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In Vitro assessment of the Anti-viral effect of *Curcumin longa* on Herpes Simplex Virus Type 1

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ABSTRACT

Antiviral drug resistance has become more prevalent in recent years. Therefore, this research aimed to investigate the antiviral effect of the of hydroalcoholic extract of *Curcumin longa* against human herpes simplex virus type 1. In this study, *Curcumin longa* hydroalcoholic extract was provided by the clevenger system. Cytotoxicity of *Curcumin longa* was determined on the Vero cell line. The antiviral effect of extract was evaluated by plaque reduction assays. The mean number of herpes simplex plaques was lower in group that received *Curcumin longa* extract with concentration of 2 mg/mL compared to that of the control group. Moreover, In contrast, acyclovir prevented 94.4% of virus plaque formation at the concentration of 2500 µg/mL. So, the finding of the present study showed the significant inhibitory effect of the *Curcumin longa* hydroalcoholic extract on HSV-1 replication. *Curcumin longa* hydroalcoholic extract or preparation can be recommended as a pharmaceutical source for controlling HSV-1 related diseases. However, it is necessary to investigate in-vivo the potential side effect of *Curcumin longa*.

Key words: Herpes simplex type1, Curcumin longa, Extract, in-vitro

1. INTRODUCTION

Human herpes simplex virus (HSV) type 1 and 2 are among the most prevalent and important pathogenic viruses in humans. The virus is transferred via respiratory system, direct contact of person-to-person, saliva, sexual contact and through the birth canal in women who are infected with genital virus (1). Primary infections usually appear in the form of integrated blisters on the skin or mucous membranes, which are also observed in the form of inflammation of the mouth, gingivitis and inflammation of the pharynx. Also, HSV can infect the liver, adrenal glands, and eyes and can be followed by blindness. It can also result in encephalitis, meningitis and, death by attacking the central nervous system and brain (1). In recent years, some reports on the emergence of drug-resistant mutants have been reported following the long-term treatment with acyclovir. The mutants without thymidine kinase enzyme that is the original location of the effects of medicine have been separated from patients with immune disorder and may appear as a major problem in the coming years (2). Some adverse effects, such as renal failure, are seen due to the use of acyclovir and vidarabine as well (2-4). Regarding the above point, it seems necessity to find new medications with low side effects. Nowadays, since herbal medicines have low side effects and much of their effective ingredients cannot be synthetized artificially due to their complex chemical structure, many herbal medicines are considered in the treatments of diseases (5). Likewise, due to the lack of topical acyclovir for intra-oral consumptions, the treatment of recurrent intraoral herpes infection (RIH) has been limited to symptomatic treatment and there is no certain topical treatment to control this disease yet (4). One of the recent treatments which is highly common among patients is the herbal topical treatment and patients tend to use such medicines because of their fewer adverse effects. *Curcumin longa* is from the family of Zingiberaceae and a yellowish orange substance with 3 feet height and with

significant effects in medical sciences. According to previous studies, the effective concentration of *Curcumin longa* should between 4/5 -3/0 in order to obtain the mentioned effects of this substance. Its general properties, which can be mentioned, are antioxidant, anti-inflammatory, protection of the liver cells, anti-mutation effect, anti-microbial property, and etc. The antioxidant effect of *Curcumin longa* is reported to be more than that of vitamin E. Arora *et al*. pointed out the anti-inflammatory activity of *Curcumin longa* in different branches of petroleum ether. The anti-inflammatory properties of alcoholic pure extract of *Curcumin longa* has been proved to be effective in acute inflammation of oral consumption of *Curcumin longa* such as cortisone or phenylbutazone but its effect is reduced to the half in oral chronic inflammation. Previously, different anti-JNK agonist compounds of *Curcumin longa* were showed including gamma, ultra violet –c, anisomycin, ionomycin, phorbol12-myristate 13-acetate, sodium orthovanadate, tumor necrosis factor - alpha, and radiation. The repressive effect of CCN2 expression has been proved in the activity of liver cells (5). The effect of this substance in dentistry is the reduction of pain and swelling but no study has been conducted on the final effect of *Curcumin longa* on the herpes virus particularly HSV-1 for its application in intra-oral herpes lesions. The purpose of present study was to investigate the inhibitory effect of *Curcumin longa* hydroalcohlic extract on the pathogenic effect of HSV-1 in cell culture system.

2. MATERIALS AND METHODS

2.1. Plant extract, cell line and virus sample

Curcumin longa which is not powdered was prepared and codified for the verification of its scientific name in the herbarium department of medicinal plants in Pharmacy College of Shiraz University of Medical Sciences and verified with a herbarium number of PM669. In the present study, Vero cell line was used for the replication of the HSV-1 virus. For cell culture, DMEM medium (Dulbecco's Modified Eagle's Medium) containing 10% fetal calf serum, 100 IU/mL penicillin and 100 mg/mL of streptocine was used. The HSV-1 virus which was used in the present study was provided by the Department of Bacteriology and Virology, Shiraz University of Medical Sciences, Shiraz, Iran.

2.2. Determination of the toxicity threshold of Curcumin longa extract on Vero cell

Trypan Blue Exclusion Method was used to determine the toxicity threshold of the extract on Vero cells. In this method, after the formation of a single layer cell in the 96-well micro plate, the concentrations of 0.1, 0.2, 0.5, 1, 2 mg/mL of *Curcumin longa* extract of DMEM medium was added, without fetal calf serum, to the micro plate wells. For each concentration, a row of a micro plate was considered and the medium was only added in the final well as a control cell. Micro plate was placed at 37 °C and 5 percent of CO_2. After 24 hours, cells of the two wells with any concentration was detached and stained with 10%. Trypan blue stains dead cells and live cells remained colourless. Then, living and the dead cells were counted and this procedure was repeated until the fourth day. In the fourth day, cytotoxic concentration 50% (CC50) was estimated as the concentration of the extract at which 50% of cells were died.

2.3. Investigation of the Virucidal effect of Curcumin longa extract on HSV-1

100 TCID50 viruses were treated with CC50 concentration of extract. After 1, 2, 3, 4 hours, the above suspension was added to the Vero cell monolayer. After an hour, cells were washed 3 times to remove extract residuals. After 24 hours, the reduction of virus infectivity was determined by quantal method.

2.4. Detection of the inhibitory effect of Curcumin longa extract, in different concentrations, on the replication of HSV-1

To determine the concentration of the extract with 50% inhibitory effect on the replication of the virus, at first, after the formation of a single layer of cells in plate wells, 100 TCID50 viruses was added to all wells except the virus-free control wells at the end of the row. After an hour, wells were washed with PBS buffer to remove unabsorbed viruses. Different concentrations of *Curcumin longa* hydroalcoholic extract were added to the DMEM medium without fetal calf serum to the micro plate wells. 12 wells were allocated for each concentration. Controls consisted of 0.1 mL of virus suspension and 0.1 mL of culture medium without extract and normal cell monolayers and monolayers without exposure to the extracts and inoculated with the virus. Subsequently, the monolayers were overlaid with 1% *carboxymethyl cellulose (*CMC) in the maintenance medium with the same increasing concentrations of CSE, and were incubated at 37°C with 5% CO2 for four days. After 96 hours, the contents of each series of wells were removed and virus infectivity was determined by the direct plaque count. HSV-1 plaques formed on cells were fixed with methanol for 10 min and stained with0.5% crystal violet solution. Experiments were performed three times.

2.5. Statistical methods

One-sample repeated measures (one-sample RM ANOVA) were applied to compare antiviral effect of *Curcumin longa* extract. T-test (student's t-test) was run to compare the antiviral effect of Curcumin and acyclovir, using the SPSS version 15 software package (SPSS Inc., Chicago, IL, U.S.A.). A p-value lower than 0.05 was considered significant.

3. RESULTS AND DISCUSSION

As it can be seen in Table 1, a one-way analysis of

variance showed a significant difference (P <0.001) for the mean number of plaques (PC) among studied groups. Tombane t2 post hoc test showed that the average PC in group 2 was significantly lower than other groups (P <0.05) but there was no difference between the PC mean of other groups.

Table 1. Mean and standard deviation of HSV-1 plaque numbers

Groups	Concentration	Mean	Standard Deviation	Minimum	Maximum
1	0.1 mg/mL	37.8	10.8	22.3	41
2	0.2 mg/mL	33	7.8	24	37.5
3	0.5 mg/mL	32.3	8.5	22.5	37.3
4	1 mg/mL	27.2	3.2	24.5	37.8
5	2 mg/mL	15.9	6.2	10.7	22.8
6	Virus control without extract	37.7	11.1	25	45.7

According to the Kruskal-Wallis test, there is no significant differences in the recipient groups of Hydroalcoholic Curcumin extract (P value = 0.15). According to ANOVA analysis, the P value is equal to P <0.001 which shows the inhibitory effect of herpes simplex virus in the groups with Curcumin extract at concentrations of 2 mg/mL compared with 1 mg/mL with p = 0.048 , compared with 0.2 mg/mL, with p = 0.014, compared with 0.5 mg/mL, with p = 0.030, compared with 1 mg/mL with p = 0.038, compared with the control group, virus-free extract with p = 0.024 was significant, and had more inhibitory effect on virus. According to Figure 1, the average number of herpes simplex virus plaque was high in the control group and the lowest rate was seen in the group receiving Curcumin extract with the concentration of 2 mg/mL. This concentration resulted in viral inhibition of plaque formation. There was no HSV-1 plaque formation at the concentration of 2500 mg/mL of acyclovir.

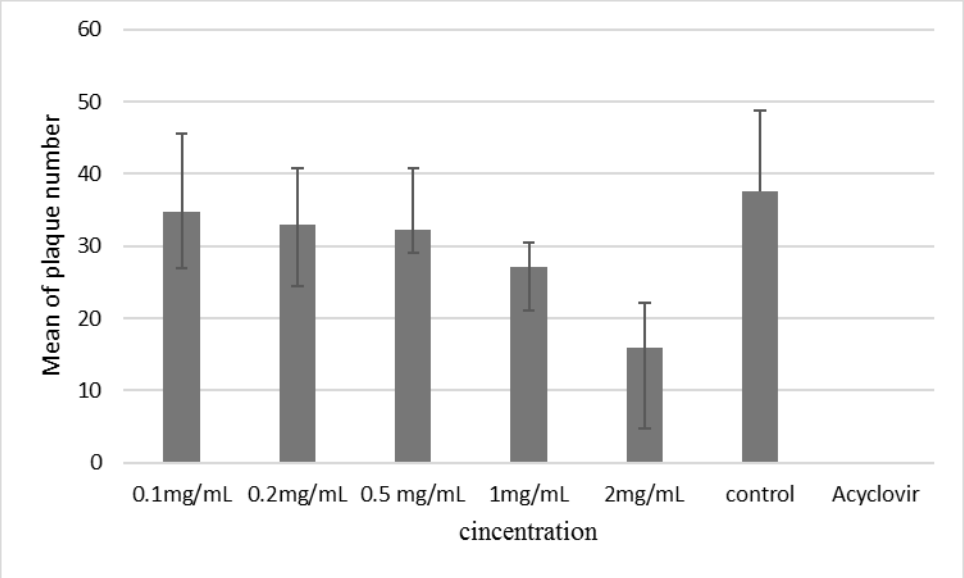

Figure 1. Diagram of the average number of viral plaques in different concentrations of Curcumin extract

Cytotoxicity assays showed that Curcumin extract at concentrations of 0.1-2 mg/mL was safe for vero cells. The results of viable cell counting of cell toxicity assays with Trypan blue test for acyclovir solutions for 48 hours with 3 concentrations of 625, 1250, 2500 µg /mL and % of living cells which obtained, were as follows: 625 µg/mL: 18/17: 94.4% -1250 µg/mL: 15/14: 93.3% and 2500 µg/mL: 9/12: 75%. Researchers have considered the expansion of chemotherapy against viruses, but there are restrictions in this regard that prevent the progression of chemotherapy against viruses. One of these obstacles is that viruses replicate intracellular. Therefore, useful antiviral compounds should discriminate between virus and host activities with a high degree of specificity to make the least damage to the host cells. There are several effective antiviral drugs against herpes infections most which prevent the viral DNA synthesis; acyclovir is an optional drug (6). In recent years, Clinicians have concerns about long-term treatment with acyclovir, due to resistant mutants to the drug and drug toxicity (6). The use of medicinal plants for the treatment of diseases has centuries of history and is the main method of treatment in developed countries (7). Curcumin is the most important, effective substance of turmeric with the scientific name

Longa Curcumin (Curcuma Longa) which has remarkable antioxidant properties, and causes the reduction of free radicals, inhibition of lipid peroxidation and increase of superoxide dismutase activity. It has anti-inflammatory effect, reduces blood fat and blood sugar (8), and improves memory in an experimental model of Alzheimer's disease caused by intra ventricular streptozotocin. In addition, its analgesic effect in thermal hyperalgesia in an experimental model and chronic diabetes induced by strep Strepozotocin in ---- model (9) and analgesic effect in an experimental model of neuropathic pain by using chronic nerve compression have been confirmed (10). Turmeric extract with the scientific name of curcuma longa is an extracted polyphenol complex of turmeric pigment with anti-inflammatory, anti-oxidation capabilities and also prevents cell death (11). In recent years there has been a global tendency to use medicinal plants because of fewer side effects and low cost. For example, in Greek medicine, lemon balm extract is used orally for the treatment of the influenza virus and herpes simplex virus (12). Mazhrum (mashroom) extract has also a very effective ability against herpes simplex virus. The anti-viral activities of Licorice extract have been investigated in the viral hepatitis so far (13). According to the conducted survey in Bushehr, extract of one type of green algae called "Color Sertolarivaidis" has a significant inhibitory effect on one side of this virus in "Vero" cell culture (14). One of the understudied plants in this field is eucalyptus. In a study to investigate the effect of eucalyptus extract on the reduction of cytopathic effect (CPE) in cells infected with HSV1, it was found that eucalyptus extract has a positive role in reducing the CPE obtained from the virus in the direct and indirect effect phases, but the best time for the effect of the extract was after the virus absorption by cell (15). In this study, turmeric extract caused the inhibition of the viral plaques. Garlic is among herbal medicines which have anti-fungal and bacterial effect. It is introduced as an effective anti-viral compound in many articles and the Allicin in garlic extract is the main active compound that has in vitro and in vivo anti-viral properties. Cytomegalovirus, influenza B, HSVI, HSVII, Para influenza virus III, Vaccine virus, and human rhinoviruses type III are among sensitive viruses to garlic extract (16). Armaka *et al.* in 1999 in Greece Aristotle University had a study on Izoporneol, which is a component of essential oils in many plants (including oil H-Balm), and showed that this substance has a double Virusidal effect against HSVI. Thus, during 30 minutes, after it was exposed to them, HSVI virus was inactivated completely in l0.06 mg/mL concentration of virus replication (17). In a study conducted in 2000 at the University of Oveido, Spain, after investigation of cytotoxic effect of watery extract of Filantus Euripicolaris in MTT method and the CPE investigation, its anti-viral effect was tested and its antiviral effect was determined against herpes simplex type I and ii (18). In an investigation on the extracts of 24 plants, Lopez and Hudson (2001) found out that some of these extracts had inhibitory effect on herpes simplex. Complete prevention of growth and replication in non-cytotoxic concentrations was reported by the application of CPE observation method (19). Armaka et.al in 1999, in Greece Aristotle University, had a study on Izoporneol, which is a component of essential oils in many plants (including oil H-Balm) and showed that this substance has a double Virusidal effect against HSVI. Thus, during 30 minutes, after it was exposed to them, HSVI virus was deactivated and stopped completely in l0.06 mg /m concentration of virus replication (17). Considering the high consumption of turmeric in Iran and different compounds of this plant in other areas, it is necessary to study the anti HSV-1 effect of *Curcumin longa*, determine the percentage of its major compounds and anti-viral effect of it on appropriate cell culture conditions. Given the importance of the HSV-1 virus and its widespread around the world, researchers are always looking for new medicinal compounds with minimal side effects against the virus. Therefore, for better comparison of the effect of different plants on this virus, it is suggested that the results of this study to be compared simultaneously and under the same conditions. According to the conducted survey, *Curcumin longa* extract has an inhibitory effect on HSV-1 and thus it is recommended to be used as anti-HSV-1 as an herbal product, replaced with the classical therapeutic compounds that the virus may be resistant to. Although, before that, it seems necessary to perform in vivo experiments to ensure the pharmacological properties and the potential toxicity of this extract on animals' bodies and other consumers. In recent years, many studies have been done on the inhibitory effects of natural substances against microorganisms. In this regard, it is essential to use compounds that are non-toxic for humans and have no side effects.

4. CONCLUSION

This study showed remarkable anti-HSV-1 effect of hydroalcoholic extract of *Curcumin longa,* as a medicinal plant. Our findings indicate a significant inhibitory effect of *Curcumin longa* on the HSV-1 virus, and it can be recommended to be used as a medicinal plants source to control HSV-1. However, detection of its potential toxicity for the consumer seems to be essential before conducting any clinical trial for further investigation.

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AUTHORS CONTRIBUTION

This work was carried out in collaboration among all

authors.

CONFLICT OF INTEREST

The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this article.

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