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Original Article

Evaluation of Potential Antiviral Activity of the Hydroalcoholic Extract of Lemon Balm L. against Herpes Simplex Virus Type 1

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Abstract

Background and Aims: Lemon Balm L (Lamiaceae) has been used in a variety of practical applications in medical sciences. Its antiviral activity against herpes simplex virus type-1 (HSV-1) was investigated in cell culture. Lemon Balm hydroalcoholic extract was found to be non-toxic to Vero cells up to concentration 800 ug/ml and inhibited the growth and development of HSV-1 in dose-dependent manner in Vero cells.

Methods: In order to study the possible mechanisms of the antiviral activity of Lemon Balm extract, cells were treated with extract before, during and after infection, and the viral titers were tested by TCID50 assay.

Results: The antiviral effects in treatment of post and during virus infection were more remarkable than the treatment of preinfection. For further investigation indirect immunofluorescence technique was used to elucidate the antiviral mechanism of the extract by infecting Vero cells at different times and monitoring the synthesis of viral proteins.

Conclusion: Although the precise mechanism has not yet to be defined, our work indicated that lemon Balm L. extract could inhibit growth and development of HSV-1 in cells in vitro.

Keywords: Lemon Balm L; HSV-1; Antiviral activity; vero cell; TCID50

Introduction

Herpes simplex virus is capale of causing a widespread spectrum of mild to sever disorders. These include acute primary and recurrent mucocutaneous diseases. HSV -1 causes several neuronal diseases; it spreads in sensory axons and infects sensory neurons in the ganglia of the peripheral nervous system stablishing latent infection (1, 2).

Today, in the treatment of herpes virus infections, antiviral drugs like acyclovir (ACV), vidara-bine (ara-A) and ganciclovir (DHPG) are used (3, 4). The mechanism of action of these drugs is basically dependent on their abilities to inhibit the virus-specific enzymes, thymidine kinase and the+ DNA polymerase (5, 6).Because of their cytotoxic effects, these drugs except of acycloir are not widely used (7). In recent years, however, acyclovir and other drugs have been reported to be inefficient in treating genital herpes infections. HSV-1 has also been reported to acquire resistance to these drugs (4, 6). For all these reasons, the search for new antiviral drugs active against HSV is on the increase. In this direction, the results of studies with plant extracts have been especially promising.

One of the plant derivatives that has been occupied an important place in the field of chemotherapyeutic research in recent years is Lemon Balm L. Lemon Balm L. (Lamiaceae)

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is a plant growing in various areas, incluing the Mediterranean region and western Asia (8, 9). The extract of Lemon Balm L. (Lamiaceae) has been reported to inhibit protein synthesis (10). In Iran this plant is widely cultivat-ed through out Isfahan and Fars. Lemon Balm is an aromatic perenial sub shrub. The leaves contain at least 0.05% (v/w) volatile oils based on the dried herb. Main components assre tannins unique to the Lamiaceae, such as triterpenylic acid, rosmarinic acid and flavonoids (11).

Subsequent studies demonstrated that the antiviral activity of Lemon Balm was due to tannin and also to a non-tannin polyphenilic fraction (12, 13).

Methods

Plant materials

Leaves of Lemon Balm L. (Lamiaceae) were collected from a farmland in Isfahan and identified at the department of pharmacology of Isfahan university of Medical Sciences. The dried leaves were pulverized and 200gr of pulverized sample was extracted with 500ml of 80% methanol by maceration for 72hr. The methanol extract was concentrated in a rotary evaporator, lyophilized and thereafter preserved for further use.

Cell culture and Virus

Vero cells (African green monkey kidney cells) were cultured with Dulbeccos Modified Eageles Medium (DMEM) supplemented with 10% heatinactivated Fetal Bovine Serum (FBS), 100 IV/ml penicillin and l Ouug/ml streptomycin. The cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 and were subcultured two or three times a week.

HSV -1 was isolated from patients and identified by specific monoclonal antibodies. Viruses were quantified in terms of the 50% tissue culture infective dose (TCID50) by endpoint dilution, with the infectious titer determined by the method of Reed and Meunch (14), ad stored in small aliquots at -70°C until use.

Cytotoxicity assay

In order to test the effect of the Lemon Balm L. extract on vero cells, $5\times l0^4$ cells, (in l ml DMEM, supplemented with 10% FBS) were seeded in to each well of microplates, cultured for 6hr at 37°c, cells were allowed to grow for additional 48hr in the presence of increasing amounts of extract (10, 100, 200, 400, 600, 800 and 1000 ug/ml), The cytotoxicity of the extract was determined on a conventional hemocytometer using the trypan blue exclusion method (18). The 50% cytotoxic concentration (CC_{50}) was defined.

As the concentration, which caused a 50% reduction in the number of viable cells.

Virus yield inhibition assay

or the virus yield inhibition assay, semi-confluent Vero cell monolayers in 24-well plates (Falcon) were treated with Lemon Balm extract before, during and after virus infection as described in the following section.

Incubation of cells with the extract before, during and after virus infection

Lemmon Balm L. hydroalcoholic extract was dissolved in serum free DMEM and incubated with semi-confluent cell in 24well tissue culture plates in increasing concentration from 10 to 400 ug/ml for 2h at 37°C. After removal of the extract, the cells were washed with phosphate buffered saline (PBS) and then infected with HSV-1 at multiplicity of infection (MOI) 1. After l hr incubation, the unabsorbed virus was removed, the cell monolayer was washed with PBS and further incubated in DMEM with 2% FBS. Controls consisted of Vero cells untreated and Vero cells infected with HSV1.

For determination of antiviral activity of the extract during and post virus infection the assay was performed as described above, with the exception that the extract was added together with the virus and after adsorption, correspondingly. After 48hr incubation at 37°C, virus titer was determined by the endpoint dilution method and expressed as TCID50/ml.

EC_{50} , the concentration needed to restrain 50% virus infection compared to untreated infected cells, were determined directly from the curve obtained by plotting the inhibition of the virus yield against the concentration of the samples.

Immunofluorescence

Confluent Vero cells grown on 13mm cover slips were infected with 100-fold TCID50/ml HSV-l for 16h in the presence of 400 ug/ml of Lemon Balm L. extract compared to untreated infected cells. Cover slips were removed, washed in PBS and fixed in acetone at 4°C then they were stained indirectly with fluorescein conjugated anti-rabbit IgG using specific viral antiserum. Cover slips were mounted in glicerol buffer and examined in a VV equipped microscope.

Results

Cytotoxicity

Trypan blue exclusion method showed that the Lemon Balm extract had no serious effect on the proliferation of cells, up to concentration of 800 ug/ml (data not shown). Therefore, we could draw a conclusion that the CC_{50} (the concentration which causes 50% cytotoxic effect) was more than 800 ug/ml (Table 1).

Antiviral activity of the extract by TCID50 assay the inhibition of virus yield by Lemon Balm L. extract was evaluated by TCID50 assay in Vero cells. Lemon Balm extract showed strong antiviral activity against HSV - 1 when added during the early stages of viral infection (Fig. l B&C).

The degree of inhibition showed to be proportional to the concentration of the extract and when the concentration was higher Than 200 ug/ml, Lemon Balm L. extract inhibited almost completely the virus yield (Fig. 1C). As shown in Fig. lA the extract had no significant inhibitory effect when pre-incubated with the cells.

The EC_{50} value of the extract when treated during infection was 800 ug/ml and when treated after infection the EC_{50}decreased to l Sflug/ml (Table. 1), then the extract was more effective at low concentrations when present after viral infection.

Immunofluorescence

Semi-confluent monolayers of vero cells infected with HSV -1 in the presence and absence of Lemon Balm L. extract, were fixed and processed for indirect immuno-fluorescence. The expression of HSV-l proteins was evident after 18 hr of infection correlating with the appearance of its charactristic cytopathic effect (Fig. 2b, c).

When the same experiment was repeated in the presence of the extract, expression of the HSV-

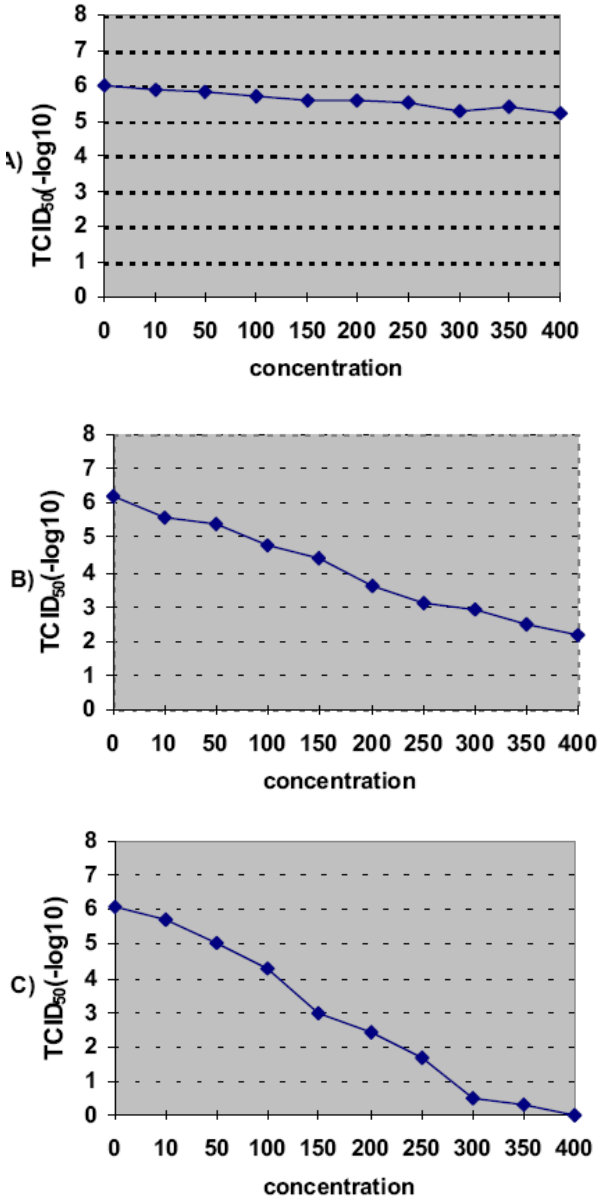

Fig. 1. Effect of increasing concentration of Lemon Balm L. extract on the titer of HSV-1 infected Vera cells by TCID50 assay. The multiplicity of infection was 0.1. The extract was present in before (A), during (B) and after (C) HSV-1 infection. The data were reported on the horizental axis in - log10 units as mean values ± SD for at least three separate expriments. Not significantly effective than VC; P> 0.05 (student's t-test).

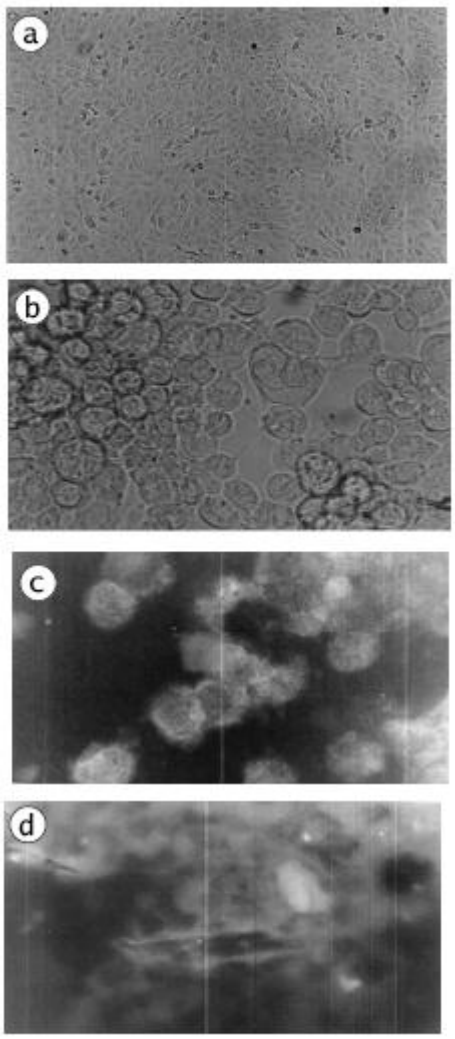

Fig. 2. Vera monolayers infected with HSV-1 were processed for light microscopy (b) or immunofluorescence (c), in compared with infected cells in the presence of the 400 ug/ml Lemon Balm extract (a and d), correspondingly.

1 antigens were very weak and did not show fluorescence apparently (Fig. 2d).

Discussion

Current chemotherapeutic antiviral drugs have been characterized as having in many cases limited clinical efficiency, suboptimal pharmacokinetics and toxic side effects (15, 16). New antiviral agents from the plant origin can have easy acceptability being non-toxic and inexpensive. Lemon balm (Lamiaceae) contains the the flavonoids quercitrin and rhamnocitrin, and the 7 -glucosides of apigenin and luteoline; phenolic acids and tannins, chiefly rosmarinic acid (up to 4%), and glycosidically bound caffeic and chlorogenic acids; triterpens; volatile oil (0.05- 0.375%), of which the monoterpenoid citronellal is 30-40%, geranial and neral nare 10-30% (17).

In this study the effects of different concentrations of the extract on Vero cells after a 72 hr incubation were determined. Lemon Balm L. extract had no cytotoxicity effect on Vero cells up to concentration of 800IJg/ml. In the preliminary screening test for anti-HSV-l activity by the CPE inhibition. The extract inhibited the appearance of CPE in HSV -1 infected Vero cells with EC_{so} of 150 ug/ml (Table. 1). Therefore Lemon Balm L. extract exhibits a potent anti -herpetic activity with SI more than 8.

It is well-known that the antiviral activities of Lemon Balm L. extract is due to tannins and polyphenolic fraction which inhibit the early stages of viral infection such as attachment and penetration (13). Vero cell monolayer in 24-well plates was treated with the extract before, during and after virus infection. The efficiency of protection against virus infection by pre-incubation in presence of the extract was negligible as compared to that achieved at post-incubation and during viral infection Fig. l. A significant reduction in infection virus yield could only be found with when the extract was added post-virus infection. It is possible in this case that Lemon Balm L. extract inhibits growth and maturation of viral progeny by inhibition of viral macromolecules synthesis or virus assembly. In conclusion, Lemon Balm showed strong antiviral activity against HSV -1 in Vero cells. These results imply that the effective antiviral concentrations of Lemon Balm L. are far from the cytotoxicity threshold, and, consequently, this plant extract possess good SIs and has a potential to be used as antiviral agent.

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Table 1. Distribution of HI antibody titer among the target groups before and after the vaccination (group a and b).

Virus	CC_{50} [a]	EC_{50} [b]			SI [c]		
		A	B	C	A	B	C
HSV-1	>800	*	248	150	*	4.8	8

A: the extract was present before viral infection in Vero cells; B: the extract was present during viral infection; C: the extract was present after viral infection.
a CC50 is the concentration which causes 50% cytotoxic effect.
b EC50 is the concentration of the drug required to inhibit 50% of virus-induced CPE.
c Selectivity index (SI) = CC50/ EC50
* The extract was not effective when present that time.

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