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Chemical properties, mode of action, and *in vivo* anti-herpes activities of a lignin–carbohydrate complex from *Prunella vulgaris*

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Abstract

The chemical nature, the mode of action, and the *in vitro* and *in vivo* anti-HSV activities of the polysaccharide from *Prunella vulgaris* were characterized. The polysaccharide was isolated by ethanol precipitation, dialysis, CTAB precipitation, and gel exclusion chromatography. The isolated compound (PPS-2b) was a lignin–carbohydrate complex with a molecular weight of 8500. The carbohydrate moiety was composed of glucose, galactose, mannose, galacturonic acid, rhamnose, xylose, and arabinose with glucose as the major sugar. In plaque reduction assay, PPS-2b showed activities against HSV-1 and HSV-2. The anti-HSV activity could be abolished by periodate oxidation. Mechanism studies showed that PPS-2b inactivated HSV-1 directly, blocked HSV-1 binding to Vero cells, and inhibited HSV-1 penetration into Vero cells. A similar inhibition was observed with a gC-deficient strain of HSV-1. The *in vivo* activities of a *Prunella* cream formulated with a semi-purified fraction was assessed in a HSV-1 skin lesion model in guinea pigs and a HSV-2 genital infection model in BALB/c mice. Guinea pigs that received the *Prunella* cream treatment showed a significant reduction ($P < 0.01$) in skin lesions. Mice that received the *Prunella* cream treatment showed a significant reduction ($P < 0.01$) in mortality. In conclusion, the anti-HSV compound from *P. vulgaris* is a lignin–polysaccharide complex with potent activity against HSV-1 and HSV-2. Its mode of action appears to be inhibiting viral binding and penetration into host cells.
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Keywords: Anti-herpes; *Prunella*; Animal models

1. Introduction

Herpes infections are common. In the USA, about 80 and 35% of the adult population are infected by or sero-positive for HSV-1 (herpes simplex virus-1) and HSV-2 (herpes simplex virus-2), respectively (Cassady and Whitley, 1997). In addition, the incidence and severity of infections caused by HSV have increased over the last decade. The increase is due to an increasing number of immuno-compromised patients produced by aggressive chemotherapy regimens, expanded organ transplantation, and a greater occurrence of human immunodeficiency virus infections. With this change in disease pattern and the increase in drug use frequency, acyclovir-resistant HSV infections have emerged (Field and Biron, 1994). Thus there is an urgent need for novel anti-HSV agents, especially those with a different mode of action than acyclovir.

Xia-Ku-Cao, the fruitspikes of *Prunella vulgaris* L., is used in Chinese medicine as a remedy for the treatment of some 'heat-like' symptoms (Chang and But, 1987). The spike of this herb in the form of hot water infusion has been used to treat sores in the mouth and throat (Grieve, 1974), as an astringent for internal and external purposes (Grieve, 1974), as a crude anti-cancer drug (Lee and Lin, 1988), and as an herbal remedy to lower high blood pressure (Namba, 1994). Kurokawa et al. (1993) reported that the hot water extract of *P. vulgaris* contained anti-herpes activity in the plaque reduction assay. Zheng (1990) previously reported the use of a crude aqueous extract of

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P. vulgaris in clinical treatment of herpetic keratitis with some success. Of the 78 patients who received eye drops containing crude extracts of *P. vulgaris* and *Pyrrosia lingua*, 38 were cured, 37 showed improvement, and 3 did not respond. We previously described the isolation and preliminary characterization of an active anti-herpes polysaccharide from *P. vulgaris* (Xu et al., 1999). By hot water extraction, ethanol precipitation, and gel permeation chromatography, we have isolated a semi-pure polysaccharide from the spike of *P. vulgaris* that showed good activity against HSV-1 and HSV-2, including known acyclovir-resistant strains and clinical isolates (Xu et al., 1999). The polysaccharide appeared to inhibit HSV binding to cell receptors as well as by some unknown mechanisms after the virus has penetrated the cells. The polysaccharide was anionic but showed no anti-coagulant activity and was not cytotoxic to mammalian cells.

In the present study, the chemical nature, mode of action, and *in vivo* anti-HSV activities of the *Prunella* polysaccharide were described. The results showed that the *Prunella* anti-herpes compound is a lignin–carbohydrate complex and contains therapeutic effects in HSV-1 cutaneous infection and HSV-2 vaginal infection in guinea pig and mouse model, respectively.

2. Materials and methods

2.1. Plant materials, cells, and viruses

The fruitspikes of *P. vulgaris* L. were purchased from a local herbal market in Hong Kong and authenticated at the Institute of Chinese Medicine, the Chinese University of Hong Kong. A voucher specimen (No. 2399) was deposited in the museum of Chinese Medicine at the same institute for reference. Vero cells were grown in the Dulbecco's Modified Eagle's medium (MEM) with 10% fetal calf serum (FCS) as described previously (Xu et al., 1999). HSV-1 (strains BW-S and DM2.1) and HSV-2 (strain 8702) were provided by Jack Hill, The Wellcome Research Laboratories. Strain BW-S and 8702 are acyclovir-sensitive strains, while DM2.1 is an acyclovir-resistant strain with the thymidine kinase-deficient phenotype. The gC-negative deletion HSV-1 mutant (ΔgC2-3) was provided by C. Brandt, University of Wisconsin. The above strains were used in *in vitro* assays. HSV-1 strain SM44 was obtained from the American Type Culture Collection and used in the *in vivo* experiment. HSV-2 strain 333, batch no. 200104010, was obtained from the Department of Virological Diagnosis, Institute of Virology, Chinese Academy of Preventive Medicine, China.

2.2. Extraction and isolation of the Prunella lignin–carbohydrate complex

The extraction process was based on methods described previously (Xu et al., 1999; Zhang et al., 1997). Briefly, the fruitspikes of *P. vulgaris* (500 g) were decocted for 1 h with 10 l of distilled water and the residue was decocted again for 1 h with 8 l of distilled water. The extracts were freeze-dried (PPS-0, yield: 53 g). PPS-0 was re-dissolved in 1.5 l of distilled water and precipitated with three volumes of ethanol. The resulting precipitates were dissolved and dialyzed against distilled water. The retentate was clarified by centrifugation and freeze-dried to obtain PPS-1 (15 g).

PPS-1 was fractionated into acidic polysaccharide (PPS-2, 10.5 g), weakly acidic polysaccharide (PPS-3, 0.51 g) and the neutral polysaccharide (PPS-4, 1.9 g) fractions by cetyltrimethylaminium bromide (CTAB) precipitation (Yamada et al., 1984). PPS-2 was further fractionated by gel-permeation chromatography on a Sepharose CL-6B column (2.2 cm × 90 cm) using 0.2 M NaCl as the eluant. Two carbohydrate fractions, PPS-2a and PPS-2b, eluted at 1/4 and 1/2 of the bed volume, respectively, were obtained. The fractions were dialyzed against distilled water and consequently purified by chromatography on a Sephadex G-100 column (2.2 cm × 90 cm) to obtain 6.3 g of PPS-2a and 4.2 g of PPS-2b.

2.3. General methods of chemical characterization

Total carbohydrate and uronic acid were determined by the phenol–sulfuric acid assay (Dubois et al., 1956) and the method of Blumenkrantz and Asboe-Hansen (1973), using galactose and galacturonic acid as the standard, respectively.

High pressure liquid chromatography (HPLC) analysis of the purified samples was performed on the Shodex sugar KS-850 and KS-840 columns (0.8 cm × 30 cm, Showa Denko Co., USA) connected in tandem using 0.2 M NaCl as the eluant. The carbohydrate was detected by a HP 1047A refractive index (RI) detector and a HP 1040A ultraviolet diode array detector. The molecular weights of samples were estimated by comparison with pululan standards (P-800, 400, 100, 50, 20, 10, and 5 with the corresponding molecular weights of 758, 338, 194, 95.4, 46.7, 20.8, 12, and 5.3 kDa). The pululans were gifts from Profs. H. Yamada and H. Kiyohara (Kitasato University, Tokyo, Japan).

The lignin contents in samples were determined colorimetrically by an improved acetyl bromide method (Dence, 1992). In addition, lignin was subjected to alkaline nitrobenzene oxidation (Chen, 1988) and the resulting benzaldehyde derivatives (vanillin, syringaldehyde and *p*-hydroxybenzaldehyde) were identified by gas-liquid chromatography–mass spectrometry (GLC–MS) as described by Kiyohara et al. (1999) on a HP-5 capillary column (0.32 mm i.d. ×30 m, 0.25 μm, Hewlett-Packard) under the following conditions: 60 °C (1 min), 60 → 140 °C (15 °C/min), and 140 → 250 °C (5 °C/min). Helium (2 ml/min) was used as a carrier gas, and the injector and detector temperatures were 200 and 270 °C, respectively.

To analyze the constituent sugars in PPS-2b, 100 μg of the sample was hydrolyzed in 2 M trifluoroacetic acid at 121 °C for 1.5 h. Neutral sugars and galacturonic acids in the hydrolysate were converted to alditol acetates and trimethylsilyl methylglycoside derivatives, respectively, and analyzed by GLC on a HP-1 capillary column (0.25 mm i.d. ×30 m, 0.2 μm, Supleco, USA, York et al., 1986) under the following conditions: 140 °C for 1 min, 140 → 180 °C (2 °C/min), 180 → 275 °C (1 °C/min), and 275 °C for 5.8 min.

Elemental analysis was conducted by MED AC, Ltd. UK.

2.4. Periodate oxidation

Periodate oxidation of the carbohydrate was performed using methods as described previously by Zhang et al. (1997). PPS-2b (20 mg) was oxidized with 40 ml of 50 mM $NaIO_4$ in 50 mM sodium acetate buffer, pH 4.5, at 4 °C for 96 h in the dark. After the reaction had been terminated with ethylene glycol, the product was reduced with $NaBH_4$ (40 mg) and dialyzed against distilled water to obtain the periodate oxidized product.

2.5. Plaque reduction assay

Plaque reduction assay was performed according to the standard method described by Hill et al. (1991). Briefly, monolayers of Vero cells grown on six-well culture plates were infected with 100–200 PFU (plaque-forming unit) of virus. After incubation for 1 h to allow viral adsorption, the inoculum was aspirated and the cultures were overlaid with 0.8% methylcellulose in maintenance medium (MM [MEM with 2% FCS]) containing dilutions of the *Prunella* sample. After 72 h of incubation at 37 °C, the plates were fixed with formalin, stained with crystal violet, air dried, and the number of plaques counted. Plates overlaid with methylcellulose medium without the *Prunella* sample were used as controls. The percentage of inhibition of plaque formation was calculated as follows: [(mean # of plaques in control − mean # of plaques in test)/(mean # of plaques in control)] × 100.

2.6. Virucidal assay

The direct effect of PPS-2b on HSV-1 was assayed as follow: 0.1 ml of virus containing 4.4×10^6 PFU was mixed with 50 μg of PPS-2b in 0.1 ml of MEM or with 0.1 ml of MEM (control) and incubated at 37 °C for 1 h. The mixture was promptly diluted (10,000 folds) and assayed for residual virus. Diluted virus was adsorbed for 1 h at 37 °C. Inoculum was removed and Vero cell cultures were washed twice prior to receiving overlaid medium to allow plaque formation.

2.7. Viral binding assay

HSV-1 stock was diluted in pre-chilled medium and mixed with an equal volume of pre-chilled medium containing 100, 50, 25, 12.5 and 6.25 μg/ml of PPS-2b or with medium (control). The mixtures in 0.25 ml volumes containing *ca*. 100 PFU were immediately inoculated onto Vero cell cultures at 4 and 37 °C. After 1 h adsorption, inocula were removed from the cultures followed by two washes with medium and plaques were allowed to form at 37 °C in methylcellulose medium. The percentage of inhibition was calculated as follows: [(mean # of plaques in control − mean # of plaques in test)/(mean # of plaques in control)] × 100.

2.8. Penetration assay

The penetration assay was conducted using the method as described by Herold et al. (1991) with modifications. Confluent Vero cell monolayers in 25 ml tissue culture flasks were chilled to 4 °C for 45 min. The monolayers were infected with *ca.* 300 PFU of HSV-1 in 2.5 ml of cold MEM medium. Viral attachment was synchronized for 1 h at 4 °C. The monolayers were washed twice with 4 ml of cold phosphate buffered saline (PBS) to remove any unbound virus. The monolayers were covered with 5 ml of MM and shifted to 37 °C to allow viral penetration to proceed. At set time intervals, the medium was removed and the cells received one of the following treatments: (a) PPS-2b, (b) heparin, (c) citrate buffer, pH 3 (0.04 M Na_2HPO_4, 0.08 M citric acid), and (d) no treatment control. For the PPS-2b- or heparin-treated groups, 5 ml of fresh MM containing 100 μg/ml PPS-2b or 1000 μg/ml heparin was added to the monolayers. The flasks were incubated at 37 °C for 90 min, after which time the monolayers were washed twice with 4 ml of PBS and covered with 10 ml of 0.8% methylcellulose in MM. Plaque formation was allowed at 37 °C for 72 h. For the citrate buffer treated group, 5 ml of buffer was added to the monolayers for 1 min. The cells were washed twice with PBS and methylcellulose-overlay medium was then added to allow plaque formation. For the no treatment control group, following medium removal, the monolayers were washed twice with PBS and methylcellulose-overlay medium added to allow plaque formation.

2.9. Prunella topical cream

The base cream was provided by the Department of Virological Diagnosis, Institute of Virology, Chinese Academy of Preventive Medicine. The composition of the base cream was: 18% (wt/wt) mineral oil, 5% isopropyl palmitate, 2% hexadecanol, 2% steric acid, 0.8% tween-20, 0.4% alpha-propylene glycol, 1.8% diethylamide, 0.0005% *Nocardia* cell wall, and 66.1% distilled water. PPS-1 (150 g) was homogenized with 200 ml of distilled water at room temperature for 6 h and mixed with 150 g of base cream to give a 30% (wt/wt) *Prunella* cream. Lower concentration *Prunella* creams were prepared from the 30% formulation by mixing with the base cream.

2.10. Skin irritation test

The dermal toxicity of the *Prunella* creams was tested in guinea pigs. Guinea pigs (half male and half female, 200–250 g) were supplied by the Animal Center of the Chinese Academy Medical Sciences. Hair was removed from the dorsal side of the animal with 8% BaS. The naked skin (6 cm × 7 cm) was washed with warm water, dried with tissue papers and abraded with dermal (Seven-Star) needles (Suzhou Medical Appliance Factory, Suzhou, China). The *Prunella* creams (30, 15, and 7.5%) were applied to the abraded area of cohorts of animals ($n = 5$). The amount of cream applied was 2 g per animal. After 24 h, the creams were removed with warm water and the animals were examined for erythema and edema 1 h later. The animals were observed for the next 72 h.

2.11. HSV-1 cutaneous lesion in guinea pigs

The dorsal skin of guinea pigs was prepared and abraded as above. The abraded area was divided into four quadrants and

each of the quadrants was infected with 30 μl of 10-fold diluted HSV-1 (strain SM44). The animals ($n = 5$) were observed for 10 days for lesion development. Typical herpes lesions appeared on the infected skin on the 4th day of infection. Based on this initial result, it was determined that 150 μl of the stock virus (10^8 PFU) was required to infect 42 cm^2 and to obtain consistent lesion development. Cohorts of animals ($n = 15$) were infected as above and treated with the *Prunella* cream, 3% (wt/wt) acyclovir cream, or base cream on day 4 post-infection twice daily for a 6-day period. The creams were applied to the infected area with cotton swabs. The amount of creams given to each animal was 1.5 g per dose. The extent of lesion was scored daily as follows: 1.0–1.6, lesions on 1/4 of infected area; 1.7–2.4, lesions on 1/2 of infected area; 2.5–3.2, lesions on 3/4 of infected area; and 3.3–4.0, lesions on the entire infected area.

2.12. HSV-2 genital herpes in mice

BALB/c mice (female, 18–20 g) were supplied by the Peking Union Medical College. HSV-2 (strain 333) stock was 10-fold diluted to 10^{-3}. The diluted virus suspension (30 μl) was inoculated to the vagina by a size 12 needle. Ten animals were inoculated with each dilution of HSV-2. The mice were kept under observation for 12 days, by which time the animals developed vaginitis or lethality occurred. The median lethal dose (LD_{50}) was determined to be $10^{-2.35}$.

In the experiment to test the efficacy of the *Prunella* cream, the animals were infected with a dose equivalent to 10 LD_{50} (10^5 PFU) using the manner as described above. Following inoculation, a vaginal cotton swab sample was collected from each animal and transferred to 0.5 ml of PBS and stored at −20 °C. The mice were divided into three test groups (5, 10, and 15% *Prunella* cream), one positive control group (3% acyclovir), one negative control group (base cream), and one no treatment (virus control) group. An additional group of animals that were not infected with HSV-2 and received no treatment served as the uninfected control group. Symptoms of viral vaginitis were observed on the third day of infection. The symptoms were topical edema of the vaginal tract with turbid secretions. Treatment began on day 3 post-infection. This was achieved by applying the cream to the vaginal tract with cotton swabs. The creams were given at a dose of 2 mg per mouse twice daily for a 6-day period. Mortality and the number of days for mortality to occur were recorded. One day following the completion of the treatment, vaginal swab samples were obtained. Swab samples were also obtained from the deceased animals immediately following their death. The vaginal samples were diluted five times in MEM and used to infect Vero cells. Samples that gave positive cytopathic effects were considered positive for HSV-2.

2.13. Statistical analysis

The results were analyzed by Student's *t*-test, with a P of <0.05 considered statistically significant.

3. Results

3.1. Isolation and chemical characterization of the Prunella lignin–carbohydrate complex

The *Prunella* hot water extract was subjected to ethanol precipitation, CTAB precipitation, and gel permeation chromatography. The anti-HSV-1 activity of the fractions was followed by the plaque reduction assay. Following CTAB precipitation, the anti-HSV-1 activity was found in the acidic (PPS-2) and the weakly acidic (PPS-3) polysaccharide fractions. Because PPS-2 was obtained in a larger quantity than PPS-3, it was used as a source for subsequent purification. Following gel permeation chromatography, PPS-2 was separated into PPS-2a and PPS-2b. Plaque reduction assay showed that PPS-2b displayed anti-HSV activity with an IC_{50} (50% inhibitory concentration) of 18 μg/ml while PPS-2a lacked any activity. HPLC analysis showed that PPS-2b contained a single homogenous peak suggesting purity (data not shown). The molecular weight of PPS-2b was estimated to be 8500.

PPS-2b contained 39% carbohydrate (expressed as mg galactose per mg of compound), and 11% uronic acid (expressed as mg galacturonic acid per mg). Elemental analysis showed that PPS-2b contained 37.4% carbon, 3.76% hydrogen, 0.6% nitrogen and 0.92% sulfur. The carbohydrate moiety of PPS-2b was found to consist of glucose, galactose, mannose, galacturonic acid, xylose, rhamnose, and arabinose in a molar ratio of 3.4:1.0:0.7:0.5:0.3:0.3:0.1.

In addition to carbohydrate, PPS-2b also contained 24% (wt/wt) lignin by the acetyl bromide method. The presence of lignin was confirmed by two other results. PPS-2b showed a UV spectrum with absorption maxima at 207, 270, and 320 nm, which was similar to a commercial lignin (Sigma–Aldrich). GLC–MS analysis of PPS-2b following alkaline nitrobenzene oxidation showed the presence of vanillin, syringaldehyde, and *p*-hydroxybenzaldehyde in the sample. The above results collectively indicate that the anti-HSV compound is a lignin–carbohydrate complex.

3.2. In vitro anti-HSV-1 activities of PPS-2b

PPS-2b was assayed for anti-HSV-1 and anti-HSV-2 activities by the plaque reduction assay. The IC_{50} against HSV-1 (strain BW-S) and HSV-2 (strain 8702) was 18 μg/ml for both strains. PPS-2b showed a similar IC_{50} against the gC-deficient HSV-1 and an IC_{50} of 17 μg/ml against the acyclovir-resistant strain of HSV-1 (strain DM2.1). In contrast, heparin showed an IC_{50} of 300 and >1000 μg/ml against the wild type (strain BW-S) and the gC-deficient mutant of HSV-1. As a control, the IC_{50} for acyclovir against all the strains, except the DM2.1, was 0.1 μg/ml.

To determine whether the carbohydrate moiety in PPS-2b was required for activity, PPS-2b was treated with $NaIO_4$. The IC_{50} of the periodate oxidized PPS-2b against HSV-1 strain BW-S was found to be >100 μg/ml, a 5.6-fold increase in IC_{50} over the untreated PPS-2b. When a commercial lignin (Sigma–Aldrich) was assayed at 100 μg/ml, it did not exhibit any anti-HSV-1 activity.

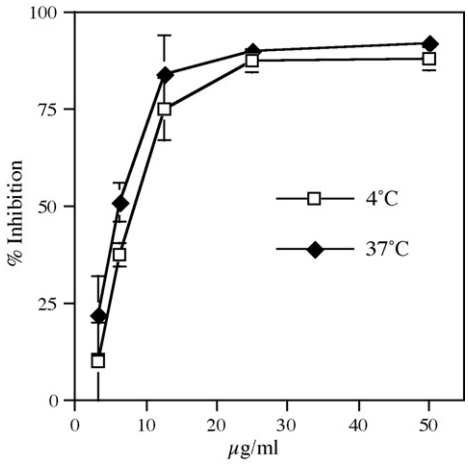

Fig. 1. Effects of the *Prunella* lignin–carbohydrate complex on HSV-1 binding to Vero cells.

When PPS-2b (50 μg) was mixed with HSV-1 (4.4×10^6 PFU) at 37 °C for 1 h, a 86% reduction in viral infectivity was observed when compared to the untreated controls. This result suggests that PPS-2b has virucidal activity. The effect of PPS-2b on viral binding to Vero cells at 4 and 37 °C was investigated. A dose-dependent inhibition of PPS-2b to virus adsorption was observed at 4 °C (Fig. 1). A similar dose-dependent inhibition on virus adsorption was also observed at 37 °C.

In addition to viral binding, the effect of PPS-2b on HSV-1 penetration into Vero cell was also investigated. Fig. 2A showed the effect of PPS-2b on HSV-1 strain BW-S penetration into Vero cells. The citrate buffer curve represented the penetration kinetics of HSV-1 into Vero cells. The no treatment curve showed that the penetration process occurred very rapidly and after 20 min, virtually all the viral particles had completed the penetration process. When the cultures were treated with PPS-2b, a reduction in viral penetration was clearly observed. This reduction in penetration was also observed, but to a lesser degree, when heparin was used. In the case of the gC-deficient mutant, the rate of penetration was slower than that displayed by the wild type HSV-1 strain BW-S (Fig. 2B). By the end of 60 min incubation, all the virus particles appeared to have penetrated. The prevention of penetration by PPS-2b was again observed. In contrast, heparin had no apparent effects on the penetration by the gC-deficient mutant.

3.3. Therapeutic effects of Prunella creams in a HSV-1 skin infection model in guinea pigs

The dermal toxicity of the *Prunella* creams was assessed by application of the creams to the dorsal skin of guinea pigs. No sign of edema or erythema was observed in any of the animals indicating that the creams have no dermal toxicity.

The *in vivo* anti-HSV-1 activity of the *Prunella* lignin–carbohydrate was tested in guinea pigs. All the animals developed typical herpes lesions on day 4 post-infection and the lesion scores on day 4 were very similar in all groups (Table 1). The lesion scores for animals that received base cream or no treatment (virus control group) were very similar throughout

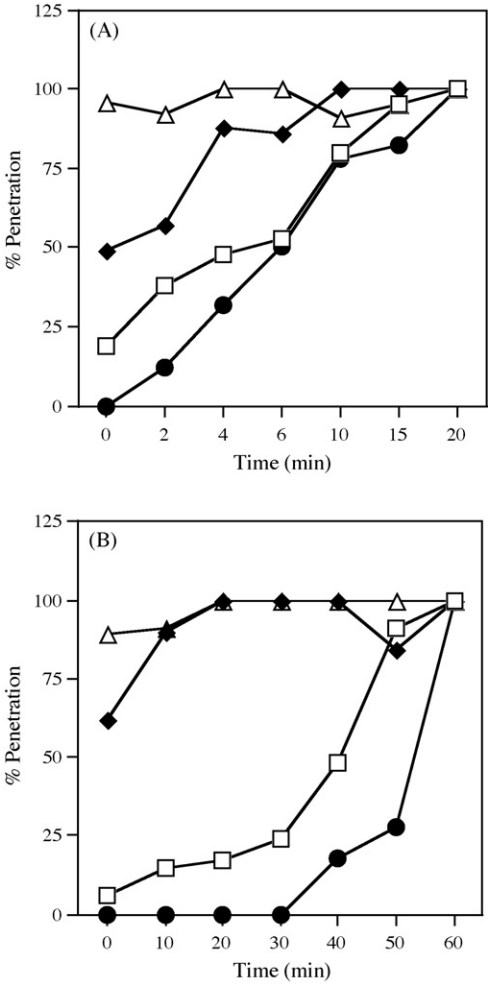

Fig. 2. Effects of the *Prunella* lignin–carbohydrate complex on penetration of wild type HSV-1 (A) and a gC-deficient mutant of HSV-1 (B) into Vero cells. Triangles: no treatment control; diamonds: heparin (1 mg/ml); squares: PPS-2b (100 μg/ml); circles: pH 3 citrate buffer. Percentage penetration is calculated as (number of PFU/number of PFU at time 20 or 60 min) × 100.

the experiment. On day 11 post-infection, lesions still could be found on half of the infected area for animals in these two groups. In contrast, animals that received the acyclovir cream showed a dramatic reduction in lesion scores by day 8 post-infection. On day 11, lesions were almost completely disappeared. More importantly, animals that received the *Prunella* cream showed a dose-dependent reduction in lesion scores with animals receiving 15% *Prunella* cream showing similar results to the acyclovir group.

In addition to the cutaneous lesions, a clear sign of the therapeutic effect of the *Prunella* cream was noted. In the virus control group, seven animals showed signs of paralysis in their hind limbs. Such signs were not observed in the *Prunella* and acyclovir treated groups.

3.4. Therapeutic effects of Prunella creams in a genital HSV-2 infection model

Three days following HSV-2 inoculation, all animals except the uninfected control group developed symptoms of vaginitis.

Table 1
Therapeutic effects of *Prunella* creams on HSV-1 skin lesions in guinea pigs

Testing group	Concentration (%)	No. of animals	Lesion scores (mean ± S.D.)							
			Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11
Prunella	15	15	3.85 ± 0.15 (0)[a]	3.78 ± 0.14 (5)	3.39 ± 0.10 (14)	2.74 ± 0.16 (25)	2.10 ± 0.26 (36)	1.32 ± 0.36 (54)	0.69 ± 0.24 (71)	0.20 ± 0.17 (90) $P<0.01$[b]
	10	15	3.83 ± 0.16 (0.3)	3.83 ± 0.14 (3)	3.57 ± 0.19 (9)	3.05 ± 0.43 (16)	2.57 ± 0.28 (22)	1.87 ± 0.55 (35)	1.28 ± 0.53 (47)	0.86 ± 0.36 (58) $P<0.01$
	5	15	3.84 ± 0.15 (0)	3.85 ± 0.11 (3)	3.75 ± 0.09 (5)	3.39 ± 0.17 (7)	3.13 ± 0.27 (5)	2.71 ± 0.36 (6)	2.17 ± 0.50 (10)	1.37 ± 0.26 (33) $P<0.01$
	2.5	15	3.81 ± 0.18 (0.8)	3.78 ± 0.16 (5)	3.71 ± 0.18 (6)	3.41 ± 0.20 (6)	3.15 ± 0.16 (4)	2.85 ± 0.16 (1)	2.55 ± 0.30 (0)	1.93 ± 0.34 (6) $P>0.05$
	Base cream	15	3.82 ± 0.02 (0.5)	3.82 ± 0.02 (3)	3.99 ± 0.01 (0)	3.99 ± 0.01 (0.2)	3.58 ± 0.04 (3)	3.16 ± 0.01 (4)	2.66 ± 0.03 (5)	2.06 ± 0.03 (7) $P>0.05$
Acyclovir	3	15	3.87 ± 0.12 (0)	3.54 ± 0.22 (11)	2.77 ± 0.40 (30)	2.02 ± 0.30 (44)	1.21 ± 0.38 (63)	0.82 ± 0.16 (71)	0.38 ± 0.18 (84)	0.09 ± 0.19 (96) $P<0.01$
Virus control		15	3.84 ± 0.14	3.96 ± 0.05	3.94 ± 0.07	3.63 ± 0.20	3.29 ± 0.26	2.87 ± 0.35	2.40 ± 0.34	2.06 ± 0.30

[a] Number in parenthesis refers to % inhibition, which is calculated as [(lesion score of virus control group − lesion score of treatment group)/(lesion score of virus control group)] × 100.
[b] P value from t-test between the test group and the virus control group for day 11.

The animals that were infected but received no treatment (virus control) had a very low survival rate and days-to-mortality (Table 2). Similar results were observed for animals that received the base cream. In contrast, the survival rate for the acyclovir group was 80%. The six dead animals lived for an average time of 11.6 days, which was significantly longer than those in the virus control group. The animals that received 15% *Prunella* cream showed similar survival rate and days-to-mortality to the acyclovir group. In groups that received 5 and 10% *Prunella* cream a significantly higher survival rate and days-to-mortality than that of the virus control group were also observed.

HSV-2 was detected in the vaginal samples from all animals except the uninfected control group following inoculation confirming that the animals were infected (Table 2). A large number of samples recovered from the animals following treatment or post-mortem was positive for HSV-2. It is interesting to note that the number of samples positive for HSV-2 was similar to the number of death except in the 5 and 10% *Prunella* cream groups. In these two groups, the number of HSV-2-positive samples was considerably higher than the number of death.

4. Discussion

In this study, the active anti-HSV principle was isolated from the herbal plant, *P. vulgaris*, by hot water extraction, precipitation, and gel permeation chromatography. The active principle is a lignin–carbohydrate complex with a molecular weight of 8500. The carbohydrate moiety is required for anti-HSV activity as demonstrated by the periodate oxidation experiment. Whether the lignin moiety is needed for activity is unclear at this time, although a commercial lignin did not show any anti-HSV activity. The lignin–carbohydrate complex isolated in this study shows similarities to the semi-purified anti-HSV polysaccharide reported previously by our group (Xu et al., 1999). The similarities include the CHNS ratio and sugar compositions. PPS-2b has a CHNS ratio of 37.40:3.76:0.60:0.92 and the polysaccharide reported previously has a ratio of 30.78:3.05:0.66:2.69. The slight difference in CHNS ratio may be attributed to the difference in molecular masses with the previous polysaccharide being smaller (3.5 kDa). In addition to the CHNS ratio, the two compounds show similar constituent sugar compositions (glucose, galactose, xylose, and galacturonic acid) with glucose being the major sugar. The additional sugars (mannose, rhamnose, and arabinose) detected in PPS2-b, but not in the 3.5 kDa polysaccharide, may be attributed to the use of GLC–MS instead of paper chromatography as the detection method. Therefore, it is possible that the 3.5 kDa polysaccharide was a fraction of PPS-2b, although such a claim cannot be made conclusively without solving the structures of the two compounds.

The anti-HSV PPS-2b shows some similarities, but not identical, to the polyanionic polysaccharide, prunellin, previously isolated by Tabba et al. (1989), which has anti-HIV activity (Tabba et al., 1989; Yao et al., 1992). Prunellin was described to be 10 kDa in size, showed an adsorption peak at 370 nm which extended to 500 nm, and contained 0.14% sulfur adsorption peak at 370 nm which extended to 500 nm, and contained 0.14% sulfur and glucose and galactose as the major constituent sugars and

Table 2
Efficacy of *Prunella* creams in a mouse HSV-2 genital infection model

Treatment	No. of animals (*n*)	No. of survival	No. of death	Survival (%)	Days-to-mortality (mean ± S.D., days)[a]	No. of vaginal samples containing HSV-2	
						Before treatment	After treatment/death
Prunella							
15%	30	22	8	73.3 ($P<0.01$)[b]	11.2 ± 1.33 ($P<0.01$)	30	9 ($P<0.01$)
10%	30	19	11	63.3 ($P<0.01$)	10.7 ± 1.82 ($P<0.01$)	30	16 ($P<0.01$)
5%	30	14	16	46.7 ($P<0.01$)	9.50 ± 2.50 ($P<0.01$)	30	21 ($P<0.01$)
Acyclovir (3%)	30	24	6	80 ($P<0.01$)	11.6 ± 0.96 ($P<0.01$)	30	8 ($P<0.01$)
Base cream	30	4	26	13.3 ($P>0.05$)	7.20 ± 2.07 ($P>0.05$)	30	27 ($P>0.05$)
Virus control	30	2	28	6.7	6.40 ± 1.73	30	28
Uninfected control	30	30	0	100	NA[c]	0	0

[a] Average time for mortality to occur following infection.
[b] *P* value from *t*-test between test group and virus control group.
[c] Not applicable.

xylose and galactosamine as minor sugars. PPS-2b is 8.5 kDa, showed a UV spectrum with absorption maxima at 207, 270, and 320 nm, and contained 0.92% sulfur and glucose as the major constituent sugar. It may be possible that the PPS-2b is a fragment of prunellin or it can also be a completely different carbohydrate synthesized by *P. vulgaris*. This question can only be answered by comparing the structure of prunellin and the anti-HSV polysaccharide, which is not available.

Polysaccharides are known to affect the growth of animal viruses (Shannan, 1984). In particular, anionic polysaccharides, such as heparin, dextran sulfate, carrageenans, pentosan polysulfate, fucoidan, and sulfated xylogalactans, are potent inhibitors of herpes virus binding to host cells (Baba et al., 1988; Damonte et al., 1996; Gonzalez et al., 1987). Herold et al. (1995, 1996) showed that *N*-sulfations and the presence of carboxy groups on heparin are key determinants for HSV-1 and HSV-2 interactions with host cells since *N*-desulfation and carboxyl reduction abolished heparin's anti-viral activity. The presence of sulfur in PPS-2b suggests that some of the sugar residues may contain SO_4 to confer the anionic nature of the polysaccharide. Hence, our finding that the carbohydrate moiety is required for activity is consistent with the literature.

The results from the viral binding assay showed that PPS-2b prevented HSV-1 binding to Vero cells. The 3.5 kDa polysaccharide described previously also prevented HSV binding (Xu et al., 1999) further indicating the similarity between the two compounds. In this study, we also demonstrate that PPS-2b exerts its anti-HSV effect by inhibiting viral penetration into Vero cells. Therefore, it appears that PPS-2b can block these two early steps in the HSV infection cycle. The adsorption of HSV-1 to host cells is known to be mediated mainly by the envelop glycoprotein gC (Campadelli-Fiume et al., 1990; Herold et al., 1991; Tal-Singer et al., 1995) and to a lesser extent by gB (Herold et al., 1994). Penetration, however, requires the activities of three virion glycoproteins, gB, gD, and gH (Cai et al., 1988; Fuller et al., 1989; Highlander et al., 1988). Whether these glycoproteins (particularly, gC and gD) are the targets of PPS-2b remains to be determined.

It is worthy to note that heparin has little effects on the penetration of the gC-deficient HSV-1 mutant into Vero cells. This is likely due to the fact that the binding targets of heparin are gC and gB (Herold et al., 1991, 1994). In the absence of its major binding target (gC), heparin therefore fails to prevent penetration. In contrast, PPS-2b is equally effective in preventing penetration by the mutant and wild type HSV-1 suggesting that its targets are different from heparin. The same argument holds for the differences in IC_{50} against the wild type and the gC-deficient HSV-1 displayed by heparin and PPS-2b.

The *in vivo* anti-HSV activities of the *Prunella* lignin–carbohydrate are demonstrated in two animal infection models. In both models, the efficacy of the *Prunella* cream is clearly evident as the therapeutic effect of the *Prunella* cream is observed in a dose-dependent manner while the base cream did not exhibit any therapeutic effects. At 15% *Prunella*, the therapeutic effects were equivalent to 3% acyclovir. The concentration of PPS-2b in the 15% *Prunella* cream is estimated to be 4.2% based on the yield of 4.2 g of PPS-2b from 15 g of PPS-1. Since PPS-2b is a much larger compound than acyclovir, PPS-2b appears to be a more effective compound on a per molar basis. One less-than-ideal aspect of the animal results is the use of PPS-1 in the cream. Unfortunately, the use of the purified PPS-2b in the cream is not possible due to the immense resources needed to produce enough of the pure compound. Despite this, we are confident that the observed therapeutic effect is due to the anti-HSV effect of PPS-2b as PPS-2b accounts for 28% (wt/wt) of PPS-1 and the majority of the anti-HSV activity.

In conclusion, we have isolated a lignin–carbohydrate complex from the spike of *P. vulgaris* that exhibits strong activity against HSV-1 and HSV-2 including a strain of acyclovir-resistant HSV-1. This lignin–carbohydrate complex appears to exert its anti-HSV effect by inhibiting viral binding and penetration. The *in vivo* anti-HSV activity of the lignin–carbohydrate complex is demonstrated and suggests that the complex can be developed into an effective anti-herpes drug.

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