APPROACHES TO AN ANTI-HSV-1 GEL FROM THE LEAVES OF *Gynura procumbens* EXTRACT

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Abstract

Gynura procumbens Merr. is a decumbent perennial herb prevalent mainly in Southern and Southeastern Asia. Local Thai people usually call it Pae Tam Peung. In previous research, it was shown that a crude 50% ethanol extract inhibited *Herpes simplex* serotype 1. Therefore, the possibility of an anti-HSV-1 gel was studied stepwise: Step 1) Soxhlet extraction of air-dried ground *G. procumbens* was done sequentially with hexane and 50% ethanol, until the refluxing solvent was colorless. Then, the 50% ethanol was evaporated to obtain a 50% ethanol crude extract. Step 2) Anti-HSV-1 gels were prepared for three compositions containing 0.1, 0.5 and 1.0 g of the 50% ethanol extract of *G. procumbens*. Step 3) Stability of three anti-HSV-1 gels were tested. It was found that all of them showed their greatest stability at 4°C. Step 4) Anti-HSV-1 and cytotoxic activities were analyzed. The results showed that the gel was inactive for anti-HSV-1 activity, but was non-cytotoxic against Vero cells. The maximum concentration of tested sample was 50 µg/mL.

Keywords: *Gynura procumbens*, *Herpes simplex*, anti-HSV-1, Asteraceae

Introduction

In Thailand, the incidence of *Herpes simplex* virus (HSV) serotypes 1 and 2 infection that occur respectively at the oropharyngeal and genital mucosa, or uticaria and is one of the important domestic diseases despite patients who manage to cure themselves of symptoms of this disease. Usually, the patient's skin at first becomes inflamed after getting HSV and then a liquid forms within a lump. After that, if the patient has good health and immunity [1-2], the lump breaks to form a scab and/or a scar.

Currently, HSV treatment uses acyclovir, valacyclovir and famciclovir medicines approved by the Food and Drug Administration (FDA), USA. These medicines selectively inhibit DNA replication of HSV with low toxicity to the host cells. Nevertheless, there is still demand for an alternative anti-HSV drug due to the occurrence of drug-resistant strains [3]. Therefore, herbs that have anti-HSV-1 activity are often chosen. Ethnomedical use of plants proves to be a useful guideline for plant selection which is further scientifically evaluated for their relevant medicinal properties. *Gynura procumbens* Merr. (Asteraceae) is one such choice although others are also known.

G. procumbens Merr. is a decumbent perennial herb prevalent mainly in Southern and Southeastern Asia. Local Thai people call it Pae Tam Peung or Jaknarai or Jin Jere Mao Yaea [1, 4]. In Thai folk medicine, patients with HSV infection have applied an ethanol extract of *G. procumbens* leaves on inflamed skin for relief [1]. The use of this traditional Thai medicinal plant to suppress the herpes virus inspired our study on the anti-HSV-1 activity of this plant. As an aside, this research was also interested in convenience and easy application during use. A product in gel form was first selected for this study. We also hoped that this research might be useful in decreasing the importation of HSV medicine from abroad.

Aims

To study the quantity of an ethanol extract of *G. procumbens* necessary in a gel to be effective against *Herpes simplex* virus serotype 1. In addition, the cytotoxicity and stability of the gel was studied as well as microbes that might contaminate the gels.

Materials and methods

Plant material: Fresh leaves of *G. procumbens* were collected at the Agricultural Research and Development Center, Nongpaklong Tampon, Nakhon Pathom Province, Thailand.

Extraction: The air-dried and ground leaves (78.8 g) of *G. procumbens* were extracted using a Soxhlet extractor sequentially with hexane and 50% ethanol (EtOH) until the extracting solvent was colorless. Then, the solvent was evaporated with a rotatory evaporator under reduced pressure to obtain a 50% EtOH crude extract of *G. procumbens* (24.8 g, 32%yield).

Preparation of anti-HSV-1 gel: Firstly, part A was stirred at room temperature using a Heidon instrument with a dispersion turbine at 650 rpm/min for 40 min or until a gel had been completely formed. Carbopol^{TM} in part A was neutralized to pH 7 with sodium hydroxide (NaOH). Sequentially, parts B, C and D were added with stirring. The prepared anti-HSV-1 gel, formulae I, II and III, including gel control, formula IV, were continuously stirred for 10 min. After that, all of them were measured for pH (Table 1).

Stability testing: All prepared anti-HSV-1 gels (formulae I, II and III) were tested for their stability, color, odor, texture, flowing, pH and viscosity, at room temperature (RT), 4^{o} and 60^{o}C [5], compared with gel IV as a control formulation. The analysis of color and viscosity used a Hunter lab and a Texture analysis instrument, respectively. All tested samples were contained in 250 mL of a glass bottom flask with a tightly closed covering. The analyses were recorded every week for 3 months.

Total microbe: Anti-HSV-1 gels (formulae I, II and III) and control gel (formula IV) were tested at four concentrations, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} % (v/v), during one week for a total microbe assay against fungi, bacteria and yeast and compared to gel formula V that contained the same components as formulae I, II and III but without MB:PB (1:1) as a preservative [6]. The total microbes were counted as Colony Forming Unit (CFU)/g. When the amount of CFU was too dense to count, it was recorded as TNTC (Too Numerous to Count).

Table 1 The components of the anti-HSV-1 (formulae I, II, III) and control (formula IV) gels

Part	Components	Weight (g) of each formula			
		I	**II**	**III**	**IV**
A	1. carbopol^{TM} as a substance to form gel	1.00	1.00	1.00	1.00
	2. NaOH as a base	0.36	0.36	0.36	0.36
	3. DI water	qs 100	qs 100	qs 100	qs 100
B	4. 50%EtOH *Aloe vera* extract as a healing wound healer	0.50	0.50	0.50	0.50
C	5. methyl paraben (MB) and propyl baraben (PB) (1:1) as preservatives	0.80	0.80	0.80	0.80
D	6. 50%EtOH *G. procumbens* crude extract	**0.10**	**0.50**	**1.00**	**-**
	pH*	7.0	6.9	6.8	7.0

*The pH of the anti-HSV-1 gel was desired to fall into the range of 6-7.

Bioassay:

- ***Anti-Herpes simplex virus type-1 (HSV-1) assay***

  Method**:** Green fluorescent protein (GFP) detection [7]

  Positive control: Acyclovir

  Negative control: 0.5%DMSO

  Final test concentration: 50 µg/mL

  Description: Before performing the anti-viral assay, compounds were tested over a range of what were considered to be non-cytotoxic concentrations to evaluate any cytotoxic effect to host cells. IC_{50} values are derived from dose-response curves generated by the SOFTMax Pro software (Molecular device) using 6 concentrations of 2-fold serially diluted samples. Acyclovir and 0.5% DMSO are used as a positive and negative controls, respectively.

- ***Cytotoxicity against primate cell line (Vero) assay***

  Method: Green fluorescent protein (GFP) detection [7]

  Positive control: Ellipticine

  Negative control: 0.5%DMSO

  Maximum test concentration: 50 µg/mL

  Description: The GFP-expressing Vero cell line was generated in-house by stably transfecting the African green monkey kidney cell line (Vero, ATCC CCL-81), with pEGFP-N1 plasmid (Clontech). The cell line is maintained in minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1.5 g/L sodium bicarbonate and 0.8 mg/ml geneticin, at 37^{o}C in a humidified incubator with 5%CO_2. IC_{50} values are derived from dose-response curves, as in the anti-HSV assay. Ellipticine and 0.5% DMSO are used as a positive and negative controls, respectively.

Results and discussion

The preparation of a model anti-HSV-1 gel was firstly formulated as a basified gel. An amount of NaOH used to neutralize each gel differed, because carbopol^{TM} as the gel-forming substance normally has a pH of 3-4 [8]. We desired the pH of the anti-HSV-1 gel to be in the range of 6-7.

The stability of three anti-HSV-1 gel formulae (I, II and III) were determined by comparison with the gel formula IV as a control. The color of gels I, II and III began to darken after 2 weeks at all temperatures and continued darkening at RT and 60^{o}C even after 3 months. Similarly, the odor of formulae I, II and III varied slightly at 4^{o}C, but all gave off a bad odor at RT especially at 60^{o}C after 3-4 weeks. The texture of the formulae I, II and III began to differ at RT and 60^{o}C after 6 weeks and developed a watery texture after 10 weeks. All of them provided different textures at 4^{o}C. After 2 weeks all anti-HSV-1 formulae had a greater rate of flowing at 4^{o}C than RT, whereas at 60^{o}C the flow rates were reversed. The pH of the formulae I, II and III were within specification under all conditions except gel IV had changed to a pH slightly greater than 7 only at RT. The viscosity of all gel samples were determined before and after 3 months and were found not to have changed at RT or 4^{o}C as compared to 60^{o}C. As a consequence of these studies, the anti-HSV-1 gels containing the *G. procumbens* extract should be kept continually in a refrigerator.

Only the gel III comprising 1% of the *G. procumbens* crude extract was tested for anti-*Herpes simplex* serotype 1 and cytotoxicity in Vero cells having the greatest amount of *G. procumbens* extract over the others. The concentration of the tested sample was 50 μg/mL. Unfortunately, both the gel and the crude extract itself were inactive for anti-HSV-1 activity at <50% of viral inhibition as compared with acyclovir as a positive control that had IC_{50} at value of 4.402 μg/mL. However, both displayed non-cytotoxicity against Vero cells comparing to ellipticine as a positive control that had IC_{50} at value of 0.564 μg/mL (Table 2).

Table 2 The results of anti-HSV-1 and cytotoxicity activities of the anti-HSV-1 gel formula III

The tested sample[1]	Anti-HSV-1 activity[2, *]	%Viral inhibition	Cytotoxicity Activity[3, #]	%Cell growth
Anti-HSV-1 gel III	Inactive	<50	non-cytotoxic	>50
50%EtOH *G. procumbens* extract	Inactive	<50	non-cytotoxic	>50

[1]The maximum concentration of the tested sample was 50 μg/mL; [2]The IC_{50} of 4.402 μg/mL of acyclovir as a positive control; [3]The IC_{50} of 0.564 μg/mL of ellipticine as a positive control; [*]Anti-HSV-1 activity: <50% (inactive), ≤50% (active); [#]Cytotoxicity against Vero cells: >50% (non-cytotoxic), ≤50% (cytotoxic)

Sriripen *et al*. first reported that a 95% EtOH *G. procumbens* extract showed antiviral activity against *Herpes simplex* virus serotypes 1 and 2. Also, 3,5- and 4-5-di-*O*-caffeoylquinic acids were isolated from *G. procumbens* and shown to be important active compounds for this virucidal activity

[1]. Normally, 50% EtOH is more polar than 95% EtOH. Therefore, 50%EtOH solvent have successfully extracted any 3,5- and 4-5-di-*O*-caffeoylquinic acids. Nevertheless the source of collected *G. procumbens* and/or age of the leaves of *G. procumbens* are likely key factor in their activity.

Finally, all anti-HSV-1 gels including gel formula IV as the control and formula V comprising 50% EtOH *G. procumbens* and *A. vera* extracts but without MB:PB (1:1) were evaluated for total microbes, including fungi, bacteria and yeast. Consequently, it was found that the total microbes of formulae I, II, III and IV displayed after a week values of 110×10^3, 160×10^3, 180×10^3 and 180×10^3 CFU/g, respectively, whereas the gel formula V showed a value of 910×10^3 CFU/g. The total microbe count of all formulae was TNTC after 24 hours. It should be noted that MB:PB (1:1) would not inhibit a Gram negative (-ve) bacteria, because the paraben esters group are be effectiveness at pH 8 [9], not pH 7 of our gels.

Conclusions

A possibility creating an anti-HSV-1 gel was studied in 4 stages. The air-dried and ground leaves of *G. procumbens* were extracted with 50% EtOH to obtain a 50% EtOH crude extract which was added in dfferent amounts to a suitable basified gel for the preparation of three anti-HSV-1 gel formulae. These anti-HSV-1 gels showed their greatest stability of color, odor, texture, flowing, pH and viscosity at 4°C. Unfortunately, the anti-HSV-1 gel was inactive for inhibition of *Herpes simplex* virus type 1, but was non-cytotoxic against Vero cells at the maximum concentration of 50 µg/mL. Furthermore, the analysis of total number of microbes after one week gave a result that had a value of $>1\times10^3$ CFU/mL. We plan to continue this research using 2-methyl-4-isothiazolin-3-one hydrochloride as a preservative for inhibition of a Gram -ve bacteria and will look for for a marker compound to judge the anti-viral quality of *G. procumbens*.

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