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Antiviral traditional medicines against herpes simplex virus (HSV-1), poliovirus, and measles virus in vitro and their therapeutic efficacies for HSV-1 infection in mice

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Summary

One hundred forty-two kinds of traditional medicines, which have been historically used in China, Indonesia, and Japan, were examined for the antiviral activity of their hot water (HW) extracts against herpes simplex virus type 1 (HSV-1), poliovirus type 1, and measles virus by plaque reduction assay. Thirty-two, 55, and 30 HW-extracts of them showed anti-HSV-1, anti-poliovirus, and anti-measles virus activities, respectively. Among the 32 HW-extracts with anti-HSV-1 activity, 3 HW-extracts had anti-HSV-1 activity alone and the others showed anti-HSV-1 activity with anti-poliovirus and/or anti-measles virus activities. The 32 HW-extracts were further examined for their therapeutic efficacies of HSV-1 infection in mice. The mice were infected cutaneously with HSV-1 and HW-extracts were orally administered three times daily. Twelve HW-extracts, currently used for the treatment of various diseases other than viral infection, were found to be significantly effective in limiting the development of skin lesions and/or in prolonging the mean survival times of HSV-1-infected mice. These results suggested that 12 of 142 HW-extracts that exhibited therapeutic efficacy in an animal infection model were possible candidates for anti-HSV-1 traditional medicine.

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Introduction

Traditional medicines utilizing natural products have been shown to contain antiviral compounds in vitro (Amoros et al., 1987; Chang and Yeung, 1988; Fukuchi et al., 1989; Hayashi et al., 1992; Hudson, 1989; Ito et al., 1987; Okada and Kim, 1972; Sydislis et al., 1991; Tabba et al., 1989; Tang et al., 1990; Yamamoto et al., 1989; Yao et al., 1992). Plants have their own metabolism of nucleic acids, proteins etc. Some of them might recognize the differences between viral and host metabolism resulting in antiviral activity; for example, some component (antibiotics) of fungus recognizes the differences of metabolism between human and bacteria and has been used for antibacterial chemotherapy. Thus the traditional medicines appear to be useful sources to search new antiviral agents (Herrmann, 1961; Shihman Chang and Yeung, 1988).

Traditional medicines in the form of hot water (HW) extracts have been used orally for the treatment of various diseases. Therefore we hypothesized that some of HW-extracts would exhibit direct antiviral activity in vitro and in vivo at the concentration used for therapy. In this study, we assayed typical and easily available 142 traditional medicines which are currently used for the treatment of various diseases in China, Indonesia, and Japan. Those traditional medicines can be also easily obtained as a source for antiviral medicines. We first examined possible antiviral activities and specificities of their HW-extracts for herpes simplex virus type 1 (HSV-1) by plaque reduction assay at their putative concentrations absorbed from alimentary tracts. Then we evaluated their therapeutic efficacies in an animal infection model. Twelve of 142 HW-extracts were found to show anti-HSV-1 therapeutic activity in vivo.

Materials and Methods

Viruses and cells

HSV-1 [Seibert strain (Shiraki et al., 1991) or 7401H strain (Kumano et al., 1987) provided from Dr. R. Mori, Kyushu University, Japan], poliovirus type 1 (Sabin strain), and measles virus (Tanabe strain) were propagated in Vero E6 cells. The infected cultures were frozen and thawed three times, and centrifuged at 3000 rpm for 15 min. Their supernatants were stored at $-80^{\circ}C$ until use (Shiraki and Rapp, 1988). Vero cells were grown and maintained in Eagle's minimal essential medium (MEM) supplemented with 5% and 2% calf serum, respectively.

Preparation of HW-extracts

We used typical and easily available traditional medicines which have been orally administered for the treatment of various diseases. These traditional medicines have been authenticated and stocked in Research Institute for Wakan-Yaku (Traditional Sino-Japanese Medicines), Toyama Medical and Pharmaceutical University, Japan and some of them were purchased in China and Indonesia.

HW-extracts were prepared from dried traditional medicines according to the standard methods with minor modification as reported by Tabba et al. (1989), Shihman Chang and Yeung (1988), and Yao et al. (1992). Dried traditional medicines (100 g) were boiled under reflux in 1500 ml of distilled water for 3 h. The aqueous extract was filtered (No. 2, Toyo Roshi Co., Ltd., Japan), concentrated under reduced pressure, and lyophilized. The lyophilized materials were suspended in distilled water at concentrations as indicated in the text. The suspension was boiled for 10 min and centrifuged at 3000 rpm for 15 min. The sterilized supernatant (HW-extract) was used for the following assays.

Acyclovir

Acyclovir (ACV) was purchased as tablets from Nippon Wellcome K. K. A tablet (200 mg) was powdered and suspended in distilled water.

Plaque reduction assay

Duplicate cultures of Vero cells in 60 mm plastic dishes were infected with 100 plaque forming units (PFU)/0.2 ml of HSV-1 (Seibert strain), poliovirus, or measles virus for 1 h. Then the cells were overlaid with 5 ml of nutrient methylcellulose (0.8%) medium containing 100 and 300 or 500 μg/ml of HW-extracts. The HSV-1-, poliovirus-, and measles virus-infected cultures were incubated for 2-days, 3-days and 5-days at 37°C, respectively. The infected cells were fixed and stained, and the number of plaques was counted (Shiraki et al., 1991).

Cytotoxicity of HW-extracts was evaluated by the extent of omission of uninfected cells from the surface of stained dishes in plaque reduction assay (visible cytotoxicity). Strong (++), intermediate (+), and weak (±) cytotoxicity were scored as omission of more than 50%, 50–10%, and less than 10%, respectively, of uninfected cells as compared with untreated-dishes (controls) as shown in Table 1.

Cytotoxicity of HW-extracts was also monitored by measuring their effects on the growth of Vero cells and on the incorporation of [*methyl*-^{3}H]thymidine into the cellular DNA according to the methods with minor modification as reported by Osterhaus et al. (1984), Palu et al. (1986), and Snoeck et al. (1991). Vero cells were seeded at a concentration of 2.5×10^4 cells/well in 24-well plates and grown at 37°C for 2 days. The culture medium was replaced to fresh medium containing 100 μg/ml of HW-extracts and then cells were further grown for 2 days. The monolayer cells were treated with trypsin and the cell number was determined by Trypan blue exclusion test.

TABLE 1

Antiviral assay of hot water extracts by plaque reduction assay

Traditional medicines	Used part	Plaque formation (%)[a]						Cytotoxicity				
		100 μg/ml			300 or 500 μg/ml			100 μg/ml			300 or 500 μg/ml	
		HSV	Polio	Measles	HSV	Polio	Measles	Visible[b]	DNA[c] (%)	Growth[d] (%)	Visible	DNA (%)
Ainsliaea fragrans Champ.	whole plant	55.7	94.2	87.0	51.7	84.4	88.6	-			-	
Alisma plantago-aquatica L. var. *orientale* Samuelsson	rhizome	98.2	79.9	83.8	81.7	72.7	105.1	-			-[e]	
Allolofophora caliginosa trapezoides Ant. Druges	whole body	82.6	86.9	91.9	79.2	93.3	61.1	-			-	
Alocasia odora K. Koch	rhizome	96.8	89.1	64.7	88.1	81.4	90.5	-			-	
Alpinia officinarum Hance[f,g]	rhizome	106.8	58.9	107.7	0.0[h]	11.5	90.2	-	73.5	87.6	±	62.2
Alyxia stellata Roem.	bark	59.8	74.6	87.0	57.9	75.4	95.3	-			-	
Andrographis paniculata Nees	bark	61.7	88.1	87.9	63.6	55.1	105.1	-			-	
Anemarrhena asphodeloides Bunge[g]	rhizome	84.1	44.2	89.1	95.2	30.1	94.1	±			±[e]	
Angelica acutiloba Kitagawa var. *sugiyame* Hikino	root	108.5	63.5	123.5	116.9	66.7	307.8	-			±	
Arctostaphylos uva-ursi (L.) Sprengel	leaf	111.0	63.3	110.6	74.0	54.6	67.1	-			±	
Areca catechu L.[f,g,i]	seed	0.0	86.5	93.6	0.0	0.0	0.0	±	64.7	91.2	+[e]	52.8
Aristolochia contorta Bge.	fruit	88.8	108.8	88.1	99.1	120.2	67.6	-			-	
Artemisia capillaris Thunb.	seedling	79.1	71.8	97.5	59.7	52.5	91.3	-			-	
Artemisia princeps Pamp.[f,g,i]	leaf	65.0	84.3	10.8	0.0	40.5	13.6	-	47.8	84.7	-[e]	47.5
Asiasarum heterotropoides F. Maekawa var. *seoulense* F. Maekawa[g]	whole plant	95.1	73.2	104.3	98.6	43.0	118.1	-			-[e]	
Aster tataricus L. f.	root	109.8	109.2	84.9	83.3	99.5	105.9	-			-	
Astragalus membranaceus (Fisch.) Bge.	root	86.8	88.4	74.9	81.3	92.4	82.9	-			-	
Atractylodes lancea (Thunb.) DC.	rhizome	74.7	83.7	87.5	106.6	82.7	99.7	-			-	
Atractylodes ovata DC.	rhizome	91.5	104.9	92.2	91.5	104.6	88.2	-			-[e]	
Aucklandia lappa Dcne.	root	103.4	77.6	86.5	100.0	63.8	83.7	-			-	
Belamcanda chinensis (L.) DC.[i]	rhizome	110.5	97.4	100.0	86.3	55.1	0.0	±			±[e]	
Bletilla striata (Thunb.) Reichb. fil.	rhizome	123.7	109.9	89.2	98.3	77.7	105.9	-			-[e]	
Brainia insignis (Hook.) J.Sm.[f,i]	rhizome	62.7	61.0	109.7	0.0	54.6	5.1	-	87.3	90.7	±	73.8
Brucea javanica (L.) Merr[f,g,i]	seed	6.3	0.0	37.2	0.0	0.0	0.0	±	9.7	45.0	±	4.5
Buthus martensi Karsch	whole body	104.7	81.4	83.2	84.4	85.5	84.8	-			-	
Caesalpinia sappan L.[f,g,i]	bark	0.0	0.0	0.0	0.0	0.0	0.0	+	2.8	11.1	+ +	0.0
Camellia japonica L.	leaf	89.7	85.3	88.2	54.8	59.0	102.3	-			-	
Cannabis sativa L.	fruit	91.2	100.5	87.8	82.4	73.4	93.7	-			-	
Cassia fistula L.[f,g,i]	bark	67.0	52.1	100.0	0.0	2.8	14.1	±	6.7	76.5	±	3.6
Chrysanthemum morifolium Ramat.	capitulum	92.1	98.2	98.2	86.8	81.7	68.7	-			-[e]	
Cimicifuga heracleifolia Komarov	rhizome	84.1	84.6	86.9	79.4	82.1	103.6	-			-	

Cinnamomum sintok Blume[f,g,i]	bark	0.0	102.5	153.0	0.0	0.0	0.0	±	71.9	109.3	+	47.4
Citrus unshiu Marc.	fruit peel	79.1	103.5	110.0	62.8	77.7	106.8	-			-	
Clematis chinensis Osbeck	root	99.2	98.7	76.9	100.0	64.1	83.3	-			-	
Cnidium monnieri (L.) Cuss.	fruit	100.0	78.9	100.0	85.8	81.7	100.0	-			-	
Coix lacryma-jobi L. var. *ma-yuen* Stapf	seed	74.5	93.6	77.1	98.0	100.5	71.2	-			-	
Coptis chinensis Franch.[f,g,i]	rhizome	74.1	89.7	5.6	0.0	0.0	0.0	±	52.3	65.8	+[e]	22.3
Cornus officinalis Sieb. et Zucc.	fruit	102.1	82.2	100.0	97.9	57.2	100.0	-			-[e]	
Corydalis yanhusuo W.T. Wang[g]	rhizome	101.7	77.4	358.8	78.0	6.4	380.4	-			±[e]	
Curculigo orchioides Gaertn.[g]	fruit	77.6	88.1	104.7	77.6	42.4	126.0	-			-	
Curcuma aeroginosa Roxb.[g]	rhizome	70.1	34.0	136.7	78.5	29.7	112.6	±			±	
Curcuma xanthorrhiza Roxb.[g]	rhizome	77.6	39.0	103.7	84.1	35.6	113.5	±			±	
Cyperus rotundus L.	rhizome	81.4	89.9	73.2	78.4	104.6	84.4	-			-	
Cyrtomium fortunei J. Sm.[f,g,i]	rhizome	59.6	66.5	112.1	0.0	0.0	0.0	±	97.5	101.7	+	49.8
Dictamnus dasycarpus Turcz.	root bark	134.2	121.4	74.8	135.6	130.6	78.9	-			-	
Dioscorea hispida Dennst.	rhizome	50.0	95.8	69.4	58.3	94.8	72.3	-			-	
Drynaria fortunei (Kunze) J. Smith[f]	rhizome	87.4	90.5	108.5	0.0	64.2	107.7	-	45.6	106.2	±	15.5
Dryopteris crassirhizoma Nakai[g]	rhizome	62.1	93.8	89.9	51.7	48.4	91.7	-			-	
Elaeocarpus grandiflorus Smith[f,g,i]	fruit	0.0	0.0	0.0	0.0	0.0	0.0	+	21.4	7.8	+ +	6.4
Elephantopus scaber L.[g,i]	leaf	85.8	20.8	85.0	59.2	17.7	47.8	±			±	
Ephedra sinica Stapf[g]	stem	92.7	43.2	100.9	68.1	100.0	96.6	-			±	
Epimedium sagittatum (Sieb. et Zucc.) Maxim.[f]	leaf	65.1	91.7	84.3	42.3	92.6	77.3	-	96.8	87.0	-	81.5
Equus aninus L.	gelatin	91.8	64.3	81.7	91.8	66.3	89.4	-			-	
Euphorbia kansui Liou	root	105.5	83.7	94.7	105.5	75.0	97.6	-			-	
Evodia ruteacarpa Hook. f. et Thoms.[g]	fruit	83.8	82.1	94.4	92.1	41.1	94.9	-			-	
Ficus edelfeltii King.	bark	104.4	108.3	87.9	85.4	68.5	111.2	-			-	
Forsythia suspensa Vahl.[g,i]	fruit	98.6	72.9	26.1	60.8	30.7	6.7	-			±[e]	
Fritillaria thunbergii Miq.	rhizome	87.3	56.0	89.8	70.6	90.4	89.3	-			-	
Galium aparine L.	whole plant	65.6	81.4	75.4	78.1	78.5	83.2	-			-	
Gardenia jasminoides Ellis	fruit	112.6	87.4	94.0	93.7	58.8	112.1	-			-[e]	
Gentiana macrophylla Pall.	root	84.3	87.2	72.2	94.1	78.0	95.6	-			-	
Gentiana scabra Bunge	root, rhizome	95.8	99.5	57.2	90.8	86.2	79.5	-			-	
Geranium thunbergii Sieb. et Zucc.[f,g,i]	whole plant	91.6	30.0	104.7	23.5	21.4	0.0	±	76.1	76.4	±	35.3
Geum japonicum Thunb.[f,g]	whole plant	81.7	90.4	76.1	0.0	0.0	79.9	-	88.9	65.0	±[e]	40.7
Ginkgo biloba L.	leaf	84.2	98.9	128.8	86.8	92.8	54.6	-			-[e]	
Ginkgo biloba L.	fruit	103.4	108.1	133.3	100.0	85.8	337.3	-			-[e]	
Houttuynia cordata Thunb.	whole plant	69.8	81.2	101.9	68.2	64.4	105.6	-			-	
Juglans mandshurica Maxim.[f,g,i]	bark	73.6	35.5	96.5	0.0	4.8	3.5	-	60.1	73.4	+	51.1
Lasiosphaera fenzlii Reich.	fruit body	89.2	100.4	76.7	81.6	105.0	77.3	-			-	
Lithospermum erythrorhizon Sieb. et Zucc.[g]	root	97.5	61.9	87.9	107.6	50.0	88.4	-			-	
Lonicera japonica Thunb.	flower bud	114.7	105.2	95.8	109.8	106.4	96.6	-			-[e]	
Loranthus parasiticus (L.) Merr.[g]	aerial part	60.0	83.3	91.0	67.5	29.2	93.7	-			-	
Lycium chinense Mill.	fruit	86.8	95.0	106.2	73.6	82.2	94.4	-			-	
Lycopus lucidus Turcz.	whole plant	93.3	91.2	95.1	87.9	90.1	101.6	-			-	
Machilus thunbergii Sieb. et Zucc.[f,g]	bark	98.6	55.6	111.8	17.8	0.0	62.2	-	96.2	83.3	±	89.4

Magnolia fargesii Cheng	flower bud	90.8	79.0	87.4	119.3	81.4	83.7	-			-	
Magnolia obovata Thunb.	bark	94.1	70.5	77.2	94.1	79.5	97.2	-			-	
Magnolia officinalis Rehd. et Wils.[3]	bark	71.7	122.7	71.6	95.5	120.6	34.1	-			±	
Matteuccia orientalis (Hook.) Trev.	rhizome	79.6	68.6	70.2	70.8			-			-	
Matteuccia orientalis (Hook.) Trev.	spore	81.7	56.9	93.2	72.1		75.3	-			-	
Matteuccia struthiopteris (L.) Todaro[g]	rhizome	49.7	65.9	86.6	60.9	34.6	96.2	-			-	
Melia toosendan Sieb. et Zucc.	fruit	99.2	91.0	94.1	98.4	90.4	86.4	-			-	
Miscanthus sinensis Anderss.	spike	98.9	87.4	85.8	82.4	86.7	75.8	-			-	
Nandina domestica Thunb.	leaf	91.5	71.3	189.2	96.6	63.5	251.0	-			-[c]	
Oldenlandia diffusa (Willd.) Roxb.	whole plant	88.8	99.0	91.9	78.1	90.0	61.1	-			-[c]	
Ophiopogon japonicus Ker-Gawler	root	147.5	105.2	103.0	127.1	109.9	123.5	-			-[c]	
Paeonia suffruticosa Andrews[f,g,i]	root bark	96.5	124.2	119.1	0.0	35.5	0.0	±	93.3	98.2	±[c]	73.4
Parameria laevigata Moldenke[g]	bark	52.5	43.8	102.0	68.3	32.3	88.1	±			±	
Parkia roxburghii Benth.	fruit	99.3	100.0	85.0	70.8	78.7	105.8	-			-	
Patrinia villosa Juss.	root	99.3	99.5	78.9	78.9	80.3	99.9	-			-[c]	
Phellodendron amurense Ruprecht[f,g,i]	bark	85.3	103.4	17.2	29.4	43.3	0.6	±	57.5	59.7	±[c]	32.6
Physalis angulata L.[g]	aerial part	102.9	17.6	108.7	86.1	0.0	105.8	±			±	
Picrorrhiza kurrooa Royle ex. Benth.	rhizome	98.4	71.8	62.4	112.7	67.9	74.2	-			-	
Pinellia ternata Breitenbach	rhizome	109.6	106.6	100.0	71.2	106.1	50.8	-			-	
Pistacia lentiscus L. (?)	resin	105.5	70.5	100.7	89.0	82.2	90.7	-			-	
Plagiogyria matsumureana Makino[g]	rhizome	76.1	73.9	90.8	50.7	0.0	84.6	±			±	
Platycodon grandiflorum (Jacquin) A. DC.[1]	root	86.3	123.0	95.1	105.5	97.4	0.0	-			±	
Polygala tenuifolia Willd.[1,g,i]	root	118.6	86.4	100.0	0.0	0.0	0.0	±	49.8	67.6	+[e]	38.1
Polygonum cuspidatum Sieb. et Zucc.[1,g]	root, rhizome	93.4	88.0	112.0	0.0	38.0	105.7	-	75.9	78.4	-[e]	70.0
Portulaca oleracea L.	aerial part	93.7	95.1	80.2	75.5	72.2	154.3	-			-[e]	
Prunella vulgaris L. subsp. *asiatica* Hara[1]	spike	66.1	83.5	111.8	0.0	64.6	111.8	-	92.4	102.6	-[e]	75.3
Prunus armeniaca L.	seed	95.1	103.1	104.2	101.4	116.5	108.9	-			-[e]	
Prunus mume Sieb. et Zucc.[g]	fruit	88.0	75.8	73.1	102.1	38.9	74.4	-			-	
Prunus persica (L.) Batsch.	seed	130.5	89.9	130.4	93.2	73.3	323.5	-			-[e]	
Pueraria lobata (Willd.) Ohwi	root	96.5	89.6	102.6	82.5	84.8	98.3	-			-[e]	
Pulsatilla chinensis (Bunge) Regel	root	100.0	74.6	100.0	105.6	101.5	102.3	-			-	
Punica granatum L.[f,g,i]	root bark	0.0	79.5	0.0	0.0	0.0	0.0	±	103.5	67.4	+[c]	38.7
Quercus salicina Blume[g]	leaf	74.0	48.0	95.9	98.6	30.6	89.0	-			-	
Quisqualis indica L.[g]	fruit	100.0	28.2	100.0	105.2	16.9	127.3	-			-	
Rheum palmatum L.[1,g,i]	rhizome	93.2	13.7	108.1	0.0	0.0	0.0	-	64.4	64.6	+	50.3
Rhus javanica L.[1,g,i]	gall	0.0	65.1	3.4	0.0	4.3	0.0	±	20.1	41.7	±[c]	12.4
Rosa laevigata Michx[g]	fruit	100.0	25.4	108.2	80.9	69.0	97.6	-			-	
Salvia miltiorrhiza Bunge	root	89.9	87.1	98.7	58.6	83.3	65.8	-			-[c]	
Sarcandra glabra (Thunb.) Nakai	leaf	67.7	129.8	92.6	51.6	140.3	76.7	-			-	
Schizandra chinensis (Turcz.) Baill.	fruit	99.3	121.4	110.6	101.4	119.0	108.5	-			±[c]	
Scolopendra subspinipes multidens L. Koch.	whole body	101.6	93.8	94.8	81.3	76.4	81.7	-			-	
Scutellaria baicalensis Georgi[g,i]	root	78.3	66.0	12.3	66.4	14.7	5.0	-			-[c]	
Senecio kirilowi Turcz.	whole plant	103.1	105.9	90.9	86.1	96.6	87.5	-			-	
Sida mysorensis Wight et Arn.[g]	seed	100.0	67.6	100.0	100.3	33.8	53.1	-			-	

Sinomenium acutum Rehd. et Wils.	root	108.0	100.0	94.7	112.4	64.8	99.5	-			-	
Sophora japonica L.[g]	flower bud	81.3	67.1	97.2	65.4	49.5	98.1	-			-	
Sophora subprosarata Chun et Chen	root	96.5	99.5	103.4	88.1	57.7	131.9	-			-[e]	
Spatholobus suberectus Dunn[f,g,i]	rhizome	92.2	80.2	75.4	0.0	0.0	0.0	-	82.8	61.7	±	35.4
Stellera chamaejasme L.	root	81.6	87.0	80.0	80.1	81.7	83.9	-			-	
Stemona japonica Miq.	root	79.4	106.9	72.7	91.2	103.2	80.0	-			-	
Strobilanthus crispus L.	leaf	107.3	109.3	90.3	93.4	94.4	114.6	-			-	
Struthiopteris nipponica (Kunze) Nakai[g]	rhizome	57.1	66.0	96.1	56.5	33.4	94.6	-			-	
Strychnos nux-vomica L.[g]	seed	60.8	39.6	89.1	88.3	47.9	87.8	-			±	
Syzygium aromaticum (L.) Merr. et Perry[f,g,i]	flower bud	79.4	55.1	98.6	0.0	0.0	0.0	±	70.6	66.8	+	
Taraxacum mongolicum Hand.-Mazz.	whole plant	101.4	113.5	84.3	83.9	125.7	78.8	-			-[e]	
Terminalia arjuna Wight et Arn.[f,g,i]	bark	56.3	22.5	100.0	0.0	0.0	0.0	±	67.5	76.0	+	40.9
Terminalia belerica Roxb.[f,g,i]	fruit peel	70.8	7.4	104.4	0.0	0.0	0.0	±	82.2	73.0	+	22.4
Terminalia chebula Retzus[f,g,i]	fruit	99.6	32.6	152.0	0.0	0.7	0.0	-	77.5	66.9	+[e]	18.3
Uncaria gambir Roxburgh[g]	leaf	97.4	85.3	104.7	89.5	24.2	92.7	-			±	
Usnea misaminensis Vain.	whole plant	117.8	111.1	86.9	84.7	53.7	93.2	-			-	
Viscum album L. var. *coloratum* (Komar.) Ohwi	stem, leaf	72.1	80.2	93.2	73.6	86.6	102.5	-			-	
Woodfordia floribunda Salisb.[f,g,i]	flower, leaf	0.0	0.0	0.0	0.0	0.0	0.0	+	86.1	61.6	+ +	13.8
Zanthoxylum bungeanum Maxim.[f,g]	fruit peel	107.7	80.4	100.0	20.3	14.3	100.0	-	72.2	93.8	±[e]	49.9
Zanthoxylum schinifolium Sieb. et Zucc.	fruit peel	90.1	75.8	103.1	82.4	77.9	98.6	-			-	
Zea mays L.	stigma	77.6	91.0	73.9	80.1	86.0	85.3	-			-	
Zingiber officinale Roscoe	rhizome	79.4	87.6	94.6	79.4	76.6	101.5	-			-	
Ziziphus jujuba Mill. var. *inermis* (Bge.) (Rehd.)	fruit	85.7	101.7	88.6	80.3	104.2	92.6	-			-	

[a]The range of plaque numbers of untreated controls was 50–150. Plaque formation represents the percentage compared to untreated controls. Percent plaque formation of HSV-1 was 0% at both 100 and 300 μg/ml of ACV.
[b]+ +, +, ±, and - indicate strong, intermediate, weak, and no cytotoxicity, respectively, as described in the text.
[c]Percent of [^{3}H]thymidine-uptake into DNA of HW-extracts-treated cells to that of untreated-cells. The cells were treated with 100 or 300 μg/ml of HW-extracts. Traditional medicines indicated by footnote (f) in this Table 1 were tested.
[d]Percent of the number of cells grown in the presence of HW-extracts (100 μg/ml) to that in its absence. Traditional medicines indicated by footnote (f) in this Table 1 were tested.
[e] Plaque reduction assay was performed at 500 μg/ml.
[f]HSV-1-inhibitory traditional medicines selected in this assay.
[g]Poliovirus-inhibitory traditional medicines selected in this assay.
[h]Underlines represent the percentages less than 50% in plaque reduction assay.
[i]Measles virus-inhibitory traditional medicines selected in this assay.

The Vero cells were grown in 24-well plates for 2 days as described above for [^{3}H]thymidine-uptake assay. The culture medium were replaced with fresh medium containing 37 kBg of [*methyl*-^{3}H]thymidine (3.1 TBg/mmol, Amersham) and 100 or 300 μg/ml of HW-extract. After a 18 h exponential growth period of the cells, the cells were lysed with 20 mM Tris, (pH 8.0), 5 mM EDTA, 0.5% (w/v) SDS and 100 μg/ml of proteinase K at 37°C for 3 h. The lysates were spotted onto filters (514A paper filters, Toyo Roshi Co., Ltd., Japan) and then the filters were washed three times with cold 5% TCA and then once with ethanol. Radioactivity on the dried filters was determined in a liquid scintillation counter.

Therapeutic efficacy in mouse HSV-1 infection model

Female BALB/c mice (6-week-old) were purchased from Sankyo Labo Service Co., Ltd., Tokyo, Japan. The right midflank of each mouse was clipped and depilated with a chemical depilatory, Hair Remover (Shiseido, Co., Ltd., Tokyo, Japan). One or two days later, the naked skin was scratched using a 27-gauge needle and 7 μl of HSV-1 (7401H strain) suspension containing 1×10^6 PFU was applied to the scarified area (Kumano et al., 1987; Simmons and Nash, 1984). HW-extracts (1–10 mg) or ACV (2.5 or 5 mg/kg) was orally administered in a volume of 0.25 ml/mouse once at 8 h before and three times daily for 10 successive days after HSV-1 infection. The development of skin lesions and mortality were continuously monitored every 8 h daily and scored as follows: 0, no lesion; 2, vesicles in local region; 4, erosion and/or ulceration in local region; 6, mild zosteriform lesion; 8, moderate zosteriform lesion; 10, severe zosteriform lesion; and death. The infected mice were held at least for a month after infection. The Student's *t*-test was used to evaluate the significance of differences between control and HW-extract-treated mice in mean survival times and mean times at which skin lesions were initially scored as 2 or 6 after infection. Differences in the mortality between control and HW-extract-treated mice were evaluated using the Kaplan-Meier method and the Wilcoxon test.

Results

Effects of HW-extracts on the plaque formation

To evaluate the antiviral activity of 142 HW-extracts, we examined their inhibitory effects on the plaque formation of HSV-1, poliovirus, or measles virus (Table 1). HW-extracts that reduced the plaque forming ability of each virus to less than 50% were selected as virus-inhibitory HW-extracts. Thirty-two HW-extracts exhibited anti-HSV-1 activity; eight of them were effective at 100 μg/ml and the other 24 HW-extracts were active at 300 or 500 μg/ml as indicated in Table 1. For poliovirus, 55 HW-extracts were effective; twenty-one of them were effective at 100 μg/ml and the other 34 were effective at 300 or 500 μg/ml. Thirty HW-extracts were effective against measles virus; eleven of them

were similarly effective at 100 μg/ml and the other 19 were effective at 300 or 500 μg/ml.

Alpinia officinarum, *Geum japonicum*, *Machilus thunbergii*, *Polygonum cuspidatum*, and *Zanthoxylum bungeanum* inhibited the plaque formation of both HSV-1 and poliovirus. *Brainia insignis* was effective against both HSV-1 and measles virus. *Drynaria fortunei*, *Epimedium sagittatum*, and *Prunella vulgaris* were selectively inhibitory to HSV-1 only. All of HSV-1-inhibitory HW-extracts except for 9 HW-extracts described above were also effective against both poliovirus and measles virus. Thus the 32 HW-extracts with anti-HSV-1 activity could be classified into 4 groups based on antiviral activities for poliovirus and measles virus.

Some HSV-1-inhibitory HW-extracts showed visible cytotoxicity and reduced the incorporation of [^{3}H]thymidine into cellular DNA at 300 or 500 μg/ml but less at 100 μg/ml. Cytotoxicity of most HSV-1-inhibitory HW-extracts in vitro was evaluated similarly by visible cytotoxicity assay, growth-inhibition assay, and [^{3}H]thymidine-uptake assay at 100 μg/ml. However there was discrepancy between [^{3}H]thymidine-uptake assay and the other two assays (cell-growth inhibition and visible cytotoxicity); [^{3}H]thymidine-uptake was reduced in the presence of *Artemisia princeps*, *Brucea javanica*, *Cassia fistula*, *Drynaria fortunei*, or *Rhus javanica* but cells treated with them were significantly viable in the other two assays. Thus those HW-extracts possibly contain some compounds which may compete with [^{3}H]thymidine, so that [^{3}H]thymidine-uptake might be reduced. Although there were HW-extracts with intermediate visible cytotoxicity, all of these HW-extracts were also effective to inhibit the plaque formation of HSV-1. Therefore we selected all of 32 HSV-1-inhibitory HW-extracts including HW-extracts with intermediate cytotoxicity in vitro as candidates for HSV-1-inhibitory medicines.

Therapeutic efficacies of HW-extracts on a mouse HSV-1 infection model

Therapeutic efficacies of 32 HW-extracts selected were examined in a cutaneous HSV-1 infection model in mice (Table 2). In this model, 2.5 mg/kg of ACV was not effective in limiting the development of skin lesions, in prolonging the survival times, and in reducing the mortality. However the treatment of 5 mg/kg of ACV significantly prolonged mean survival times and delayed the development of skin lesions although the mortality under the treatment was similar to that of the controls (water-administered mice). In groups treated with HW-extracts (30 mg/mouse/day) of *Brucea javanica*, *Drynaria fortunei*, *Elaeocarpus grandiflorus*, *Geranium thunbergii* or *Juglans mandshurica*, most of mice were dead before skin lesions could be scored, because of the possible toxicity of the extract being administered. Twelve HW-extracts significantly prolonged mean survival times and/or delayed the development and progression of skin lesions. *Phellodendron amurense* reduced the mortality significantly.

TABLE 2

Effects of HW-extracts on cutaneous HSV-1 infection in BALB/c mice

Exp. No.	Treatment	Dose (mg/mouse)	Mean time (days ± S.D.)			
			Score 2[a]	Score 6[a]	Survival[b]	Mortality[c]
1	Control (water)	0	3.39 ± 0.51	5.15 ± 0.38	6.92 ± 0.64	13/14
	ACV (2.5 mg/kg)	0.05	3.62 ± 0.51	5.02 ± 0.87	7.73 ± 1.49	11/14
	ACV (5 mg/kg)	0.1	4.08 ± 0.29[d]	5.70 ± 0.48[d]	9.00 ± 1.33[d]	9/12
2	Control	0		6.83 ± 0.41	8.17 ± 0.41	6/6
	Drynaria fortunei	10		6[e]	9[e]	8/8
	Geranium thunbergii	10		ND[f]	ND	7/7
	Geum japonicum[g]	10		7.67 ± 0.58[h]	9.75 ± 0.50[d]	4/5
	Juglans mandshurica	10		7[e]	8.5[e]	5/6
3	Control	0	3.00 ± 0.96	5.29 ± 0.47	6.86 ± 1.10	13/15
	Alpinia officinarum[g]	10	4.20 ± 0.42[d]	5.78 ± 0.44[h]	8.00 ± 1.56[h]	10/10
	Brainia insignis	10	2.67 ± 0.87	5.33 ± 0.87	6.89 ± 0.60	9/10
	Brucea javanica	10	ND	ND	ND	10/10
	Cassia fistula	10	3.67 ± 0.50	5.67 ± 0.50	7.56 ± 1.13	9/9
	Elaeocarpus grandiflorus	10	2.5[e]	ND	6[e]	11/11
	Polygala tenuifolia[g]	10	3.40 ± 0.84	6.40 ± 1.17[d]	7.60 ± 1.27	10/10
	Syzygium aromaticum[g]	10	3.78 ± 0.44[h]	6.25 ± 0.46[d]	7.67 ± 1.12	9/9
	Terminalia arjuna[g]	5	3.25 ± 1.29	6.00 ± 1.13	8.00 ± 1.41[h]	11/12
	Terminalia belerica	10	3.20 ± 0.63	5.73 ± 0.91	7.46 ± 1.04	11/12
4	Control	0	2.70 ± 0.95	5.71 ± 0.76	7.57 ± 0.98	7/11
	Artemisia princeps	5	3.70 ± 1.25	5.83 ± 0.75	8.14 ± 1.46	7/10
	Caesalpinia sappan[g]	5	3.73 ± 1.27	6.43 ± 0.98	9.00 ± 1.29[h]	7/11
	Cinnamomum sintok	1	3.00 ± 1.16	6.43 ± 0.54	9.00 ± 1.58	5/7
	Coptis chinensis	5	3.40 ± 1.08	6.10 ± 0.88	7.67 ± 1.50	9/10
	Paeonia suffruticosa[g]	5	3.91 ± 1.70	6.43 ± 0.54	9.57 ± 1.40[d]	7/11
	Phellodendron amurense[g]	5	4.40 ± 0.84[d]	7.00 ± 0.63[d]	7[e]	1/10[i]
	Polygonum cuspidatum[g]	5	4.00 ± 1.16[h]	5.50 ± 0.54	7.90 ± 1.44	10/10
	Punica granatum[g]	5	3.10 ± 0.88	5.50 ± 0.53	9.11 ± 1.17[h]	9/10
	Rhus javanica[g]	5	4.10 ± 0.99[d]	6.50 ± 0.54[h]	8.33 ± 1.23	9/10
	Woodfordia floribunda	5	3.67 ± 1.16	6.33 ± 0.58	7.67 ± 0.58	3/3
5	Control	0	3.09 ± 0.30	5.27 ± 0.47	6.36 ± 0.51	11/11
	Areca catechu	5	3.20 ± 0.42	5.50 ± 0.53	6.80 ± 1.23	10/10
	Prunell vulgaris subsp.*asiatica*	5	3.30 ± 0.48	5.11 ± 0.33	6.60 ± 0.84	10/10
6	Control	0	3.35 ± 0.50	5.56 ± 0.73	7.00 ± 0.76	8/9
	Terminalia chebula[g]	5	4.00 ± 0.89	6.50 ± 0.58[h]	8.75 ± 1.50[h]	4/6
	Spatholobus suberectus	5	3.78 ± 0.67	5.75 ± 0.71	7.50 ± 1.69	8/9
7	Control	0	3.40 ± 0.52	5.50 ± 0.53	6.50 ± 0.71	10/10
	Cyrtomium fortunei	5	3.67 ± 0.71	5.44 ± 0.53	6.78 ± 0.44	9/9
	Epimedium sagittatum	5	3.50 ± 0.53	5.60 ± 0.52	6.70 ± 0.95	10/10
	Rheum palmatum	5	3.50 ± 0.53	5.00 ± 0.50	6.40 ± 0.52	10/10
	Machilus thunbergii	5	3.60 ± 0.52	5.50 ± 0.53	6.60 ± 0.52	10/10
	Zanthoxylum bungeanum	5	3.60 ± 0.70	5.30 ± 0.68	6.60 ± 0.70	10/10

[a]Mean time at which score 2 or 6 was first observed after infection. The infected mice which were not scored were excluded from the calculation of mean times.
[b]Surviving mice were not included for the calculation of mean survival times.
[c]Number of dead mice number of mice tested.
[d]Mean times are significantly prolonged ($P < 0.01$ vs. control by Student's t-test).
[e]The number of scored mice was less than 2.
[f]ND, not detected.
[g]HW-extracts exhibiting significant therapeutic efficacy in this HSV-1 infection model.
[h]Mean times are significantly prolonged ($P < 0.05$ vs. control by Student's t-test).
[i]Mortality is significantly decreased ($P < 0.05$ vs. control by the Kaplan-Meire method and the Wilcoxon test).

Discussion

We examined the anti-HSV-1 activities of 142 HW-extracts in vitro and in vivo. The selectivity of HW-extracts for anti-HSV-1 activity was simultaneously assessed in plaque reduction assay by testing antiviral activities for poliovirus and measles virus. The model of cutaneous HSV- 1 infection in mice used in this study was helpful in defining those HW-extracts which had potential for inhibiting HSV-1 infection. Twelve HW-extracts were found to be the potential therapeutic medicines against HSV-1 infection. Many herbs contain antiviral compounds as reported by Amoros et al. (1987), Chang and Yeung (1988), Fukuchi et al. (1989), Hayashi et al. (1992), Ito et al. (1987), Sydislis et al. (1991), Tang et al. (1990), Yamamoto et al. (1989), and Yao et al. (1992). However, the therapeutic antiviral efficacies of HW-extracts in vivo have not been reported. Thus, our results are the first evidence demonstrating that HW-extracts of 12 traditional medicines exhibit therapeutic antiviral activity in vivo.

Plaque reduction assay, as an initial test procedure, would not be suitable for demonstrating antiviral activity of compounds which may be found in low concentrations in HW-extracts, compounds which are active only when activated metabolically, and those which may act antivirally through biological response modification (BRM). We did not examine each antiviral activity of traditional medicines extracted by other procedures, because most of traditional medicines have been used clinically in the form of HW-extracts and thus our purpose in this study is to find HW-extracts with antiviral therapeutic efficacy.

Glucose is one of typical chemicals which are rapidly absorbed from alimentary tracts in human. Its maximal serum concentration is reported to increase by approximately 300 mg/dl (300 μg/ml) after 100 g (10 g) oral administration in human with diabetes mellitus (Kosaka et al., 1977). Some of the components in HW-extracts may be absorbed as rapidly and efficiently as glucose. Since HW-extracts (approximately 3–30 g) prepared from approximately 30–100 g of dried traditional medicines have been orally administered in human, a dose (100 μg/ml) of HW-extracts in plaque reduction assay may correspond to their putative concentrations in serum after oral administration. We also used the higher concentrations (300 or 500 μg/ml) to survey the possible antiviral activities in all HW-extracts which were not selected at 100 μg/ml. Therefore, plaque reduction assay using these doses is designed to examine antiviral activity at the putative concentration of HW-extracts in serum and none of possible antiviral traditional medicines for conventional oral use would be lost in this assay.

The concentrations of HW-extracts which reduce plaque forming ability to less than 50% in plaque reduction assay would be expected to be effective in vivo. We used a 50% reduction in plaque assay as our definition of antiviral activity in this antiviral screening study. It was postulated that a 100 μg/ml concentration which was active in the plaque assay may also achieve an

adequate serum concentration in mice to also render an in vivo antiviral activity. It was on this basis that 32 in vitro-active HW-extracts were selected for the in vivo experiments.

Some of the 32 HW-extracts with anti-HSV-1 activity also exhibited anti-poliovirus activity, anti-measles virus activity, or activity against both viruses. Since each of these viruses have different structures and replication cycles, their difference in sensitivity to the various HW-extracts may be due to the different modes of antiviral action of the active compounds in the extracts. Where all three viruses were inhibited by the same extracts, it is probable that antiviral activity was a manifestation of sublethal cytotoxicity.

Twelve of 32 virus-inhibitory HW-extracts showed intermediate cytotoxicity in plaque reduction assay. However, *Caesalpinia sappan*, *Polygala tenuifolia*, *Syzygium aromaticum*, *Terminalia arjuna*, and *Terminalia chebula* among them were found to exhibit significant therapeutic efficacy in vivo in an HSV-1 infection model in mice (Table 2). These results suggest that antiviral components in their HW-extracts were selectively absorbed from alimentary tracts and exerted an HSV-1-inhibitory effect which was not associated with toxicity.

HW-extracts contain various kinds of compounds. Some compounds may be absorbed from alimentary tracts, and others may not. Even if the antiviral compounds are absorbed, their concentrations in vivo vary possibly by the rate of absorption, metabolism, and excretion. However the concentrations of antiviral compounds in HW-extracts are much more constantly retained for assay period in vitro than in vivo. Alternatively some compounds in HW-extracts may act antivirally through BRM in vivo but not in vitro. Therefore the antiviral activity of HW-extracts observed in an in vitro assay would not always correlate to their therapeutic antiviral efficacy in vivo.

HW-extracts have been traditionally used for the treatment of various diseases in human. The information on their adverse reactions have been accumulated in their history. We can refer to this information when we use them. It was decided, for these screening experiments, that it would be more important to follow up the in vitro anti-HSV-1-inhibitory materials with animal studies rather than pursue more detailed cytotoxicity experiments. The early deaths of mice treated with certain of these extracts was generally unexpected, since all the HW-extracts studied have been used apparently safely in humans. We have not yet followed up on the lethally toxic materials to determine if lower, more tolerated doses would exhibit an antiviral effect in this mouse model. *Brucea javanica*, *Geranium thunbergii*, and *Juglans mandshurica* have been used as antibacterial agents, and are historically known to contain possible toxic compounds. Thus their lethal toxicity in mice as seen in this study should have been anticipated. Caution has been indicated for their use in the clinic when they are used as described in the Jiangxu New Medical College, 1978.

HW-extracts of *Alpinia officinarum*, *Geum japonicum*, *Polygonum cuspidatum*, *Punica granatum*, and *Rhus javanica* showed significant therapeutic

TABLE 3

Traditional medicines with anti-HSV-1 activity and their traditional clinical application

Name	Clinical application
Alpinia officinarum	Parotiditis, Gastric and duodenal ulcer
Caesalpinia sappan	Swelling and pain, Subcutaneous hemorrhage
Geum japonicum	Bruise, Diuresis, Empyema
Paeonia suffruticosa	Hypertension, Allergic rhinitis
Phellodendron amurense	Epidemic meningitis, Pneumonia
Polygala tenuifolia	Acute mastitis
Polygonum cuspidatum	Chronic myelitis, Burn, Arthritis
Punica granatum	Amebic dysentery
Rhus javanica	Gastric and duodenal ulcer, Empyema
Syzygium aromaticum	Acute gastroenteritis
Terminalia arjuna	Heart diseases, Hypertension
Terminalia chebula	Chronic pharyngolaryngitis, Diarrhea

efficacies in a mouse HSV-1 infection model (Table 2). These HW-extracts exhibited anti-HSV-1 activities in plaque reduction assay but did not exhibit anti-poliovirus or -measles virus activity (Table 1). Therefore, it is suggested that these HW-extracts show therapeutic efficacies for HSV-1 infection in vivo possibly based on their anti-HSV-1 specific activity observed in plaque resection assay.

The 12 HW-extracts which exhibited significant inhibitory effects on HSV-1 infections in mice are shown in Table 3 with their current traditional clinical application indicated. Since these plant extracts are already being used clinically for treatment of other human diseases, it may be reasonable to expect these materials to thus be able to be used safely also for therapy of human HSV-1 infections. Thus for practical use of these medicines, we are now evaluating the efficient combinations of those HW-extracts with one another or with ACV and elucidating the mechanism of their actions.

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