Natural Product Sciences
5(2) : 107~111 (1999)

Anti-Varicella Zoster Virus Activity of Water Soluble Substance from *Elfvingia applanata* Alone and in Combinations with Acyclovir and Vidarabine

Soo-Dong Kim, Seong-Kug Eo, Young-So Kim and Seong-Sun Han*

College of Pharmacy, Chungbuk National University, Cheongju 361-763, Korea

Abstract – To investigate less toxic antiviral agents from Basidiomycetes, EA, the water soluble substance, was isolated from the carpophores of *Elfvingia applanata* (pers.) Karst. Anti-varicella zoster virus (Oka strain; anti-VZV/Oka) activity of EA was examined in MRC-5 cells by plaque reduction assay *in vitro*. And the combined antiviral effects of EA with nucleoside anti-VZV agents, acyclovir and vidarabine, were examined on the multiplication of VZV/Oka. EA exhibited a concentration-dependent reduction in the plaque formation of VZV/Oka with a 50% effective concentration (EC_{50}) of 464.14 μg/ml. The results of combination assay were evaluated by the combination index (CI) that was calculated by the multiple drug effect analysis. The combination of EA with acyclovir showed more potent synergism with CI values of 0.18~0.62 for 50~90% effective levels than that of EA with vidarabine with CI values of 0.67~1.04.

Key words – *Elfvingia applanata*, varicella zoster virus (Oka strain; VZV/Oka), acyclovir, vidarabine, combination index (CI).

Introduction

The carpophores of *Elfvingia applanata* (Pers.) Karst. (Polyporaceae) in Basidiomycetes have been used to treat various ailments including cancers in oriental folk medicine, as have been the carpophores of *Ganoderma lucidum* (Kim and Kim, 1990). *E. applanata* was reported to contain some biologically active components such as bitter triterpenoid (Nishitoba *et al.*, 1988), alusenone and friedelin (Protiva *et al.*, 1980), α-D-glucan and β-D-glucan (Mizuno *et al.*, 1981; Usui *et al.*, 1983). Recently, FDP isolated from *E. applanata* was reported to modulate humoral immune response (Kim *et al.*, 1994a), and the aqueous extract was also reported to show the antibacterial and antiviral activities on pathogenic microorganism (Kim *et al.*, 1994b; Rym *et al.*, 1999), and not to display any toxicity in the acute toxicity test (Kim *et al.*, 1994c).

Varicella zoster virus (Oka strain; VZV/Oka), which belongs to herpesvirus, causes chickenfox in childhood, and a latent virus, on reactivation it causes shingles. It is characterized by latent and prolonged infection, and has become a problem with AIDS patients (Lapucci *et al.*, 1993). Nucleoside anti-VZV agent such as acyclovir (ACV) is a well-known drug which exhibits selective toxicity against infected cells by phosphorylating virus-derived thymidine kinase. However, prolonged therapies with acyclovir, the most successful antiviral drug, have resulted in some undesirable complications (Richman *et al.*, 1987) and also induced the emergence of drug-resistant viruses (Larder *et al.*, 1989). Therefore, it is necessary to develop new anti-VZV agents, and combination therapy with currently available drugs is attractive.

In this study, we investigated the anti-VZV/Oka activity of EA, the water soluble substance isolated from the carpophores of *E. applanata*, and combined anti-VZV effect of EA with nucleoside anti-VZV agents, ACV and vidarabine (ara-A).

Experimental

Material – The carpophores *Elfvingia applanata* (Pers.) Karst. (Polyporaceae) were purchased from a local herbal drug store and authenticated by Dr. Wan Hee Park, Seoul National Industrial University. A voucher specimen (No. CPM 319) has been deposited at the Medicinal Plants Herbarium of our college.

Cells and virus – HEL299 cell (Lung, embryonic, Human diploid), ATCC CCL 137), MRC-5 cell (Lung, Human diploid) ATCC CCL 171, varicella

*Author for correspondence.

zoster virus (Oka strain; VZV/Oka) ATCC VR 795 were obtained from the American Type Culture Collection (Rockville, USA). HEL299 and MRC-5 cells were cultured with DMEM supplemented with 10%(v/v) heat inactivated fetal bovine serum (FBS), 100 IU/ml penicillin, 100 μg/ml of streptomycin and 0.25 μg/ml of amphotericin B. The cell associated VZV/Oka was prepared from infected confluent HEL299 cells at an input multiplicity of infection (MOI) of 0.01 pfu/cell. The infected HEL299 cells were incubated for 3-4 days until 70~80% of cells were cytopathic by viruses, dissociated by trypsin-EDTA solution, collected by centrifugation, suspended in FBS containing 10% dimethyl sulfoxide (TEDIA, Fairfield, OH, USA), and eliquots of cell associated virus were stored at −70°C.

Isolation of water soluble substance – The carpophores of *E. applanata* (500 g) were extracted with hot water for 8 h. The extract was concentrated, lyophilized, and EA (21.1 g) as a dark brownish substance was obtained.

Cytotoxicity assay – For cytotoxicity assay, the cells were seeded in 96 well plates (Falcon, NJ, USA) at an initial density of 3.5×10^4 cells per well. After incubation of the cells for 16-18 h at 37°C, various concentrations of EA were added, and the incubation was continued for 48 h. Viable cell yield was determined by MTT reduction assay according to reported procedure (Scubiero *et al.*, 1988). The cytotoxicity was expressed as the 50% cytotoxic concentration (CC_{50}) which is the concentration of substances to inhibit the growth of cells up to 50% by regression analysis.

Anti-VZV activity assay – Anti-VZV/Oka activity was evaluated by plaque reduction assay (Hondo *et al.*, 1992). Host cell monolayers grown in 6 well plates (Falcon, NJ, USA) were infected with about 150 pfu of cell associated VZV/Oka per well. After 1 h adsorption, agar overlay medium containing EA at various concentrations was overlaid. After incubation at 37°C until plaques formed, virus plaques were counted. The degree of inhibition was expressed as the 50% effective concentration (EC_{50}) which was calculated as the concentration of EA required to reduce virus plaque by 50% using regression analysis. Anti-VZV/Oka activity for EA was evaluated by selectivity index (SI) which was calculated by dividing the CC_{50} by EC_{50}. Acyclovir (ACV, Sigma, USA) and vidarabine (ara-A, Sigma, USA) are clinically used for the treatment of VZV diseases. These drugs were used as a positive control under this assay system.

Combination assay – The combination assay was essentially performed according to the published method (Schinazi *et al.*, 1982) with some modifications. Confluent MRC-5 cells in 6 well culture plates were infected with about 150 pfu of cell associated VZV/Oka per well. After 1 h adsorption of virus, EA at concentrations of 80~2,400 μg/ml, and anti-VZV agents, ACV and ara-A, at concentrations of 0.08~2.40 μg/ml alone, and a constant ratio (1000:1) mixture of EA and anti-VZV agents were overlaid. The above plates were incubated at 37°C until the formation of plaques. The formed virus plaques were counted.

Calculation and analysis of drug interaction – For the determination of synergistic or antagonistic drug interactions using the multiple drug effect analysis procedure (Chou and Talalay, 1984), data were expressed as the fraction affected relative to the untreated control cultures. Multiple drug effect analysis involves plotting the results obtained for each drug alone, or when combined at multiple fixed-ratio drug concentrations, in the form of a dose-effect curve defined by the median effect equation $f(a) / f(u) = (C/C_m)^m$. The f(a) and f(u) are the fractions affected and unaffected, respectively, by the concentration C. C_m is the concentration required to produce the median effect (i.e. 50% effective concentration), and m represents the sigmoidicity of the curve. If the slopes (m) of the dose-effect curves for each drug alone and in combination are all parallel, such drugs are said to be mutually exclusive (similar mode of action). Conversely, a mutually nonexclusive case (different mode of action) is defined by parallel gradient for each drug alone, and with the gradient for the drug combinations being non-parallel. A combination index (CI) can be calculated for either mutually exclusive or nonexclusive assumptions. The CI values less than 1.0 indicate synergism, CI values greater than 1.0 represent antagonism, and CI values equal to 1.0 indicate additive effect.

For the analysis of combinations of EA with ACV and ara-A by multiple drug effect analysis, only data with high linear correlation coefficients ($r>0.8$) as determined by the median effect plots were used in this analysis.

Statistical analysis – Statistical significance of the differences between control and treated groups was calculated using Student's *t*-test. $P<0.05$ was considered to be significant.

Results and Discussion

In an attempt to find anti-VZV substances to reduce the adverse side effects of associated with long term therapy and limit the emergence of resistant virus, water soluble substance, EA, was isolated from the carpophores of *E. applanata*. Antiviral activity of EA against VZV/Oka was evaluated in MRC-5 cells by plaque reduction assay. The inhibitory effects of EA on plaque formation of VZV in MRC-5 cells are shown in Table 1. EA inhibited plaque formation of VZV at its EC_{50} of 464.14 μg/ml, and had the CC_{50} on MRC-5 cells at a concentration of 5,874.43 μg/ml. Therefore, EA exhibited anti-VZV activity with SI of 12.66. *E. applanata* in Basidiomycetes was reported to contain some biologically active components such as polysaccharides, proteins and nucleoside analogue substances (Mizuno *et al.*, 1981; Usui *et al.*, 1983; Kim *et al.*, 1994a), and nucleic acid isolated from *E. applanata* showed to induce the secretion of interferon-like substances in spleen cell of mice (Kandefer *et al.*, 1979). Therefore, these water soluble components contained in EA may be responsible for inhibition of the replication of VZV/Oka, although it remains to be clarified.

The various reasons for combination chemotherapy for viral infections include synergy of antiviral effects, antagonism of toxicities, distribution of toxicities among organ systems, prevention of emergence of resistant variants, enhancement of immune functions (Schinazi, 1991). To determine whether any antiviral synergism between EA and nucleoside anti-VZV agents, combined effects of EA with ACV or ara-A were examined on the plaque formation of VZV/Oka in MRC-5 cells. Combinations of EA with ACV or ara-A showed a potent inhibition on the plaque formation of VZV/Oka than when tested for individual drugs (Fig. 1, 2). Analysis of combinations of EA with ACV or ara-A by multiple drug effect analysis showed the following parameters : for EA, m = 1.14, r = 0.98; for ACV, m = 0.70, r = 0.92; for ara-A, m = 0.63, r = 0.98. EA showed the inhibitory effect on VZV at its EC_{50} of 672.69 μg/ml, and when combined, the parameters appeared with m = 1.58 and r = 0.92 for ACV, and m = 1.17 and r = 0.90 for ara-A (Table 2). Since the slopes (m) of the dose-effect curves for each drug alone and in combinations were not parallel to each other, the exclusivity of the combined effects could not be established. Therefore, the CI values were calculated under both mutually exclusive and mutually nonexclusive assumptions. The CI values of EA with ACV or ara-A on VZV/Oka for 50%, 70% and 90% effect levels were given

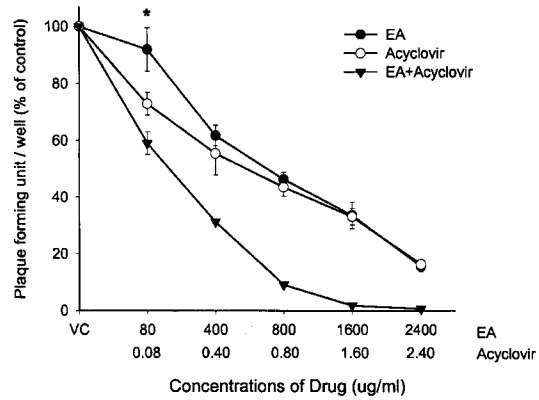

Fig. 1. Inhibitory effects of plaque formation of varicella zoster virus (Oka strain) in MRC-5 cells by EA, acyclovir, and their combination at a fixed ratio of 1000:1. Results are expressed as percent with respect to virus control (VC) group. Each value is the mean±S.D. of quadruplicate determinations. *Not significantly effective than VC; $P>0.05$ (Student t test)

Table 1. Antiviral activity of EA[a], acyclovir and vidarabine on varicella zoster virus (Oka strain) in MRC-5 cells by plaque reduction assay.

Antiviral substances	CC_{50}[b] (mg/ml)	EC[c] (mg/ml)			SI[d]		
		EC_{50}	EC_{70}	EC_{90}	SI_{50}	SI_{70}	SI_{90}
EA	5,874.43	464.14	1,274.21	3,499.28	12.66	4.61	1.68
Acyclovir		0.36	1.04	3.03			
Vidarabine		1.71	7.95	36.01			

[a]Water soluble substance isolated from the carpophores of *Elfvingia applanata*.
[b]50% cytotoxicity concentration (CC_{50}) is the concentration of the 50% cytotoxic effect.
[c]Effective concentration (EC) is the concentration of antiviral substance required to reduce plaque formation of virus by 50%, 70%, and 90%.
[d]Selectivity Index (SI) = CC_{50} / EC_{50}.
The values represent the means of quadraplicate experiments.

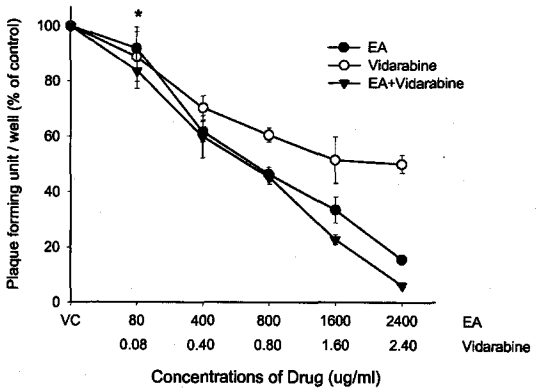

Fig. 2. Inhibitory effects of plaque formation of varicella zoster virus (Oka strain) in MRC-5 cells by EA, vidarabine, and their combination at a fixed ratio of 1000:1. Results are expressed as percent with respect to virus control (VC) group. Each value is the mean±S.D. of quadruplicate determinations. *Not significantly effective than VC; $P>0.05$ (Student t test)

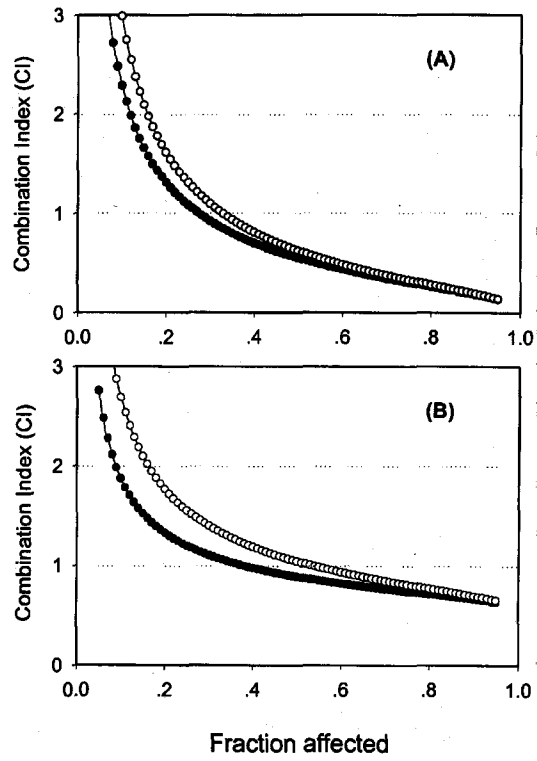

Fig. 3. CI values for combinations of EA with IFN alpha (A) or IFN gamma (B) on varicella zoster virus (Oka strain) corresponding to fraction affected ranging from 0.05 to 0.95. CI values were determined under both mutually exclusive (●) and mutually nonexclusive (○) assumptions.

in Table II, and CI values for combinations of EA with ACV or ara-A on VZV in MRC-5 cells corresponding to fraction affected are shown in Fig. 3. CI values were in the range of 0.18~0.62 for a combination of EA with ACV, and in the range of 0.67~1.04 for a combination of EA with ara-A.

In conclusion, EA was reported to have no toxicity when administered *in vivo* (Kim *et al.*, 1994c), and the combinations of EA with ACV or ara-A showed synergistic or additive effect on the replication of VZV/Oka in this study. This discovery in Basidiomycetes is the first report as far as we know. Based on these studies, EA may be able to use both as a potential anti-VZV agent and combination therapy agents that can permit a significant reduction in the dosage of the toxic anti-VZV agents without compromising antiviral activity.

Table 2. Median effective concentrations and CI values of combinations of EA[a] with acyclovir and vidarabine on the plaque formation of varicella zoster virus (Oka strain) in MRC-5 cells.

Antiviral substances	Parameters[b]			CI at f(a) of :[c]		
	m	EC_{50} (μg/ml)	r	0.50	0.70	0.90
EA	1.14	672.69	0.98			
Acyclovir	0.70	0.42	0.92			
Vidarabine	0.63	1.87	0.98			
EA/Acyclovir (1000:1)	1.58	143.21	0.92	0.55(0.62)	0.35(0.38)	0.18(0.19)
EA/Vidarabine (1000:1)	1.17	439.76	0.90	0.89(1.04)	0.77(0.85)	0.67(0.70)

[a]Water soluble substance isolated from the carpophores of *Elfvingia applanata.*

[b]m is the slope, EC_{50} is the median effective concentration, and r is the correlation coefficient as determined from the median-effect plot.

[c]CI<1, synergism; CI = 1, additive effect; CI>1, antagonism. f(a) is a component of the median-effect equation referring to the fraction of the system affected (e.g., 0.5 means the CI at a 50% reduction of activity). CI values were determined under both mutually exclusive and mutually nonexclusive(numbers in parentheses) assumptions.

The values represent the means of quadraplicate experiments.

References

Chou, T. C. and Talalay, P., Quantitative analysis of dose-effect relationships; The Combined effects of multiple drugs or enzyme inhibitors. *Adv. Enzyme Regul.* **22**, 27-55 (1984).

Hondo, R., Shibuta, H. and Matumoto, M. An improved plaque-assay for varicella virus. *Arch. Virology.* **51**, 355-359 (1976).

Kandefer, S. M., Kawecki, Z. and Guz, M., Fungal nucleic acids as interferon inducers. *Acta Microbiol. Pol.* **28**, 277-291 (1979).

Kim, S. S. and Kim, Y. S., *Korean Mushrooms*, Yupoong Publishing Co, Seoul, 1990, pp. 300-301.

Kim, Y. S., Ryu, K. H., Mo, Y. K., Lee, C. K. and Han, S. S., Effects of the antitumor component, F-D-P, isolated from *Elfvingia applanata* on the immune response. *Kor. J. Pharmacog.* **25**, 348-355 (1994a).

Kim, Y. S., Rym, K. H., Lee, C. K. and Han, S. S., Antimicrobial activity of *Elfvingia applanata* extract alone and in combination with some antibiotics. *Yakhak hoeiji* **38**, 742-748 (1994b).

Kim, Y. S., Kang, J.K., Lee, C. K. and Han, S. S., Effect of *Elfvingia applanata* extract on the acute toxicity in mice. *Yakhak Hoeji* **38**, 756-762 (1994c).

Lapucci, A., Macchia, M. and Parkin, A., Antiherpes virus agents: A review. *Il Farmaco*, **48**(7), 871-895 (1993).

Larder, B. A., Darby, G. and Richman, D. D., HIV with reduced sensitivity to zidovudine (AZT) isolated during prolonged therapy. *Science* **243**, 1731-1734 (1989).

Mizuno, T., Hayashi, K., Arakawa, M., Shinkai, K., Shimizu, M. and Tanaka, M., Host-mediated antitumor polysaccharides. III. Fractionation, chemical structure, and antitumor activity of water-soluble homoglucans isolated from kofukisarno koshikske, the fruit body of *Ganoderma applanatum*. *Shizuoka Daigaku Nogakubu Kenkyu Hokoku* **31**, 65-82 (1981).

Nishitoba, T., Goto,S., Sato, H. and Sakamura, S., Bitter triterpenoids from the fungus *Ganoderma applanatum*. *Phytochemistry* **28**, 193-197 (1988).

Protiva, J., Skorkovska, H., Urban, J. and Vystrcil, A., Triterpenes LXIII. Triterpenes and steroids from *Ganoderma applanatum*. *Collect. Czech Chem. Commun.* **45**, 2710-2713 (1980).

Richman, D. D. Fischi, M. A., Grieco, M. H., The toxicity of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex. *N. Engl. Med.* **317**, 192-197 (1987).

Rym, K. H., Eo, S. K., Kim, Y. S., Lee, C. K. and Han, S.S., Antiviral activity of water soluble substance from *Elfvingia applanata*. *Kor. J. Pharmacogn.* **30**(1), 25-33 (1999).

Scubiero, D. A., Shoemaker, R. H. and Paull, K. D., Monks, A., Tierney, S., Nofziger, T. H., Currens, M.J., Seniff, D. and Boyd, M.R., Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in cutlure using human and other tumor cell lines. *Cancer Res.* **48**, 4827-4833 (1988).

Schnazi, R. F., Combined therapeutic modalities for viruses-rationale and clinical potential, in Chou, T. C. and Rideout, D. C. (eds.), *Molecular mechanism of chemotherapeutic synergism, potentiation and antagonism*, Academic Press, Orlando, FL., 1991, pp. 110-181.

Schinazi, R. F., Peters, J., Williams, C. C., Chance, D. and Nahmias, A. J., Effect of combinations of acyclovir with vidarabine or its 5'-monophosphate on herpes simplex viruses in cell culture and in mice. *Antimicrob. Agents Chemother.* **22**(3), 499-507 (1982).

Usui, T., Iwasaki, Y., Mizuno, T., Tanaka, M., Shinkai, K. and Arakawa, M., Isolation and characterization of antitumor active β-D-glucans from the fruit bodies of *Ganoderma applanatum*. *Carbohydr. Res.* **115**, 273-280 (1983).

(Accepted June 21, 1999)