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Inhibition of Cytopathic Effect of Human Immunodeficiency Virus-1 by Water-soluble Extract of *Ganoderma lucidum*

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To examine components of *Ganoderma lucidum* for anti-human immunodeficiency virus (HIV) activity, the aqueous extracts of its basidiocarps were separated into high-molecular-weight (HMF) and low-molecular-weight (LMF) fractions. These fractions were used in XTT [2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide] antiviral assay which can quantitatively measure cytopathic effects of HIV-1 on CEM, human T lymphoblastoid cell line. The CEM cell line added with serial diluted HMF or LMF was cultured in the absence or presence of HIV-1. The results showed that the LMF of the aqueous extract strongly inhibited cytopathic effect of the target cell induced by HIV-1. When two-fold serially diluted LMF ranging from 0.97 μg/ml to 125.00 μg/ml was added to the virus-free culture system, no toxicity on the target cells was detected in all the concentrations tested. However, when it was added to the HIV-infected culture system, the viabilities of the target cell reached a plateau recovering its viabilities to 71.7% and 82.5% in experiment-1 and -2 at 15.60 μg/ml, respectively. The cell viabilities were then gradually decreased but maintained at more than 50% above 31.20 μg/ml concentration. On the contrary, HMF did not prevent any HIV-induced cytopathic effect at any concentrations tested on this cell line. From these results, negligible toxicities were observed by both HMF and LMF of *G. lucidum*, and recovery of cell viability in HIV infected target cell was induced only by LMF of the carpophores.

Key words : *Ganoderma lucidum*, Anti-HIV-1 activity, XTT

INTRODUCTION

Fruiting bodies of the fungus *Ganoderma lucidum* (Fr.) Karst. (Polyporaceae) have attracted much attention as a popular folk medicine in the Orient to treat various human diseases such as hepatopathy, chronic hepatitis, nephritis, gastric ulcer, hypertension, arthritis, neurasthenia, insomnia and bronchitis.

Investigations on biological activities of the components from this mushroom have been made possible by the success of artificial cultivation of this mushroom (reviewed by Jong and Birmingham, 1992). Water extract of *G. lucidum* showed various biological activities such as adrenaline-induced lipolysis (Kubo *et al.*, 1980), inhibition of platelet aggregation (Shimizu *et al.*, 1985), secretion of insulin (Kimura *et al.*, 1983) and growth-promotion of mouse hair (Miyamoto and Abe, 1985).

The first elucidated compounds from *G. lucidum* were ganoderic acids A and B that have bitter tastes (Kubota *et al.*, 1982). The bitter tastes were found mainly in ganoderic acids, ganoderenic acids and lucidenic acids (Hirotani *et al.*, 1985; Nishitoba *et al.*, 1985). The fact that *G. lucidum* was traditionally used to treat bronchitis was confirmed scientifically by the inhibition of histamine release from mast cells by ganoderic acids (Kohda *et al.*, 1985). Ganoderic acids also inhibited angiotensin converting enzyme that is responsible for the hypertension (Komoda *et al.*, 1985). Some ganoderic acids inhibited growth of liver cancer cells (Toth *et al.*, 1983).

On the other hand, polysaccharides from *G. lucidum* have various biological activities such as, anticancer activity (Kim *et al.*, 1980; Miyazaki *et al.*, 1981), antihypertension (Morinaka Milk Industry Co., 1981; Park *et al.*, 1987) and decrease of blood glucose (Hikino *et al.*, 1985). From this fungus, immunomodulatory protein, Ling Zhi-8, was also isolated (Kino *et al.*, 1989), its complete amino acid was sequenced (Tanaka *et al.*, 1989), cDNA for Ling Zhi-8 was cloned (Murasugi *et al.*, 1991) and biological activities including prevention of insulitis (Kino *et al.*, 1990) and inhibition of antibody production by Ling Zhi-8 (Kino *et al.*, 1991), were reported. Moreover extracts of *G. lucidum* did not show any conceivable toxicities (Sugiura *et al.*,

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1977; Kim *et al.*, 1986), thus it considered as a very safe edible mushroom.

Anti-encephalitis virus activities of Basidiomycetes have been reported with a virus-like RNA particle of *Lentinus edodes* (Takehara *et al.*, 1979), anti-HIV effects of lentinan sulfate (Hatanaka *et al.*, 1989) and a lignin-glycoprotein complex from *Lentinus edodes* (Shiitake) (Suzuki *et al.*, 1990). Various inhibitors of HIV replication have been proved to be effective clinically for the AIDS patients. However long term use of the anti-HIV drugs has evoked serious side effects such as pancreatitis (Levin *et al.*, 1997) or neurotoxicities (Schmued *et al.*, 1996) by dideoxyinosine (ddI), occurrence of AZT-resistant HIV mutants (Larder *et al.*, 1989) and neutropenia or anemia by nucleoside drugs such as alovudine, zalcitabine, zidovudine, ganciclovir, stavudine, didanosine, lamivudine, and acyclovir (Dornsife and Averett, 1996). Therefore, it is urgent to identify and develop new anti-HIV agents without having adverse effects on the AIDS patients clinically. As various biological active components have been reported from *G. lucidum*, we investigated whether partially purified fractions of this fungus have antiviral activities against human immunodeficiency virus. Here we report anti-HIV activities of this mushroom.

MATERIALS AND METHODS

Fractionation of *G. lucidum*

Artificially grown carpophores (1.7 kg) of *G. lucidum* were minced and extracted with hot water for 5 hrs. The water extract was filtered and concentrated to 1/10 of the original volume, and added with 3 volumes of ice-cold ethanol to precipitate high molecular weight components. The resulting precipitates were dissolved in small volume of distilled water and dialyzed with Spectra/Por membrane (Spectrum, Houston, U.S.A.) for 7 days at 4°C against streaming distilled water to remove contaminated low molecular weight components. The dialysate was freeze-dried to obtain 10.0 g and named HMF. On the other hand, the left solution by removing the precipitates was evaporated and freeze-dried to obtain 50.3 g and named LMF.

Maintenance of human immunodeficiency virus

The human immunodeficiency virus-1 IIIb variant, used in this investigation was maintained in H9 cell line. The H9 cell was cultured in RPMI 1640 containing 10% fetal calf serum and 50 μg/ml gentamicin. The H9 cell was replaced by new batch every two months to maintain consistent condition for virus sensitivity. The medium of the HIV-1 infected H9 cell was replaced once a week. The infected H9 cell was adjusted to a concentration of 0.1×10^6 cells/ml for the initial culture. After three days of the split, 20% of the medium was replace with fresh medium. After five days of the split, uninfected H9 cells were added to the culture to provide cells for virus multiplication. The culture supernatant was harvested by centrifugation at 4,000 rpm for 30 min after 7 day-culture to obtain virus stock solution, which was frozen in liquid nitrogen tank. Virus titer assay was performed by syncytium assay (Nara *et al.*, 1987). Its assay result was expressed as syncytium-forming unit (SFU)/ml. The average titer of the frozen virus was about 3×10^4 SFU/ml.

Anti-HIV activity assay

Target cell for anti-HIV activity was CEM T cell line which grows well in the absence of interleukin-2. The viability of the target cell was assessed by XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide] assay in which yellow XTT formazan produced by the viable cell was measured by optical density at 450 nm (Weislow *et al.*, 1989). The CEM cell was cultured in neutral red free RPMI-1640 medium containing 10% fetal calf serum and 50 μg/ml gentamicin to reduce background color. For anti-HIV assay, 100 μl of HIV-1 infected CEM cells (1,000 cells) in 96-well culture plate were mixed with 100 μl of serially diluted fractions of *G. lucidum* were cultured for 7 days in 5% CO_2 and 37°C incubator. On day 8, each 50 μl of the mixture of XTT tetrazolium (Sigma, St. Louis, U.S.A.) and 20 uM N-methylphenazonium methosulfate (PMS) was added to the culture wells and the plate was further incubated for 4 hrs to develop color. Then the homogenated culture solutions were measured for optical density at 450 nm. The viability (%) was calculated by dividing the optical density at 450 nm of sample-treated cell with optical density of virus-free target cell as shown below:

$$\text{Viability (\%)} = \frac{OD_{450} \text{ of sample treated cell}}{OD_{450} \text{ of virus-free target cell}} \times 100$$

RESULTS

Fractionation of *G. lucidum*

Hot water extraction method of the minced carpophores of *G. lucidum* was employed to obtain water-soluble components from this fungus. Addition of the cold ethanol to the extractive produced white flake-like precipitation. The precipitate was separated by centrifugation after 12 hr at 4°C. HMF from the precipitate was partially purified by dialyzing against distilled water, and LMF was obtained by evaporation and lyophilzing of the ethanol-nonprecipitable solution. From 1.7 kg of the carpophores, 10.0 g (0.58% yield) and 50.3 g (2.95% yield) were obtained as HMF

Table I. Extraction yield of the water-soluble fraction from *Ganoderma lucidum*

Fraction	Weight (g)	Yield (%)
Fruit bodies	1,700.0	-
HMF	10.0	0.58
LMF	50.3	2.95

Carpophores of *G. lucidum* were extracted with hot water for 5 hrs, filtered, concentrated with to 1/10 of the original volume and followed by adding three-volume of ice-cold ethanol to precipitate high molecular weight components. The precipitates were dissolved in small volume of distilled water and dialyzed for 7 days at 4°C against streaming distilled water to remove contaminated low molecular weights. It was finally lyophilized and named HMF. On the other hand, ethanol-nonprecipitable solution was evaporated and freeze-dried and named LMF.

and LMF, respectively (Table I).

Effects of HMF on HIV-infected cell viability

HIV-1 infected CEM cells (1,000 cells/well) were cultured for 7 days in the absence or presence of serially diluted fractions of *G. lucidum*. The experiment was done twice separately in duplicate. When the HMF was added to the target cells in the absence of virus, the viability of the cells were above 84.2% in experiment-1 or 75.2% in experiment-2 when compared with that of the virus uninfected target cell. No dead cells were observed below 62.5 μg/ml concentration in experiment-1, indicating that the HMF of *G. lucidum* did not have any toxicity on the target cells. When we assayed the viability of the target cells in the presence of both HIV-1 virus and various concentrations of the HMF, viable cells were below 10% in almost every concentrations except 62.5 μg/ml concentration in experiment-2 (Table II). This suggests that the high-molecular weight fraction did not have any anti-viral activity.

Effects of LMF on HIV infected cell viability

The LMF of water extract of *G. lucidum* was added to the culture system in the absence or presence of HIV-1. When two-fold serially diluted LMF ranging from 125.00 μg/ml to 0.97 μg/ml was added to the virus-free culture system, no toxicity on the target cells

Table II. Anti-HIV effect of the water-soluble HMF from the fruit bodies of *G. lucidum*.

Concentration (μg/ml)	Experiment-1		Experiment-2	
	OD[a]	Viability[b] (%)	OD	Viability (%)
Virus uninfected[c]				
Target cell alone	1.40	-	1.69	-
0.97	1.37	98.5	1.66	98.2
1.95	1.40	100.6	1.78	105.2
3.90	1.59	113.9	1.75	103.7
7.81	1.38	99.0	1.57	92.9
15.60	1.35	96.8	1.77	104.7
31.20	1.56	111.6	1.72	101.8
62.50	1.41	101.1	1.27	75.2
125.00	1.18	84.2	1.48	87.7
Virus infected				
Target cell alone	1.40±0.12[e]	-	1.69±0.05	-
0.00	0.11±0.02	7.6	0.15±0.04	8.7
0.97	0.12±0.01	8.6	0.10±0.05	6.0
1.95	0.11±0.04	7.7	0.11±0.02	6.2
3.90	0.07±0.01	5.3	0.09±0.01	5.2
7.81	0.06±0.02	3.9	0.12±0.02	6.8
15.60	0.11±0.01	7.8	0.09±0.01	5.3
31.20	0.07±0.03	5.0	0.99±0.01	5.2
62.50	0.06±0.01	4.4	0.18±0.10	10.4
125.00	0.13±0.05	9.3	0.13±0.01	7.8

The CEM cell line, target cell for anti-HIV activity, was cultured in neutral red-free RPMI-1640 medium containing 10% fetal calf serum and 50 μg/ml gentamicin. For anti-HIV assay, 100 μl of HIV-1 infected CEM cells (1,000 cells) in 96-well culture plate were mixed with 100 μl of serially diluted fractions of *G. lucidum* were cultured for 7 days in 5% CO_2 and 37°C incubator. On the eighth-day, each 50 μl mixture of XTT tetrazolium and 20 uM PMS was added to the culture wells and the plate was further incubated for 4 hrs to develop color. Then the homogenated culture solutions were measured optical densities at 450 nm.

[a]OD: optical density at 450 nm

[b]$\text{Viability (\%)} = \frac{OD_{450} \text{ of sample treated cell}}{OD_{450} \text{ of virus-free target cell}} \times 100$

[c]Done in one well

[d]Done in duplicate

[e]Mean±SD

Table III. Anti-HIV effect of the water-soluble LMF from the fruit bodies of *G. lucidum*

Concentration (μg/ml)	Experiment-1		Experiment-2	
	OD[a]	Viability[b] (%)	OD	Viability (%)
Virus uninfected[c]				
Target cell alone	1.51	-	1.52	-
0.97	1.59	105.2	1.75	114.7
1.95	1.64	108.3	1.70	111.5
3.90	1.42	93.8	1.75	114.9
7.81	1.69	111.5	1.77	116.2
15.60	1.56	103.1	1.70	111.5
31.20	1.53	101.4	1.53	100.7
62.50	1.60	106.0	1.74	114.3
125.00	1.56	102.9	1.46	96.2
Virus infected[d]				
Target cell alone	1.51±0.10[e]	-	1.52±0.20	-
0.00	1.14±0.04	9.2	0.14±0.05	9.0
0.97	0.17±0.00	11.0	0.20±0.02	12.9
1.95	0.17±0.01	11.4	0.16±0.00	10.3
3.90	0.17±0.04	11.2	0.35±0.12	23.2
7.81	0.56±0.13	37.2	0.37±0.22	24.3
15.60	1.09±0.00	71.7	1.25±0.44	82.5
31.20	0.65±0.01	43.1	0.90±0.00	58.8
62.50	0.83±0.11	54.7	1.13±0.16	74.0
125.00	0.83±0.02	54.6	0.87±0.00	57.0

See Table II legend for specific experimental procedure.
[a]OD: optical density at 450 nm

[b]Viability (%) = $\frac{OD_{450} \text{ of sample treated cell}}{OD_{450} \text{ of virus-free target cell}} \times 100$

[c]Done in one well
[d]Done in duplicate
[e]Mean±SD

was detected in all the concentrations tested. However, when two-fold serially diluted LMF ranging from 125.00 μg/ml to 0.97 μg/ml was added to the HIV-infected culture system, the viabilities of the target cell were increased with the added LMF at a concentration-dependent manner below 15.60 μg/ml concentration of LMF. Thus the maximal viabilities of the target cell at this concentration was 71.7% and 82.5% in experiments-1 and -2, respectively. The cell viabilities were then gradually decreased above 31.20 μg/ml concentration. More than half of the cells were still viable at 125.00 μg/ml, which was the highest concentration tested in this system (Table III).

DISCUSSION

In this experiment, we demonstrated anti-HIV activities of the hot water-extracted fractions of *G. lucidum*. The low molecular weight, not the high molecular weight, fraction of this fungus showed strong anti-HIV activities as measured by XTT assay. The microtiter XTT assay used in this experiment led to quantitative HIV infected cytopathic assay with safety and convenience. In order to prevent medium color due to the ingredient of neutral red, we used neutral red-free medium for optical density measurement.

As most of the mushrooms of Polyporaceae including *G. lucidum* have very hard carpophores, the extraction yields were very low in our experiment, yielding 0.58% and 2.95% as HMF and LMF, respectively. Ethanol or acetone is frequently used to precipitate high molecular weight components from the water extracted solution. Therefore the major components of HMF might be polysaccharides, proteins and DNA/RNA. Among them proteins could be denatured by the added ethanol possibly losing their biological activities and none of the DNA/RNA itself could exert biological activity except virus. Accordingly major biological activities of HMF might be due to the polysaccharides. The polysaccharides are not denatured by ethanol or acetone and maintain their three dimensional structures. Many of the biologically active polysaccharides were isolated by ethanol precipitation method (Kim *et al.*, 1993, Sone *et al.*, 1985). Many of the antitumor β-(1→3)-D-glucans isolated by organic solvent precipitation were reported and only glucans having right-triple stranded helixes have antitumor activities, but glucan having a right-single stranded helix does not show any biological activities, indicating that addition of organic solvent to the glucan may not

modify its three-dimensional structure (Mizuno, 1983).

The HMF of *G. lucidum* did not damage the target cell in virus-free cell culture in most of the concentrations tested except the highest one, 125.00 μg/ml as shown in Table II, indicating that HMF does not have any toxicity to the cell line used. To prevent any residual organic solvent in the HMF and LMF, they were repeatedly dissolved in distilled water and lyophilized. The HMF did not lead to the recovery of the cell viability in the virus-infected target cell culture system in all the concentrations tested. Therefore no anti-HIV activity was observed in the high-molecular-weight fraction of *G. lucidum.*

The water-soluble low-molecular-weight fraction of this fungus did not show any toxicity on the virus-free target cell culture in all the concentrations tested. The viability of 96.2% at 125.0 μg/ml in experiment-2 does not seem to be significant since the viability in experiment-1 at that concentration was 102.9% viability (Table III). The target cell viabilities began to increase steeply from 7.81 μg/ml concentration of LMF by recovering viability from 11.2% at 3.90 μg/ml to 37.2% at 7.81 μg/ml concentration in experiment-1. However, in experiment-2, the recovery of the cell viability occurred at 3.90 μg/ml, which was lower concentration than that of experiment-1. The maximal recovery of the viability in HIV-1 infected cell was observed at 15.6 μg/ml in experiments-1 and -2. The cell viabilities over that concentration decreased but remained between 43.1% and 74.0%. Despite of the no toxicities in the virus-free target cells, the LMF failed recovery of the cell over 31.2 μg/ml concentration. This might be due to the presence of interfering components in LMF, since it was the total low-molecular-weight extractive of *G. lucidum.* In order to express quantitative indices of the fractions, we employed IC_{50} (50% inhibitory concentration representing 50% cytotoxicity in HIV-free target cell, μg/ml), EC50 (50% effective concentration representing 50% protection from HIV infection, μg/ml) and TI (therapeutic index, IC_{50}/EC_{50}) from the computer plotted graphics by using the data of Table II and Table III (Weislow *et al.*, 1989).

Table IV. Quantitative anti-HIV activity of the water-soluble fractions of *G. lucidum*

Fraction	Experiment-1			Experiment-2		
	IC_{50}[a] (μg/ml)	EC_{50}[b] (μg/ml)	TIc	IC_{50} (μg/ml)	EC_{50} (μg/ml)	TI
HMF	>125.0	>125.0	-	>125.0	>125.0	-
LMF	>125.0	11.0	>11.3	>125.0	11.2	>11.1

[a]IC_{50}: 50% Inhibitory concentration representing 50% cytotoxicity in HIV-free target cell

[b]EC_{50}: 50% Effective concentration representing 50% protection from HIV infection

[c]TI: Therapeutic index (IC_{50}/EC_{50})

The IC_{50} values of HMF and LMF were higher than 125.0 μg/ml indicating no toxicity on the target cells. The values of EC_{50} of the HMF and LMF represented over 125.0 μg/ml and 11.0 μg/ml, respectively, indicating that only LMF protected from HIV infection (Table IV). These indices might be convenient in case of comparison of the activities with large number of samples. The TI value (over 11.3) of LMF seems to be much smaller than that of AZT (1,027) (Weislow *et al.*, 1989). Considering most of the known compounds of the *Ganoderma lucidum* belong to triterpenoids whose average molecular weight is approximately 500, the TI value of LMF is not too small. By using the average molecular weight, EC_{50} of LMF is 22 uM. When we compare EC_{50} value of LMF (22 uM) with those of AZT (0.186 uM) (Weislow *et al.*, 1989), 3-allyl-AZT (0.9 uM) (Kitade *et al.*, 1992) or gleditsia triterpenoid (1.1 uM) (Konoshima *et al.*, 1995), our EC_{50} of LMF is comparable to those compounds since our LMF is total extract of the mushroom. The fact that the absence of toxicities both in HMF and LMF on our T cell culture has great significance for public health, since this mushroom has been consumed as folk medicine for curing several diseases in the Orient including China, Korea, Taiwan, Malaysia and Japan (Mizuno *et al.*, 1996) and its non-toxicity is coincidence with our previous animal experiment (Kim *et al.*, 1986). In our experiment, we used total extracts for the cytopathic assay by using HIV-1. Antiviral activities of total extract from natural product frequently cause false-positive due to the presence of interfering substances such as tannin (Tan *et al.*, 1991). However, as our LMF is a low molecular fraction, tannins that belong to high molecular compounds could not be contained in the fraction. Fractionation and purification of LMF are in actively progress to identify the anti-HIV-1 agents by using cytopathic assay.

Anti-encephalitis virus activities of Basidiomycetes have been reported from virus-like RNA particles of *Lentinus edodes* (Takehara *et al.*, 1979). The water-soluble lignin from the submerge-cultured mycelia from this mushroom showed anti-cytopathic effect of HIV (Suzuki *et al.*, 1989 and 1990). Unmodified lentinan, (1→3)-D-glucan, from *L. edodes* had no anti-HIV activity, but interestingly heavily sulfated lentinan effectively prevented HIV-induced cytopathic effect (Hatanaka *et al.*, 1989). Therefore it is necessary to perform anti-HIV activity by sulfating our polysaccharide of HMF. The finding of our anti-HIV activity of LMF from *G. lucidum* is the first report as far as we know, and currently purification of the LMF is in progress.

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