SYNERGISTIC EFFICACY OF EUCALYPTOL WITH ACYCLOVIR AGAINST HSV-2

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The increasing drug resistance in herpes viruses in recent years brings with it new treatment approaches. In recent years, it has been tried to overcome drug resistance, especially with the use of herbal products in combination with existing drugs. In this study, we aimed to investigate the effectiveness of eucalyptol in combination with acyclovir. A Vero cell line was used for toxicity tests and viral culture isolation studies in the study. The non-toxic concentrations of eucalyptol and acyclovir were determined by the MTT method. Antiviral efficacy studies were performed within non-toxic concentrations. Antiviral activity was determined by calculating the IC50 values of the compounds against HSV-2. In addition, it was evaluated by the RT-PCR method. The 50% inhibitory concentration (IC50) and Fractional inhibitory concentration (FIC) index values determined during 24 hours and 48 hours of action showed that Eucalyptol exhibited a potent activity. This efficacy was found to be stronger when used in combination with acyclovir. These results show that the combination of Eucalyptol and acyclovir may be beneficial against resistant HSV infections. We suggest that the results of these studies, which are planned as in-vitro, be supported by in-vivo studies.

Keywords: Eucalyptol, antiviral, HSV-2, acyclovir, synergy.

INTRODUCTION

Herpes simplex virus (HSV) infections are shared all over the world and cause serious complications in immunocompromised patients. The drug of choice for treatment is acyclovir, a nucleoside analog of guanosine that needs to be phosphorylated three times. Initial phosphorylation is completed by the virally encoded thymidine kinase protein, which allows acyclovir to be activated only in virus-infected cells. Cellular thymidylate kinases provide second and third phosphorylations. Acyclovir triphosphate is a DNA chain terminator that acts by inhibiting viral DNA polymerase (Groves, 2016; Tognarelli *et al*., 2019).

The widespread use of acyclovir has resulted in the emergence of acyclovir-resistant HSV strains. It has been reported that acyclovir-resistant strains can occur mostly in patients whose immune system is suppressed (Sadowski *et al*., 2021; Schnitzler, 2019).

A wide variety of synthetic and natural products are being studied against HSV to solve the increasing drug resistance in herpesviruses. Eucalyptol is one of the phytochemicals on which a wide variety of studies have been conducted.

HSV infections with reduced susceptibility to acyclovir are mainly reported in immunocompromised patients. The prevalence of drug resistance in these patients varies between 3.5-10%. It has been reported that acyclovir resistance may be even higher in bone marrow or transplant recipients (>25%) (Ho *et al*., 2020; Sadowski *et al*., 2021).

It has been reported that HSV resistance to acyclovir is less than 1% in immunocompetent patients. However, the presence of acyclovir-resistant HSV has been reported among immunocompetent individuals, especially in cases of recurrent herpetic keratitis. It has also been reported that acyclovir prophylaxis predisposes to antiviral-resistant recurrent herpetic keratitis. Eucalyptol (1,8-cineol) is a naturally occurring monocyclic monoterpene ether. It has an aromatic scent. Due to its pleasant spicy aroma

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and taste, eucalyptol is used as a flavoring agent and in cosmetics (Juergens *et al*., 2020). In this study, we aimed to investigate the effectiveness of eucalyptol in combination with acyclovir.

MATERIALS AND METHODS

Determination of Cytotoxic Dosage of Eucalyptol

First of all, non-toxic concentrations of eucalyptol in healthy cells were determined. Vero (African green monkey kidney cell line) was used for this purpose. Antiviral activity studies were performed on the concentrations of the molecule determined as non-toxic on Vero cells. The MTT method was used for cytotoxicity tests.

Cell Culture Experiments

In cell culture studies, RPMI-1640 cell growth medium containing 10% fetal calf serum (FBS), 10 mM HEPES, 100 IU/ml penicillin/streptomycin with 4mM glutamine was used. Cultures were incubated at 37 °C in a cell incubator with 5% carbon dioxide.

The cell concentration was set to be $1x10^6$ cells/ml for cell proliferation and generation experiments, and 1x105 cells/ml for activity studies. Incubation of the cells was continued until they covered the surface of the culture dish for 3-7 days.

The passage of cells covering the culture dish surface was performed using a 0.25% trypsinization solution. Cells were removed from the culture dish surface with the versen-trypsin solution and incubated at 1500 rpm for 15 min collected by centrifugation. Cell viability was then determined by trypan blue staining.

Proliferation Assays

Proliferation experiments were performed in 12, 24, 48, and 96-well flat-bottom culture plates. In the experiments, activity studies were carried out by adjusting the wells to 1x105 cells per ml with RPMI-1640 medium containing 10% fetal calf serum. Dimethyl sulfoxide (DMSO) was used to dissolve chemical compounds in the culture medium. The selected concentration of DMSO used as the solvent in the experiments was included in the experiments as a negative control.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Method

The MTT method allows living cells to be detected colorimetrically and quantitatively (Mosmann *et al*., 1983). This method is based on the principle that the MTT dye of intact mitochondria can cleave the tetrazolium ring in cells. MTT is reduced to colored, water-insoluble formazan by a mitochondrial-dependent reaction, which is actively absorbed into cells. The MTT-reducing property of the cells is taken as a measure of cell viability. The dye density obtained as a result of MTT analysis correlates with the number of viable cells. In the experiments, different concentrations of eucalyptol were treated with virus-infected cells and their effects on viral replication were investigated. In the study, negative (DMSO) and positive control (Acyclovir) were also studied together in the MTT method. At the end of the incubation, 10 µl of MTT was added to each well and the plates were incubated for 4 hours under the same

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conditions. Absorbance measurements were performed at 570 nm with a spectrophotometer.

HSV-2 Isolation

RPMI 1640 medium containing 10% fetal calf serum was used as a viral production medium. The vero cell line was used as the cell line for viral isolation. HSV-2 strain was obtained from Hatay Mustafa Kemal University Faculty of Medicine Cell Culture Laboratory virus stocks. The viral strain was reactivated by removing the virus strain from the deep freezer, thawing it in a water bath at 37°C, and inoculating the Vero cell culture covering the culture dish's surface. Following inoculation, the herpes simplex virus began to determine the characteristic CPE patterns from the 3rd day. Incubation was continued until day 7. The collected cells were centrifuged at 4000 rpm for 20 minutes after the freeze-thaw process. The sample taken from the supernatant was confirmed for herpes simplex virus type 2 by RT-PCR.

Titration of HSV-2

For this purpose, the HSV-2 strain removed from -80 °C was thawed at 37 °C and incubated in Vero cell lines for 96 hours. Then, the viral culture vessel was freeze-thawed and the cells were blasted. The cells were collected from the culture dish and centrifuged at 4000 rpm for 20 minutes and the supernatant was organized as a virus solution. Subsequently, the virus suspension was infected with Vero cells in 96-well flat-bottomed microplates, and the infectious dose calculation was performed as stated in the literature. Vero cells were produced by inoculating ($1x10^5$ cells/ml) into 96-well microplates. When cell growth covered the surface of the wells, the medium was taken from each well and 50 µl of 10-fold diluted stock virus solution (from to $10^{-1}$to 10^{-6}), prepared in serum-free RPMI-1640 medium, was added to the cells. For viral adsorption, after incubation at 37 °C for 2 hours, 50 µl of medium containing 5% FBS was added to each well and incubated at 37 °C for 7 days in an atmosphere of 5% CO_2.

At the end of the incubation, viral dilutions were examined for the presence of CPE. Viral titer was calculated according to the Reed and Muench method. All experiments were performed independently of each other in triplicate (Reed and Muench, 1938; Allahverdiyev *et al*., 2004).

Viral DNA Isolation from Cell Culture

HSV-2 infected cell cultures incubated with treatment compounds were collected by centrifugation at 1250 rpm for 10 min. Viral DNA isolation was performed from the collected cell pellets using the Zymo Research Viral DNA Kit according to the test package insert as follows. Collected cell pellets were suspended in 800 µl of ZR Viral DNA Buffer with the help of a vortex. The cell suspension, which was kept at room temperature for 10 minutes, was transferred into the Zymo-Spin IC Column and centrifuged at 15000 rpm for 1 minute. After pouring the supernatant, 300 µl of DNA Wash Buffer was added into the column and centrifuged for 1 min. This step was repeated once. 10 µl of DNA Elution Buffer was added to the Zymo-Spin IC Column and placed in a sterile eppendorf, and after 1 minute of incubation at room temperature, it was centrifuged at 15000 rpm for 1 minute. DNA samples obtained in sterile eppendorf were stored at -20 °C until the PCR study.

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Determination of Viral Load by Real-Time PCR

In viral load determination studies, 1 µl of DNA was used from DNA samples kept at -20°C. GoTaq PCR Mastermix (Promega, USA) was used for RT-PCR analyses. The reaction protocol was performed in the following steps using the Montana 4896 RT-PCR (Anatolia, Turkey). Denaturation was applied for 2 minutes, at 95 °C for 15 seconds, and at 60 °C for 60 seconds, for a total of 40 cycles. Each sample was worked in duplicate. The results were evaluated logarithmically considering the CT values of the samples. The primer sequences used are given below. (Forward 5'-ATCAACTTCGACTGGCCCTT -3', Reverse 5'- CCGTACATGTCGATGTTCAC -3') (Cunningham *et al*., 1996; Lakeman *et al*., 1995).

RESULTS

While the non-toxic concentration of eucalyptol on Vero cells was determined as >62.5 µg/ml, this value for Acyclovir was found to be toxic at a concentration of 62.5 µg/ml after 48 hours of incubation (Figures 1 and 2).

The 50% inhibitory concentration (IC_{50}) and Fractional inhibitory concentration (FIC) index values determined during 24 hours and 48 hours of action showed that Eucalyptol exhibited a potent activity. This efficacy was found to be stronger when used in combination with acyclovir.

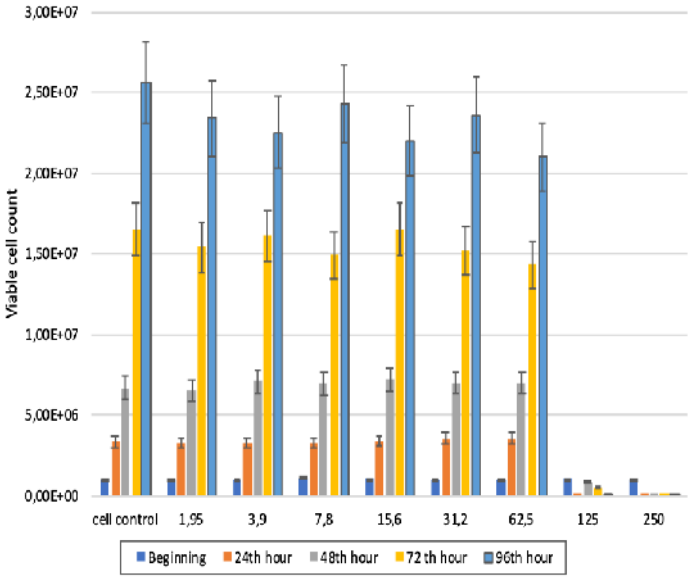

Figure 1. The non-toxic concentration of eucalyptol on Vero cells

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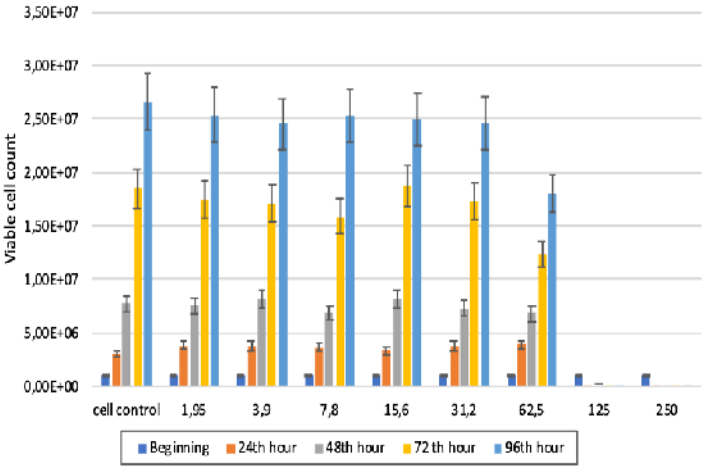

Figure 2. The non-toxic concentration of Acyclovir on Vero cells

The antiviral activities of eucalyptol at a concentration of 62.5 and acyclovir at a concentration of 31.2 µg/ml against the virus at a titer of 100 TCID50 of HSV-2 are given in Figure 3. According to this figure, it was determined that eucalyptol was quite potent, and the efficacy was significantly increased in the case of the acyclovir combination. ($p<0.05$).

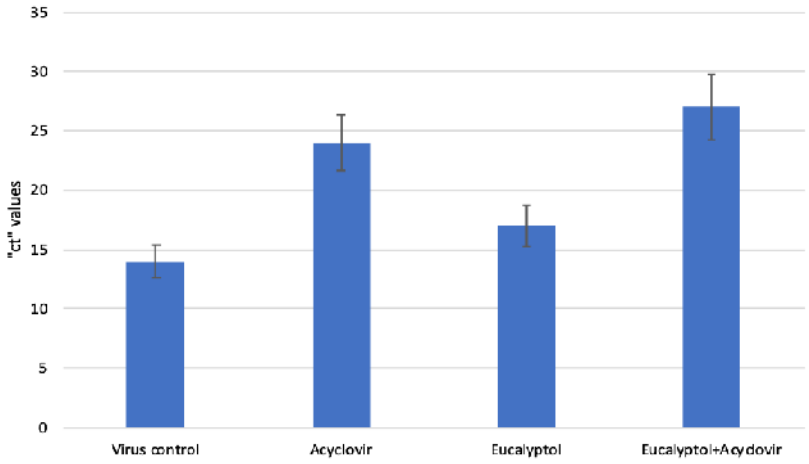

Figure 3. Single and combined activities of eucalyptol and acyclovir against HSV-2

The antiviral activities of acyclovir and eucalyptol at different concentrations were evaluated by considering the percentage of CPE. Similarly, it was determined that the combination of eucalyptol and acyclovir exhibited extreme antiviral activity (Figure 4).

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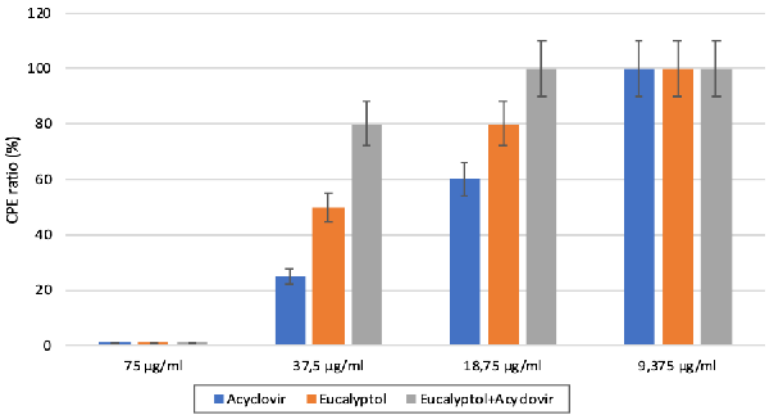

Figure 4. Evaluation of the efficacy of eucalyptol and acyclovir against HSV-2 in terms of CPE

CONCLUSION

These results show that the combination of Eucalyptol and acyclovir may be beneficial against resistant HSV infections. We suggest that the results of these studies, which are planned as *in-vitro*, be supported by *in-vivo* studies.

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