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Investigating the Safety and Efficacy of the Synthetic Drug Herbix on Immune Responses Involved in the Treatment of a Mouse Model of Herpes Simplex Virus

Mohammad Ali Khodadoust[1], Gholam Reza Keramatian[2], Najmeh Kaffash Farkhad[1], and Jalil Tavakol-Afshari[1]

[1] Department of Immunology, Immunology Research Center, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
[2] Department of Medicine, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

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ABSTRACT

Herpes simplex virus-1 (HSV-1) infections can cause significant harm to individuals, including blindness, congenital defects, genital herpes, and even cancer, with no definitive cure .so, finding new treatment strategies is crucial.

In this study, 25 male BALB/c mice were used to conduct a mouse model of herpes by subcutaneously injecting an HSV-1 suspension (100 μL of 1×10^9 PFU/mL). The mice were divided into 5 groups with groups 1 to 3 designated as intervention groups, and groups 4 and 5 serving as positive and negative control groups, respectively. After 2 days of virus inoculation, the mice were treated with different concentrations of Herbix (100, 200, and 300 mg/mL) via subcutaneous injection. Mice Blood samples (0.5 to 1 mL) were taken from the mice before and after the experiments, and after three-week follow-up period, the mice were sacrificed and the spleens were removed for lymphocyte analysis.

we found that administration of Herbix at a dose of 300 mg/mL showed the greatest efficacy, characterized by a delay in skin lesion formation, an increment in survival rate and lymphocyte proliferation, upregulation of the gene expression of interferon alpha (*IFN-a*) and tumor necrosis factor alpha (*TNF-a)*, and an increase in the polarization of cytotoxic and helper T lymphocytes compared to the control group.

These results suggest that Herbix at a dose of 300 mg/mL is effective in treating murine herpes and stimulating immune responses, making it a potential candidate for further investigation as an antiherpetic drug.

Keywords: Immune responses; Herpes simplex virus-1; Viral infection

INTRODUCTION

Herpes simplex virus 1 (HSV-1) is a member of the Herpesviridae family, affecting 60% to 95% of adults worldwide.[1,2] Among the known types of herpes viruses, some are pathogenic to humans, the most significant of

Corresponding Author: Jalil Tavakol-Afshari, PhD; Department of Immunology, Immunology Research Center, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran.
Tel: (+98 915) 1134 220, Fax: (+98 51) 3711 2596, E-mail: Tavakolaj@mums.ac.ir, tavakolafsharijalil@gmail.com

which include HSV-1, which primarily causes mouth and face infections and is characterized by painful blisters. This virus can also infect the central nervous system (CNS) and the cornea of the eye.[2] HSV-2 is the primary cause of genital infections and has a high rate of mother-to-child transmission. HSV infections may lead to blindness, congenital disabilities, genital herpes, and malignancies.[3] Upon entering the body, the viral genetic information remains in the posterior root of the brain ganglia for the rest of a person's life, where it can transform from a latent to an active state under certain circumstances.[4,5]

There are currently no definitive therapies for herpes; nevertheless, certain medications and gene therapies have shown to be effective, each carrying its own risks.[6] For instance, acyclovir, a synthetic drug, that can be used topically, intravenously, or orally, is effective in reducing symptoms and limiting the virus's spread. However, the virus can develop resistance to acyclovir through mutations in the thymidine-kinase encoding gene, as seen in the case of human immunodeficiency virus (HIV). Adverse reactions, such as nausea, dizziness, diarrhea, vomiting, headache, and sensitivity to light, have also been reported.[7]

The results of genetic engineering and gene therapy to remove the viral gene *γ-134.5* that could prevent replication of the virus in brain cells (after mitosis) have been promising, particularly in the treatment of glioblastoma multiforme. But the use of vectors and gene therapy methods is very expensive and, in some cases, carries a high risk, limiting their use.[8]

Moreover, there are limited clinical trials on antiherpetic drugs due to the challenge of detecting the virus in the brain ganglia of infected patients. This requires a biopsy of the spinal cord or brain ganglia, which can only be done through an autopsy. The virus is typically found in lower amounts in the blood compared to the nervous system, making it difficult to measure related factors.[9] Animal models and laboratory research play a significant role in advancing our knowledge in this field.

Considering the limited information and the lack of a definitive treatment for herpes, finding new and effective medicinal solutions (herbal, chemical, or combined) with lower costs and fewer side effects can be very effective in treating herpes. In this regard, the combination drug "Herbix" (consisting of herbal and chemical components) can be considered a novel solution in this field. It should be noted that no domestic or foreign analogs of this medication is currently available.

MATERIALS AND METHODS

Cell and Virus Stocks

Vero cells were obtained from the Iranian Biological Resource Center (IBRC) cell bank in Tehran, Iran. They were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Gibco-BRL Co., Germany) and 1% penicillin/streptomycin. The HSV-1 strain was procured from the virus repository of the Virology Department of Tarbiat Modares University, Tehran, Iran. Virus stocks were propagated in Vero cells, and had an initial titer of 10^6 plaque-forming units (PFU). After 3 passages, the virus titer was raised to 10^9 PFU and used at a concentration of 10^9 PFU/mL in the experiment based on the Tissue Culture Infectious Dose 50 assay. The final virus titer was selected based on the fastest and most efficient appearance of cytopathic effect (Figure 1).

Preparation of Herbix

Herbix is a novel synthetic drug synthesized by a research team led by Dr. Keramatian for the first time and neither a national nor an international equivalent exists. The drug was prepared using a combination of compounds such as jelly powder, calcium bicarbonate, distilled water, sulfur, and calcium oxide. The appropriate amounts of each compound were determined through several adjustments and calculated separately. The preferred sterilization method for this product was membrane filtration.

MTT Assay to Determine the Safety and Cytotoxicity of the Extract

The cytotoxicity of Herbix was determined by investigating its effect on the Vero cells. Vero cells were cultured in 96-well plates at 2×10^5 cells/mL and incubated at 37°C with 5% CO_2 for 24 hours. Three different concentrations of the extract were added to cell culture plates (100, 200, and 300 mg/mL in each well) at a total volume of 200 µL in triplicate. Untreated Vero cells and H_2SO_4-treated cells were used for negative and positive controls, respectively. The cell culture plates were incubated at 37°C with 5% CO2 for 24, 48, and 72 hours, and the methyl thiazolyl tetrazolium test (MTT) reagent (30 µL) was added to the wells. After 4 hours of

incubation at 37°C, the purple sediment was solubilized in 100 µL of dimethylsulfoxide (DMSO). The absorbance was read at 570 nm with a reference wavelength of 630 nm by an ELISA reader. The following formula was used to calculate the percentage of cell viability: [(sample absorbance − cell-free sample blank)/mean media control absorbance)]*100%.[10]

Cutaneous Mouse HSV-1 Infection Model

Male, 6 weeks old BALB/c mice were purchased from Pasteur Institute of Iran, Tehran, Iran. All mice were housed in the animal house of Mashhad University of Medical Sciences under standard conditions for 1 week to adapt to the environment, and had free access to food and water. An electronic hair remover was used to shave the right mid-flank of each mouse.

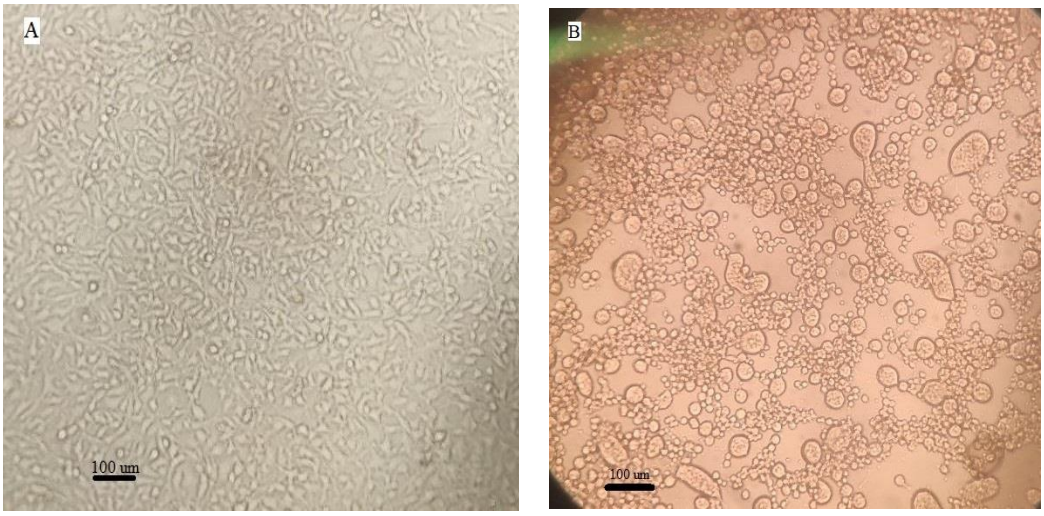

Figure 1. Normal Vero cells (A) and the cytopathic effect of herpes simplex virus 1 on Vero cells causing giant round cells (B)

According to multiple pilot studies conducted by our team, the cutaneous injection of 100 µL of HSV-1 suspension (1×10^9 PFU/mL) was applied to the hairless area of mice to create swollen skin lesions within 2 days. Either Herbix or acyclovir (100 µL; 5 mg/kg per dose) was cutaneously injected into the mice.[11] Also, 0.5 to 1 mL of blood was taken from all mice at baseline and at the end of the study with a follow-up time of 2 weeks. This procedure adhered to the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Experimental Groups and Monitoring of the Mice

In this experimental study, 25 male HSV-1–infected BALB/c mice were divided into 5 groups (5 mice per group) as follows:

Group 1: treated with Herbix (100 µL; 100 mg/mL); Group 2: treated with Herbix (100 µL; 200 mg/mL); Group 3: treated with Herbix (100 µL; 300 mg/mL); Group 4 (negative control): treated with topical Vaseline; and Group 5 (positive control): treated with subcutaneous acyclovir (100 µL; 5 mg/kg).

After HSV-1 infection, the development of skin lesions and mortality were continuously observed 3 times daily for at least 2 weeks. At the end of 3 weeks, blood samples were taken from the mice via the caudal vein, the mice were euthanized and their spleens were removed and prepared for further flow cytometric analysis.

RNA Extraction

Total RNA was extracted from whole blood according to the kit manufacturer's instructions (Parstous, Iran). Briefly, 150 µL of whole blood was added to a 1.5-mL tube, and 750 µL of red blood Lysing (RL) solution was added to remove red blood cells. After 5 minutes of pipetting at room temperature, 150 µL of chloroform was added to the mixture, shaken for 15 seconds, and incubated for 3 minutes at room temperature, and centrifuged for 12 minutes (13000 rpm, at 4°C). 400 µL of the supernatant was transferred into a new 1.5 mL tube, mixed with 400 µL of 70% ethanol, filtered through a spin column by centrifuging for 1 minute at 13000 rpm. The flow-through was collected and 700 µL of Pars-toos Wash buffer (PW) was added, followed by another centrifugation for 1 minute at 13000 rpm. This step was repeated with 500 µL of PW to

further purify the RNA, and was centrifuged once more for 2 min at 13000 rpm to remove the remaining wash buffer. The spin column was then transferred to a new 1.5-mL tube and eluted with 50 µL of diethylpyrocarbonate (DEPC)-treated water by incubating for 3 minutes at room temperature, and centrifuging for 1 minute at 13000 rpm. The RNA solution was then prepared for the cDNA synthesis.

cDNA Synthesis

cDNA synthesis was done according to the manufacturer's instructions (Parstous, Iran). Briefly, the work solution was prepared in a solution containing 100 ng of total RNA, 10 µL of 2×buffer solution, 2 µL of Master-Mix, and 6 µL of DEPC-treated water. The final reaction volume was 20 µL. The incubation times were 10 minutes at 25℃, 60 minutes at 47℃, and 5 minutes at 85℃.

Real-time PCR Analysis

Gene expression was analyzed with real-time PCR. Real-time PCR with SYBR® Green master mix was used to determine the interferon-alpha (*IFN-α*), tumor necrosis factor-alpha (*TNF-α*), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene expression. Furthermore, reactions were performed in a volume of 10 µL containing 50 PM of each forward and reverse primer, 50 ng cDNA, 5 µL Master Mix, and 2 µL of DEPC water according to the manufacturer's instructions. After 10 min at 94°C as the activation stage, 35 cycles, including 15 Seconds at 95°C, 20 seconds at 60°C for IFN-α and TNF-α and 50°C for GAPMO, and 20 seconds at 72°C, were carried out by Bio-Rad Real-Time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA). Primer specifications and PCR product sizes are shown in Supplementary Table.

Isolation of Splenocytes

After anesthesia (with 10% ketamine [50 mg/kg] and 2% xylazine [5 mg/kg]; Sigma-Aldrich, USA), the mice were sacrificed, their spleens were removed, and lymphocytes were isolated. In brief, single-cell suspensions were prepared by crushing the tissue between two slides and centrifuging at 3000 rpm for 10 minutes at 4°C. Erythrocytes were lysed in ammonium chloride lysis solution [0.5 M ammonium chloride (NH_4Cl), 10 mM potassium bicarbonate ($KHCO_3$)] and 0.1 mM disodium ethylenediaminetetraacetic acid (EDTA), pH 7.2) for 5 minutes. Lymphocytes were obtained and then washed with phosphate-buffered saline. The viability of the cells was then checked by the trypan blue test. For each sample, the final cell count was adjusted to 20×10^5 cells/mL in DMEM (Gibco, USA) containing 10% fetal bovine serum and 1% penicillin/streptomycin[12] and Cell Activation Cocktail (with Brefeldin A, a premixed cocktail of optimized concentrations of PMA [phorbol 12-myristate-13-acetate] and ionomycin; Sigma-Aldich, USA.2 µL/mL) was added to stimulate cytokine production.

Flow Cytometry

The homogeneous suspension of splenocytes was incubated for 4 to 6 hours in a cell culture incubator at 37℃ under 5% CO_2. Analysis of CD3, CD4, and CD8 markers was performed (BD FACS-Calibur, USA) according to the defined protocols. In summary, FITC-labeled anti-mouse CD4 and CD8, PerCP/cyanine5.5-labeled anti-mouse CD3, and PE-conjugated anti-mouse IFN-γ antibodies (All from Biolegend, USA) were incubated with splenocytes (1×10^6 cells per mL). FlowJo software version 7.6 (Treestar, Ashland, OR, USA) was used to analyze the results.[12]

Lymphocyte Proliferation Assay

Splenocytes, isolated from each group, were seeded in triplicates in a 96-well culture plate (20×10^4 cells/well). PMA (50 ng/mL) and ionomycin (1 µg/mL) were added to each well separately for priming the T lymphocytes. Plates were incubated at 37°C in 5% CO_2 for 72 hours. Afterward, the proliferation of cells was determined by the MTT assay.[13] Sample absorption was measured by an ELISA reader at 570 nm with a reference wavelength of 630 nm.

Statistical Analysis

The normal distribution of the data was checked using the Shapiro-Wilk test. To compare the effect of various drug doses on quantitative variables before and after the intervention (for data with normal distribution), one-way analysis of variance and Brown-Forsythe analysis were performed, followed by the posthoc LSD test and the posthoc Dunnett T3 test, respectively. In the case of a non-normal distribution, the nonparametric Wilcoxon test was used. $p<0.05$ was considered significant in all calculations.

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RESULTS

Herbix Drug Safety on Vero Cells: Dose Analysis

The proliferation rate of Vero cells was examined using the MTT assay after 24-, 48-, and 72-hour treatments with different doses of Herbix (100, 200, and 300 mg/mL). The findings showed that all three concentrations of Herbix had no cell toxicity and that there was no significant difference in the optical absorption results of the different drug concentrations compared to the negative control group (untreated cells). Cell viability increased, although not significantly, more at the highest concentration (300 mg/mL) compared to the other doses, as determined by cell suspension staining with the trypan blue method. Additionally, no specific side effects were observed in mice after this extract's administration in the animal model.

Formation of Skin Lesions and Survival Rate of Mice Over Time

In groups 1 (100 mg/mL), 2 (200 mg/mL), and 4 (negative control), skin lesions appeared approximately 2 days after virus injection (with a titer of 1×10^9 PFU/mL). Most of the mice in these groups died within 5 to 7 days (2 to 3 days after receiving Herbix) and showed little therapeutic effect in increasing survival. In group 3 (300 mg/mL), skin lesions appeared 4 days after virus injection (with delay) in mice, but after receiving Herbix, the mice survived. Over the course of 3 weeks, no signs of infection were observed in the mice, and their survival rate increased significantly ($p<0.01$) compared to the other groups. Additionally, in group 5 (treated with acyclovir), a significant increase in lifespan was observed compared to groups 1, 2, and 4. It is important to note that in 1 case, a mouse receiving acyclovir experienced paralysis in one leg, which was determined to be a side effect of the drug.

Effect of Herbix on T Lymphocyte Proliferation

The T-lymphocyte proliferation rate was assessed using an MTT assay after 72 hours of treatment with varying doses of Herbix in different groups. The results indicated a significant increase in the percentage of lymphocytes in groups 3 (%152.61±4.1) and 4 (%124.57±3.6) at the end of the experiments, compared to the control group (%67.01±1.9). Conversely, cell proliferation in groups 1 and 2 did not show a significant difference compared to the control group (Figure 2).

Effect of Herbix on *IFN-α* Gene Expression

The expression level of the *IFN-α* gene in Vero cells was evaluated using real-time PCR. Before the intervention, there was no significant difference in IFN-α levels in all mice. However, treatment with different doses of Herbix in groups 1 and 2 resulted in a significant decrease ($p<0.01$) in cytokine levels compared to the control. In contrast, at the end of the experiment, there was no significant difference in group 3, compared to the positive control group (Figure 3).

Effect of Herbix on *TNF-α* Gene Expression

At the baseline, *TNF-α* gene expression was not significantly different between groups. However, after treatment with 100 and 200 mg/mL of Herbix (in groups 2 and 3) and treatment with the commercial acyclovir drug (in group 4), a significant increase in *TNF-α* gene expression was observed at the end of the study (Figure 4).

Effect of Herbix on CD8+ T Lymphocytes (CTL)

The CTL percentage at the end of the experiment did not show a significant difference in the groups, except for group 3 (23.6±3.6), which showed a significant increase compared to the control group (14.87±1.9) (Figure 5).

Effect of Herbix on T_H1 Lymphocytes

In experimental groups 1 and 2, a non-significant difference in the percentage of TH1 lymphocytes compared to the control group (29.8±2.1) was observed. In groups 3 (37.2±4.3) and 4 (38.4±3.6) a non-significant increase in the percentage of T_H1 lymphocytes was observed at the end of the experiment (Figure 6).

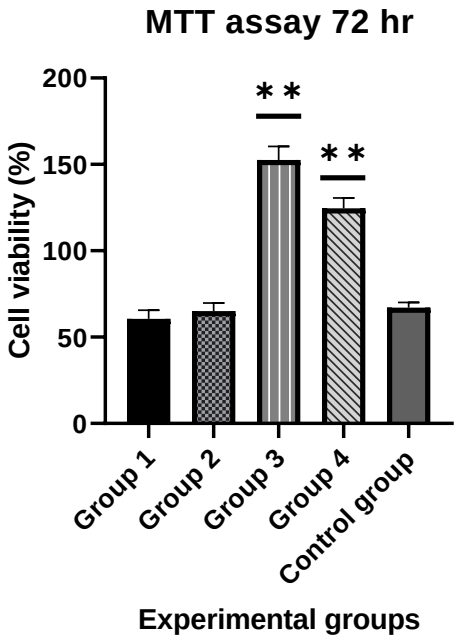

Figure 2. The proliferation rate of T-lymphocytes after 72 hours of incubation with various doses of Herbix. All data were presented as mean±SEM of triplicate determinations. **p<0.01 compared to the control group;
Group 1: treated with Herbix (100 µL; 100 mg/mL); Group 2: treated with Herbix (100 µL; 200 mg/mL); Group 3: treated with Herbix (100 µL; 300 mg/mL); Group 4 (negative control): treated with topical Vaseline; and Group 5 (positive control): treated with subcutaneous acyclovir (100 µL; 5 mg/kg). (n=5 HSV-1–infected mouse in each group).

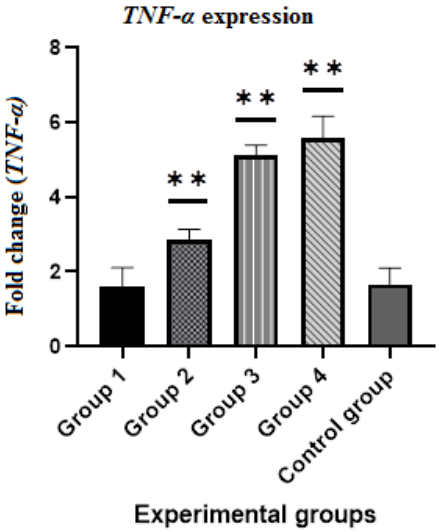

Figure 3. Interferon-alpha (*IFN-α)* gene expression (fold change) after treatment with different doses of Herbix compared to the control group; data presented as mean±SEM of triplicate determinations. **p<0.01).
Group 1: treated with Herbix (100 µL; 100 mg/mL); Group 2: treated with Herbix (100 µL; 200 mg/mL); Group 3: treated with Herbix (100 µL; 300 mg/mL); Group 4 (negative control): treated with topical Vaseline; and Group 5 (positive control): treated with subcutaneous acyclovir (100 µL; 5 mg/kg). (n=5 HSV-1–infected mouse in each group).

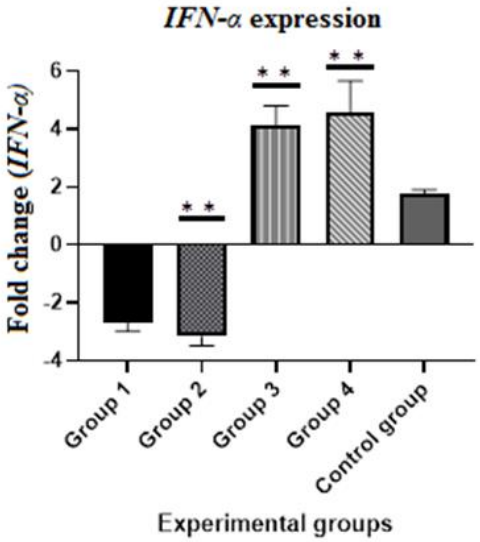

Figure 4. Tumor necrosis factor-alpha (*TNF-α*) gene expression (fold change) after treatment with different doses of Herbix compared to the control group; data presented as mean±SEM of triplicate determinations. **p<0.01
Group 1: treated with Herbix (100 µL; 100 mg/mL); Group 2: treated with Herbix (100 µL; 200 mg/mL); Group 3: treated with Herbix (100 µL; 300 mg/mL); Group 4 (negative control): treated with topical Vaseline; and Group 5 (positive control): treated with subcutaneous acyclovir (100 µL; 5 mg/kg). (n=5 HSV-1–infected mouse in each group).

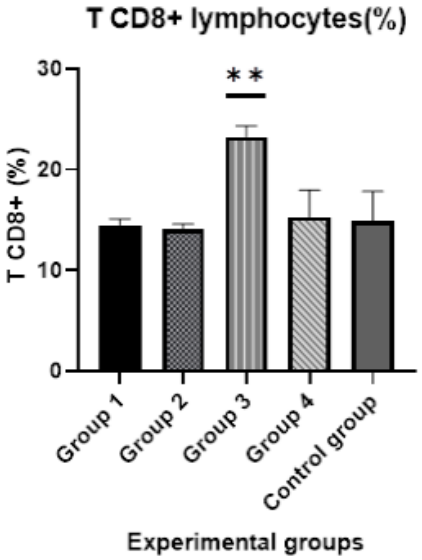

Figure 5. Percentage of cytotoxic (CD8+) T lymphocytes in different study groups.
Data presented as mean±SEM of triplicate determinations. **p<0.01 compared to the control group.
Group 1: treated with Herbix (100 µL; 100 mg/mL); Group 2: treated with Herbix (100 µL; 200 mg/mL); Group 3: treated with Herbix (100 µL; 300 mg/mL); Group 4 (negative control): treated with topical Vaseline; and Group 5 (positive control): treated with subcutaneous acyclovir (100 µL; 5 mg/kg). (n=5 HSV-1–infected mouse in each group).

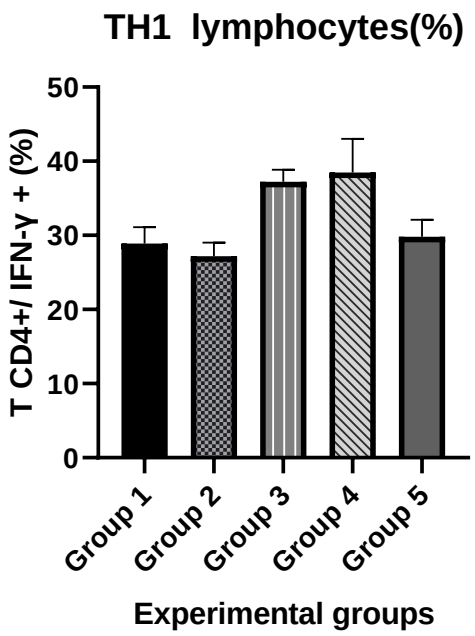

Figure 6. Percentage of helper T lymphocyte (T_H1) in the control and intervention groups. IFN-γ, interferon-gamma. Group 1: treated with Herbix (100 µL; 100 mg/mL); Group 2: treated with Herbix (100 µL; 200 mg/mL); Group 3: treated with Herbix (100 µL; 300 mg/mL); Group 4 (negative control): treated with topical Vaseline; and Group 5 (positive control): treated with subcutaneous acyclovir (100 µL; 5 mg/kg). (n=5 HSV-1–infected mouse in each group). No significant difference was observed between groups regarding TH1 lymphocytes' percentage.

DISCUSSION

This study aimed to investigate the safety and efficacy of the synthetic extract Herbix in an animal model of herpes induced by HSV-1. Our preliminary results from an in vitro study on Vero cells showed that all 3 doses of Herbix were safe for virus-infected cells. The survival of cells at the dose of 300 mg/mL was higher than in other doses; thus, this dose was chosen for animal intervention. Additionally, the results showed that this extract dose (300 mg/mL) was highly effective in increasing T-cell proliferation, the percentage of CTL, and the expression of *IFN-α* and *TNF-α* genes.

Many animal models have been used to investigate the antiviral effect of various herbal and chemical extracts on HSV, and some have reported effective results. For example, a study by Kurokawa et al, investigated the antiviral effect of 142 plant extracts collected from China, Japan, and Indonesia in a mouse animal model of herpes[14] created through skin inoculation of HSV-1. The mice were treated daily with different doses of hot water extract from the mentioned plants, and the reduction in the formation time of skin lesions was used to measure the effectiveness of the extracts. The study showed that 12 extracts effectively treated mouse skin lesions and increased their survival time. In another study, the effect of different doses (5, 10, 15 mg/mL) of *Myrtus communis L.* herbal oil was investigated in the herpes mouse model for 10 days. The rate and time of skin lesion healing and the mice's mortality rate were investigated.[15] The results showed that the dose of 15 mg/ml of the oil effectively reduced pustule formation time compared to other doses. These results are consistent with those we found in our analysis.

On the other hand, it has been proven that IFN-α prevents the replication of HSV-1 virus genes in mice by preventing rapid translation.[16] Additionally, IFN-α activates the host's defense against the herpes virus, including the activation of natural killer cells, which play a crucial role in controlling infectivity and pathogenicity.[17] IFN-α also limits disease progression from peripheral tissues to the central nervous system.[18]

Our study showed that Herbix at a dose of 300 mg/mL/kg body weight (group 3) caused a significant increase in IFN-α levels compared to other groups. No significant difference was observed compared to the

positive control group (treatment with acyclovir). This indicates the extract's effect on activating and stimulating the immune system against the virus.

Studies have shown that T_H1 lymphocytes among T lymphocyte subtypes can maintain immunity against intracellular infections like viruses and bacteria.[19,20] Our study's flow cytometric analysis of T lymphocytes showed that Herbix at a dose of 300 mg/mL could increase the polarization of T_H1 lymphocytes, though not significantly, compared to the control group.

Some cytokines, such as TNF-α and IL-12, which play crucial roles in innate and adaptive immune responses, have been proven to be responsible for the polarization of T_H1 lymphocytes.[12,21] Furthermore, T lymphocyte-mediated immunity is a key component of TNF-α responses in activating CTL and natural killer cells to kill virus-infected cells in a positive feedback loop.[22]

Nakanishi et al.'s study found that a group of CD8+ and special CD4+ T lymphocytes directly inhibit the expression of some viral proteins (about a third of the proteins) in each individual. Some CD4+ effector T lymphocytes also enhance the migration of CD8+ T lymphocytes to herpes virus-infected tissues in an IFN-γ–dependent pathway, enhancing antiviral responses.[23,24]

CD4+ effector T lymphocytes support antiviral responses of CD8+ T lymphocytes in other ways, such as facilitating the entry of CD8+ T lymphocytes into lymph nodes and activating CD8+ T lymphocyte function, leading to increased secretion of IFN-γ and TNF-α cytokines without increasing cytotoxicity.[25,26,27]

The results of our study show an increase in the percentage of CTLs and TNF-α after treatment with Herbix (300 mg/mL). These results are consistent with existing studies[28,29] in this field which confirms the effective role of this extract in stimulating immune responses.

To summarize, the results of our study indicate that the plant-chemical synthetic drug "Herbix" in a dose of 300 mg/mL has no harmful cellular effects and has positive therapeutic outcomes in mouse herpes animal models. These outcomes include a delay in the development of skin lesions, an increase in mouse survival rate, an increase in the expression of *IFN-α* and *TNF-α* genes, increase in T-cell proliferation and CTL response. Based on these results, a phase-1 clinical trial with a limited number of patients is recommended to validate these findings.

STATEMENT OF ETHICS

This research is approved by the Ethics Committee of Mashhad University of Medical Sciences (approval code: IR.MUMS.MEDICAL.REC.1400.695).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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