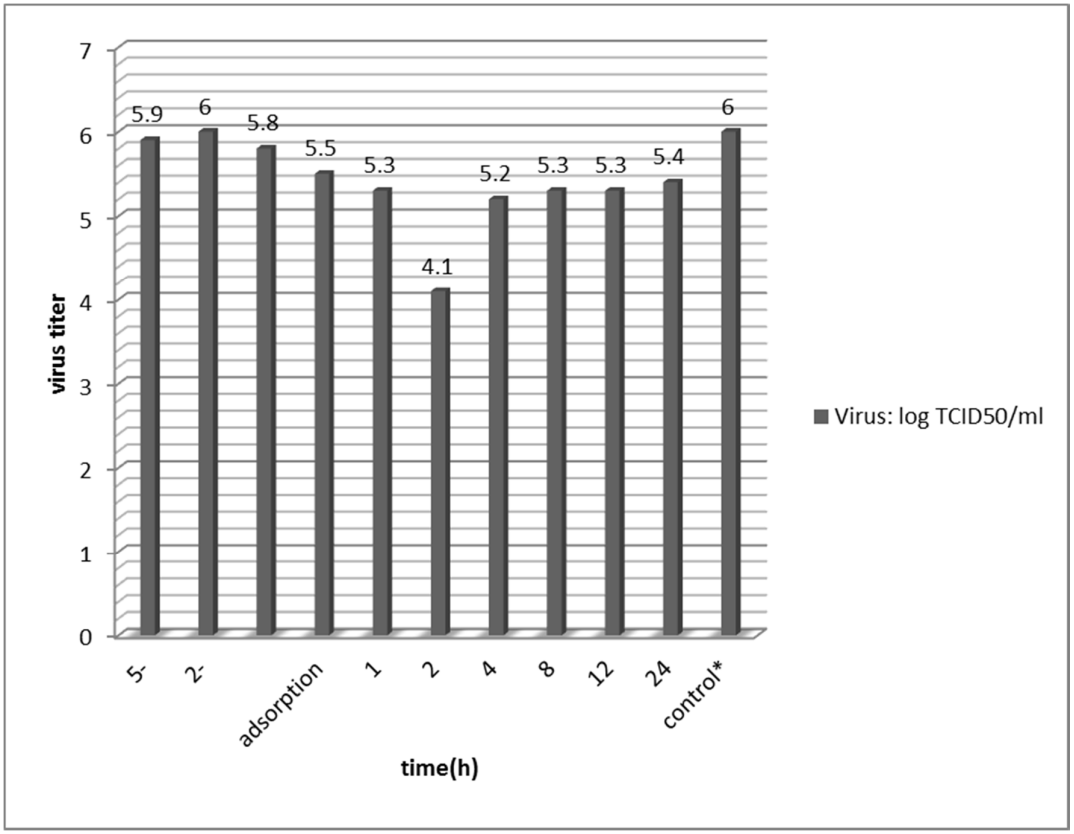

Chart 2. Effect of 0.125 µg/ml Rutagraveolens extract on virus proliferation at different times of adsorption
Control*: without extract treatment

DISCUSSION

Herpes simplex virus is responsible for various diseases among humans. Early infection caused by the virus lead to rise immune responses and produce antibody, but these antibodies are not able to immunize individuals, therefore viruses do not eliminate by immune system. In contrast, they continue their life cycle in nerves system of infected person [15]. Mechanism of common antiviral drugs are DNA polymerase inactivation. Antiviral drugs such as Acyclovir convert to monophosphate form *in vivo* by Thymidine kinase. Then it was converted to di or tri phosphate active form by cell enzymes. Active Acyclovir enter to the DNA double helix and inhibit HSV DNA polymerase, thus it inhibits replication of virus's DNA. Although approximate successful treatments are achieved, worries about consumption of these drugs are being increased regarding to incidence drug resistance mutants. Besides, complications and side effects are being noticed. Considering to all mentioned above, researchers look for new methods for treating patients suffer from HSV-1infections. Since herbal medicines have less complications and their effective components are not known well to synthetize, researchers tend to impart profit of medicinal plants [5-7]. Recent evidences determined that some plants have antiviral effects, among them, plants rich of tannins, flavonoids and alkaloids have significant anti-bacterial, anti-viral and anti-fungal effects [16, 17].In 2008, Wei Wang *et al* studied activity of antioxidant and antimicrobial *Phellodendrohamurense Rupr* extract component, belongs to Rutaceae family, showed that Acridone has great activity against HSV-1 and HSV-2 in low concentration of extract and decreases viral plaques [18]. In this study, antiviral effect of *Rutagraveolens* extract was investigated. After performing trypan blue and MTT assay to check extract toxicity, it is determined that the most non-toxic concentration of extract was 0.125 µg/ml, although Binazir .J.F *et al* in 2011, asset the influence of this extract on Hep2 cells, reported 0.039 mg/ml as a non-toxic dose[19]. One reason for this difference was correlated to variations among Hep2 and Hela cells resistance. Our findings from antiviral extract effect at different time of virus infection or adsorption showed that inhibitory effect of extract was done 2 hours since virus adsorption. This was in accordance with monavari *et al*. that examined effects of 86 types of *Glycyrrhizaglabra* extracts on HSV-1 and concluded these extracts have effects after 1 hour of adsorption[20]. In another study done by Zandi *et al* indicated that green algae extract prohibit viruses proliferation before their attachment to cells. This resultis different from our findings that show our hydro alcoholic extract affects virus replication potently at early time of infection *in vitro*[21]. Moller *et al* reported pre-treatment of HSV particle with *Brackenridgea Zanguebarica* extract decreases viral plaques formation up to 99% just 1 hour before cells exposure. In this study, we observed 27% reduction of viral plaques formation when extract added to cells before infection however only insignificant diminution was detected after infected cells exposing to extract. Thus, it is concluded that extract and its components have no effects on virus proliferation [22].

Herpes simplex virus expresses its immediate early gene almost after 2 hours post infection phase [23]. Regarding to HSV-1 proliferation cycle, alpha genes expressed at early 4 hours of proliferation cycle and *Rutagraveolens* probably interfered with propagation. It is obvious this genes prohibit virusto attachment and permeate cells. Further researches are needed to find out this mechanism better and in details. Other studies done by Kostova *et al* in 1999 and Mallikharjuna *et al* in 2007 showed *Rutagraveolens* extract has alkaloids and flavonoids and antiviral effect of extract associates to these components [24, 25]. Further researches should be done to fractionate extract components and to detect the effect of each ingredients on virus replication and then investigated *in vivo*.

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