

# Treatment of Herpes Simplex Virus with Lignin-Carbohydrate Complex Tablet, an Alternative Therapeutic Formula

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## 1. Introduction

Herpes simplex virus type 1 (HSV-1) commonly infects the mucosa and skin epithelial cells, and the virus remains latent in sensory neurons mainly in the trigeminal ganglia. Once a patient has been infected, the infection continues for life (Hunt, 2011a). Differences in HSV-1 prevalence have been reported around the world. According to Smith & Robinson (2002), the incidence in lower socioeconomic countries is higher. Primary infection, occur mainly in infants and young children, infections are usually mild or subclinical. Acute gingivostomatitis is characterized by the appearance of multiple vesicular and ulcerative painful lesions in oral mucosa, with inflammation and bleeding of the gums, may also be associated with systemic symptoms (Arduino & Porter, 2008). Once the clinical infection concludes, the virus reaches peripheral nerves which supply sensation to the skin, migrating along the nerve axon to the dorsal root ganglia of the trigeminal or facial nerves and goes into latency stage (Esmann, 2001)

Recurrences of HSV-1 can be triggered by internal and external factors. The reactivation mechanism is unknown, the virus begins to replicate within the ganglion and grows down the nerves and out into the skin or mucous membranes (Koelle & Corey, 2008). After a prodromal of tingling, warmth or itching, the clinical lesion appear (Fatahzadeh & Schwartz 2007). The recurrence of oral HSV- 1 is developed almost always in the vermilion border of the lips but lesions can appear elsewhere around perioral skin (Siegel, 2002).

Prevention of infection can be achieved by avoiding the physical contact, kissing when the lesions are present, touching or using the articles that the patient has used (eating or drinking utensils, glasses, or straws). However, in order to prevent the recurrences, the control of external factors is recommended; avoiding the exposure to wind burn and ultraviolet radiation, using labial protectants and controlling the emotional stress (Paterson & Kwong, 2008).

On the other hand, internal factors that are related to the recurrence outbreaks such as, fever, illness, menstruation, gastrointestinal and respiratory infections, diseases as diabetes and hyperthyroidism, fatigue and factors that depress the immune system are difficult to control (Siegel, 2002; Paterson & Kwong, 2008).

Although different clinical assays have been developed in order to assess the efficacy of topical or oral antivirals, its effectiveness has not been demonstrated due to the immediate and complete termination of viral replication, the restoration of previously infected cells, and the inactivation of free virions (Hamuy & Berman 1998).

There is no treatment that can eradicate herpes virus, even though antiviral medications can reduce the frequency, duration, and severity of outbreaks (Emmert, 2006; Siegel, 2002; Sprurance, et al., 2003; Sprurance, et al., 2005)

### 1.1 Conventional treatment

According to Hunt (2011b), there are different phases of life cycle of virus; adsorption and penetration of the virus in the host cell, and early transcription, in which DNA polymerase, DNA binding proteins, thymidine kinase and ribonucleotide reductase are synthesized. These proteins are virally-coded, not host-coded enzymes, and therefore potentially weak in the virus life cycle, making them promising targets for anti-viral drugs.

The nucleoside analogues acyclovir, valacyclovir, are phosphorylated initially by viral thymidine kinase to eventually form a nucleoside triphosphate, and these molecules inhibit herpes simplex virus (HSV-1) polymerase, inhibiting replication of HSV-1 (Balzarini, 1994). The best anti-viral drugs are nucleoside analogs such as acyclovir (acycloguanosine). It gets into the cell across the plasma membrane as the nucleoside form and is then specifically phosphorylated inside the cell by herpes virus thymidine kinase to an active form. The advantages of nucleoside analogs are that they are only activated by the virus-infected cell and the activated form of the drug is rendered even more specific as a result of the viral DNA polymerase being more sensitive to the drug than the host enzyme (Hunt, 2001b)

In general, acyclovir compounds are safe and effective for treatment of HSV-1 reactivation and have good oral bioavailability (Chon & Elliott, 2007). However, topical administration of acyclovir at the acute stage of the lesion disease seems to be ineffective. On the other hand, its capability to avoid the recurrent episodes produces controversial efficacy (Elish, 2004; Sprurance, et al., 2002). Emmert (2000) suggested that patients with mild and infrequent recurrences are not benefited with acyclovir treatment. There is general consensus that the therapy is most effective when started soon after symptoms occur.

Rare adverse effects include: coma, seizures, neutropenia, leukopenia, tremor, ataxia, encephalopathy, psychotic symptoms, crystalluria, anorexia, fatigue, hepatitis, Stevens-Johnson syndrome, toxic epidermal necrolysis and/or anaphylaxis (United States Food and Drug Administration, FDA, 2011). Also the appearance of virus strains resistant to frequently used anti-herpes virus drugs (Greco, et al., 2007 ; Stránská, et al., 2005; Ziyaeyan, et al, 2007)

### 1.2 Alternative treatment

It has been suggested that there are insufficient scientific evidences to support the use of alternative medicine in HSV-1 infection. Even though, anecdotal reports of alternative

remedies claimed to be beneficial in the treatment of herpes infection, arguing that alternative medicine could be beneficial in the treatment of herpes infection through enhancing the immune system. The interest in alternative drugs having antiviral effect is increasingly, since HSV-1, might develop resistance to commonly used antiviral agents.

Besides, despite the fact that some patients manifested side effects, the majority of the natural remedies did not show a high prevalence of adverse or severe reactions.

There are a number of natural remedies used in the HSV-1 treatment; some of them have been the subject of scientific analysis, demonstrating *in vitro* and *in vivo* satisfactory results.

### 1.2.1 Some examples of traditional medicine

In an experimental-placebo study, the application of zinc oxide/glycine cream showed shorter duration of cold sore lesions and reduction in overall severity of signs and symptoms (Godfrey, et al., 2001). In a pilot study, Femiano, et al. (2005) reported similar results and a reduction of number of episodes of herpes labialis. Singh, et al. (2005) in a clinical trial, evaluated a combination of L-lysine with a mixture of botanicals and other nutrients, with satisfactory results. Since the early 90s, Kümel et al. (1990a, 1991b) explained that the zinc ions inactivate virus by inhibition of the virion glycoprotein's function after a nonspecific accumulation of zinc into many virion membrane components, thus inhibiting viral adsorption and penetration. Also, Arens & Travis (2000) demonstrated that zinc salts inactivated the clinical isolates of HSV *in vitro*.

Mårdberg et al., (2001), demonstrated that viruses with mutations at residues Arg129,130, Ile142, Arg143,145, Arg145,147, Arg151,155 and Arg155,160 had significantly impaired the heparan sulfate (HS) binding. Impairment of the HS-binding activity of glycoprotein C, by these mutations had profound consequences for virus attachment and infection of cells in which amounts of HS exposed on the cell surface had been reduced.

Recently Katsuyama et al., (2008) established that Butyroyl-arginine, an arginine derivative, strongly inactivates the enveloped virus, as HSV-1. The authors suggest that the ability of arginine to bind membranes may be responsible for the inactivation of viruses. Naito et al., (2009) also has demonstrated the inhibition of HSV-1 multiplication by arginine.

Huleihel & Isanu (2002) reported that propolis could block the cell membrane receptors for HSV-1, blocking the penetration of viral particles into the cells and/or inducing the intracellular metabolic changes of host cells, which would in turn affect the viral replication cycle *in vitro*.

Ascorbic acid has been shown to inactivate HSV-1, prevent the virus reactivation, have anti-inflammatory properties and to enhance the immune function (Gaby, 2006; Hovi, et al., 1995; Yoon et al., 2000). Supplementation with flavonoids further increases the effectiveness of vitamin C (Terezhalmay, et al., 1978). According to Narayana, et al., (2001), flavonoids showed strong antiviral activity against HSV-1. Essential oils of ginger, thyme hyssop and sandalwood have been demonstrated to inactivate HSV-1 before it enters into the cells, even in acyclovir resistant HSV-1 (Schnitzler, et al., 2007). *Melissa officinalis* (*lemon balm*) contains rosmarinic acid, phenolic acids and tannins; rosmarinic acid has been reported to show anti-inflammatory and potent antioxidant action. Schnitzler, et al. (2008), demonstrated that balm oil affected the viruses *in vitro* before adsorption, although the mechanism of action is

unclear. They suggest that the balm oil could bind the viral proteins involved in the host adsorption and penetration, or damage the virions envelop. Carson, et al. (2006) have reported that tea tree oil of *Melaleuca alternifolia* showed the greatest effect on free virus.

Surveys of alternative treatment for HSV-1 are difficult to perform, since numerous patients are needed the regular contact for long period until the completion of the study. Even so, we have applied the vitamin C-supplemented tablet of lignin-carbohydrate complex (LCC) prepared from the pine cone of *Pinus parviflora* Sieb et Zucc., to a sample of HSV-1 patients, and investigated its clinical effect for the first time, with satisfactory results (González et al., 2009). The inhibitory effect of pine cone lignin and ascorbic combination treatment depend on antioxidant and immunopotentiating activities of lignin and ascorbic acid (Sakagami, et al., 1992).

The goal of both conventional and alternative treatment is promoting faster healing, reduction of symptoms, as well as decreasing the frequency of recurrent episodes. There are different phases in the viral cycle, to which the medicaments could be applied, according to the properties and mechanism of action of each medication (Fig. 1)

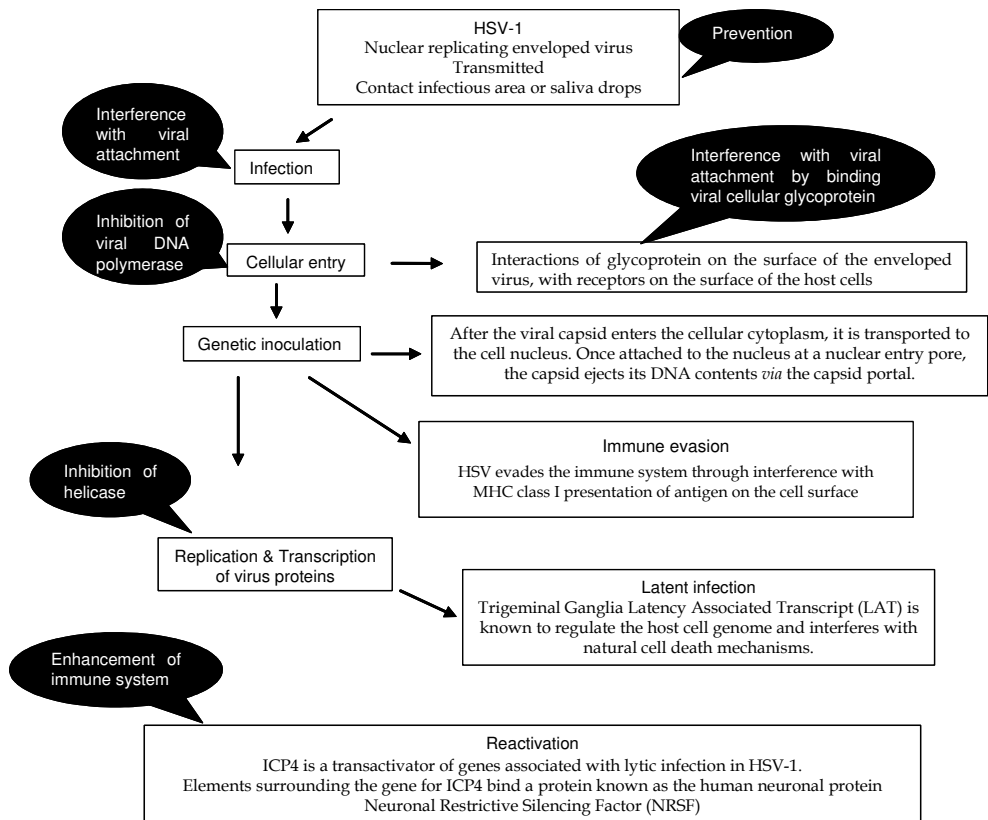


Fig. 1. A desirable effect of alternative treatment on HVS-1 virus cycle (Everett, 2006; Frick, 2003; Mettenleiter, et al., 2006; Newcomb, et al., 2007, Pinnoji, et al, 2007).

## 2. Functionality of LCC as alternative medicine

Lignin (polymers of phenylpropenoids), tannin and flavonoid are three major polyphenols in the natural kingdom (Table 1). So far, thousands of tannin and flavonoid-related compounds have been isolated from the methanol extracts of various plants and their complete structures have been elucidated. In contrast, lignins, extracted with alkaline solution, have been bound to polysaccharides (composed of glucose, arabinose, mannose, galactose, fucose, or uronic acids) to form lignin-carbohydrate complex (LCC) (Fig. 2).

	Component unit	MW (kDa)
Tannin	Hydrolysable Tannin	Esters of gallic acid and its oxidative derivatives with glucose or related sugars
	Condensed tannin	Flavan oligomers or polymers where their constituent monomeric flavans are connected mainly by C-4 - C-8 or C-4 - C-6 linkages
Flavonoid	Oxygen containing cyclic structure between two benzene rings	0.3~1
Lignin carbohydrate complex (LCC)	Complex of phenylpropenoid polymers and polysaccharide	10~200

Table 1. Representative polyphenols present in the natural kingdom.

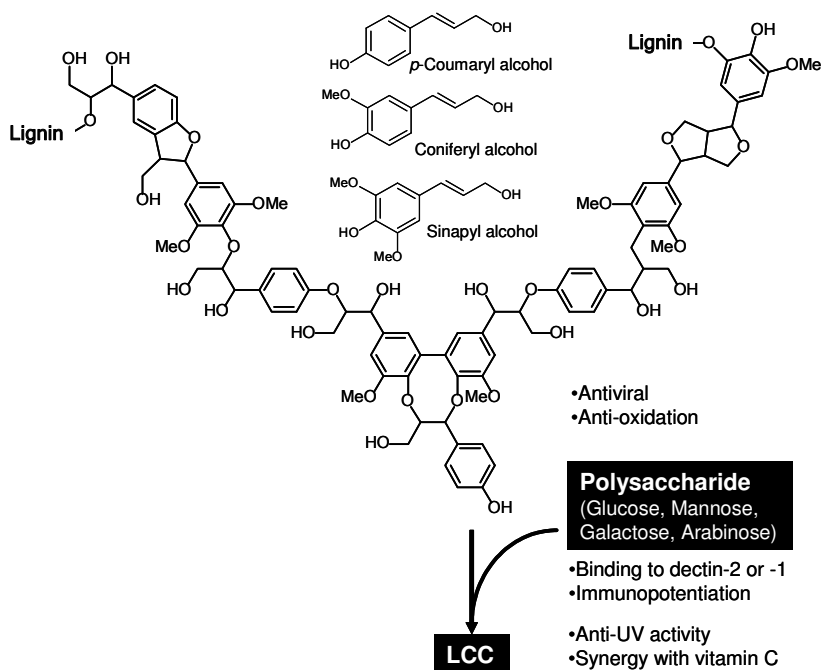


Fig. 2. Structure and function of lignin-carbohydrate complex (LCC)

This structural complexity of LCC has made it difficult to elucidate its complete structure. Varying the ratio of polysaccharide to phenylpropanoid polymer produces heterogeneity in the acidity, water-solubility, ethanol-insolubility, and molecular weight of LCC that might strongly affect its antiviral potency (Sakagami, et al., 2005, 2010b). However, this possibility has not been tested yet by any investigators. LCC was recoverable at much higher yield from the alkaline solution, in contrast to tannins and flavonoids (Fig. 3). The higher yield of LCC is very convenient for the mass-production in the factory.

## 2.1 Identification of LCC as an active antitumor principle of pine cone extract

We have paid attention to the folklore that intake of the hot water extract of pine cone of *Pinus parviflora* Sieb. et Zucc is effective for gastroenterological tumors. We isolated various polysaccharide fractions (Fig. 3), and investigated their antitumor activity against ascites sarcoma-180 cells (Sakagami, et al., 1987).

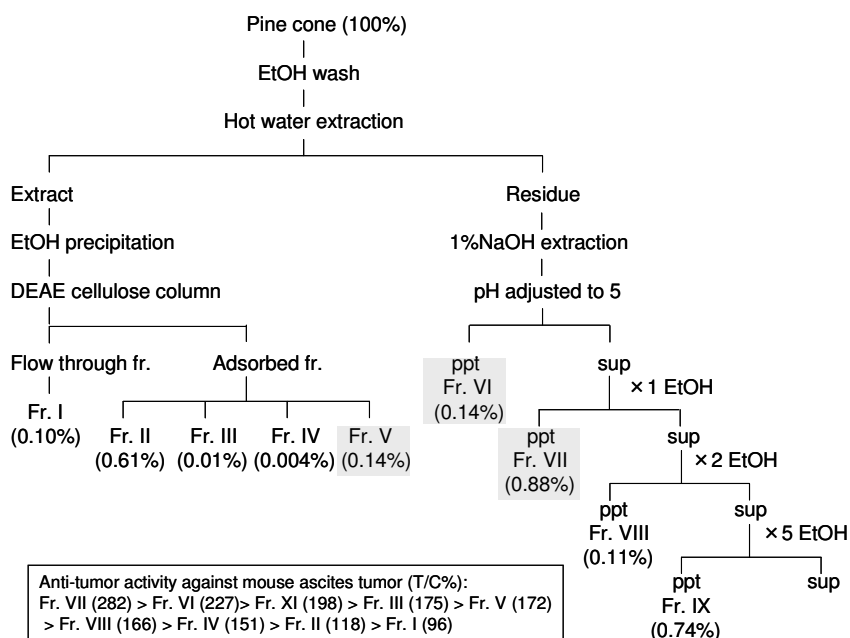


Fig. 3. Fractional preparation of LCC fractions from *Pinus parviflora* Sieb. et Zucc. Cited from Sakagami et al. (1987), with permission.

Pine cone was treated with ethanol to remove the sticky resin that contains cytotoxic substances, and then extracted with hot-water and then alkaline solution (1% NaOH). Polysaccharides in the hot water extract were precipitated by 86% ethanol, and then applied to DEAE-cellulose column chromatography. Neutral polysaccharide fraction (Fr. I) passed through the column, and then acidic polysaccharide fractions (Fr. II and III) rich in uronic acid were eluted from DEAE cellulose column chromatography with 0.5 and 2 M NaCl, respectively. The most acidic polysaccharides (Fr. VI and Fr. V) were eluted with 0.15M NaOH. The anti-tumor activity [evaluated by the survival ratio of treated group to control

group (T/C%) increased with the acidity: Fr. I (T/C=98%) < Fr. II (T/C=118%) < Fr. III (T/C=175%), Fr. IV (T/C=151 %) < Fr. V (T/C=172%) (Sakagami, et al., 1987). Higher antitumor activity was recovered by extraction with 1%NaOH, precipitated by acidification (pH 5) (Fr. VI) (T/C=227%), and also by ethanol precipitation (Fr. VII) (T/C=282%).

The most active acidic polysaccharide (IV) was subjected to spectral analysis, and identified as lignin-carbohydrate complex (LCC), based on the following evidences (Sakagami, et al., 1989). (i) UV absorption spectra : minimum absorption at 260 nm, maximum absorption at 280 nm, broad maximum absorption at 500 nm. (ii) IR spectra: hydroxyl group with hydrogen bonding (3400  $\text{cm}^{-1}$ ), aliphatic C-H (2700  $\text{cm}^{-1}$ ), carbonyl group conjugated to  $\pi$ -electron system (1700 ~ 1600  $\text{cm}^{-1}$ ), aromatic double bond (1600, 1500  $\text{cm}^{-1}$ ), C-O expansion and contraction (1400 ~ 1000  $\text{cm}^{-1}$ ), no ester bonding.(iii) ESR: one strong signal at  $g=2.003$  under solid state at room temperature. Signal intensity was significantly reduced by oxidation and reduction.(iv)  $^1\text{H-NMR}$  : When measured in 0.2%NaOD- $\text{D}_2\text{O}$ , the presence of hydrogens in aromatic CH ( $\delta 6.5\sim 7.5$  ppm),  $>\text{C}=\text{C}<$  ( $\delta 4.5\sim 5.5$  ppm) and  $-\text{O}-\text{CH}-\text{CH}$  ( $\delta 3.0 \sim 4.0$  ppm) was suggested. When the sample was acetylated by pyridine-acetic acid anhydride and dissolved in  $\text{CDCl}_3$ , the presence of acetyl group bound to phenolic OH ( $\delta 2.3$  ppm) or bound to alcoholic OH ( $\delta 2.1$  ppm) was confirmed. (v) Thin layer chromatography: Rf value of Fr. VI was the same with that of commercial alkali-lignin in various solvent systems (Table 2).

UV Absorption spectra	absorption peak at 260, 280, 500 nm (broad)
IR spectra	3400, 2700, 1700~1600, 1600, 1500, 1400~1000 $\text{cm}^{-1}$
ESR spectra	$g=2.003$ (strong signal)
$^1\text{H-NMR}$ spectra	$\delta 6.5\sim 7.5$ , $4.5\sim 5.5$ , $3.0\sim 4.0$ ppm
TLC	The same Rf value with alkali-lignin
Elementary analysis	C (43.21%), H (3.96%), N (2.61%), S (not detectable)
Neutral sugar/uronic acid	11.0%/1.7%
Composition of neutral sugar	Gal (44.7%), Glc (26.9%), Man (19.0%), Fuc (9.4%)
Molecular weight	10 kDa on gel filtration chromatography

Table 2. Identification of Fr. IV from pine cone of *Pinus parviflora* Sieb. et Zucc. as LCC, based on chemical analyses. Cited from Sakagami et al. (1987), with permission.

## 2.2 Distribution of LCC in the natural kingdom

Frs. VI and VII, prepared from the pine cones of *Pinus parviflora* Sieb. and Zucc. (T/C=227, 247%) showed higher antitumor activity in mice, than those prepared from *Pinus densiflora* Sieb. et Zucc (T/C=155, 245%), *Pinus thunbergii* Parl. (T/C=218, 191%), *Pinus elliottii* var. Elliotti (T/C=170, 217%), *Pinus taeda* L. (T/C=196, 179%), *Pinus caribaea* var. Hondurenses (T/C=114, 147%), *Pinus sylvestris* L. (T/C=180, 135%), or the pine seed shells of *Pinus parviflora* Sieb. et Zucc. (T/C=194. 220%), and *Pinus armandii* Franch (T/C=125%). Furthermore, the yields of Frs. VI and VII, prepared from *Pinus parviflora* Sieb. et Zucc. (0.51, 0.91%) were much higher than those from other pine cone sources [ $0.19\pm 0.18$  (0.001~0.48),  $0.31\pm 0.16$  (0.06~0.48) %] (Harada, et al., 1988). These data suggest that acidic polysaccharides (Frs. VI and VII) are responsible for the legendary antitumor potential of the pine cone of *Pinus parviflora* Sieb. et Zucc.

LCCs from pine cone from *Pinus parviflora* Sieb et Zucc., and *Pinus elliottii* var. *Elliotti* [SI (selectivity index for measuring anti-HIV activity) =14, 28), bark of *Erythroxylum catuaba* Arr. Cam. (SI=43) (Manabe, et al., 1992), husk and mass of *Theobroma cacao* (SI= 311, 46) (Sakagami et al., 2008, 2011) and cultured extract of *Lentinus edodes* mycelia (LEM) (SI=>94) (Kawano et al. 2010) and mulberry juice (SI=7) (Sakagami et al., 2007, 2010b; Sakagami & Watanabe, 2011) showed higher anti-HIV activity than lower molecular weight polyphenols, such as tannins (SI=1-11) (Nakashima, et al., 1992b) and flavonoids (SI=1) (Fukai, et al., 2000), and natural and chemically modified glucans [*N,N*-dimethylaminoethyl paramylon, *N,N*-diethylaminoethyl paramylon, 2-hydroxy-3-trimethylammonioethyl paramylon, sodium carboxymethyl paramylon, carboxymethyl-TAK) (SI=1) except for sulfated polysaccharide (such as paramylon sulfate and dextran sulfate) (Koizumi, et al., 1993). Limited digestion of lignin structure by NaClO<sub>2</sub> resulted in significant loss of anti-HIV activity (from SI=14 to 3), whereas removal of the monosaccharide residues by acid-catalyzed hydrolysis did not significantly affect the anti-HIV activity (from SI= 14 to 13) (Lai et al., 1992) suggesting that that phenylpropanoid polymer, but not sugar moiety, is important for anti-HIV activity. This was confirmed by our finding that dehydrogenation polymers of phenylpropanoids without carbohydrate showed generally higher anti-HIV activity (SI=105) than LCCs (Nakashima, et al., 1992a). On the other hand, phenylpropanoid monomers (*p*-coumaric acid, ferulic acid, caffeic acid) were inactive, suggesting the importance of highly polymerized structure (Nakashima, et al., 1992a). The mechanism of anti-HIV activity induction has been suggested to be mediated by the inhibition of HIV adsorption to the cells (Nakashima, et al., 1992a). *In vitro*, LCCs have also been reported to inhibit the HIV-1 reverse transcriptase activity (Lai, et al., 1990, 1992) and HIV-1 protease activity (Ichimura, et al., 1999).

## 2.3 Anti-HSV activity *in vitro*

### 2.3.1 Inhibition of HSV-1 infection by Fr. VI

Inhibition of HSV infection was determined by plaque assay. Cells were inoculated with HSV-1 (200-400 plaques per well (3.5 cm diameter) 2 days after infection. Fr. VI showed potent anti-HSV-1 activity. Addition of Fr. VI during and after adsorption significantly reduced the number of plaques without affecting the morphology of the CV-1 cells. The plaque formation of HSV-1 was significantly inhibited by Fr. VI at a concentration of more than 0.1 µg/ml and completely inhibited by Fr. VI at more than 10 µg/ml (Fig. 4) (Fukuchi, et al., 1989a). Fr. VI inhibited the cytopathic effect of two different HSV-1 strains (HF and F) and HSV-2 strain G on two samples of cultured monkey kidney cells (CV-1 and Vero) and one sample of human adenocarcinoma cells (A-549). From the dose-response curves, the doses of Fr. VI that inhibited plaque formation by 50% (50% effective dose) in these cells were calculated to be 0.1-0.3 µg/ml (Fig. 4). When Fr. VI was adsorbed on and eluted from Sephadex LH-60, anti-HIV activity was slightly enhanced (Fr. VIb in Fig. 4).

Neither the growth rate nor the saturation density of CV-1 cells was significantly affected by up to 100 µg/ml of Fr. VI (Fig. 5). This indicates that the anti-HSV-1 effect of Fr. VI was not merely due to toxicity for the host cell.



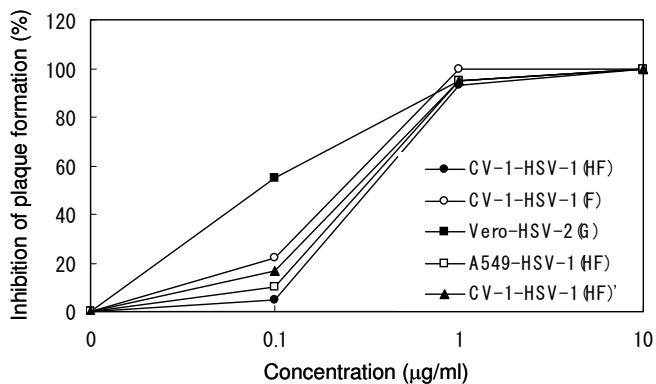


Fig. 4. Inhibition of HSV-1 plaque formation by Fr. VI and Fr. VIb in various cultured cells. The indicated doses of Fr. VI were added to the CV-1 cells at the time of infection with HSV-1 (HF) (●) or HSV-1 (F) (○), to the Vero cells at the time of infection with herpes virus simplex type 2 (G) (■), or to the A549 cells at the time of infection with HSV-1 (HF) (□). The CV-1 cells were also infected with HSV-1 (HF) in the presence of the indicated doses of Fr. VIb (▲). After washing with DME, these infected cells were overlaid with agarose, further incubated for 2 days in the presence of the same amounts of Fr. VI or Fr. VIb, and the number of plaques was then determined. Each value represents mean of triplicate determinations. Cited from Fukuchi, et al., (1989a), with permission.

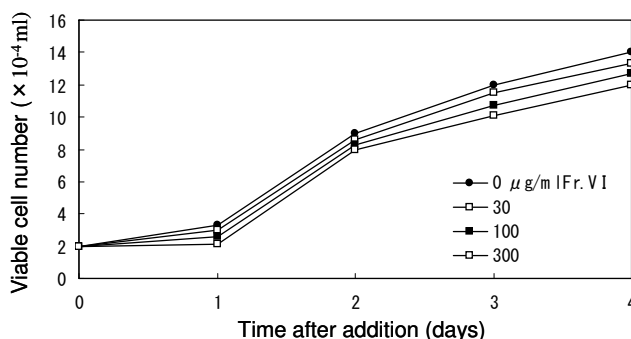


Fig. 5. Effect of Fr. VI on the growth of CV-1 cells. Each value represents mean of triplicate assays. Cited from Fukuchi, et al., (1989a), with permission.

### 2.3.2 Anti-HSV activity of LCCs from other plants, natural and chemically modified polysaccharides

Neutral polysaccharide (Fr. I) and uronic acid-rich polysaccharide (Fr. II) from pine cone extract of *Pinus parviflora* Sieb. et Zucc. had no anti-HSV activity at 10 µg/ml. Similarly, popular antitumor polysaccharides, such as paramylon, PSK, Schizophyllan, and chemically modified glucans (*N,N*-dimethylaminoethylparamylon, sodium carboxymethylparamylon, sodium paramylon sulfate, carboxymethyl TAK) were all inactive at 10 µg/ml. On the other hand, Fr. V tightly bound to DEAE-cellulose chromatography, and four LCC fractions (Frs.

VI-IX) almost completely inhibited the HSV-infection at 10 µg/ml (Table 3). LCC fractions (Frs. VI, VII) obtained from cones of other Japanese pine trees (*Pinus densiflora* Sieb. et Zucc., *Pinus thunbergii* Parl.), three Brazilian pine trees (*Pinus elliottii* var. *Elliottii*, *Pinus taeda* L., *Pinus caribaea* var. *Hondurensis*) and one Finnish pine tree (*Pinus sylvestris* L.), and from the seed shells of *Pinus parviflora* Sieb. et Zucc., and *Pinus armandii* Franch, also showed potent anti-HSV activity (Table 3).

Sample	Fr.	No. of plaques	% of inhibition
Neutral polysaccharide from cone of <i>Pinus parviflora</i> Sieb et Zucc.	I	310	0
Acidic polysaccharide from cone of <i>Pinus parviflora</i> Sieb et Zucc.	II	321	0
LCCs from pine cone of <i>Pinus parviflora</i> Sieb et Zucc.	V	0	100
	VI	0	100
	VII	33	89
	VIII	0	100
	IX	0	100
LCCs from cone of <i>Pinus densiflora</i> Sieb. et Zucc.	VI	0	100
	VII	9	97
LCCs from cone of <i>Pinus thunbergii</i> Parl.	VI	0	100
	VII	0	100
LCCs from cone of <i>Pinus elliottii</i> var. <i>Elliottii</i>	VI	2	99
	VII	0	100
LCCs from cone of <i>Pinus taeda</i> L.	VI	0	100
	VII	12	96
LCCs from cone of <i>Pinus caribaea</i> var. <i>Hondurensis</i>	VI	0	100
	VII	0	100
LCCs from cone of <i>Pinus sylvestris</i> L.	VI	0	100
	VII	0	100
LCCs from seed shell of <i>Pinus parviflora</i> Sieb et. Zucc.	VI	0	100
	VII	0	100
LCC from seed shell of <i>Pinus armandii</i> Franch	VI	0	100
Paramylon		310	0
PSK		320	0
Schizophyllan		325	0
<i>N,N</i> -dimethy aminoethylparamylon		306	0
Sodium carboxymethylparamylon		312	0
Sodium paramylon sulfate		299	2
Carboxymethyl-TAK		320	0
Alkali-lignin		0	100
Tannic acid		0	100
Saline (control)		305	–

Table 3. Anti-HSV activity of LCCs, natural and synthetic polysaccharides, added at 10 µg/ml. Each value represents mean of triplicate assays. Cited from Fukuchi, et al., (1989a), with permission.

### 2.3.3 Interference by Fr. VI of HSV-1 cellular adsorption

To determine the point of inhibition of HSV-1 infection, cells were treated with Fr. VI at various times before and after HSV-1 infection. Table 4 shows that: (i) no protective effect was observed when Fr. VI was not present in the adsorption medium, and (ii) pretreatment of the cells with Fr. VI for 6 days did not decrease the number of plaques.

Fr. VI ( $\mu\text{g/ml}$ )		After adsorption	No. of plaques	% of inhibition
Before adsorption	During adsorption			
0	0	0	229	–
10 (6 days)	0	0	204	11
0	10	0	0	100
0	0	10	230	0
10 (6 days)	0	10	188	18

Table 4. Dependence of anti-HSV activity induction by Fr. VI on treatment schedule. CV-1 cells were infected with HSV-1 strain HF. Fr. VI was added at the indicated stages. Each value represents mean of three separate assays. Cited from Fukuchi, et al., (1989a), with permission.

The results suggest that the protective effect of Fr. VI might be caused by its inhibition of virus adsorption. To test this possibility, the CV-1 cells were incubated with higher concentrations of radiolabeled virus particles (20,000 PFU) in the presence of Fr. VI, and the cell-bound radioactivity was measured. Table 5 shows Fr. VI at 10  $\mu\text{g/ml}$  significantly inhibited the binding of the radiolabeled virus particles, even when the cells were incubated with higher concentrations of virus. Lignin similarly inhibited virus adsorption, but its effect was slightly lower than that of Fr. VI (Table 5).

Sample	Dose ( $\mu\text{g/ml}$ )	Cell-bound radioactivity	
		cpm	% of inhibition
Fr. VI	0	3007 $\pm$ 168	–
	10	438 $\pm$ 230	85
Lignin	0	5367 $\pm$ 184	–
	10	2774 $\pm$ 491	48
	100	193 $\pm$ 53	96

Table 5. Inhibition of radiolabeled virus adsorption by Fr. VI and lignin. CV-1 cells were incubated for 1 hour at 37°C with the radioactive virus particle equivalent to 60,000 cpm (20,000 PFU/well) in the absence or presence of the indicated amounts of Fr. VI or lignin, and the cell-bound radioactivity was then determined. Each value represents mean  $\pm$  S.D. of triplicate assays. Cited from Fukuchi, et al., (1989a), with permission.

We next investigated the effect of Fr. VI on virus penetration. Cells were first adsorbed for 1~2 hours with virus (200-400 PFU/well) at 4°C, a condition that allows virus adsorption but not virus penetration. The cells were treated with Fr. VI and the temperature was then raised to 37°C to initiate virus penetration. Fr. VI (1  $\mu\text{g/ml}$ ) did not inhibit plaque formation after completion of virus adsorption (data not shown). From the results, it was concluded that Fr. VI inhibits virus adsorption on target cells, but does not inhibit virus penetration.

We have previously reported that LCC also inhibited the adsorption of HIV to the cells (Nakashima, et al., 1992a, 1992b).

Recently, carboxylated lignins, synthesized using 4-hydroxy cinnamic acid scaffold by enzymatic oxidative coupling inhibited the entry of HSV-1 into the cells (Thakkar et al., 2010). Sulfated LCL (PPS-2b) (MW8500) also showed anti-HSV activity possibly by inhibiting the viral binding and penetration into host cells. Prunella cream formulated with a semi-purified fraction significantly reduced the skin lesion and mortality induced by HSV-1 infection in Guinea pigs (Zhang et al., 2007) The anti-HSV activity of sulfated lignins depended on their molecular weight, with the maximum at 39.4 kDa (Raghuraman et al. 2007).

## 2.4 Clinical effect of LCC-ascorbic acid tablet

The combination of alternative products can provide an effective therapy. To evaluate anti-HSV-1 activity of a pine cone LCC and ascorbic acid treatment, a clinical pilot study was carried out. We have modified the extraction method of LCC to achieve the mass production at the factory level (Fig. 6). Each LCC-ascorbic acid tablet contained a mixture of 50 mg pine cone extract powder JS, 50 mg ascorbic acid, 83 mg maltitol, 13 mg potato starch and 13 mg calcium stearate.

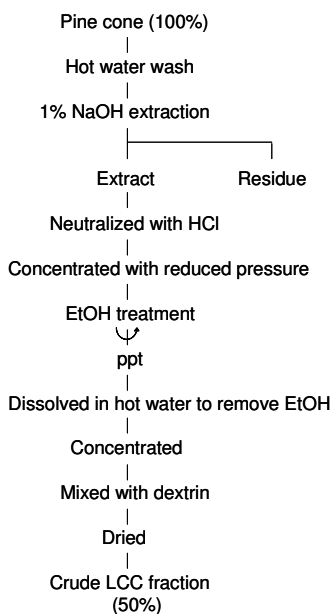


Fig. 6. Modified method for mass production of LCC at the factory level.

A pilot clinical study with pine cone lignin and ascorbic acid complex treatment against HSV-1-patients was carried out to evaluate the reduction of the duration with lesions, and the decrease of symptoms. A convenience sample of forty eight healthy patients of both genders between 4 and 61 years old (mean:  $31 \pm 16.12$  years), with active lesions of HSV-1,

took part in the study. The patients were classified into the prodromic (16 patients), erythema (11 patients), papule edema (1 patient), vesicle/pustule (13 patients) and ulcer stages (7 patients). One mg of LCC-ascorbic acid tablet or solution was orally administered three times daily for a month. Clinical evaluations were made at least three times a week during the two first weeks after the onset and every six months during the subsequent year to identify recurrence episodes. The patients who began the LCC-ascorbic acid treatment within the first 48 hours did not develop HSV-1 characteristic lesions, whereas those patients who began the treatment later experienced a shorter duration of cold sore lesions and a decrease in the symptoms compared with previous episodes. The majority of the patients reported a reduction in the severity of symptoms and a reduction in the recurrence episodes after the LCC-ascorbic acid treatment compared with previous episodes, suggesting its possible applicability for the prevention and treatment of HSV-1 infection. Figs. 7, 8 and 9.

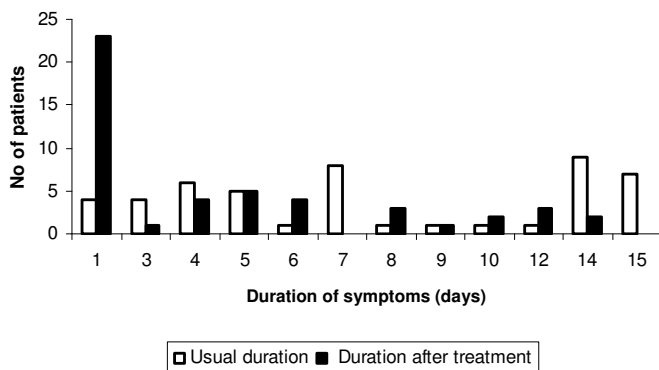


Fig. 7. Previous and new duration of lesions after pine cone ascorbic acid treatment. A significant difference in the duration of lesions was found between before and after treatment. Usual duration is based on patient report. Student's  $t$  4.202  $p = 0.001$ . Cited from González et al. (2009) with permission.

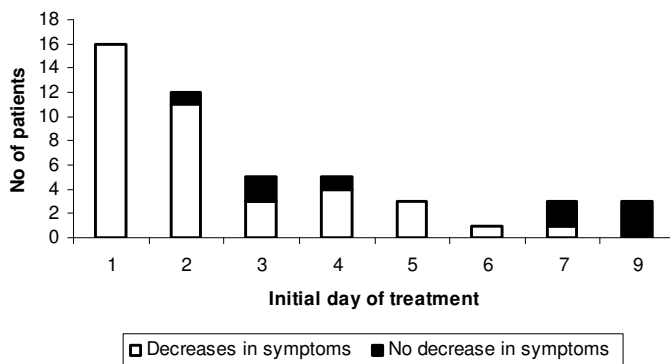


Fig. 8. Symptoms reduction according to the day of starting pine cone lignin and ascorbic acid complex treatment after onset. Symptoms were reduced notoriously when the treatment was taken in the first 48 h. after onset Kendall's  $\tau$ -b 0.456  $p = 0.001$ . Cited from González, et al., (2009) with permission.

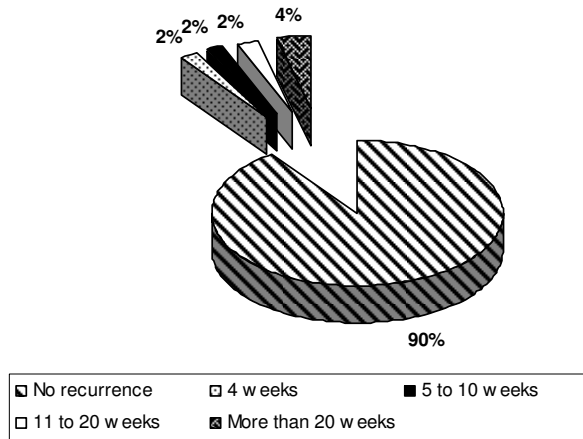


Fig. 9. Recurrence rate after pine cone lignin ascorbic acid complex treatment. Cited from González et al. (2009) with permission.

Majority of the patients reported the reduction in the severity of symptoms and in the recurrent episodes. This pilot study suggests possible applicability of LCC-vitamin C tablet for the prevention and treatment of HSV-1 infection (González, et al., 2009). However, it is not clear whether this clinical effect is due to the antiviral action of LCC itself or due to the combination effect of LCC + vitamin C.

We found significant differences between the usual and current duration after the pine cone lignin and ascorbic acid combination treatment and differences in the reduction of symptoms, taking into account the historical data provided by the patients. However, the small number of patients and the lack of a control group limited our ability to generalize the results to a larger population. To evaluate the effectiveness of pine cone lignin and ascorbic acid combination treatment, subsequent double-blind randomized controlled clinical trials must be done in a representative sample of patients who suffer from recurrent HSV-1.

### 2.5 Clinical effect of LCC tablet without vitamin C

In order to evaluate the effect of LCC obtained from pine "*Pinus parviflora* Sieb et Zucc." without ascorbic acid, on the treatment of infection by HSV type I, we already started a cross-sectional study on a sample of the patients, from the School of Dentistry of the Mexico State University. It has been planned to develop a double blind randomized clinical study in a larger, heterogeneous sample of captive population, incorporating students, professors and working personal of the Schools of Health Sciences of the University, the study will be carried out during three years.

Patients aged 18 or more who gave informed consent will enter into the study; they will be initially identified through an interview, and they will be asked to answer a questionnaire that includes signs and symptoms, the triggers and the treatments used. The patients will be randomized for treatment or placebo. No other antiviral drugs will be permitted during the study

The patients will be seen as soon as possible after the start of the outbreaks. The tablets will be delivered to patients with the instruction of taking a tablet 3 times a day for 30 days. The patients will be seen daily or at least three times during the episode until the complete healing had occurred. Any possible adverse effects will be recorded. When a recurrence occurs, the patients will repeat the treatment as previously described. The placebo group treatment protocol will be the same, but tablets without LCC will be administrated.

Until now we have identified 18 patients with a history of infection by HSV-1, who accepted to participate in the study, they signed the informed consent and answered the questionnaire. Among them only six patients have attended the clinic at the early stage of the disease, we carried out a clinical follow-up of patients to register the evolution of the disease (Table 6). In general the patients reported a reduction of the duration of the lesions and of the symptoms, according to their previous experience (Fig. 10-14).

One patient presented a light rash at the tenth day of LCC treatment. This disappeared without treatment, once the patient suspended the treatment. At present, it is not clear whether the reaction could be related with the LCC treatment. Alternatively, this may suggest that the combination of LCC and vitamin C is necessary to reduce such incidence.

Patient	Gender/Age	Date of initiation of treatment	Decrease of symptoms	Edema	Eritema	Vesicle	Erosion	Seudomebrane	Scab
49	Female	Mar, 31	Apr, 03	Mar, 31	-	-	-	Apr, 06	Apr, 06
23	Female	Mar, 01	Mar, 02	-	Mar, 01	-	Mar, 08	Mar, 09	Mar, 09
21	Male	July, 10	July, 10	-	July, 11	July, 12	July, 12	July, 13	July, 13
53	Female	Feb, 02	Feb, 02	Feb, 02	Feb, 03	Feb, 04	Feb, 04	Feb, 05	Feb, 05
57	Female	Jan 10	Jan 10	-	-	Jan 12	Jan 12	Jan 13	Jan 14
24*	Female	Mar, 29	Mar, 30	Mar, 29	Mar, 31	Apr, 01	Apr, 01	Apr, 02	Apr, 02

\*The patient presented a light skin rash.

Table 6. Evolution of the lesions of HSV-1 in five patients treated with LCC tablet without vitamin C.

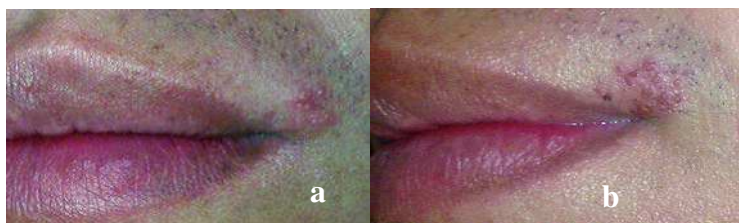


Fig. 10. Male 21 years old, a) Vesicles, b) Scab early stage

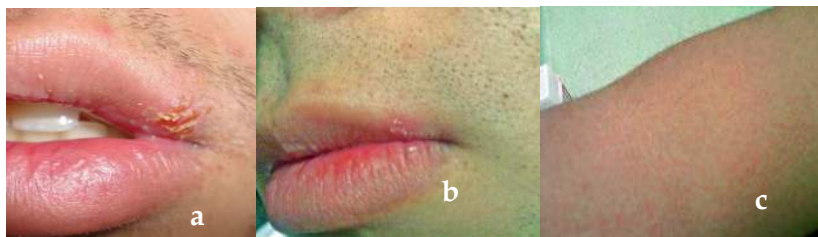


Fig. 11. Male 24 years old, a) Ulcerative stage, c) Seudomembrane stage, d) Skin rash



Fig. 12. Male, 25 years old. a) Erosion stage, b) Late scab stage, c) Complete healthy stage



Fig. 13. Female 57 years old, a) Ulcerative stage, b) Scab early stage



Fig. 14. Female 53 years old, a) Vesicle stage, b) Ulcerative stage, b) Seudomembrane stage

Fig. 10-14. Stages in the evolution of HSV-1 lesions in patients who received treatment with LCC. Only representative photographs were shown.



### 3. Conclusion

We have demonstrated that LCC from pine cone of *Pinus parviflora* Sieb. Zucc. showed anti-HSV activity *in vitro*, by inhibiting the viral adsorption to the cells. LCC shows broad antiviral spectrum. LCC exhibited high affinity with influenza virus, and in contact to LCC, influenza virus rapidly lose the virulence (Sakagami, et al., 1992). LCC showed much higher anti-HIV activity than tannins (Nakashima, et al., 1992a, 1992b), whereas both LCC and tannins showed potent anti-HSV activity (Fukuchi, et al., 1989a, 1989b). Since virus is one of major risk factor of oral cavity cancer (Sakagami, 2010a), anti-viral action of LCC may reduce the incidence of virus-triggered diseases such as cancer.

LCC shows also immunopotentiating activity. Administration with LCC induced antitumor, antimicrobial and anti-parasite activity, and enhanced the endogenous TNF production. At present, the receptors for LCC have not been identified. Recently, we have found LCC fraction isolated from *Lentinus edodes* mycelia extract (Fr4) enhanced the expression of dectin-2 (4.2-fold) and toll-like receptor (TLR)-2 (2.5-fold) prominently, but only slightly modified the expression of dectin-1 (0.8-fold), complement receptor 3 (0.9-fold), TLR1, 3, 4, 9 and 13 (0.8- to 1.7-fold), spleen tyrosine kinase (Syk) $\beta$ , zeta-chain (TCR) associated protein kinase 70kDa (Zap70), Janus tyrosine kinase (Jak)2 (1.0- to 1.2-fold), nuclear factor (Nf) $\kappa$ b1, Nf $\kappa$ b2, reticuloendotheliosis viral oncogene homolog (Rel)a, Relb (1.0- to 1.6-fold), Nf $\kappa$ bia, Nf $\kappa$ bib, Nf $\kappa$ bie, Nf $\kappa$ bi12 Nf $\kappa$ biz (0.8- to 2.3-fold). On the other hand, LPS did not affect the expression of dectin-2 nor TLR-2. These data suggest the significant role of the activation of the dectin-2 signaling pathway in the action of LCC on macrophages (Kushida, et al., 2011). Identification of dectin-2 as LCC receptor awaits further confirmation with siRNA and gene over expression experiments.

The other intriguing property of LCC is the synergy with vitamin C. Ascorbate derivatives that produced the doublet signal of ascorbate radical (sodium-L-ascorbate, L-ascorbic acid, D-isoascorbic acid, 6- $\beta$ -D-galactosyl-L-ascorbate, sodium 5,6-benzylidene-L-ascorbate) induced apoptosis in HL-60 cells, whereas ascorbate derivatives that did not produce radicals (L-ascorbic acid-2-phosphate magnesium salt, L-ascorbic acid 2-sulfate and dehydroascorbic acid) did not induce apoptosis (Sakagami, et al., 1996a, 1996b). High concentrations of LCC from the pine cone of *Pinus parviflora* Sieb et Zucc., pine cone of *Pinus elliottii* var. *Elliotti*, leaf of *Ceriops decandra* (Griff.) Ding Hou and, thorn apple of *Crataegu Cuneata* Sieb. et Zucc enhanced the radical intensity and cytotoxicity of sodium ascorbate. On the other hand, lower concentrations of LCC stimulated the superoxide anion (O<sub>2</sub><sup>-</sup>), hydroxyl radical and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of sodium ascorbate (Sakagami et al., 2000, 2005, 2008) . This suggests the possible application of LCC as stimulator of vitamin C action, especially in the field of UV protection and anti-aging research.

Solvent fractionation of alkaline extract of the leaves of *Sasa senanensis* Rehder (SE) demonstrated that (i) chlorophyllin in SE was recovered from the water layer, that contains majority of compounds (more than 81%) and inhibited the NO production by macrophages more potently than other *n*-hexane, diethyl ether and ethylacetate layers (Sakagami, et al., 2010c). Three-dimensional HPLC analysis demonstrated that the majority of SE components are recovered from one major peak. Furthermore, LCC isolated from SE showed the unique greenish color of chlorophyllin (absorption maximum = 452 nm) (Sakagami, et al., 2010c).

These data strongly suggest the possible association of chlorophyllin with LCC in the native state or during extraction with alkaline solutions. Biological significance of such association remains to be investigated.

#### 4. Acknowledgment

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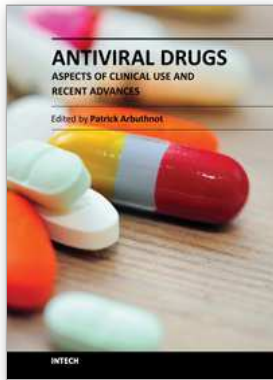
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The articles that appear in Antiviral Drugs - Aspects of Clinical Use and Recent Advances cover several topics that reflect the varied mechanisms of viral disease pathogenesis and treatment. Clinical management and new developments in the treatment of virus-related diseases are the two main sections of the book. The first part reviews the treatment of hepatitis C virus infection, the management of virus-related acute retinal necrosis, the use of leflunomide therapy in renal transplant patients, and mathematical modeling of HIV-1 treatment responses. Basic research topics are dealt with in the second half of the book. New developments in the treatment of the influenza virus, the use of animal models for HIV-1 drug development, the use of single chain camelid antibodies against negative strand RNA viruses, countering norovirus infection, and the use of plant extracts to treat herpes simplex virus infection are described. The content of the book is not intended to be comprehensive, but aims to provide the reader with insights into selected aspects of established and new viral therapies.

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