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ORIGINAL ARTICLE

In vitro anti-HSV1 activity of aqueous extract of *Areca catechu* L.

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ABSTRACT

Background and Objectives: HSV-1 is known as a very contagious virus and the main cause of cold sores or fever blisters. Herein, the aqueous extract of *Areca catechu* L. was evaluated for its anti-HSV-1 activity, compared to the standard control (acyclovir). Also, the effect of extract on the expression of UL46 and US6 genes that accumulate late in viral infection, was studied.

Materials and Methods: The aqueous extract was obtained by the maceration of powdered plant in boiling water. Its antiviral activity was evaluated on Vero cells infected with HSV-1 at different times: 2 h pre-infection, simultaneous infection, and 4 h post-infection, using MTT assay. The effect of extract on the expression of genes was investigated with quantitative real-time PCR.

Results: The aqueous extract of *A. catechu* induced the inhibition of infection with the IC_{50} value of 110.52 ± 1.36 µg/ml. Also, it reduced the expression of UL46 when it was added 2 h pre-infection at 100 µg/ml. Moreover, reduction of expression of US6 was observed at the same concentration when the extract was used simultaneously with the occurrence of infection and 4 h post-infection.

Conclusion: *A. catechu* can be considered an essential element of natural-based anti-HSV-1 agents.

Keywords: *Areca catechu*; Betel nut; Gene expression; Herpes simplex virus type 1; Medicinal plant

INTRODUCTION

Herpes simplex virus (HSV) is a double-stranded DNA and contagious virus that is classified into two types; HSV-1 (type 1) and HSV-2 (type 2). HSV-1 is mostly transmitted by oral-to-oral contact leading to cold sores. HSV-2 is a sexually transmitted infection that causes genital herpes. Although HSV-1 and HSV-2 encode many equivalent proteins, many minor changes have been recorded (1). The virus is also able to create a lifelong; latent infection in trigeminal or lumbosacral ganglia, which can be reactivated by

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physiological and psychological stress (2). A study estimated that 3583.5 million of the 5632.6 million global population of 0-49-year-olds were infected orally with HSV-1 (63.6%) in 2016. The largest number belonged to WHO South-East Asia Region (3).

Blisters, erythema, and ulcers are the main symptoms of herpes labialis infection (4). On the other hand, symptoms of herpes genitalis infection are indicated by macules and papules in the anal or genital region that may lead to the formation of pustules, vesicles, and ulcers (5). Thus, HSV infections prevention and control are crucial to improve the general health of people with recurrent infections. There is no certain cure for HSV infection, however, antiviral medications are frequently prescribed to prevent or reduce symptoms and recurrent outbreaks (6). Acyclic guanosine analogs are recognized as the first-line therapy for HSV infection control that aim viral DNA replication. This class includes acyclovir (Acv) which has been considered the gold standard for both treatment and prophylaxis since the 1980s (7). The chronic nature of HSV infections following long-term use of antiviral medications has caused drug resistance, particularly in immunocompromised individuals (7). Consequently, development of effective therapeutic strategies such as medicinal plants seems to be necessary. In this respect, a wide range of plant extracts have been found to be a strong tool against herpes which were reviewed by Garber et al. (8). Also, good results have been obtained by clinical trials of *Melaleuca alternifolia* oil gel (9), an herbal mixture containing *Ganoderma lucidum* (10), leaves of *Salvia officinalis* as well as sage and rhubarb extracts (11). Natural remedies also have depicted lower toxicity, side effects, and resistance to medications than the synthetic drugs (12).

A. catechu belongs to the family Arecaceae, is a medium-sized palm tree, known for its seeds that are called betel nuts or areca nuts. Various compounds such as alkaloids, tannins and polyphenols have been identified in the phytochemical analysis of the plant (13). It has depicted various biological activities such as anti-Alzheimer's (14, 15), antibacterial and antioxidant (16), anti-inflammatory (17), anti-allergic (18), and anthelmintic properties (19). Apart from valuable medicinal properties of the betel nut, it is globally abused and chewing or smoking betel nuts especially in south-east Asia is popular due to the presence of acrecoline. It is commonly used as a psychoactive substance and has multiple neurological side effects (20). To profit from biological activity of *A. catechu* and its constituents, comprehensive research is needed to investigate the molecular mechanisms.

Encouraged by the efficacy of herbal remedies for the treatment of HSV-1 infection, we focused on the aqueous extract of *Areca catechu* L. and evaluated it's *in vitro* antiviral activity against HSV-1 followed by the study of expression of UL46 and US6 genes due to their role in the pathogenesis and virulence of HSV-1 infection.

UL46 has been demonstrated to downregulate TBK1-dependent antiviral innate immunity and has a definite role in activating the PI3K/AKT host path. Promotion of cell survival connection with the host causes the degradation of immune-boosting mechanism to inactivate T cells that plays an important role in pathogenesis of infection. Glycoprotein D (gD) from HSV-1 is coded by HSV gene US6 that is an important receptor for the virus in epithelial cell and lymphocyte infections (21, 22). However, the expression of UL46 and US6 in the presence of medicinal plants has not been widely studied (23).

MATERIALS AND METHODS

Betel nuts (*Areca catechu* L.) were obtained from the Tehran market, Iran, identified, and deposited in the herbarium of the Faculty of Pharmacy of Tehran University of Medical Sciences using the code PMP-691.

For replication of HSV-1, an African green monkey kidney cell line (Vero) was selected as it is considered the most used continuous cell line for the propagation of HSV-1 and purchased from the Genetic Resource Center of the Academic Center for Education, Culture, and Research (Accession Cell No: IBRC C10001). HSV-1 was obtained from the virology laboratory of the High Institute for Research and Education in Transfusion Medicine. MTT reagent ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and the all chemicals used in the test were obtained from Aldrich and Gibco. The quantitative PCR (qPCR) was performed using the Favorgen (FavorPrep™ Tissue Total RNA Mini Kit). cDNA synthesis was performed using the YTA (YT4500).

Preparation of the aqueous extract of *A. catechu*. The powdered plant (betel nuts, 20.0 g) was boiled in water (200 mL) for 15 min, the resulting mixture was centrifuged for 5 min at 3000 rpm, the solvent

was evaporated under vacuum, and the residue was lyophilized to give the corresponding extract (7.7 g, 38.6%) (14, 15).

Cell culture. The cell culture was performed using the DMEM culture medium with 10% FBS, and 1% P/S in an incubator with 5% CO_2 at 37°C (23).

Virus replication. Cell lines were infected with a multiplicity of infection (MOI) of 1:10 (virus: Vero cell) in a 25 cm^2 dish, then incubated at 37ºC and 5% CO_2 for 24 h. After the onset of the Cytopathic Effect (CPE), it was kept at -80°C (23).

Virus titration. Cell culture was conducted in a 96-well plate and 10 logarithmic dilutions were provided from the viral stock. Four Vero cell monolayers were used as cell control, virus control, acyclovir, and each viral dilution. The related viral dilution (100 µl) was added to each well and the serum culture medium (2%, 300 µl) was added to the Vero cells after 1 h. Finally, the onset of CPE was daily screened and the virus titration was calculated based on the Reed-Muench method (23).

Quantitative assessment of cell survival using the MTT assay. The survival rate of the cells treated with the aqueous extract of *A. catechu* was measured at four concentrations (400, 200, 100, and 50 µg/ml, prepared in DMSO/methanol: 40/60) replicated three times, using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay, compared with acyclovir as the positive control and normal Vero cells. Herein, 2×10^4 cells were cultured in each well of the 96-well plate (the cell number was calculated considering the volume of cell culture plate) after adding trypsin to Vero cells and counting them. After reaching 80-90% cell density in each well, the plant extract with different concentrations of 400, 200, 100, and 50 µg/ml was administered (the extract exhibited high antiviral activity with no toxicity), and after 18 h exposure (time to start the MTT assay), 10 µl of the MTT solution was added to each well and incubated at 37ºC in 5% CO_2 for 3-4 h. Then, 100 µl of the MTT solvent was added to each well and it was placed on a shaker for 15 min to dissolve the sediment particles. Then, the absorption was measured at 570 nm. The ratio of living cells was calculated by the following formula:

Percentage of survival = ([absorption of infected cells – empty] / [absorption of infected control cells – empty]) * 100

Then, based on the non-cytotoxic effect of the tested concentrations of the plant extract, an MTT assay was performed in cell culture at different times of occurrence of infection with HSV-1. Also, the IC_{50} values were calculated by using GraphPad Prism8.

Quantitative Real-time PCR (qPCR). The RNA extracted from the Vero cells infected with HSV-1 and then treated with the plant extract, was investigated at various concentrations of 200, 100, and 50 µg/ml and different times of occurrence of infection with HSV-1 (MOI = 0.1) to study the gene expression using the corresponding kit. Then, RNA quality and concentration were measured using NanpDrop Microvolume Spectrophotometers. cDNA synthesis was also performed using the related kit. To investigate the expression of UL46 and gD (US6), Real-time PCR was performed by the Sybr Green Kit developed by Yekta Tajhiz and specific primers developed by the Gene Fanavaran Company (the exclusive representative of TAG Copenhagen, Denmark) (Table 1). The sequence and position of the primers were selected based on the NCBI genetic database (Table 1) and were confirmed using the online software of the NCBI site (PRIMER BLAST) and offline software such as gene runner.

According to the temperature profile, the initial denaturation was achieved at 95°C for 10 min. Then, 35 cycles of denaturation and annealing occurred at 95°C and 51.5°C, respectively, for 10 and 20 s. Next, the primer extension step occurred at 72°C for 20 s. The final cycle was performed at 72°C for 5 min and melting curve analysis was conducted by a Rotor-gene to determine the reaction's specificity. UL46, US6, and GAPDH gene-specific primers were used at 10 µM.

Statistical analysis. A one-way ANOVA test was performed using Prism graph pad software and the related charts were plotted. A p-value less than 0.05 was statistically significant.

RESULTS

Microscopic observations (Fig. 1) indicated that acyclovir at concentrations above 50 µg/ml was toxic to the cells. However, the plant extract did not induce toxicity up to 400 µg/ml. It should be noted that the concentration of DMSO as the extract solvent was below 1%.

Table 1. HSV-1 virus UL46 and US6 genes primers details and GAPDH (reference gene)

Name	Primer Sequence (5′ to 3′)	Length (n)	PCR Product Size (bp)
UL46 F	GTTTTCGTAGACCCGCATCC	20	182
UL46 R	ATGGAAGCCACGTATCTGACG	21	
US6)gD(F	CTATGACAGCTTCAGCGCCGTCAG	24	112
US6(gD)R	CGTCCAGTCGTTTATCTTCACGAGC	25	
GAPDH F	ACGGATTTGGTCGTATTGGG	20	210
GAPDH R	TGATTTTGGAGGGATCTCGC	20	

Inhibition of infection evaluated using MTT assay. According to results obtained from the MTT assay, the best cell survival rate and inhibition of replication of HSV-1 toward Vero cells at a MOI equal to 0.1 with a titer of 3.4×10^4, was observed at 37.5 µg/ml for acyclovir and 200 µg/ml for the aqueous extract of *A. catechu* (Figs. S1 and 2). The extract showed anti-HSV-1 activity with IC_{50} value of 110.52 ± 1.36 µg/ml, compared with acyclovir (IC_{50} = 28.65 µg/ml) (Fig. 2).

Quantitative real-time PCR (qRT-PCR). The expression levels of UL46 and US6 were measured by qRT-PCR, compared by untreated viruses as the positive control. As shown in Fig. 3, treatment of viral particles (MOI = 0.1) with the extract led to the reduction of UL46 and US6 genes expression (Fig. 3). It reduced the UL46 gene expression 2 h pre-infection at the concentration of 100 µg/ml (fold change = 12.5). However, the gene expression was reduced at the same time as the infection occurred and 4 h post-infection with fold change equal to 6.0 and 6.3 respectively. The most significant reduction of US6 gene expression was observed at simultaneous and 4 h post-infection by the plant extract at 100 µg/ml with fold change values of 31.7 and 33.5, respectively.

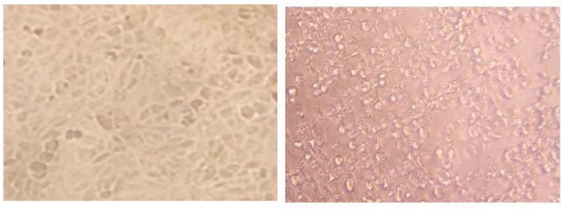

Fig. 1. Microscopic observation of Vero cells. A: Normal cells and B: toxic cells

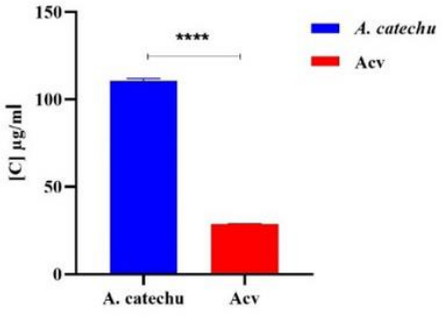

Fig. 2. The activity of aqueous extract of *A. catechu* (IC_{50} = 110.52 µg/ml). The extract showed a significant effect on HSV-1 ($p<0.0001$) comparing with Acv (IC_{50} = 28.65 µg/ml).

DISCUSSION

Focusing on the successful results reported on the anti-HSV-1 activity of medicinal plants (8), the aqueous extract of *A. catechu* was investigated in this study. In this respect, antiviral activity of the plant aqueous extract was evaluated at different concentrations and times (2 h pre-infection, simultaneous infection, and 4 h post-infection) as well as survival rate of the cells using MTT assay. It was found not to be toxic up to 400 µg/ml and showed the best activity against HSV-1 with IC_{50} value of 110.52 ± 1.36 µg/ml.

Addition of the plant extract to the cells at the same time as the infection occurred (simultaneous infection), led to the statistically significant reduction of cell death ($p<0.0001$) suggesting that the extract may have effects on the virus-cell binding step and or inhibiting the HSV-1 gene expression.

As inhibition or reduction of expression of UL46 and US6 can suppress HSV replication during infection, treatment of HSV-1-infected Vero cells with the *A. catechu* aqueous extract was investigated and expression of UL46 and US6 was remarkably reduced at the concentration of 100 µg/ml, compared to the control (virus). This result demonstrates the effect of

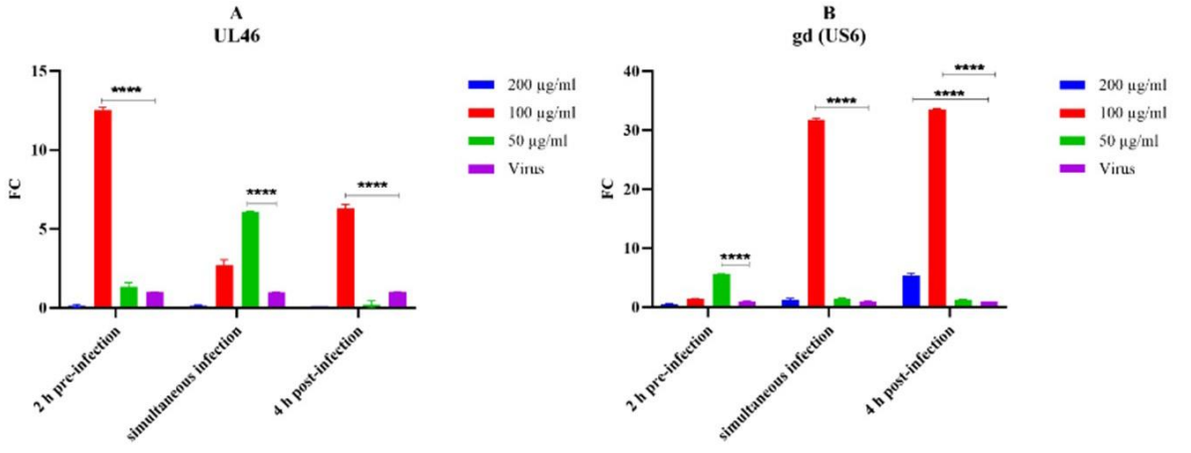

Fig. 3. Genes expression in the presence of the extract at different times, expressed by fold change (FC): A) UL46 and B) US6 (gD).

the extract on the cell surface receptors leading to the reduction of cell attachment by covering the cell surface receptors or altering their structure. Also, real-time PCR tests revealed a significant inhibition of synthesis of virus gD protein after treatment.

Previously, we reported anti-HSV-1 effect of the aqueous extract of *Artemisia aucheri* (23). It could inhibit Vero cells death with IC_{50} = 17.87 µg/ml. The expression of genes UL46 and US6 was remarkably reduced in the presence of the extract at the concentration of 100 µg/ml. Compared with our results, *A. aucheri* showed more potent activity than *A. catechu* (IC_{50} = 110.52 µg/ml).

The chloroform fraction of *Acanthospermum hispidum* and *Acanthospermum australe* depicted a dose-dependent antiviral activity via virus–cell binding and virus cycle in a post-entry mechanism by decreasing replication and the expression of early and late viral genes: Tk (an early gene), gB, gC and, gD (late genes). However, the presence of flavones was identified in this fraction (24).

The phytochemical analysis of aqueous extract of *A. catechu* has indicated the presence of flavonoids 98.19 (mg RE/gDW), phenols 47.21 (mg GAE/gDW), and alkaloids 14.29 (mg ARE/gDW) (25), which have been known for their anti-HSV-1 activity via intricate mechanisms.

Flavonoids have attracted lots of attention in *in vitro* experiments by interrupting key stages of the viral life cycle including attachment to host cells, entry, DNA replication, latency, and reactivation (26). Evaluation of 18 flavonoids by Park et al. (27), revealed that epicatechin, epigallocatechin, epigallocatechin gallate, naringin, chrysin, fisetin, and galangin induced a strong antiviral activity against HSV-1 (EC_{50} = 2.5 µM), however, naringin and galangin possessed lower toxicity on Vero cells (CC_{50} = 1000 µM). Moreover, the results from the clinical study of 68 individuals with oral herpes treated with an herbal blend enriched with quercetin (100 mg) (Gene-Eden-VIR/Novirin, administered daily for 2 to 36 months) indicated it notably safer and more effective than valacyclovir (27).

The polyphenol-rich extract mainly containing catechin, eriodictyol-7-O-glucoside, gallic acid, protocatechuic, and caffeic acid from pistachios kernels (*Pistacia vera* L.) was found to be effective against HSV-1 in a concentration-dependent manner. It reduced plaque formation at the concentration of 0.8 mg/ml, compared with acyclovir (20 µM). It showed remarkable inhibitory activity when was instantly added after the attachment step (28).

Alkaloids possessing alkaline nature have shown important antiviral activity (29), e. g. alkaloid extract of *Tripterygium hypoglaucum* demonstrated good activity against HSV-1 in Vero cells with IC_{50} value of 6.5 µg/ml, significantly more potent than acyclovir (IC_{50} = 15.4 µg/ml) (30). Also, the extract reduced plaque formation 35% higher than that of acyclovir, in a concentration range from 6.25 to 12.5 µg/ml. It merits mentioning that transcription of UL30 and UL39 (delayed early genes) as well as US6 (a late gene) of HSV-1 genome were suppressed by 74.6%, 70.9%, and 62.6%, respectively, at the concentration of 12.5 µg/ml of the extract, compared with the positive control.

CONCLUSION

In conclusion, *in vitro* evaluation of the *A. catechu* aqueous extract against HSV-1 depicted satisfactory results such as reducing UL46 and US6 genes expression, probably due to interruption in HSV-1 binding to Vero cells or inhibition of the intermediate genes expression and late virus genes expression. It seems that the plant can be considered in the development of herbal agents for the treatment of HSV-1.

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