

The Effect of Hydroalcoholic Extract of Olive Leaves against Herpes Simplex Virus Type 1

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Abstract

Background: It was shown that olive leaf extract has anti-fungal, antibacterial and antiviral activities. The effects of OLE on herpes simplex virus-1 (HSV-1) have not been systematically investigated yet. The aim of this study was to examine the *in vitro* effect of olive leaf hydroalcoholic extract (OLHE) on HSV-1.

Methods: Virucidal effect and viral replication in Vero cell line were studied in the presence of various concentrations of OLHE applied at different time intervals using a standard plaque assay method. The 50% cytotoxic concentration (CC₅₀), 50% inhibitory concentration (IC₅₀), and therapeutic index of OLHE were determined.

Results: OLHE showed virucidal effect on HSV-1 in concentrations >1 mg/mL. The CC₅₀ of OLHE for Vero cells and IC₅₀ were 1.75 and 0.66 mg/mL, respectively. When applied to cell culture infected with the HSV-1, one hour earlier, OLHE showed no antiviral activities. When applied to the cells followed by the virus infection one hour later, or to the media containing the virus and the combination was added to cell culture one hour later, OLHE showed anti-HSV-1 activities at concentrations >1 mg/mL.

Conclusion: OLHE has anti-HSV-1 activity likely due to the prevention of virus entry into the cells.

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Keywords • Olive leaves • hydroalcoholic extract • Herpes simplex type-1 • Vero cell line

Introduction

Olive tree (*Olea europaea*) is a sacred plant to which the almighty Allah swears in the Holy Quran. In Iran's traditional medicine, both olive oil, derived from olive fruit and olive leaves were of therapeutic values.

Olive leaves have been traditionally used to decrease blood pressure and glucose.¹⁻³ Recent reports using experimental techniques indicated that it has antioxidant,^{2,3} antihypertensive,^{4,5} vasodilatory,⁶ hypoglycemic,^{7,8} and anti-inflammatory activities. Moreover, olive leaves have antimalarial,⁸⁻¹⁴ antifungal, antibacterial,^{15,16} and antiviral,^{17,18} activities.

Among major viral infections, herpes simplex virus type 1 (HSV-1) continues to be a major public health problem. HSV-1 may cause oral and genital infection associated with herpes labialis or cold sore. The infection can be serious in

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immuno-compromized hosts and may involve the central nervous system, which if left untreated, may be associated with a mortality of 70%.¹⁹

Acyclovir is widely used for the treatment of primary and recurrent HSV-1 infections. It however, only acts on actively-replicating HSV, and to be active, it needs to be phosphorylated by HSV thymidine kinase. Some HSV mutants, however, lack the thymidine kinase activity, and thereby are resistant to acyclovir. Such a resistance constitutes a major problem in the treatment of HSV infections.¹⁹⁻²²

To the best of our knowledge, the effects of olive leaves on HSV have not been investigated systematically yet. Considering the potentials of herbal medications in the treatment of human diseases,²³ including viral infections, the present study was conducted to examine the effects on hydroalcoholic extract of olive leaves (OLHE) on HSV-1 in Vero cell line.

Materials and Methods

Olive leaves collection

Olive leaves were obtained from local gardens of Dashtkooh in suburbs of Shiraz, southern Iran, with an altitude of 1600–1700 m from the sea level. The species of the plant (*Olea europaea* L.) were characterised by an expert from the Department of Biology, Shiraz University, Shiraz, Iran, and a herbarium registration number (40111) was assigned to the specimen.

Preparation of OLHE

The leaves were dried in shade and ground to powder. One-hundred g of the powder was percolated with 1000 mL ethanol (70% v/v) in water. After evaporation of hydroalcoholic extract over water bath, it was dried under vacuum.²⁴ The yield of extraction was 38.6% (w/w).

Virus stock

HSV-1 was isolated from the lip lesions of a patient and its genus was confirmed by neutralization test using guinea pig anti-HSV-1 serum and monoclonal anti-HSV-1 antibodies against HSV glycoproteins D and G.

OLHE stock preparation

Ten mg of OLHE extract was dissolved in one mL distilled water which was sterilized by filtration. To use in cytotoxicity and antiviral assays, the stock solutions of OLHE was diluted in the maintenance medium consisting of DMEM with 2% fetal bovine serum, 0.14% (v/v) sodium bicarbonate, 100 U/mL penicillin, 100 µg/mL streptomycin sulphate, and 0.25 µg/mL amphotericin B.

Cytotoxicity assays

The *in vitro* cytotoxicity of OLHE was exam-

ined in Vero cell line. Briefly, confluent Vero cells in 24-well plates (Nunc, Denmark) were grown in Dulbecco's modified Eagle's growth medium (DMEM, Sigma) containing 8% fetal calf serum (Gibco), 0.14% (v/v) sodium bicarbonate, 100 U/mL penicillin, 100 µg/mL streptomycin sulphate, and 0.25 µg/mL amphotericin B. Grown Vero cell monolayers were washed twice with PBS. Three mLs of the maintenance medium with increasing concentrations of OLHE were added to each well. The cells were then incubated at 37°C under 5% CO₂ for seven days. Cells were observed microscopically every 24 hrs. The extent of cytotoxicity was confirmed by trypan blue dye exclusion method. The cytotoxic concentration 50% (CC₅₀) was estimated by Karber method.^{25,26}

OLHE treatment and antiviral assays

The antiviral effect of OLHE was evaluated using plaque reduction assay as described previously.²⁷ Briefly, confluent Vero cells in 24-well plates were washed with PBS and subsequently treated with the maintenance medium containing increasing concentrations of the extract (up to 1.25 mg/mL) at 37°C for one hr prior to virus inoculation. Treatments were then removed, and monolayers were infected with 50 PFU/mL of HSV-1 for one hr. Thereafter, the monolayers were overlaid with 1% CMC in the maintenance medium containing increasing concentrations of OLHE, and were incubated at 37°C under 5% CO₂ for four days. The control samples consisting of virus-infected untreated or treated cells and mock-infected cells were also included in each assay. The cells were then fixed with methanol for 10 min and stained with 0.5% crystal violet. The inhibitory concentration 50 (IC₅₀) was determined for OLHE using Karber method.^{25,26} Experiments were performed at least twice in quadruplicates. Also, different concentrations of OLHE were applied one hr after infection of the Vero cells with 50 PFU/mL HSV-1 and plaque assay was performed as mentioned above.

To determine whether the extract have any direct virucidal activities against HSV-1, 10⁴ PFU/mL of HSV-1 was incubated for one hr at 37°C in the maintenance medium without or with various concentrations (up to two mg/mL) of OLHE. After incubation, virus titres were determined by plaque assay. Controls were mock-infected cells with no or various concentrations of OLHE, and virus infected cells without OLHE.

Statistical analysis

The mean number of viral plaques of two different experiments was compared with one-way analysis of variance (ANOVA) using SPSS v11.5. Dunnett test was used as the

post hoc test. A $P < 0.05$ was considered statistically significant.

Results

The results of cytotoxicity assays showed that the CC_{50} , IC_{50} and the therapeutic index (CC_{50}/IC_{50}) of OLHE against HSV-1 were 1.75 mg/mL, 0.66 mg/mL, and 2.65, respectively.

When applied one hr before Vero cell infection with HSV-1, OLHE at the concentration of one and 1.25 mg/mL caused a significant ($P < 0.05$) reduction in viral plaques compared to the control samples; lower OLHE concentrations did not have such effect (figure 1). No significant inhibitory effect was observed when various concentrations of OLHE were applied one hr after Vero cells were infected with HSV-1 (data not presented).

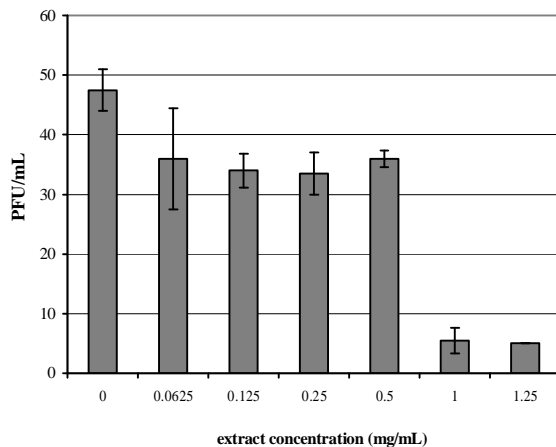


Figure 1: Growth of HSV-1 in the absence and presence of various concentrations of olive leaves hydroalcoholic extract. Data from two experiments in quadruplicate are shown as Mean ± SEM.

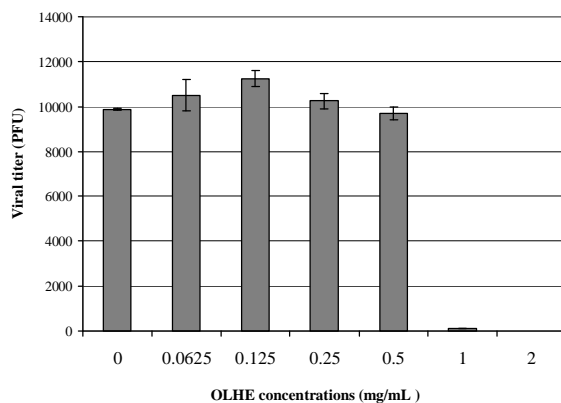


Figure 2: The HSV-1 titers in the absence and presence of various concentrations of olive hydroalcoholic extract, preincubated with the virus at room temperature for one hour. Data from two experiments in quadruplicate are shown as Mean ± SEM.

The incubation of OLHE at concentrations < 1 mg/mL with the virus at room temperature

for one hr prior to the inoculation of cell monolayers did not change the virus number significantly. However, at concentrations ≥ 1 mg/mL, it significantly ($P < 0.05$) reduced the virus number (figure 2).

Discussion

Findings of the present study indicated that OLHE has significant antiviral activity against HSV-1. To study the anti-HSV activities of OLHE, its cytotoxicity was first examined against Vero cell line. OLHE was toxic to that cell line at concentrations > 2 mg/mL. Therefore, lower concentrations were chosen for antiviral assays.

The study used three protocols to examine the antiviral activity of OLHE. When applied to cell culture which had been infected with HSV-1 one hr earlier, various OLHE concentrations had no significant inhibitory effects on HSV-1 infection of the Vero cells. This finding suggested that OLHE has no effects on the viruses that have penetrated inside the cells. In another protocol, application of OLHE to cells was followed by virus infection one hr later. At high concentrations, OLHE was able to reduce the viral plaques in this protocol. Such reduction might have been due to direct virucidal activity of OLHE or due to prevention of virus entry into the cells. Therefore, in a separate protocol OLHE was applied to the media containing virus, and the combination was added to cell culture one hr later. At a concentration similar to those of the second protocol, OLHE had virucidal activity. The similarity of OLHE virucidal activities in the third protocol, in which the virus was exposed to OLHE for one hr before the infection, and the second protocol, in which OLHE was added to the HSV-1-infected cells, might suggest that OLHE did have direct virucidal activity. Although the antiviral effect of any particular fractions of OLHE was not investigated in our study, a similar direct virucidal activity against HSV was reported for essential oils of South American plants.²⁷

The virucidal activity of OLHE is more likely to be attributed to its ability to prevent virus entry into the cells. It may be due to the interaction of OLHE with Vero cell membrane and/or HSV-1 envelope. The exact mechanism of OLHE antiviral activity is not still clear. However, it might be attributed to the prevention of attachment and adsorption of virus particles to the cell, and thereby blockade of their entry into the cells. In agreement with this hypothesis, olive leaves extract was shown to interact with the surface of phospholipid bilayer.²⁸ Moreover, it was shown that OLHE is a viral inhibitor at early stages of replication, probably

via blocking of viral envelope fusion.¹⁸ Interference with different stages of viral replication was also mentioned by other researchers.²⁹

The anti-HSV-1 activity of OLHE might be attributed to components of olive leaves including oleuropein, oleonic acid, hydroxyltryptol or calcium elenolate. These compounds were shown to have antimicrobial properties.^{15,16,29} Moreover, oleorupin was suggested to have potent antiviral activities against herpes mononucleosis, hepatitis virus, rotavirus, bovine rhinovirus, canine parvovirus and feline leukaemia virus.¹⁸ As well, it was reported to have a significant antiviral activity against respiratory syncytial virus,³⁰ parainfluenza virus,³⁰ and human immunodeficiency virus.¹⁷ Treatment with OLE was shown to reverse many of the HIV-1 infection-associated changes.¹⁷

The inhibitory effect of OLHE on HSV-1 might also be due to lack of the need to convert an active form by thymidine kinase. Therefore, viruses not containing this enzyme might be sensitive to OLHE. Thus, OLHE might be considered as a new option for treating resistant HSV-1 in clinical trials.

In conclusion, we found that OLHE has anti-HSV-1 activity. This activity was likely to be due to the prevention of virus entry into the cells.

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References

- 1 Azadbakht M. Classification of medicinal plants. Taimourzadeh Publishing Tehran; 1378. p. 234-7.(in Persian)
- 2 Mir Haidar H. Medicinal plant facts, applications in prevention and treatment of diseases. Islamic Culture Press, Tehran; 1372. p. 149-62.(in Persian).
- 3 Amin Gh. Iran's traditional medicinal plants. Undersecretary for Research Ministry of Health, treatment and Medical Education, Tehran; 1370. p. 65 (in Persian).
- 4 Ribeiro Rde A, Fiuza de Melo MM, De Barros F, et al. Acute hypotensive effect in conscious rats produced by some medicinal plants used in the state of São Paulo. *J Ethnopharmacology* 1986; 15: 261-9.

- 5 Khayyal MT, el-Ghazaly MA, Abdallah DM, et al. Blood pressure lowering effect of an olive leaf extract (*Olea europaea*) in L-NAME induced hypertension in rats. *Arzneimittelforschung* 2002; 52: 797-802.
- 6 Zarzuelo A, Duarte J, Jiménez J, et al. Vasodilator effect of olive leaf. *Planta Med* 1991; 57: 417-9.
- 7 Gonzalez M, Zarzuelo A, Gamez MJ, et al. Hypoglycemic activity of olive leaf. *Planta Med* 1992; 58: 513-5.
- 8 Cherif S, Rahal N, Haouala M, et al. A clinical trial of a titrated *Olea* extract in the treatment of essential arterial hypertension. *J Pharm Belg* 1996; 51: 69-71.
- 9 Hanbury D. On the febrifuge properties of the olive (*Olea Europaea*, L). *Pharmaceutical Journal of Provincial Transactions* 1854; 353-4.
- 10 Juven B, Henis Y. Studies on antimicrobial activity of olive phenolic compounds. *J Appl Bacteriol* 1970; 33: 721-32.
- 11 Furneri PM, Marino A, Saija A, et al. In vitro antimycoplasmal activity of oleuropein. *Int J Antimicrob Agents* 2002; 20: 293-6.
- 12 Bisignano G, Laganà MG, Trombetta D, et al. In vitro antibacterial activity of some aliphatic aldehyde from *Olea europaea* L. *FEMS Microbiol Lett* 2001; 98: 9-13.
- 13 Paster N, Juven BJ, Harshemesh H. Antimicrobial activity and inhibition of aflatoxin B1 formation by olive plant tissue constituents. *J Appl Bacteriol* 1988; 64: 293-7.
- 14 Markin D, Duek L, Berdicevsky I. In vitro antimicrobial activity of olive leaves. *Mycoses* 2003; 46: 132-6.
- 15 Reins HE. In vitro antiviral activity of calcium elenolate. *Antimicrobial Agents Chemother (Bethesda)* 1969; 9: 167-72.
- 16 Hirschman SZ. Inactivation of DNA polymerase of murine leukemia viruses by calcium elenolate. *Nat New Biol* 1972; 238: 277-9.
- 17 Lee-Huang S, Zhang L, Huang PL, et al. Anti-HIV activity of olive leaf extract (OLE) and modulation of host cell gene expression by HIV-1 infection and OLE treatment. *Biochem Biophys Res Commun* 2003; 307: 1029-37.
- 18 Micol V, Caturla N, Peterz L, Mas V. The olive leaf extract exhibits antiviral activity against viral haemorrhagic septicaemia rhabdovirus (VHSV). *Antiviral Res* 2005; 66: 129-36.
- 19 Whitley RJ. Herpes Simplex virus. In: Field BN, et al. Editors; *Fields Virology* 3rd ed. Lippincott Williams & Wilkins: Philadelphia; 1996. p. 2256-7.
- 20 Kimberlin DW, Coen DM, Biron KK et al.

- Molecular mechanisms of antiviral resistance. *Antiviral Res* 1995; 26: 369-401.
- 21 Gong Y, Raj KM, Luscombe CA, et al. The synergistic effects of betulin with acyclovir against herpes simplex viruses. *Antiviral Res* 2004; 64: 127-30.
 - 22 Sarisky RT, Crosson P, Cano R, et al. Comparison of methods for identifying resistant herpes simplex virus and measuring antiviral susceptibility. *J Clin Virol* 2002; 23: 191-200.
 - 23 Paramore LC. Use of alternative therapies: estimates from the 1994 Robert Wood Johnson Foundation National Access to Care Survey. *J Pain Symptom Manage* 1997; 13: 83-9.
 - 24 Samsam-Shariat SH. Extracting active material of medicinal plants and their identification and evaluation. 1st ed. Mani publication, Isfahan; 1002. p. 3-19. (in Persian)
 - 25 Leary JJ, Wittrock R, Sarisky RT, et al. Susceptibilities of herpes simplex viruses to penciclovir and acyclovir in eight cell lines. *Antimicrob Agents Chemother* 2002; 46: 762-8.
 - 26 Karber G. Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche (On collective treatment of serial pharmacologic studies). *Naunyn-Schmiedeberg's Arch Exp Path Pharmacol* 1931; 162: 480-3.
 - 27 Duschatzky CB, Possetto ML, Talarico LB, et al. Evaluation of chemical and antiviral properties of essential oils from South American plants. *Antivir Chem Chemother* 2005; 16: 247-51.
 - 28 Paiva-Martins F, Gordon MH, Gameiro P. Activity and location of olive oil phenolic antioxidants in liposomes. *Chem Phys Lipids* 2003; 124: 23-36.
 - 29 Yaseen Khan MD, Panchal S, Vyas N, et al. *Olea europaea*: A Phyto-Pharmacological Review. *Pharmacognosy Reviews* 2007; 1: 114-8.
 - 30 Ma SC, He ZD, Deng XL, et al. In vitro evaluation of secoiridoid glucosides from the fruits of *Ligustrum lucidum* as antiviral agents. *Chem Pharm Bull (Tokyo)* 2001; 49: 1471-3.