Assay for Antiviral Activity of Herbal Extracts Using Their Absorbed Sera

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We evaluated an antiviral assay procedure using serum obtained from guinea pigs administered 32 herbal extracts. In this assay, 21 of the 32 showed anti-herpes simplex virus type 1 (HSV-1) activity in serum. Ten of the 21 exhibited therapeutic anti-HSV-1 activity, and this was consistent with our previous results that 12 of the 32 were effective in murine infection models. Therefore, the serum pharmacological assay procedure was suitable for the selection of possible herbal extracts with biological activity *in vivo* as a pre-screening method before animal experiments.

Key words herbal extract; herpes simplex virus; serum; antiviral activity; guinea pig; serum pharmacology

Natural products, including medicinal herbs, have their own metabolites and are useful sources in the search for new antiviral agents.[1] Many antiviral compounds have been identified from them.[2-18] Some of these compounds which are not absorbed *in vivo* are difficult to predict in terms of antiviral activity *in vivo* after oral administration, even though they exhibit strong antiviral activity *in vitro*. However, there is no established procedure *in vitro* to assess the biological activity *in vivo* of components contained in herbal extracts. We have previously examined the therapeutic anti-herpes simplex virus type 1 (HSV-1) activity of 32 herbal extracts with anti-HSV-1 activity *in vitro* in a murine infection model and showed that 12 of them exhibited therapeutic anti-HSV-1 activity in infected mice.[1] Based on this result, we examined the anti-HSV-1 activity of herbal extracts to establish an assay procedure using their absorbed sera, and we evaluated this serum pharmacological assay as a screening procedure of herbal extracts before animal experiments. The advantage of serum pharmacology was discussed as a screening procedure to determine biological activity in herbal extracts.

Materials and Methods

Virus and Cells The HSV-1 strain used was a wild 7401 strain.[1,19] Virus stocks were prepared from infected-Vero cell cultures as reported previously.[19,20] Vero cells were grown and maintained in Eagle's minimum essential medium (MEM) supplemented with 5% and 2% calf serum, respectively.[19,21]

Preparation of Herbal Extracts Herbal extracts with anti-HSV-1 activity *in vitro* were prepared from 32 dried traditional herbal medicines (Table 1) as described previously.[1] These traditional herbal medicines have been authenticated and stocked at the Research Institute for Wakan-Yaku (Traditional Sino-Japanese Medicines), Toyama Medical and Pharmaceutical University, Japan. Briefly, the dried materials were boiled under reflux and the aqueous extracts were filtered and lyophilized. The lyophilized extract was suspended in distilled water at 20 mg/ml, boiled for 10 min, and then centrifuged at 3000 rpm for 15 min. The supernatant was used for the following assays.

Administration to Guinea Pigs Female Hartley guinea pigs (300—350 g, Sankyo Labo Service Co., Ltd., Tokyo, Japan) were starved for 24 h before administration in order to effectively absorb the anti-HSV-1 components of herbal extracts into serum from their alimentary tracts. Under ether anesthesia, the abdomen of each guinea pig was opened and then 10 ml each (20 mg/ml) of herbal extracts or water was administered simultaneously into the stomach, and into the small and large intestines of a guinea pig (total dose, 600 mg/guinea pig) by a syringe with a 21-gauge needle, as described previously.[18] The opened abdomen was closed by clips, and whole blood content was collected from the heart at 2 h after injection. The prepared sera were inactivated by heating at 56 °C for 30 min.

Growth Inhibition Assay of HSV-1 Anti-HSV-1 activity in the herbal extract-absorbed sera of 3 guinea pigs in each group was examined by the growth inhibition assay of HSV-1 as described previously.[18] The monolayers of Vero cells were infected with HSV-1 at 0.01 plaque forming units (PFU)/cell for 1 h and incubated in MEM containing 25% of the absorbed serum. The cultures were frozen and thawed three times after 24 h incubation at 37 °C. The virus yield in each supernatant was determined by the plaque assay using Vero cells,[19,20] and a mean virus yield and standard deviation were calculated from virus yields of three cultures treated with absorbed sera.

Results and Discussion

Thirty-two herbal extracts with anti-HSV-1 activity *in vitro*[1] were examined for their anti-HSV-1 activity absorbed into serum from the alimentary tracts of guinea pigs. Sera obtained from water-administered guinea pigs were not cytotoxic at a concentration of 25% used in the growth inhibition assay (data not shown). Table 1 summarizes the anti-HSV-1 activities in the presence of sera obtained from herbal extract-administered guinea pigs. In this assay, the mean standard deviation of virus yields was about 15% in the cultures treated with sera obtained from three guinea pigs in each group. Therefore, 21 herbal extracts that reduced HSV-1 virus yields to less than 70% of the control were considered to contain significant anti-HSV-1 activity to be absorbed.

When we compared the absorbability of anti-HSV-1 activity in 12 herbal extracts that exhibited therapeutic anti-HSV-1 activity in HSV-1-infected mice (Table 1),[1] 10 of the 12 herbal extracts reduced HSV-1 virus yields to less than 70% of the control, and were included in the 21 herbal extracts that were selected as herbal extracts with anti-HSV-1 activity to be absorbed. Most of the 12 herbal extracts were selected as possible herbal extracts with anti-HSV-1 activity *in vivo* by the serum pharmacological assay. Since the concentration of herbal extracts which reduce virus yields to 70% of the untreated control

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in the growth inhibition assay is expected to be effective *in vivo*,[1] our definitive rate for effective anti-HSV-1 activity is consistent with this reduction rate and could be reasonable for the selection of possible herbal extracts with anti-HSV-1 activity *in vivo*. However, two herbal extracts (*Rhus javanica* L. and *Terminalia chebula* Retzus) were excluded by the definition, in spite of having therapeutic anti-HSV-1 activity in infected mice.[1] This may be due to ineffective concentrations of the anti-HSV-1 compounds contained in these 2 herbal extracts in serum caused by their distribution into some organs or tissues after administration. When using the serum pharmacological assay as a pre-screening procedure before the murine infection experiments, 10 of the 32 herbal extracts examined would be selected as herbal extracts with anti-HSV-1 activity *in vivo* (Table 1). This selection ratio (10/32) was not significantly different from the ratio (12/32) selected by the murine infection model alone. Our serum pharmacological assay was ascertained to be suitable as a pre-screening procedure of possible herbal extracts with anti-HSV-1 activity *in vivo* and to be useful in estimating their possible anti-HSV-1 activity *in vivo*.

Table 1. Effects of Guinea Pig Sera on the Growth of HSV-1 in Vero Cells

Treatment	Used part	Mean virus yield (%)[a] ±S.D.
Water		100.0
Alpinia officinarum HANCE[b]	Rhizome	6.8± 1.2
Areca catechu L.[b]	Seed	57.3±23.8
Artemisia princeps PAMP.	Leaf	111.5±19.6
Brainia insignis (HOOK.) J. SM.[b]	Rhizome	45.5±20.6
Brucea javanica (L.) MERR[b]	Seed	20.4±16.3
Caesalpinia sappan L.[b]	Bark	43.4± 7.4
Cassia fistula L.[b]	Bark	45.0±16.6
Cinnamomum sintok BLUME[b]	Bark	41.9± 8.0
Coptis chinensis FRANCH.	Rhizome	78.3±22.6
Cyrtomium fortunei J. SM.	Rhizome	128.2±12.6
Drynaria fortunei (Kunze) J. SMITH[b]	Rhizome	34.0±25.8
Elaeocarpus grandiflorus SMITH[b]	Fruit	15.4±11.3
Epimedium sagittatum (SIEB. *et* ZUCC.) MAXIM.	Leaf	100.0±15.3
Geranium thubergii SIEB. *et* ZUCC.[b]	Whole plant	50.0± 1.3
Geum japonicum THUNB.[b]	Whole plant	22.5±31.1
Juglans mandshurica MAXIM.[b]	Bark	12.6± 7.7
Machilus thunbergii SIEB. *et* ZUCC.	Bark	121.4±42.7
Paeonia suffruticosa ANDREWS[b]	Root bark	61.4±11.2
Phellodendron amurense RUPRECHT[b]	Bark	30.4±17.9
Polygala tenuifolia WILLD.[b]	Root	8.3± 2.4
Polygonum cuspidatum SIEB. *et* ZUCC.[b]	Root, rhizome	65.6±11.1
Prunella vulgaris L. subsp. *asiatica* HARA[b]	Spike	58.5±24.7
Punica granatum L.[b]	Root, bark	54.3±12.5
Rheum palmatum L.	Rhizome	82.7±20.4
Rhus javanica L.	Gall	87.3±24.7
Spatholobus suberectus DUNN	Rhizome	73.7±23.3
Syzygium aromaticum (L.) MERR. *et* PERRY[b]	Flower bud	50.5±10.6
Terminalia arjuna WIGHT *et* ARN.[b]	Bark	39.0± 1.4
Terminalia belerica ROXB.[b]	Fruit peel	31.2± 4.9
Terminalia chebula RETZUS	Fruit	87.4±23.0
Woodfordia floribunda SALISB.	Flower, leaf	80.1± 8.4
Zanthoxylum bungeanum MAXIM.	Fruit peel	107.9±28.0

a) Sera obtained from three guinea pigs in each group were assayed for anti-HSV-1 activity (yield reduction) independently. Each value represents the mean percentage of the control virus yields ($0.9-2.5\times10^8$ PFU/ml). Underlines indicate herbal extracts selected by a murine HSV-1 infection model.[1] *b*) Herbal extracts selected in absorption experiments using guinea pigs.

We used 3 guinea pigs in each group for the absorption experiments. We were able to obtain enough serum from each guinea pig for the growth inhibition assay and to examine the absorption of herbal extracts pharmacologically using a possible minimal number of animals. Thus, the use of a serum pharmacological procedure may help avoid the use of many mice in animal experiments. Moreover, we may be able to evaluate herbal extracts with possible antiviral activity *in vivo* for a short time by using this pharmacological procedure as compared with animal experiments, because our serum pharmacological procedure requires only 4—5 d, whereas animal experiments usually need a much longer time. It is believed that the oral administration of herbal extracts is effective against many kinds of diseases. However, there has been no convenient assay procedure *in vitro* to search for and evaluate their biological activity. Therefore, serum pharmacology based on the absorption from alimentary tracts may be utilized not only to screen the antiviral activity of herbal extracts but also to evaluate their biological activity in general.

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