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ORIGINAL ARTICLE

Efficacy of traditional herb aqua extract of *Teucrium stocksianum* and its fractions against HSV-1 virus expression levels of genes (UL46 and US6)

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ABSTRACT

Background and Objectives: Recently, the anti-herpetic activities of different plant species have been investigated. This study evaluated the effects of *Teucrium stocksianum* aqueous extract on the HSV-1 virus-infected Vero cell.
Materials and Methods: The IC_{50} of the aqueous extract was obtained by the maceration of the plant in boiling water and has been measured with the MTT method, also the q-PCR was used to study viral gene expression reduction.
Results: Results of the MTT test indicated that the highest percentage of metabolic activity was observed in the 75 µg/ml concentration of *Teucrium stocksianum*'s aqueous extracts (IC_{50}=45.5µg/ml). Time intervals of 24 and 48 hours after viral infection revealed that the cell viability is reduced by the viral infection time (MOI=0.1), log 10^{-3}, p <0.001). Furthermore, the plant's aqueous extract concentration almost avoids cell viability reduction. Through Q-PCR results; the reduction of viral proliferation revealed that the low expression of genes UL46 and US6 were significant in the presence of different treatments utilized in the experiment.
Conclusion: *T. stocksianum*, has an anti-viral property and may be considered as a remedy for anti-HSV-1 agents.

Keywords: Lamiaceae; *Teucrium*; Herpes simplex virus type 1; Aqueous extract; Gene expression; MTT assay; Ral-time polymerase chain reaction

INTRODUCTION

The completed DNA genomic sequence of HSV-1 is 152-kbp (1, 2). Human alphaherpesviruses are among a group of viruses that produce viral infections in the majority of humans. HSV-1 is transmitted by contact with an infected person who has the virus's reactivations. HSV-1 is typically associated with oral lesions. HSV-1's genomes contain at least 74 genes (or open reading frames, ORFs).

This genome contains two extended regions of unique sequence (UL contains 56 viral genes and the US contains 12 viral genes), each of which is bounded by a pair of inverted repeat elements (TRL-IRL and IRS-TRS).

HSV genes' transcription is catalyzed by the infected host's RNA polymerase II. Through the following infection, immediate early genes are the first to be

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expressed, which encode the proteins with the regulation role of the early and late viral genes' expression. Early genes' expression, allows the synthesis of enzymes involved in DNA replication and the production of certain envelope glycoproteins. Expression of late genes occurs last; this group of genes predominantly encode proteins that form the virion particle.

Treatment against these viruses usually involves general-purpose antiviral drugs that interfere with viral replication, reduce the physical severity of outbreak-associated lesions, and lower the chance of transmission to others (3). It has been reported that daily use of antivirals such as Acyclovir and valAcyclovir can reduce reactivation rates. However, the extensive use of anti-herpetic drugs has led to drug resistance, which leads to treatment failure (4). Efforts have been made to produce a vaccine in this field (5). For this reason, the anti-herpetic activity of different plant species has been investigated (6-9).

Numerous researchers are focusing on plant-based medicines due to their strong safety profiles (10). The genus *Teucrium* belongs to the family Lamiaceae and contains 340 species. Medicinal properties of various species of *Teucrium* are reported in science showing antioxidants, antispasmodic, antimicrobial, and anti-inflammatory characteristics (11, 12).

Phytochemical screening confirms the presence of flavonoids, tannins, saponins, anthraquinone, steroids, phlorotannins, terpenoids, glycosides and reducing sugars. METS was found safe at a dose of 1000 mg/kg body weight (13).

The ethanolic extract and sub-fractions of *T. stocksianum* displayed marked to moderate anti-inflammatory, in mice. The sub-fractions, ethyl acetate fraction (EAF) demonstrated excellent anti-inflammatory action at the highest tested dose (14).

The Lamiaceae family is rich in essential oils. The main components of the essential oil reported from the genus *Teucrium* are alpha-pinene, linalool, caryophyllene oxide, Germacrene D, beta-caryophyllene, and delta cadinene. These phytochemicals possess antimicrobial, cytotoxic, phospholipase, and esterase inhibitory properties (15) and can prove very useful leads for novel drug development. *Teucrium stocksianum* is a species found in the North of Iran (Mazandaran and Semnan).

It grows in mountains in shades. This plant is used in folk medicine for treating diarrhea, cough, jaundice as well as abdominal pain (16). Crude saponins isolated from it have shown cytotoxic and anthelminthic effects (17). These scientific studies indicate the high medicinal potential of *T. stocksianum.* Its growth in nature and the presence of valuable phytochemicals in this genus prompted us to carry out the present genetic study to investigate the effect of *Teucrium stocksianum*'s aqueous extract on the HSV-1 virus-infected Vero cells derived from the kidney of an African green monkey. This is while the genetic study in the presence of medicinal plants has not been widely studied (8, 18).

MATERIALS AND METHODS

Plant material. Aerial parts of *Teucrium stocksianum* were collected in July and August from its vegetation area near Dolat Kodeh village (Versak-Abbas Abad) located in Savadkuh, Mazandaran. They were approved under the supervision of a botanist of Semnan Agricultural Jihad Research Center and deposited in the herbarium of the Biotechnology Research Tehran using the code IBRC P1006609.

Plant extract. After transfer to the laboratory, it was dried in optimal conditions (shade, room temperature, and suitable humidity). First, 30 grams of the aerial parts of the plant were completely crushed and ground, transferred to a beaker of 1000 ml and 300 ml of sterile distilled water, and covered with paraffin, and then it was left at room temperature for 72 hours. A balloon was placed on the heater at a gentle temperature until it boiled. The whole solution was then filtered through sterile gauze and filter paper. Afterwards, it was frozen at -20°C (8, 19).

Treatments. We used Acyclovir (ACV), (which is an antiviral medication and is primarily used for the treatment of herpes simplex virus infections, chickenpox, and shingles), as well as *T. stocksianum* water extract of 25, 50, 75, and 100, microgram (µg) per liter, 2 h before virus infection until 4 h after virus infection.

Cell culture. Vero cells derived from the kidney of an African green monkey, were cultured in DMEM (Dulbecco's Modified Eagle) Medium, Penicillin G (µml 100), and Streptomycin (100 µg/ml), and kept in a CO_2 incubator at 37°C. When the cell density reached over 90%, they were treated with trypsin. These cells were stained with Trypan blue dye (10%),

and the number and percentage of vital cells were determined. Later on, 105/ml cells were transferred to a culture flask.

Virus multiplications and titration. Vero cells were cultured in a 25 cm^2 flask and kept in a 5% CO_2 incubator at 37°C for 24 h. After inoculating the HSV-1 virus with MOI (1:10) and with the appearance of cytopathogenic effect (CPE), they were stored in a freezer at -80°C (18).

We prepared 10 logarithmic dilutions of the cultured virus stock. For each dilution, control Vero cells, as well as virus control, in four replications in a 96-well cell culture Plate were prepared. A 100 µl of infected viral dilution was added to each replicate and after 1 h, two ml of medium containing 5% serum was added. The CPE was daily inspected and finally, the virus titration was determined by the Reed Muench method (8).

Aqua extract effect on virus. To determine the effect of extraction on the virus, the Vero cells were cultured on 96-well plates. 200 µl of Vero cells ($1*10^5$ cells/mL) were placed in the wells and were incubated at 37°C for 24 h. Later on, the cell culture with the virus was prepared (MOI = 0.1) and incubated for 24 h, to which 10 µl of MTT was added. The MTT test was used to test the viability of the cells (8, 18).

Q-PCR of the genes UL46 and US6. Vero cells were inoculated with HSV-1 and treatment with an aqueous extract concentration of 75 µg / ml was applied for RNA extraction by FavorPrep™ Tissue Total RNA Mini Kit. Then, C-DNA synthesis was also performed by Yekta Equip Kit (Iran). Finally, the Real-Time PCR and expression of UL46 and us6 (gD) were determined by using the cyber-green kit of Yekta-Tajhiz and using specified primers for the target gene. Also, the reference gene of GAPDH was used for normalization. Details of primers for the HSV genes and the reference gene are provided in Table 1.

Statistical analysis was performed using GraphPad software and the one-way ANOVA test (p-value<0.05).

RESULTS

Cells culture. The 4.5 million cells in the 25 cm^2 flask, which were infected by HSV-1 virus with titer 1×10^3 $TCID_{50}$ (Median Tissue Culture Infectious Dose) /ml (MOI=0.1), revealed that the cell vitality is reduced by the viral infection time. Also, *Teucrium stocksianum*'s aqueous extract can reduce the viral infection rate. In the 25 (µg/ml) concentration of plant extract, after a 4-hour viral infection, the lowest number of cells occurred while the highest number of cells occurred in plant water extract in the concentration of 75 (µg/ml), 2 h before viral infection.

Table 1. Primers of US6 and UL46 genes of HSV-1 virus and the reference gene (GAPDH)*

Name	Sequences	Length
UL46 F	GTTTTCGTAGACCCGCATCC	183
UL46 R	ATGGAAGCCACGTATCTGACG	
US6(gD)F	CTATGACAGCTTCAGCGCCGTCAG	112
US6(gD)R	CGTCCAGTCGTTTATCTTCACGAGC	
GAPDH F	ACGGATTTGGTCGTATTGGG	230
GAPDH R	TGATTTTGGAGGGATCTCGC	

* Primers were used with a concentration of 10 µM

MTT test. The *T. stocksianum* aqueous extract's effects on HSV-1 activity were evaluated in comparison with acyclovir as the positive control and Vero cells as normal.

The highest percentage of metabolic activity as revealed by the MTT test was observed in the presence of 75 (µg/ml) of aqua extract, just two hours before viral infection (Fig. 1).

The lowest percentage of metabolic activity occurred in 25 (µg/ml) of aqua extract after a 4-hour viral infection.

The cytotoxicity and growth prohibition of the cells at 75 (µg/ml) were significant (P<0.001). The inhibition curves were constructed using the GraphPad program (GraphPad,San Diego, California, USA), and median inhibitory concentration (IC_{50}= 45.79 ± 0.96) values were calculated (Fig. 2).

Acyclovir was tested at concentrations between 25 -100 µg/ml, and it was found that the best cell survival rate with HSV-1 replication inhibition (0.1 MOI) in Vero cells (3.4×10^4 cells), was observed at 37.5 µg/ml concentration of the acyclovir. In three series, each extract concentration (25,50,75, and 100 µg/ml) was tested 4 times simultaneously with infection.

Real-time PCR. After pure RNA extraction, (Nanodrop showed a light absorption ratio of 280/260 =

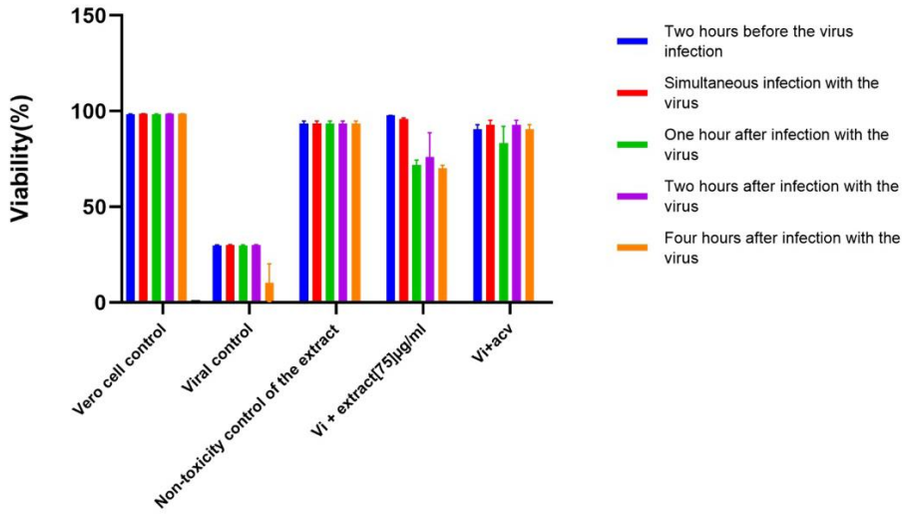

Fig. 1. MTT assay of concentration 75 µg/ml of the *T. stocksianum* aqueous extracts at different times, compared to the positive control (acyclovir) and normal (Vero cells).

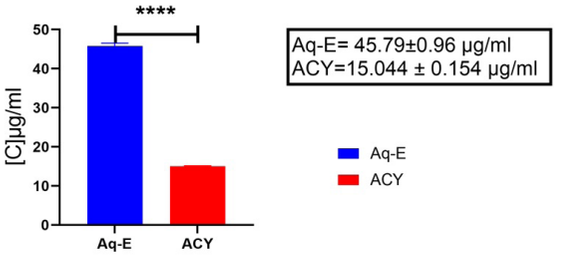

Fig. 2. The activity of aqueous extract of *T. stocksianum* (IC_{50}= 45.79 µg/ml). The extract showed a significant effect on HSV-1 (p<0.0001) compared with Acy (IC_{50} = 15.04 µg/ml).

1.89-1.99). The expression of gene UL46 in the presence of different treatments is provided in Table 1. Similarly, the expression of gene US6 in the presence of different treatments provided a significant reduction in viral gene expression in the presence of the aqueous extract of *Teucrium stocksianum*, which was observed during various hours of exposure (Table 2).

In general, the present study revealed that an aqueous extract of *T. stocksianum* significantly reduces the virus titration) (1× 10^2 50% tissue culture infectious dose, $TCID_{50}$/ ml). The level of gene expression of US6 in comparison to acyclovir was 10 times lower. However, in UL46, it was equal to the result of the acyclovir in Vero cells inoculated with HSV-1 virus at a MOI of 0.1 (P = 0.01).

DISCUSSION

The present study revealed that an aqueous extract of *Teucrium stocksianum* can significantly reduce the HSV-1 virus titration and the virus gene expression. It can be considered, that medicinal plants' extracts contain anti-viral properties.

In the present study, the 37.5 µg/ml concentration of Acyclovir with one-fold change (due to toxicity of acyclovir at higher concentrations than 37.5 µg/ml on Vero cells), is considered as the control group

Table 2. Treatments used for expression of US6 and UL46 genes

Target RNA groups	[1]FC US6*	[1]FC UL46*
Acyclovir	1.141	1.052
Plant aqueous extract 2 h before infection	10.50	0.95
Plant aqueous extract at the same time of infection	7.56	1.01
Plant aqueous extract 4 h after infection	6.55	0.67

*P-Value < 0.0001

[1]FC: Fold Change gene expression

of comparing the UL46's and US6's gene expression reduction.

The antiviral effect of *Teucrium stocksianum*'s aqueous extract on the reduction of HSV-1's UL46 gene expression (late tegument protein) was equivalent to Acyclovir ($P<0.05$ and $0.4 <$fold change<1.4) in this study.

Teucrium stocksianum's aqueous extract's effect on gD protein (effective in virus attachment) was times higher than the synthetic drug ($P<0.001$ and $3.1<$fold change<18.7). US6's fold change reduction was based on two reasons, extract efficiency on US6's gene expression's reduction and higher concentration of aqueous extract (100 µg/ml) than Acyclovir's concentration (37.5 µg/ml).

For example, some activities were correlated with the high concentration of rosmarinic acid, a phenolic compound known to possess antiviral activity (20).

Previously, we reported the anti-HSV-1 effect of the aqueous extracts of *Areca catechu* and *Artemisia aucheri* (8, 18). They could inhibit Vero cell death with IC_{50}=110.52 µg/ml and IC_50= 17.87 µg/ml. The expression of genes US6 and UL46 was remarkably reduced in the presence of the extract at the concentration of 75 µg/ml. Compared with our results, *A. aucheri* showed more potent activity than *T. stocksianum* (IC_{50}=45.5µg/ml) and *A. catechu* (IC_{50}= 110.52 µg/ml).

Ansari (2014) reported that *T. polium* was a rich source of rosmarinic acid (1.8%, w/w), weak cytotoxic activity (maximum non-toxic concentration = 1000µg/mL), and anti-HSV-1 affected by the exposure time and the concentration of the extract used. In fact, after one hour of cell infection, 93.1% of HSV-1 was inhibited by 50 µg/mL of plant extract. This percentage of inhibition reached 100% after one hour of exposure to 100 and 250 µg/mL of felty germander extract (21).

The antiviral activity of *T. polium* methanolic extract was evaluated on Coxsackievirus HSV-2. *T. polium* methanolic extract was tested. The minimal inhibitory concentrations ranged from 6.25 to 25 mg/mL for Herpes simplex virus type 2(HSV-2). The 50% cytotoxic concentration(CC50) on VERO cell lines of African green monkey's kidney, was measured at 209 µg/mL which had no antiviral activity (22).

Naturally, plants maintain the active ingredients in order to react antagonistically against bacterial and viral infections. Ullah (2018) synthesized silver NPs through chemical and biological methods from the aqueous extract of *Teucrium stocksianum* and evaluated them for anti-leishmanicidal activity (23).

Vuko in 2020 showed that some of the *Teucrium* species have improved the knowledge of the possible application of essential oils to prevent diseases caused by plant viruses. Essential oil of *Teucrium Arduini* applied on the leaves of local host plants; significantly reduced the number of lesions on both TMV and CMV-infected plants. Aside from *Teucrium Arduini*, four additional *Teucrium* species (*T. montanum*, *T. polium*, *T. chamaedrys* and *T. flavum*) are also sources of bioactive molecules with anti-phytoviral activity against CMV infection (24).

CONCLUSION

Following our experiments in antiviral test design; herein, all repeated assays exhibited anti-HSV-1 activity with *T. stocksianum* (IC_{50} =45.5µg/ml) values in the range of 75 µg/ml as the most potent inhibitor. In conclusion, the present study proposes that aqueous extracts of the medicinal plant *T. stocksianum* have antiviral properties and can be considered an anti-HSV-1 extract.

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