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Original article

Anti-herpes simplex virus activities of bioactive extracts from *Antrodia camphorata* mycelia

Yuan-Chuan He¹, Zeng-Hui Lu¹, Ping Shi¹, Jing-Chao Hao², Zong-Jie Zhao³, Hai-Tao Xie³, Ping Mao⁴, Shi-Jiang Chen^{1*}

¹Chongqing Academy of Chinese Materia Medica, Chongqing, China

²Kunming Medical University, Kunming, China

³Hong Kong Academy of Chinese Medicine Science, Hong Kong, China

⁴Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu, China

*Corresponding author e-mail: shijiangchen@163.com

Abstract:

Background: *Antrodia camphorata*, known as a Traditional Chinese Medicine (TCM), are widely used in treatment liver diseases and cancers. Besides those important activities, other biological activities, such as anti-inflammatory have been described. Herpes simplex virus infections represent one of the most serious public health concerns globally because of their devastating impact. Searching for new antiviral agents, especially those with different mechanisms of action, is a crucial goal and an unmet need for alternative and complementary therapy against HSV infection. In this study, an antiherpes screening was performed to obtained extracts from *A. camphorata* mycelia.

Methods: MTT method and FIC theorem and median-effect principle were used to evaluate anti-viral activity and calculate drug combination effect.

Results: The crude ethanol extracts and isolated constituents showed inhibition of HSV replication at very low concentration. Fraction A and antrodin A showed viral inhibitory effect with reduction of viral cell-to-cell spread. In addition, neither fraction A nor antrodin A showed interaction in combination with acyclovir.

Conclusions: *A.camphorata* mycelia and antrodin A might be potential to use as anti-HSV agents and promising for future antiviral drug design.

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Running head: Anti-HSV of *Antrodia camphorata*

Introduction

Herpes simplex viruses (HSV) are a common human pathogen that causes herpes labialis, herpes genitalis, keratitis and encephalitis. The HSV infection caused by type-1 (HSV-1) and type-2 (HSV-2) is mainly transmitted by close personal contact, and the virus can establish lifelong latent infection in sensory neurons with recurrent lesions [1]. HSV-2 remains the main cause of genital herpes and is the major etiology of genital ulcer diseases, and possibly causes life-threatening infection in

immune-compromised people and neonates [2]. HSV-2 infection has been proved to be an independent cofactor of HIV-1 sexual transmission and replication [3,4]. A recent study reported that HSV-suppressive therapy greatly reduced genital and plasma HIV RNA levels in co-infected patients [5]. Hence, reducing the spread of genital herpes could greatly decrease the risk of acquiring or transmitting HIV infection.

Long-time clinical use of acyclovir and its derivatives ganciclovir, valacyclovir as anti-virus agents could result in the development of resistance [6]. Foscarnet has effect on drug-resistant virus with severe side effects [7]. However, acyclovir also probably incorporate into the cellular DNA with inhibition of viral DNA pol, yielding adverse drug reactions, and thus unsuitable for pregnant women and neonates [8,9]. Moreover, the major determinants of effective immunity against HSV infection are not yet identified, and animal efficacy has not predicted success in humans. Furthermore, the therapeutic vaccines failed to induce antibody-specific responses to protect recipients from recurrences [10]. The development of new antiviral molecules derived from acyclovir increases the selection pressure risk of resistant strains that have been observed in vivo since the first large therapeutic trials. Therefore, Searching for a new antiviral agent, especially that with different mechanisms of action is a crucial goal and an unmet need for alternative and complementary against HSV infection.

Antrodia camphorata is a basidiomycete endemic to Taiwan, which is a fungal parasitized on the inner cavity of the endemic species *Cinnamomum kanehirae* Hayata (Lauraceae) [11]. It is well known as a TCM, and Taiwanese aborigines used this species for the prevention, or treatment, of numerous diseases including liver diseases, food and drug intoxication, diarrhea, abdominal pain, hypertension, tumorigenic diseases and itchy skin [12,13]. It was reported that ethanol extract from the mycelia of *A.camphorata* displayed anti-hepatitis B and C virus activity in dose-dependent manner without cytotoxicity and some compounds were isolated from the ethanol extract [14–17].

In this report, we have evaluated anti-virus effect of several extracts from *A.camphorata* for anti-virus activities and found that ethanol extract have potential inhibition effect on HSV. Based on traditional use in skin infections, the aim of the present work is to evaluate the in vitro antiviral activity of the crude ethanol extract, most active fractions, and the isolated compound(s) from the active fraction of *A.camphorata* mycelia, which would be the alternative and supplementary agents for anti-HSV.

Material and methods

***A.camphorata* material**

A.camphorata CCRC35396 was obtained from the ATCC, USA. Mycelia powder of *A. camphorata* kindly provided by CASW Co. LTD (Shenzhen, China) was collected from the submerged fermentation of *A. camphorata*. The mycelia were firstly freeze dried and baked in an air oven at 60°C until constant weight, then pulverized by mechanical grinder and passed through 60 mesh sieve to get the fine powder. *A.camphorata* dry mycelia powder (1kg) was extracted three times with 95% ethanol (m/v, 1:30) at 70°C water bath for 6h. The whole extracted filtrate was evaporated to dryness under reduced pressure in an Eyela Rotary Evaporator (Japan) at 50-55°C. The concentrated crude ethanol extracts

(50g) was added CHCl_3 and mixed approximately 1-5 minutes. The addition and mixing were repeated by 4 times. The CHCl_3 fraction was chromatographed on silica gel eluted with n-hexane-acetone (19:1-14:6) to give six fractions. Thin-layer chromatography (TLC) using precoated aluminium plates (E. Merck, Germany) were used to monitor all the eluted fractions and got two major condensed fractions A and B. Fraction A was chromatographed on silica gel (BW-820MH) to give compound A. The IR spectra were measured with JASCO-FT/IR-230 infrared spectrophotometer. ^1H and ^{13}C NMR spectra were measured with a Varian Unity Plus 500 spectrometer. EIMS and HREIMS were measured with a JEOL JMS-AX 505 HAD mass spectrometer at an ionization voltage of 70 eV.

Viruses and cell

African green monkey kidney epithelial Vero cells purchased from Cell Bank of Chinese Academy of Science (shanghai, China), was grown and maintained in high glucose DMEM (Invitrogen) with combination of penicillin and streptomycin (100IU ml^{-1} and $100\mu\text{g ml}^{-1}$), supplemented with 5–10% fetal bovine serum (FBS). HSV-2 G and HSV-1F obtained from the ATCC were used for the standard strain. After plaque purification, the viruses were grown and stocks were stored at -80°C for future study [18], and whenever the virus stocks required were grown firstly on Vero cells to determine the titers for next experiments.

Anti-virus effect evaluation

The cytotoxicity of extracts against Vero cell line was determined by MTT cell viability assay. Briefly, Vero cells were cultured onto 96 well plates at 1.0×10^5 cells ml^{-1} and then added different concentration extracts for 72h, with DMSO dissolved in DMEM (0.1%) as negative control. $10\mu\text{l}$ MTT reagent (5mg ml^{-1} in phosphate buffer solution) was added to each well and incubated for 4h. Then old medium containing MTT was replaced by diluted HCl (0.04mol L^{-1}) in isopropanol to dissolve formed formazan crystals, and the absorbance was read at 570 nm with a reference wavelength of 690nm by an ELISA reader. After incubation, the 50% cytotoxic concentration of each one was calculated as the concentration that reduces cell viability by 50%, when compared to the untreated control.

Subsequently, the potential antiviral activity of samples against HSV-1 and HSV-2 was evaluated by MTT assay [19]. Vero cells were seeded onto 96 well plates with a concentration of 1.0×10^5 cells ml^{-1} . After incubation at 37°C in 5% CO_2 for 6h, the viruses (2 MOI) and different concentrations of extracts were added to culture wells in triplicate at a final volume of $100\mu\text{l}$ in each well. The maximum concentration of DMSO (0.1%) was used as negative control and acyclovir as positive control throughout the study. After incubation at 37°C in 5% CO_2 for 3 days, the antiviral concentration of 50% effectiveness (EC_{50}) was required to achieve 50% inhibition of virus-induced cytotoxic effects and calculated by standard method. IC_{50} and EC_{50} values were estimated by linear regression of concentration-response curves generated from the data. The selectivity index ($\text{SI} = \text{CC}_{50}/\text{EC}_{50}$) was calculated for each sample.

Dose–response assay

To analyze the dose-dependent effect of the test drugs on infected Vero cells, different concentrations of fraction A or antrodin A was added to HSV-1 and HSV-2 infected Vero cell culture in triplicate. After 2–3 days, MTT assay was carried out to determine the inhibition of infection caused by the HSV, as described previously.

Viral plaque assay

Plaque size reduction assay evaluated the effect of the test agent on inhibition of infection of Vero cells by the free virus particles and thereby the number of viral plaques formed in cell monolayer, as every viral particle non-neutralized by the test agent will infect the cells and formed a plaque. To evaluate the antiviral activity of fraction A and antrodin A and to compare its activity with acyclovir, Serial dilutions of those in DMEM was added to the infected cells (MOI: 2) and incubated at room temperature, prior to the addition to cells. After 2 h incubation at 37°C in 5% CO₂, the cells were washed with fresh DMEM and overlaid with methylcellulose, so the virus can spread via cell-to-cell route to form plaques. The plaques that developed after 3 days of incubation were stained with crystal violet. The effective concentration of fraction A and antrodin A that inhibited the number of viral plaques by 50% (EC₅₀) was interpolated from the dose-response curves.

Time-of-addition assay

Time-of-addition assay was used to investigate the mechanism of inhibition of the infection of HSV by fraction A and antrodin A at various time periods up to 24 h. Vero cells at 1.0×10^5 cells ml⁻¹ were grown onto 96 well plates at 37°C in 5% CO₂. To detect whether the extract has effect on the virus particle, virus dilution was incubated with the different concentrations of the fraction A and antrodin A for 4h at 4°C respectively. After incubation, cells were infected with treated virus dilution and cultured in 5% CO₂ (pre-infection). After cell plates washing, Vero cell monolayers were infected with HSV (MOI: 2) and incubated at 37°C for 1h, then replaced by DMEM with 10% FBS (co-infection). Different concentration of extracts was added to the cells at intervals from 1 to 24h post-infection (post-infection). DMSO (0.1%) and acyclovir were as a negative and positive control respectively. After incubation at 37°C in 5% CO₂ for 3 days, the MTT assay was carried out as described above.

Synergistic effects in combination with acyclovir

Potential synergistic effect of fraction A combination with acyclovir and antrodin A combination with acyclovir at the ratio (1:1), was evaluated against HSV-1 and HSV-2 (MOI: 2) by MTT assay, according to experimental design [20]. The degree of interaction between fraction A, antrodin A and acyclovir was calculated through fraction inhibitory concentration (FIC), based on the median-effect principle of the mass-action law. According to the FIC theorem, FIC value indicates synergism (<0.5), additive effect (0.5-4), and antagonism (>4), respectively.

Statistical analysis

The obtained data expressed as mean ± S.D. for three independent experiments were analyzed using DPS ver. 9.50 (Zhejiang university, Zhejiang, China). To analyze the significance of experimental

groups, P-value was utilized to determine the difference compared to control group. FIC were calculated by Calcsyn ver2.0.

Results

Characterization of antrodin A

Compound A gave yellow oil and the molecular formula $C_{19}H_{23}NO_3$ was assigned by HREIMS. The mass spectra of compound A had the peak at m/z 314 (M+) with significant fragment ion peaks at m/z 246 and 131. The intense peak with highest mass number at m/z 414 is due to parent molecular ion. The IR spectrum revealed carbonyl absorption of acid anhydride at 1763 cm^{-1} . The ^{13}C NMR spectrum showed signals of four methyl carbons, two methylene carbons, and one methine carbon in the aliphatic region, as well as one benzene ring, one olefinic group, and two carbonyl carbons. The ^1H NMR spectrum showed the presence of an isobutyl moiety at δ 0.94, 2.12, and 2.59, a 3-methyl-2-butenyloxy moiety at δ 1.76, 1.81, 4.57, and 5.50, and a para-substituted benzene moiety at δ 6.95 and 7.50, which was further supported by ^1H - ^1H COSY and HMQC experiments. On the basis of the molecular formula and the ^{13}C NMR spectrum published previously by Nakamura N, this compound was defined as antrodin A [15].

Evaluation of anti-virus activity

The cytotoxicity and antiviral activity results of extracts isolated from *A. camphorata*, with selectivity index were showed in table 1. Excepted fraction B, another extracts exhibited low cytotoxic effect with significantly antiviral activity. The CC_{50} of crude ethanol extract, fraction A and antrodin A were $485\mu\text{g ml}^{-1}$, $197\mu\text{g ml}^{-1}$ and $110\mu\text{g ml}^{-1}$ respectively. Based on their EC_{50} value and selectivity index (SI), the EC_{50} of fraction A (8.2 ± 1.8 and $7.8\pm 1.6\mu\text{g ml}^{-1}$), and antrodin A (5.8 ± 1.1 and $5.5\pm 0.5\mu\text{g ml}^{-1}$) against HSV-1 and HSV-2 demonstrated stronger anti-HSV activity, compared to the crude ethanol extract ($p < 0.01$). Further, the EC_{50} and SI index indicated that fraction A and antrodin A were more active against HSV-2 than HSV-1. On the other hand, fraction B had CC_{50} and EC_{50} at higher concentration with very low SI index, indicating its inactiveness, compared to acyclovir.

Dose-dependent effect of extracts

To analyze the dose-dependent antiviral activity, different concentrations of fraction A and antrodin A were treated on HSV-1 and HSV-2 infected Vero cells, along with acyclovir and DMSO (0.1%) as positive and negative control respectively. Figure1 showed fraction A and antrodin A were found to inhibit both HSV-1 and HSV-2 infection(s) in dose-dependent manner within certain range, with an EC_{50} of $8.2\mu\text{g ml}^{-1}$ and $5.8\mu\text{g ml}^{-1}$ for HSV-1 and 7.8 and $5.5\mu\text{g ml}^{-1}$ for HSV-2 respectively. However, more than 90% inhibition of HSV-1 was recorded at $15\mu\text{g ml}^{-1}$ of fraction A and $12.5\mu\text{g ml}^{-1}$ for antrodin A, while for HSV-2 it was $14.5\mu\text{g ml}^{-1}$ of fraction A and $10\mu\text{g ml}^{-1}$ of antrodinA. However, the antiviral activity increased gently when extracts concentration out of the EC_{90} .

Assessment of plaque reduction assay

The reduction of HSV-1 and HSV-2 cell-to-cell spread was evaluated by comparing viral plaque areas between treated and untreated controls. Plaque reduction assay was also used to assess the anti-viral activity of fraction A and antrodin A, using acyclovir and DMSO (0.1%) as positive and negative control respectively. Although both fraction A and antrodin A at concentrations of 5-100 $\mu\text{g ml}^{-1}$ inhibited plaque formation by HSV-1 and HSV-2, indicating their dose dependent inhibitory activity (figure 2), antrodin A reduced more viral plaque numbers. The control drug acyclovir showed nearly 100% inhibition of plaque formation at 10 $\mu\text{g ml}^{-1}$, while no inhibition was noticed with 0.1% DMSO (data not shown).

Mechanism analysis of extracts

To investigate the mechanism of antiviral activity, time course analysis was performed with fraction A and antrodin A respectively. After 3 days of infection and expressed as percentage inhibition evaluated by MTT assay. The result showed that fraction A at 7.8 and 15 μgml^{-1} inhibited HSV-1 (Figure3 A) and HSV-2 (Figure3 B) significantly ($p < 0.01$) within 0–5h co-infection and post-infection. Before infection, the infectivity of virus treated with fraction A and antrodin A was inhibited weakly. However, the extracts and virus co-infected Vero cells for 1h, viral infectivity was significantly suppressed, probably interfering viral attachment and suppression of activation of signaling pathway. From 1 to 4h p.i, the inhibition of fraction A and antrodin A increased slowly and at peak at 4h. However, after 5h post infection, anti-herpes activity decreased with addition of fraction A or antrodin A, suggesting gently inhibition of late multiplication of virus.

Drug- extracts interaction

To evaluate whether the fraction A or antrodin A can able to increase the inhibitory efficacy of acyclovir in combination, we have tested anti-HSV activity of the mixture of fraction A and acyclovir and antrodin A and acyclovir by MTT assay, using FIC theorem and median-effect principle. Our results demonstrated that the EC_{50} of fraction A, antrodin A and acyclovir was 7.8, 5.5 and 2.9 $\mu\text{g ml}^{-1}$ respectively, but in combination with acyclovir the mean EC_{50} was 3.5 and 3.2 $\mu\text{g ml}^{-1}$ respectively. Moreover, the FIC index of 0.77 (between acyclovir and fraction A) and 0.83 (between acyclovir and antrodin A), indicated that there was additive effect without synergistic interaction between them (Table 2). Furthermore, none of these combinations exhibited cytotoxic effect against Vero cell (data not shown).

Discussion

The present study for the first time, demonstrated the anti-HSV activity of crude ethanol extract of *A. camphorata* mycelia powder, a treasure medical mushroom of Taiwan, China. Phytochemical study revealed that the crude ethanol extract contain two major fractions, fraction A and B, of which fraction A had significant anti-HSV activity, with higher antiviral activity than the crude ethanol extract, partly due to the bioactive compounds within the fraction A. The revealed that active extracts significantly suppressed virus replication when that added in Vero cells from 1 to 4 h post infection. Activation of

signal pathway is essential for herpes virus type2 replication. The previous reports that activation of ERK pathway reached peak after 4h post infection. U0126 is a repressor of signal pathway and blocked virus replication [20]. Test inhibition effect on activation of signal pathway induced by virus and the exploration of anti-HSV mechanism of antrodin A are the next works. Acyclovir specifically interfered DNA pol, inhibited virus replication and suspended extension of DNA chain, which is the main clinical anti-herpes drug and effective in the treatment of HSV infection, but reports have indicated that treatment failures result from antiviral-resistant HSV [21]. Moreover, efficacy of therapeutic vaccines against primary and recurrent HSV infection has failed [22], and thus, search for natural alternative is the top priority to control and prevent HSV infections and transmission. The earlier reports indicated that fruiting body and mycelia of *A.camphorata* contain several secondary metabolites like terpenoids, diterpenic lactones, triterpene, benzenoids, lignans, benzoquinone derivatives, succinic and maleic derivatives, with anti-cancer, anti-inflammatory, anti-oxidant, hepatoprotective and anti-hepatitis B and C replication activities [23]. Antrodin A-E belonged to succinic and maleic derivatives, showing effects of anti-cancer, anti-inflammatory and anti-hepatitis B/C [14,15,17,24,25]. In conclusion, antrodin A and extracts from *A. camphorata* have antiviral effects, presumably a new antiviral mechanism different from acyclovir and could be alternative and complementary agents.

Acknowledgement statement

We thank CASW CO. LTD for kindly providing *A.camphorata* mycelia powder for our experiments.

Disclosure statement

There are no conflicts of interest.

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Figures

Figure 1

Dose dependent activity of antrodin A, fraction A and acyclovir on HSV-1 [A] and HSV-2 [B]. Different concentrations of fraction A, antrodin A and acyclovir ($2.5-15 \mu\text{g ml}^{-1}$) were added to HSV-1 and HSV-2 infected Vero cells at 0h. Inhibition percentage was evaluated by MTT assay after 3 days of incubation at 37°C . Each point represents the mean \pm S.D. of three independent experiments.

Figure 2

Plaque reduction results of HSV-1 [A] and HSV-2 [B] with fraction A, antrodin A and acyclovir. Different concentrations of fraction A, antrodin A or acyclovir ($5-100 \mu\text{g ml}^{-1}$) were added to HSV-1 and HSV-2 infected Vero cells. After 2h incubation at 37°C , the cells were overlaid with methylcellulose and the plaques developed after 3 days of incubation were stained with crystal violet. The plaque number inhibition was calculated, and the effective concentration of fraction A and antrodin A that inhibited the number of viral plaques was interpolated from the dose-response curve.

Figure 3

Inhibitory effect of fraction A during pre-infection, co-infection and post-infection on HSV-1 [A] and HSV-2 [B]. Different concentrations of fraction A ($7.8 \mu\text{g ml}^{-1}$ and $15 \mu\text{g ml}^{-1}$) were added with the HSV-1 and HSV-2 infected Vero cells at -1h (pre-infection), 0h (co-infection) and 1-24h (post-infection). After 3 days of incubation at 37°C , inhibition was evaluated by MTT assay and expressed as the inhibition percentage. Each point represents the mean \pm SD of three independent experiments.

Table 1 Cytotoxicity and anti-HSV activity of extracts isolated from *A.camphorata*

Test drug	CC ₅₀ ($\mu\text{g ml}^{-1}$)	HSV-1 F at MOI: 2		HSV-2 G at MOI: 2	
		EC ₅₀ ($\mu\text{g ml}^{-1}$)	SI	EC ₅₀ ($\mu\text{g ml}^{-1}$)	SI
Crude extract	485.0	61.2 \pm 5.5	7.92	57.5 \pm 2.4	8.43
Fraction A	197.0	8.2 \pm 1.80	24.02	7.8 \pm 1.6	25.25
Fraction B	235.0	120.0 \pm 3.5	1.96	105.0 \pm 7.2	2.24
Antrodin A	110.0	5.8 \pm 0.5	18.97	5.5 \pm 1.1	20.00
Acyclovir	130.0	2.1 \pm 0.1	61.90	2.9 \pm 0.1	44.80

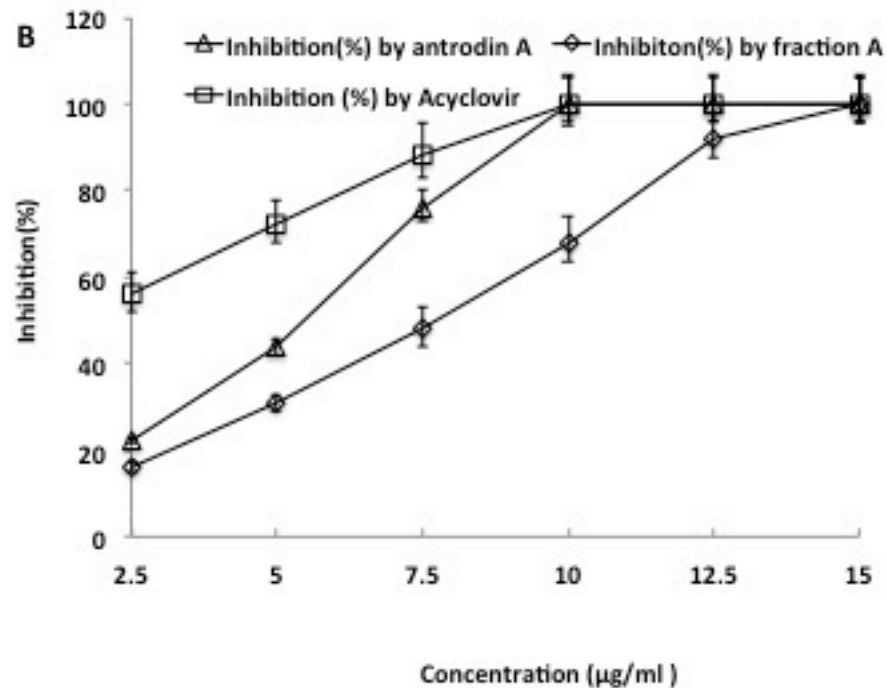
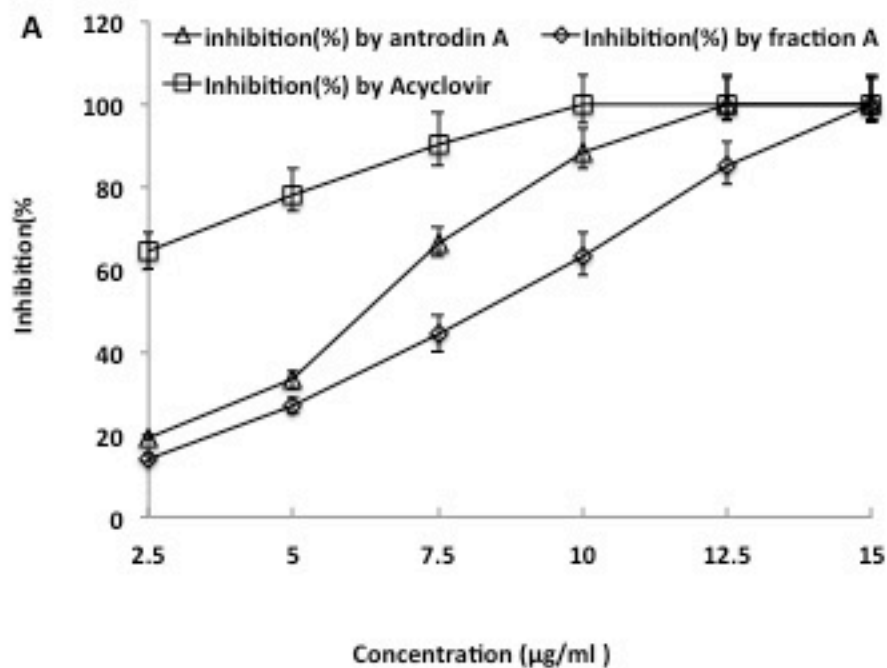
CC₅₀ is the 50% cytotoxic concentration for Vero cells ($\mu\text{g ml}^{-1}$). EC₅₀ is the 50% inhibitory concentration ($\mu\text{g ml}^{-1}$). SI is the selective index values. These values represent the mean \pm SD of three independent experiments.

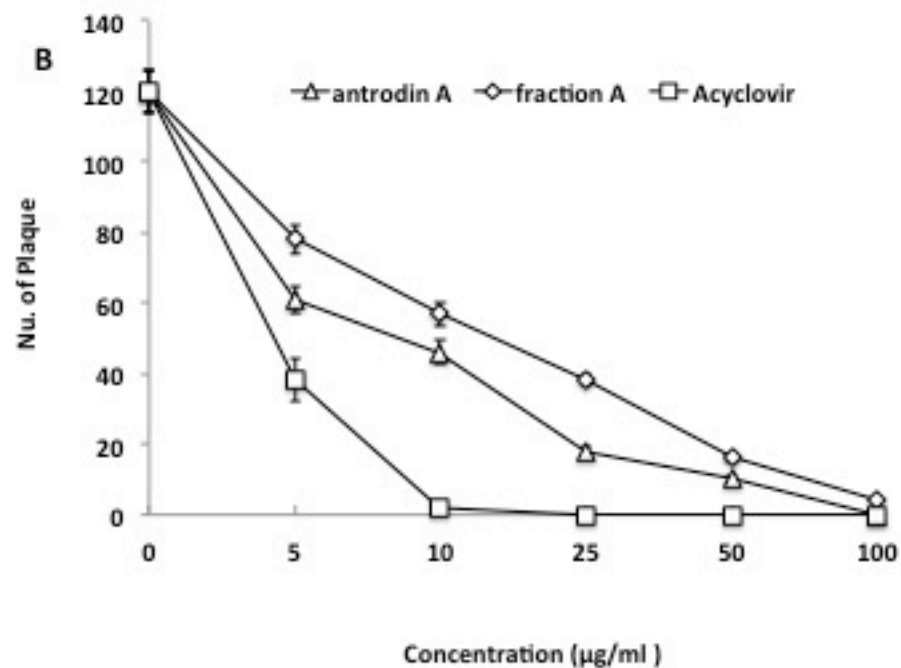
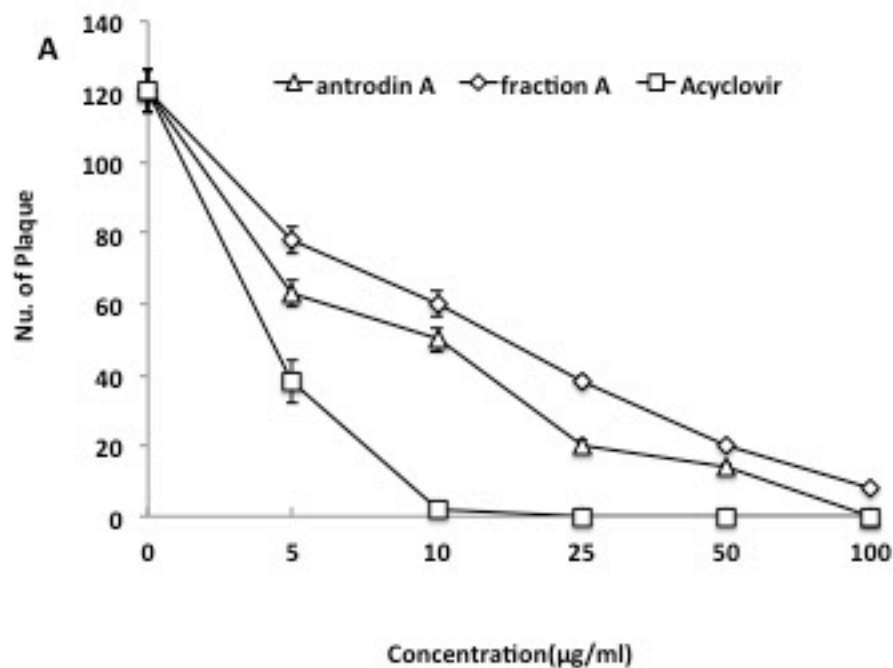
Table 2 Effects of extracts in combination with acyclovir on HSV-2 G infected Vero cells

Extracts	Mean EC \pm SD ^a	^b FIC _{fraction/compound} +FIC _{acyclovir}	Inhibitory effect
Fraction A	7.8 \pm 1.6	-	-
Antrodin A	5.5 \pm 0.54	-	-
Acyclovir	2.9 \pm 0.1	-	-
Acyclovir+Fraction A	3.5 \pm 0.15	0.83	Additive effect
Acyclovir+Antrodin A	3.2 \pm 0.18	0.77	Additive effect

^a Results are the mean of three independent experiments of MTT assay.

^b FIC_{fraction/compound} + FIC_{acyclovir} are FICs of fraction/compound and acyclovir, respectively. FIC value indicates synergism (<0.5), additive effect (0.5-4), and antagonism (>4)





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