In Vitro Antiviral Activities of Fruit Extract from *Lycium Barbarum* and Methylxanthines Extracted from *Pu-erh* and *Bancha* Tea Leaves

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**Abstract**

**Introduction:** Based on traditional medicine, many countries use various plant products (fruits, leaves and other plant parts) as food supplements or in the form of tea. The use of these plant sources has been established through the years of use and the proven benefits of their ingredients to improve human health.

**Aim:** In the present study, we have focused on the effect of *Lycium barbarum* fruit extract and methylxanthines isolated from *Pu-erh* (MXP) and *Bancha* (MXB) tea leaves on Herpes simplex virus type 1 (HSV-1), poliovirus 1 (PV1) and coxsackievirus B1 (CVB1) virus in vitro.

**Materials and methods:** We used in vitro antiviral and virus attachment assays to determine the effects of the three extracts we studied.

**Results:** None of the extracts showed significant inhibition of replication of the three treated viruses but a remarkable inhibitory effect on extracellular virions of HSV-1 was exhibited 30 minutes after exposure, especially by the *Lycium barbarum* extract. The inhibitory effect of the three extracts on the level of adsorption of the HSV-1 to sensitive cells (MDBK) was also significant, with the most pronounced effect of the MXP. The protective effect of the extracts against herpes infection on healthy cells was also determined, the MXP showing the most notable effect.

**Conclusions:** The three studied extracts can be used effectively in the treatment of herpes infections, as well as in infections with other enveloped viruses.

**Keywords**

coxsackievirus B1, green tea extracts, herpes simplex virus type 1, *Lycium barbarum* extract, poliovirus 1

**INTRODUCTION**

*Lycium barbarum*, known as wolfberry or Goji berry, has been used for more than 2,000 years as a traditional medicinal herb and food supplement in China. Constituents of *Lycium barbarum* fruits include polysaccharides and proteoglycans (23% of dried mass), carotenoids (mainly zeaxanthin dipalmitate), vitamins (riboflavin, thiamin and ascorbic acid), flavonoids, essential oil and fatty acids, free amino acids and others.¹ The polysaccharides isolated...
from *Lycium barbarum* are the most studied and are believed to contribute to a broad range of pharmacological activity, as antioxidant, immunomodulatory, anti-inflammatory, anti-tumor, antiviral and others.[2,3]

Methylxanthines, caffeine (1,3,7-trimethylxanthine), theophylline (1,3-dimethylxanthine), and theobromine (3,7-dimethylxanthine) are some of the most consumed natural products worldwide. They are found in many traditional sources, such as coffee, tea or chocolate. *Pu-erh* and *Bancha* teas are gaining wide popularity among people consuming green tea. *Pu-erh* is rich in methylxanthines, while the *Bancha* contains much less. Beside these, researchers have found a wide range of other biologically active ingredients such as flavonoids and catechins.[4] Among the known pharmacological properties of methylxanthines[5], possible antiviral activity has been adverted recently and anti-COVID-19 activity has been discussed[6].

**AIM**

The present study investigated the in vitro antiviral activity of *Lycium barbarum* fruit extract and methylxanthines fractions isolated from *Pu-erh* and *Bancha* tea leaves against viral strains of three taxonomic groups causing socially significant diseases in the human population, as poliovirus 1, Coxsackievirus B1 (Picornaviridae family) and herpes simplex virus type 1 (family Herpesviridae).

**MATERIALS AND METHODS**

**Cells**

Madin-Darby bovine kidney (MDBK) cells and human epithelial type 2 (HEp-2) cells originating from human laryngeal carcinoma were obtained from the National Bank for Industrial Microorganisms and Cell Cultures, Sofia. The cell lines were grown in DMEM medium containing 10% fetal bovine serum (Gibco BRL, USA), supplemented with 10 mM HEPES buffer (Merck, Germany) and antibiotics (penicillin 100 IU/ml, streptomycin 100 μg/ml) in CO₂ incubator (HERA cell 150, Heraeus, Germany) at 37°C/5% CO₂.

**Viruses**

Herpes simplex virus type 1, Victoria strain (HSV-1) was received from Prof. S. Dundarov, National Center of Infectious and Parasitic Diseases, Sofia. The virus was replicated in monolayer MDBK cells in a maintenance solution DMEM Gibco BRL, Paisley, Scotland, UK, plus 0.5% fetal bovine serum Gibco BRL, Scotland, UK. Infectious titer of stock virus was 10⁶₅⁰ CCID₅₀/ml.

Poliovirus 1 (LSc-2ab strain) (PV1) is from the collection of the Stephan Angeloff Institute of Microbiology, BAS (Sofia, Bulgaria), grown in HEp-2 cells (maintenance solution Dulbecco’s modified Eagles’ medium DMEM (Gibco, UK), supplemented by 0.5% bovine fetal serum (Gibco, UK), 10 mmol HEPES buffer (AppliChem GmbH, Darmstadt, Germany) and antibiotics (penicillin, 100 U/ml, streptomycin, 100 mg/ml). Infectious titer 10⁶₉⁸ CCID₅₀/ml.

Coxsackievirus B1 (Connecticut 5 strain, CVB1) is from the collection of the Stephan Angeloff Institute of Microbiology, BAS (Sofia, Bulgaria), grown on HEp-2 cells (maintenance solution DMEM (Gibco, BRL) with 10 mmol/l HEPES, 0.5% fetal calf serum (Gibco), penicillin 100 IU/ml and streptomycin 100 mg/ml); infectious titer 10⁶⁵ CCID₅₀/ml.

**Plant materials**

*Lycium barbarum* fruits (Lot No. L05042017) were provided by Paula Fruits Ltd, an official importer of Goji berries for Bulgaria with guaranteed Chinese origin, while *Pu-erh* and *Bancha* tea leaves were purchased from the local market with quality assurance. Before starting the extraction procedures, all plant materials were identified by Iliya Slavov (Associate Professor in Pharmacognosy) from the Department of Biology, Faculty of Pharmacy, Medical University of Varna, Bulgaria.

**Preparation of Lycium barbarum fruit extract (LBE)**

Initially, two fractions of *Lycium barbarum* fruits were isolated – pectin polysaccharide dry and polyphenolic liquid. After characterization of the content of the biologically active ingredients, a mixture of the two fractions was prepared with a ratio of polysaccharides to polyphenols of 1:1. All conditions regarding the extraction, the characterization of the obtained fractions and the preparation of the final extract are described thoroughly by Georgiev et al.[7]

**Preparation of extract from Pu-erh (MXP) and Bancha tea leaves methylxanthines (MXB)**

All conditions regarding the extraction of methylxanthines from the leaves of *Pu-erh* and *Bancha* tea are described in detail by Georgiev et al.[8] After characterization by HPLC analysis, it was shown that both methylxanthine fractions, from *Pu-erh* and *Bancha*, contain mainly caffeine (84.07% and 88.11%) and very small amounts of theobromine (0.16% and 0.11%, respectively). The *Pu-erh* tea sample contains a negligible amount of theophylline (<0.0001%), and none was detected in the *Bancha* tea sample.[8]

**Cytotoxicity assay**

Confluent monolayer cell culture in a 96-well plates (Costar®, Corning Inc., Kennebunk, ME, USA) was treated with 0.1 mL/well-containing a maintenance medium (untreated
control) or falling concentrations of the tested products. Cells were incubated at 37°C and 5% CO2 for 48 hours. After microscopic evaluation, the medium containing the test compound was removed, cells were washed, and they were incubated with neutral red at 37°C for 3 hours. After incubation, the neutral red was removed, and cells were washed with PBS, and 0.15 mL/well desorb solution (1% glacial acetic acid and 49% ethanol in distilled water) was added. The optical density (OD) of each well was read at 540 nm in a microplate reader (Biotek Organon, West Chester, PA, USA). The 50% cytotoxic concentration (CC50) was defined as the material concentration that reduced the cell viability by 50% when compared to untreated control. Each sample was tested in triplicate with four cell culture wells per test sample.

The maximum tolerable concentration (MTC) of the extracts is also determined, as the concentration at which they do not affect the cell monolayer and they look like the cells in the control sample (untreated with extract).

**Antiviral activity assay**

Cytopathic effect (CPE) inhibition test was used for assessment of antiviral activity of the extracts. Confluent cell monolayer in 96-well plates were infected with 100 cell culture infectious dose 50% (CCID50) in 0.1 ml (HSV-1, PV1 or CVB1). After 60 min of virus adsorption, extracts were added in various concentrations and cells were incubated for 48 hours at 37°C. The cytopathic effect was determined using a neutral red uptake assay and the percentage of CPE inhibition for each concentration of the test sample was calculated using the following formula:

\[
\% \text{ CPE} = \frac{\text{OD}_{\text{test sample}} - \text{OD}_{\text{virus control}}}{\text{OD}_{\text{untreated control}} - \text{OD}_{\text{virus control}}} \times 100
\]

where ODtest sample is the mean value of the ODs of the wells inoculated with virus and treated with the test sample in the respective concentration, ODvirus control is the mean value of the ODs of the virus control wells (without compound in the medium), and ODuntreated control is the mean value of the ODs of the wells not inoculated with virus but treated with the corresponding concentration of the test sample. The 50% inhibitory concentration (IC50) was defined as the concentration of the material that inhibited 50% of viral replication when compared to the virus control. The selectivity index (SI) was calculated from the ratio CC50/IC50.

**Viralcidal assay**

Contact samples of 1 ml containing HSV-1 (10^4 CCID50), and tested compound in its maximum tolerable concentration (MTC) in a 1:1 ratio were stored at room temperature for different time intervals (15, 30, 60, 90, and 120 min). Then, the residual infectious virus content in each sample was determined by the end-point dilution method and Δlog was compared to the untreated controls evaluated.

**Virus attachment assay**

24-well cell culture plates containing monolayer of MDBK cells were pre-chilled at 4°C and were inoculated with 10^4 CCID50 of HSV-1 for adsorption at 4°C and treated in parallel with MTC of the extract. At various intervals (15, 30, 45, and 60 min), cells were washed with PBS in individual samples in order to remove both the compound and the unattached virus, then overlaid with maintenance medium and incubated at 37°C for 24 hours. Following triple freezing and thawing the infectious virus titer of each sample was determined by the end-point dilution method. Each sample was prepared in triplicate.

**Pretreatment of MDBK cells**

Monolayers of MDBK cells pre-grown into 24-well cell culture plates (CELLSTAR, Greiner Bio-One) (2×10⁶ cells per well) were treated for 15, 30, 60, 90, and 120 min at concentration of MTC of the extract in the maintenance medium (1 ml per well). Then the extract was removed and the cells were washed with phosphate-buffered saline (PBS) and inoculated with HSV-1 (1000 CCID50 in 1 ml per well). After 60 minutes of absorption, the non-absorbed virus was removed and the cells were covered with maintenance medium. The culture plates were incubated at 37°C for 24 hours and, after triple freezing and thawing, the infectious viral titers were determined by the endpoint dilution method. Δlog was evaluated compared to viral control (untreated by compounds).

**RESULTS**

The cytopathicity of the three extracts tested was determined against two monolayer cell lines MDBK and HEP-2. This study is of primary importance for conducting antiviral experiments at non-toxic concentrations and excluding the overlapping effect of toxicity on cells. The results of cytotoxicity of the extracts are presented in Table 1.

LBE and MXP extracts have similar cytopathicity to HEP-2 cells and are about 2 times less toxic than the MXB extract. In MDBK cells, the lowest toxicity was shown by the MXP extract, which was almost twice lower than that of the LBE extract and almost three times lower than the MXB extract. The maximal tolerated concentration (MTC) of the extract in the MDBK cells was also determined. The lowest value of MTC showed the MXB extract – 100 mg/ml, and the extracts LBE and MXP showed similar values of MTC=320 mg/ml. This concentration is necessary for the proper conduct of part of the antiviral experiments.

Examining the effect of the extracts on viral replication, it was found that all three extracts did not affect the intracellular replicative cycle of HSV-1, PV1 and CVB1 (Table 1).

The lack of activity of the extracts against the intracellular replicative cycle of the studied virus strains faces us the challenge to determine the effect they have on the extracel-
Table 1. Cytotoxicity and *in vitro* antiviral activity of the extracts

| Extract | Cytotoxicity, CC₅₀ (μg/ml) | Antiviral activity |
|---------|---------------------------|-------------------|
|         | MDBK                      | HSV-1 IC₅₀ (μg/ml) | SI | PV₁ IC₅₀ (μg/ml) | SI | CVB₁ IC₅₀ (μg/ml) | SI |
| LBE     | 962                       | 1345              | -  | -                      | -  | -                      | -  |
| MXB     | 480                       | 625               | -  | -                      | -  | -                      | -  |
| MXP     | 1762                      | 1303              | -  | -                      | -  | -                      | -  |

LBE: *Lycium barbarum* extract; MXB: methylxanthines isolated from *Bancha*; MXP: methylxanthines isolated from *Pu-erh*.

Table 2. Virucidal activity against HSV-1 virions of the extracts

| Extract | Δlog 15 min | Δlog 30 min | Δlog 60 min | Δlog 90 min | Δlog 120 min |
|---------|-------------|-------------|-------------|-------------|--------------|
| LBE     | 1.25        | 3.0         | 3.0         | 3.0         | 3.0          |
| MXB     | 1.0         | 2.0         | 2.0         | 2.75        | 2.75         |
| MXP     | 1.5         | 1.75        | 1.75        | 2.25        | 2.25         |

LBE: *Lycium barbarum* extract; MXB: methylxanthines isolated from *Bancha*; MXP: methylxanthines isolated from *Pu-erh*.

Significant virion inhibition was observed in all three tested extracts at 30 minutes exposure, the most pronounced was in the LBE with a decrease in viral titer Δlog = 3.0, and this value was maintained over the remaining time intervals. The other two extracts also showed a significant virucidal activity at 30 min - MXB Δlog = 2.0 and MXP Δlog = 1.75 and this effect intensified on continuing exposure. At the last studied time interval of 120 min, the MXB extract showed suppression of viral particles by Δlog = 2.75, and the MXP – Δlog = 2.25.

The effect of the extracts on the attachment of HSV-1 virions to susceptible MDBK cells is presented in Table 3.

All three extracts showed significant inhibition of virus adsorption at 30 minutes exposure, with the strongest effect of the MXP extract with Δlog = 2.5. The effect was time dependent and increases with continuing exposure. The strongest inhibition at the stage of adsorption of the virus to the cell was observed at 45 minutes and was maintained at 60 minutes of exposure, again the most pronounced decrease in viral titers was the MXP with Δlog = 3.0.

We also investigated the protective effect of the extracts on the membrane of healthy MDBK cells before their contact to the virus (Table 4).

Table 3. Effect of the extracts on viral adsorption of HSV-1 on MDBK cells

| Extract | Δlog 15 min | Δlog 30 min | Δlog 45 min | Δlog 60 min |
|---------|-------------|-------------|-------------|-------------|
| LBE     | 1.0         | 1.75        | 2.0         | 2.0         |
| MXB     | 1.5         | 2.25        | 2.25        | 2.25        |
| MXP     | 1.0         | 2.5         | 3.0         | 3.0         |

LBE: *Lycium barbarum* extract; MXB: methylxanthines isolated from *Bancha*; MXP: methylxanthines isolated from *Pu-erh*.

The results show that the LBE extract has a protective effect at the first studied time interval of 15 min, lowering the viral titer by Δlog = 2.0. This protection effect is maintained throughout the exposure. The MXP extract showed a clear protection at 30 min Δlog = 1.75, it has enhanced at 60 min Δlog = 2.25 and maintained until 120 min of exposure. The MXB extract showed weak protection Δlog = 1.25 at all time intervals studied.

**DISCUSSION**

To develop a new antiviral drug, it must affect one or more viral reproductive stages. One of the first targets is the extracellular virion before it enters the host cell and causes subsequent disease. It can also have a protective effect on an uninfected cell so that it becomes insensitive to a viral infection. An important step is to inhibit the attachment of the virus to the host cell and prevent it from entering. The targets mentioned so far are before the onset of a viral infec-
tion and their main purpose is to prevent future infection. In most cases, however, treatment is required due to an already established infection with symptoms. In these cases, substances acting in the upper stages of virus spread are also important – to reduce the possibility of viral particles passing from cell to cell and to reduce the virulence of the virus. The inhibitors that affect the many specific viral structures required for its intracellular replicative cycle and block the production of new viral particles within the infected cell are those that are most crucial for clinical practice.\[9,10\]

The plant extracts usually are a mixture of many biologically active substances. There is a wealth of information in the literature for the biological activities of the components included in their composition. Some of these substances have antiviral activity affecting certain stages of viral replication. There is a study on virucidal activity, inhibition of the attachment of HSV-1 and HSV-2 to cells and prevention of the penetration of both types of HSV into the cells by binding to polysaccharides and/or their derivatives that are most likely to bind with HSV-specific glycoproteins and preventing complex interactions of viruses with the cell membrane.\[11,12\] The high content of polysaccharides in the study of extracts probably contributes to the manifestation of virucidal activity, which has been described by other teams.\[13\] This may also explain the more pronounced virucidal activity of polysaccharide-rich Lycium barbarum fruit extract (LBE) compared to the other fractions used. It should also be noted that Lycium barbarum polysaccharides have proven immunostimulatory functions\[2\] that could contribute to antiviral effects as well.

Of the methylxanthines – caffeine, theophylline, and theobromine, caffeine is the most studied and has the most evidence of antiviral effects against viruses such as HIV-1, HSV-1, Hepatitis C, etc.\[14-16\] In the cited studies, caffeine affects viral nucleic acid replication and viral protein synthesis. In addition, there is evidence that caffeine increases cytopathic effects and cell death of the virus-infected cells, which suggests that it could be basic for anti-HSV-1 action.\[17\] The both used methylxanthine fractions of Pu-erh (MXP) and Bancha (MXB) contain mainly caffeine\[6\], so it could be assumed that the observed effects are mediated by it. MXB and MXP have shown direct virucidal activity against HSV-1 virions, but also have exhibited an effect on the process of viral adsorption and penetration into healthy cells. The possible benefits of using methylxanthines, such as pentoxifylline and caffeine, have recently been discussed for the therapy of COVID-19 patients.\[6,18\] The reasons may lie in the fact that these compounds have complex properties, such as anti-inflammatory, antioxidant, immunomodulatory, antiviral (direct and indirect), improvement of respiratory symptoms, and therefore could be used as adjuvants in the treatment of this viral disease.

The search for natural antiviral agents, such as isolated plant extracts and fractions, could contribute to the improvement of the treatment of viral diseases because, on the one hand, such agents have complex mechanisms of actions making it difficult for viruses to build resistance, are easy to prepare and inexpensive and, on the other hand, they are well tolerated with minor side effects.

### CONCLUSIONS

Our experiments with Lycium barbarum fruit extracts and methylxanthines isolated from Pu-erh and Bancha tea leaves on the reproduction of HSV-1, poliovirus 1 and coxsackievirus 1, allow us to conclude that the studied extracts do not affect the intracellular replicative cycle of the studied viruses, but have significant effect on extracellular herpes particles, substantially inhibit the stage of attachment of the HSV-1 to the cells, and protect the uninfected cell from the HSV-1 invasion. Based on these findings, other enveloped viruses can be engaged the same way with the studied extracts.

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### Competing Interests

The authors have declared that no competing interests exist.

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Противовирусная активность in vitro экстракта плодов Lycium Barbarum и метилксантинов, извлечённых из чайных листьев Pu-erh и Bancha

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Резюме

Введение: Опираясь на традиционную медицину, многие страны используют различные растительные продукты (плоды, листья и другие части растений) в качестве пищевых добавок или в виде чая. Использование этих растительных источников было установлено за годы использования и доказанной пользы их ингредиентов для улучшения здоровья человека.

Цель: В настоящем исследовании мы сосредоточились на влиянии экстракта плодов Lycium barbarum и метилксантинов, выделенных из чайных листьев Pu-erh (MXP) и Bancha (MXB), на вирус простого герпеса типа 1 (HSV-1), полиовирус 1 (PV1) и вирус Коксаки B1 (CVB1) in vitro.

Материалы и методы: Мы использовали анализы in vitro противовирусных препаратов и анализы прикрепления вирусов для определения эффектов трёх изученных нами экстрактов.

Результаты: Ни один из экстрактов не показал значительного ингибирования репликации трёх обработанных вирусов, но заметное ингибирующее действие на внеклеточные вирусы HSV-1 проявилось через 30 минут после воздействия, особенно экстракта Lycium barbarum. Ингибитирующее действие трёх экстрактов на уровень адсорбции HSV-1 на чувствительных клетках (MDBK) также было значительным, при наиболее выраженном влиянии MXP. Также было определено защитное действие экстрактов против герпетической инфекции на здоровые клетки, наиболее заметный эффект показал MXP.

Заключение: Три исследованных экстракта могут быть эффективно использованы при лечении герпесных инфекций, а также инфекций, вызванных другими оболочечными вирусами.

Ключевые слова
вирус Коксаки B1, экстракты зелёного чая, вирус простого герпеса 1 типа, экстракт Lycium barbarum, полиовирус 1