

Evaluation of Antiviral Activity of Berberine against Herpes Simplex Viruses

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The different nucleoside analogues such as acyclovir are effective antiviral drugs against herpes simplex virus infections since its introduction. However, with the emergence of acyclovir-resistant, there is a need to develop an alternative antiherpetic drug and natural products could be the potential lead. In this study, the antiviral properties of berberine was evaluated against herpes simplex virus types 1 and 2 (HSV-1, 2) in vitro. HSV-infected Vero cells and cell-free virus suspensions were treated with berberine, and virus yield and infectivity were quantified by direct plaque assay. The results of the present study showed that berberine at 150 µg/ml provided 76.5% inhibition of plaque of HSV-1 and 80% inhibition against HSV-2. These properties suggest that this alkaloid could provide advantage as a topical prophylactic/therapeutic agent for herpes infections.

Key words: Berberine, Herpes simplex virus; Antiviral.

Berberine is an isoquinoline quaternary alkaloid isolated from many kinds of medicinal plants such as *Hydrastis canadensis*, *Berberis aristata*, *Coptis chinensis*, *Coptis rhizome*, *Coptis japonica*, *Phellodendron amurense*, *P. chinense* (Tillhon *et al.*, 2012). The medicinal properties of berberine include antibacterial, antiviral and antifungal activities. It also possesses anti-inflammatory, antioxidant, hepato- and reno-protective activities (Othman *et al.*, 2014).

Herpes simplex virus type 1 (HSV-1) is a widespread human pathogen that infects primarily epithelial tissues and causes severe diseases including mucocutaneous lesions in the oral mucosa (cold sores), encephalitis, meningitis, and

blinding keratitis (Hong *et al.*, 2014). On the other hand, *herpes simplex* virus type 2 (HSV-2) is the primary cause of genital ulcer disease worldwide. In 2003, an estimated 536 million people aged 15–49 years were living with the infection, with seroprevalence varying widely across settings and populations. Most infected individuals are unaware of their infections. In symptomatic infections, the virus causes painful ulcerative lesions that can take two to four weeks to heal in primary outbreaks, and recurrences can be frequent. The prevalence of HSV-2 infection in the general population ranges from 10 to 60 percent, with higher prevalences in female sex workers, men who have sex with men (MSM), and certain regions of the world (Ge *et al.*, 2013).

After the primary infection, HSV-2 is transported retrogradely to the lumbosacral sensory ganglia, where it establishes a lifelong latent infection that can be reactivated by stress, hormonal changes and UV light. After reactivation, HSV-2 can be transported to the primary site of

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infection causing either asymptomatic episodes, which facilitate its spread in the population, or recurrent ulcerations to the genital mucosa. These lesions are often very painful and can lead to substantial psychological morbidity (Stanberry *et al.*, 2000). The virus can also be passed from mother to child during birth with the risk of very serious neonatal infections. It has been estimated that the global prevalence in people aged 15–49 years who were living with HSV-2 worldwide in 2003 was 536 million whereas the estimated number of new HSV-2 infections among 15–49 year olds worldwide in 2003 was 23.6 million. The incidence of HSV recurrence is increased in people with an impaired immune system, such as HIV-seropositive individuals and in transplant recipients (Donalisio *et al.*, 2013). On the other hand, genital herpes may increase the risk of HIV acquisition by disrupting epithelial cells, with induction of local inflammation and production of cytokines and chemokines that activate and recruit CD4⁺ HIV target cells (Carr and Tomanek, 2006).

Currently no vaccine is available. The standard therapy for management of HSV infections is based on nucleoside analogues that target the viral DNA polymerase. These include acyclovir, penciclovir and their derivatives, valacyclovir, and famciclovir (Donalisio *et al.*, 2013). These antiviral drugs can be efficacious to treat clinical signs and symptoms of first and recurrent episodes, but their widespread use and the long term prophylactic therapy may be associated with relative high toxicity and emergence of drug-resistant virus strains especially in immunocompromised patients (Bacon *et al.*, 2002). However, extensive and long term clinical use of anti-herpesvirus agents like acyclovir, and its derivatives ganciclovir, foscarnet results severe side effects and drug-resistant viruses. Further, acyclovir is reported to incorporate into the cellular DNA, yielding adverse drug reactions and thus, unsuitable for pregnant women and neonates (Bag *et al.*, 2012). For these reasons, there is a great demand for the development of new antiviral drugs with novel mode of action. In this context, natural products are very important source of anti-HSV agents and several extracts and pure compounds from natural products have been reported to exert an anti-HSV activity (Cecilio *et al.*, 2013).

MATERIALS AND METHODS

Viruses and cell lines

Vero cells were grown in Dulbecco-modified Eagle's Minimum Essential Medium (DMEM; Gibco, Brazil) supplemented with 10% fetal bovine serum (FBS; Gibco®, Brazil) and gentamicin (80 µg/mL). The cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂ in air. The Herpes Simplex Virus type 1 and 2 were propagated in Vero cells, titrated on the basis of plaque forming units (PFU) count by plaque assay as previously described (Burleson *et al.*, 1992) and stored at –80 °C until the experiments.

Berberine preparation

Berberine chloride hydrate (CAS Number: 141433-60-5) was purchased from Sigma (St. Louis, MO, USA). For preparation, freshly berberine dissolved in DMSO [maximum concentration, 0.5% (v/v)], which was then added to complete cell culture medium prior to addition to subconfluent cells. Cells treated with vehicle only (DMSO, 0.5% in media) served as control.

Cytotoxicity assay

Confluent Vero cells were exposed to different berberine concentrations (1–1000 µg/ml) for 72 h. After incubation, cell viability was assessed by a MTT [3-(4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide) assay (Mosmann, 1983). The 50% cytotoxic concentration (CC₅₀) was defined as the concentration that reduced cell viability by 50% when compared to untreated controls.

Antiviral activity assay

The 96-well plates containing confluent cell monolayers were preincubated for 1 h with increasing non-cytotoxic concentration of berberine. Six wells were used for each concentration. Afterwards, the cell were infected with HSV-1 or 2 (10 TCID₅₀), incubated at 37 °C in a humidified atmosphere of 5% CO₂ in air and observed daily for cell cytopathic effect (CPE) using a light microscope. When CPE was observed in all virus control wells, the percentage of wells with CPE was determined for each treatment concentration, as described previously. Acyclovir at concentration of 0.05 to 2 µg/ml served as the positive control.

Viral plaque number reduction assay

This assay followed the procedures

previously described (Silva *et al.*, 2010), with minor modifications. Approximately 100 PFU of HSV types were adsorbed for 1 h at 37 °C on confluent cells and overlaid with MEM plus 1.5% carboxymethylcellulose (CMC, Sigma®, St. Louis, MO, USA) either in the presence or the absence of different concentrations of berberine. After 72 h, the cells were fixed and stained with naphthol blue-black (Sigma) and plaques were counted. The 50% inhibitory concentration (IC₅₀) was defined as the concentration that inhibited 50% of viral plaque formation when compared to untreated controls. Acyclovir (Sigma®) was used as a positive control. Results were expressed as CC₅₀ and IC₅₀ values in order to calculate the selectivity index (SI = CC₅₀/IC₅₀) of each sample (Cos *et al.*, 2006).

Statistical analysis

Data are expressed as the mean ± standard deviation from at least three separate experiments.

RESULTS AND DISCUSSION

Berberine (molecular formula C₂₀H₁₉NO₅ and molecular weight of 353.36) is the main active component of an ancient Chinese herb *Coptis chinensis* French, which has been used to treat diabetes for thousands of years. Berberine is an Over-the-Counter drug, which is used to treat gastrointestinal infections in China. Berberine hydrochloride (B·HCl·nH₂O) - the most popular form of berberine, is used in this study (Fig. 1).

Examination of the cytotoxicity of berberine was performed in the range of concentrations up to 1000 µg/ml. The maximum non-cytotoxic concentrations were read individually from the obtained survival curves.

Cytotoxicity of berberine was evaluated in cultured Vero cells by the MTT assay. According to the results of this experiment (Fig. 2), the crude berberine has cytotoxicity at 440.47 µg/ml.

The present study was carried out to test the antiviral activity of berberine against herpes simplex virus types-1 and 2 using cytopathic inhibitory assay and plaque reduction assay (Table 1). On the basis of cytopathic effect (CPE) of the virus-infected confluent monolayer of Vero cells, berberine showed strong antiviral activity against HSV-1 or anti-HSV-2 at high concentration, 200 µg/

ml. In this manner, the cytopathic effect was showed with all samples at different concentrations (from 1/4 CC₅₀ to 10 µg/ml).

Plaque inhibition assay was carried out to determine the IC₅₀. As shown in Figure 2, berberine at 150 µg/ml provided 76.5% inhibition of plaque of HSV-1 and 80.0% inhibition against HSV-2. The selectivity index (SI) of berberine was about 2.9.

Berberine has demonstrated significant antimicrobial activity against a variety of organisms, including human immunodeficiency virus (Gudima *et al.*, 1994), human cytomegalovirus (HCMV) (Hayashi *et al.*, 2007), and hepatitis B virus (Li *et al.*, 2008). It has been reported that berberine intercalates DNA, inhibiting DNA synthesis and reverse transcriptase (Gudima *et al.*, 1994; Hayashi *et al.*, 2007; Sethi, 1983). The time course of inhibition shows that the berberine alkaloids stop DNA synthesis instantly when added after the initiation of the polymerization processes (Chin *et al.*, 2010; Sethi, 1983). Inhibition of reverse transcriptase activity correlates with the structure and antileukemic activity of berberine alkaloids. In addition, berberine does not affect membrane permeability but does affect protein biosynthesis (Chin *et al.*, 2010). In consequence, these biochemical activities may mediate chemical defenses against viruses.

Chin *et al.* (2010) suggested that the synthesis of both HSV-1 and HSV-2 late genes and proteins is inhibited by berberine. Recently, Hayashi *et al.* (2007) (2007) suggested that berberine inhibits HCMV during the virus penetration and viral DNA synthesis processes. Although HCMV is a member of the subfamily Betaherpesvirinae, family Herpesviridae, when these data are taken together, our results are consistent with those of Hayashi *et al.* (2007),

Table 1. The antiviral activity of berberine

Berberine	Cell Cytopathic Effect (CPE)	
	HSV-1	HSV-2
10 µg/ml	+	+
25 µg/ml	+	+
50 µg/ml	+	+
100 µg/ml	+	+
150 µg/ml	+	+
200 µg/ml	-	-

suggesting that berberine may interfere with the viral replication cycle after virus penetration and no later than the viral DNA synthesis step, similar to a DNA synthesis inhibitor.

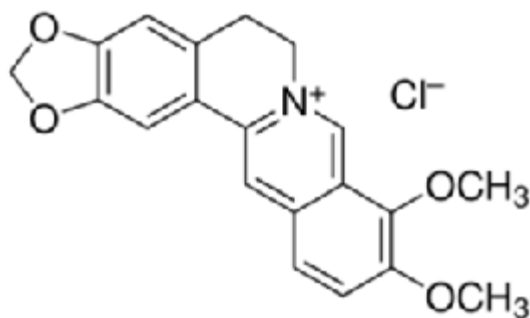


Fig. 1. Chemical structure of berberine chloride (Battu *et al.*, 2010)

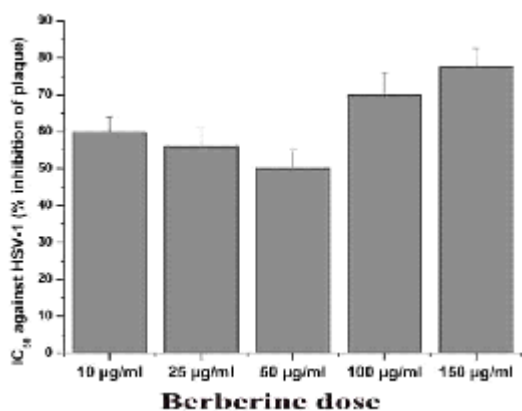


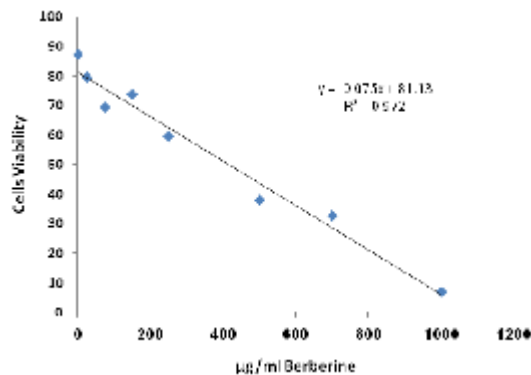
Fig. 3. The antiviral activity of berberine against HSV-1

IC₅₀ is the concentration of the sample required to inhibit 50% HSV-1 virus-induced CPE. Data are represented as Mean $\pm$ SEM of two independent experiments each performed in triplicate.

In conclusion, berberine emerges as a potential candidate in the development of effective antiviral drugs against HSV-1 and HSV-2, although further in-depth studies are needed to provide an insight into the mechanism involved and the identification of responsible target.

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CC₅₀ is the concentration of the 50% cytotoxic effect. Data are represented as Mean of two independent experiments each performed in triplicate

Fig. 2. The cytotoxic effect of berberine

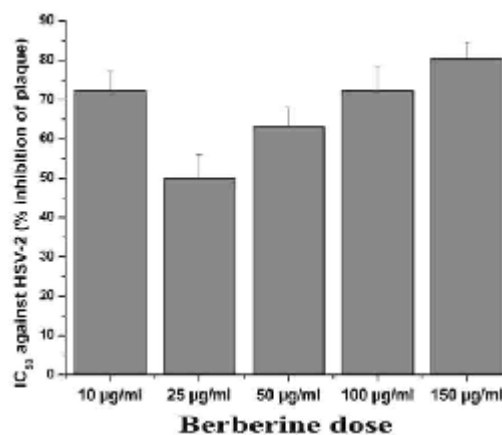


Fig. 4. The antiviral activity of berberine against HSV-2.

IC₅₀ is the concentration of the sample required to inhibit 50% HSV-2 virus-induced CPE. Data are represented as Mean $\pm$ SEM of two independent experiments each performed in triplicate.

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