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# ***In vitro* Anti HSV-1 and HSV-2 Activity of *Tanacetum vulgare* Extracts and Isolated Compounds: An Approach to their Mechanisms of Action.**

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**ABSTRACT**

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Herpes simplex viruses (HSV-1 and HSV-2) are responsible for long-term latent infections in humans, with periods of recurring viral replication associated to lesions around the lips, eyes, mucous membrane of the oral cavity or the genitals. The lack of an effective vaccine, the moderate to high toxicity of the available synthetic anti-herpes compounds and the appearance of resistant viral strains emphasise the need for new inhibitors. *Tanacetum vulgare*, commonly known as tansy, has been used for treating rheumatic pain, skin eruption, and diuretic conditions as well as an anthelmintic, antihypertensive, stimulant, emmenagogue, carminative, antiseptic, antihypertensive, antispasmodic and antioxidant agent. The anti HSV-1 activity of tansy aerial parts, ethyl acetate extract and the isolated compound parthenolide, has recently been reported. In this work, through a comprehensive mechanistic-based antiherpetic activity study, we revealed that constituents other than parthenolide are responsible for the antiviral activity of tansy.

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**KEYWORDS:** *Tanacetum vulgare*, tansy, antiviral, HSV, ethnomedicine

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## INTRODUCTION

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Herpes simplex viruses (HSV) are members of the *Herpesviridae* family, which are known to cause long-term latent infections with periods of recurring viral replication (Whitley & Roizman 2001). The two common human pathogenic HSVs are type 1 (HSV-1) and type 2 (HSV-2), which cause lesions around the lips, eyes, mucous membrane of the oral cavity and the genitals. Acyclovir (ACV) is the most commonly used chemotherapy agent against HSV-1 and HSV-2, in systemic or topical therapy. Other clinically relevant drugs include valaciclovir, penciclovir, famciclovir and vidarabine (Gupta *et al.* 2007). However, these drugs are not always well tolerated and drug-resistant strains are becoming a major problem, especially in immunocompromised patients (e.g. HIV infected patients). Moreover, the incidence of HSV-2 infections (main cause of genital herpes) not only raised in recent years but also have been linked with an increased (three-fold) risk of sexually acquired HIV infection (Freeman *et al.* 2006). Therefore, anti HSV drugs with novel modes of action are urgently needed.

*Tanacetum vulgare* L. (Asteraceae), commonly known as tansy, is an herbaceous plant growing in temperate Europe and Asia. Various preparations of tansy are known to be used for treating rheumatic pain, skin eruption, and diuretic conditions (Ognyanov & Todorova 1983; Ognyanov *et al.* 1983; Lahlou *et al.* 2007). Tansy has also been reported as an anthelmintic, antihypertensive, stimulant, emmenagogue, carminative, antiseptic, antihypertensive and as an antispasmodic agent (Grieve 1931; Barnes *et al.* 2002). Among the most studied biological activities of tansy are the antioxidant (Juan-Badaturuge *et al.* 2009), anti-inflammatory, diuretic and wound-healing effects (Williams *et al.* 1999; Xie *et al.* 2007; Lahlou *et al.* 2007; Pålsson *et al.* 2008). Antiherpes virus activity of the crude ethyl acetate extract of aerial parts of tansy of Brazilian origin has recently been reported (Onozato *et al.* 2009). This study further revealed that parthenolide was one of the major anti HSV-1 principles of tansy aerial parts. Our present study on tansy aerial parts, however revealed the presence of known antiviral agents including 3,5-dicaffeoylquinic acid (3,5-DCQA) which was isolated as a major constituent of the plant (Juan-Badaturuge *et al.* 2009). Through a

comprehensive mechanistic-based antiherpetic activity study, we now reveal that constituents other than parthenolide are responsible for the antiviral activity of tansy.

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## MATERIALS AND METHODS

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### *Plant material and extraction procedures.*

The extraction and isolation of antioxidant compounds from the aerial parts of *Tanacetum vulgare* were described previously (Juan-Badaturuge *et al.* 2009). Briefly, *T. vulgare* from an authenticated source grown at Hadlow Agricultural College medicinal gardens (Hadlow, UK) was harvested during its flowering stage (July 2006). The dried plant material was extracted by cold methanol and fractionated using solvents of ascending polarity: petroleum ether, chloroform, ethyl acetate, butanol and water. Further activity-directed fractionation of the most antioxidant fraction (ethyl acetate) resulted in the isolation of the most abundant constituent 3,5-DCQA together with axillarin and luteolin (Juan-Badaturuge *et al.* 2009). The underground rhizome of the plant was harvested from the same source in March 2009 and allowed to air-dry. A portion of the plant material (1 kg) was soaked in 7.5 L methanol and left in the dark for two weeks. The extract was collected and dried using a rotary evaporator to yield 132 g of the extract residue.

### *Quantification of parthenolide by HPLC.*

An Agilent 1200 series gradient HPLC system composed of a degasser (G1322A), a quaternary pump (G1322A), an auto sampler (G1329A), a thermostat column compartment (G1316A) maintained at 25°C and a diode array detector (G1315) was used. Solutions of the standard parthenolide (Sigma-Aldrich, Dorset, UK) and various plant extract fractions were made in acetonitrile. Samples (20 µL) were injected onto a reverse phase column (Agilent-Eclipse XDB-C18, 5 µm, 4.6 x 150 mm). The mobile phase was a mixture of acetonitrile (A) and 10 mM potassium phosphate buffer (pH=3.0) (B). The composition of the mobile phase at a flow rate of 1.5 min/ml was maintained at 41% A for the first 8 minutes and then raised to 70% A in 5 minutes. The parthenolide concentrations in the crude extract and fractions were quantified from the standard curves constructed from three-fold standard parthenolide dilutions made from a stock solution of 5 mg/ml. All experiments were repeated at least six times and the amount of parthenolide was calculated based on the peak area at  $\lambda = 210$  nm using

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3 GraphPad InStat software (GraphPad, San Diego, USA). The calculated  $r^2$  values of the  
4 standard curves were in the range 0.998 to 0.999.  
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#### 8 *Viruses and cells.*

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10 HSV-1 and HSV-2 clinical isolates were kindly provided by Dr. María Oña (Servicio de  
11 Microbiología, Hospital Universitario Central de Asturias. Spain) and propagated in  
12 African green monkey kidney (Vero) cells (ECACC No. 84113001). Virus titrations  
13 were performed using both the endpoint dilution method described by Reed and  
14 Muench (1938), and by plaque assay. Vero cells were propagated in Dulbecco-modified  
15 Eagle's minimal essential medium (DMEM) (Gibco BRL) supplemented with 10%  
16 foetal bovine serum (Sigma) and maintained in DMEM without bovine serum.  
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#### 24 *Cytotoxicity assay.*

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26 Vero cells were seeded into 96-well plates at a density of  $2 \times 10^4$  cells/well, and  
27 incubated at 37 °C in a 5% CO<sub>2</sub> atmosphere for 48-72 h, until 90% or greater  
28 confluency of the monolayers was reached. Increasing concentrations of the test extract  
29 were added to cells, with a replicate number of six wells per concentration. After a 3-  
30 days incubation period, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide  
31 (MTT) solution was added (final concentration 0.5 mg/mL) and the plates were  
32 incubated for another 4 h to allow formazan production. The solid precipitate was  
33 dissolved with absolute 2-propanol and the absorbance at 570 nm was measured using a  
34 96-well  $\mu$ Quant Spectrophotometer (Biotek Instruments) at 620 nm. The resulting cell  
35 viability was calculated as described previously (Mosmann, 1983).  
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#### 46 *Antiviral activity assay*

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48 Ninety-six-well plates containing confluent cell monolayers were preincubated for 1 h  
49 with increasing non-cytotoxic concentrations of the extracts. Six replicate wells were  
50 used for each analyzed sample. Afterwards, 10 Tissue Culture Infectious Dose<sub>50</sub>  
51 (TCID<sub>50</sub>) of HSV-1 or HSV-2 were added into each well. The plates were incubated at  
52 37 °C in a 5% CO<sub>2</sub> humidified atmosphere and observed daily for cytopathic effect  
53 (CPE) using a light microscope. When CPE was observed in all virus control wells, the  
54 percentage of wells with CPE was determined for each treatment concentration.  
55 Acyclovir (ACV) at concentrations varying from 0.1 to 50  $\mu$ g/mL served as positive  
56 control.  
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5 *Time-of-addition assay.*

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7 The effect on HSV replication of tansy extracts or purified fractions, added to cell  
8 cultures at different times before, during or after infection was investigated as described  
9 previously (Alvarez *et al.* 2009) with some modifications. The analyzed sample was  
10 added to the first series of wells at a fixed non-cytotoxic concentration (100 µg/mL for  
11 ethyl acetate and petroleum ether; 500 µg/mL for rhizome extract; 300 µg/mL for  
12 DCQA; and 5 µg/mL for parthenolide) and incubated for 1 h. All wells in the plate were  
13 then infected with 200 pfu of HSV-1 or HSV-2 and the same concentration of plant  
14 sample was added to the second series of wells at the time of infection. After 1 h of  
15 adsorption, the medium was removed from each well and fresh medium containing the  
16 plant extract was added to the first, the second and the third series of wells  
17 corresponding to -1, 0 and 1 h post-infection (hpi). Subsequent additions were  
18 performed 2, 4 and 6 hpi. After the last addition of compound the culture medium was  
19 replaced by a 1.5% methylcellulose overlay containing an identical concentration of the  
20 test extract. The plates were further incubated at 37°C in a 5% CO<sub>2</sub> atmosphere for 96 h,  
21 or until the appearance of evident viral lysis plaques in the mock-treated (virus control)  
22 wells, observed using a light inverted microscope. The cells were fixed with 500  
23 µL/well 10% formaldehyde solution and stained with 0.5% crystal violet in 20%  
24 methanol. For each time of addition the percentage of inhibition (%I) was calculated as  
25 follows: %I= (1-number of viral plaques in treated wells/number of viral plaques in  
26 virus control) ×100.  
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44 *Adsorption inhibition assay.*

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46 Twelve-well plates containing confluent Vero cells monolayers were incubated for 1 h  
47 at 4°C. Increasing non-cytotoxic concentrations of the test extract were added in  
48 duplicate and 200 pfu of HSV-1 or HSV-2 were immediately added. The plates were  
49 further incubated at 4°C for 3 h to allow virus adsorption. After this treatment, the  
50 medium was removed, the cell monolayers were gently washed with DMEM without  
51 bovine serum, 1.5% methylcellulose overlay was added to each well and the plates were  
52 finally incubated at 37°C in a 5% CO<sub>2</sub> atmosphere for 96 h or until complete  
53 development of viral lysis plaques in mock-treated (virus control) wells. The percentage  
54 of inhibition (%I) was calculated as follows: %I= (1-number of viral plaques in treated  
55 wells/number of viral plaques in virus control) ×100.  
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*Virucidal assay.*

Separate HSV-1 and HSV-2 suspensions were mixed with identical volumes of the plant extracts or the equivalent quantity of culture medium (negative control) and then incubated at room temperature for 30 min. The residual virus infectivity was investigated as described previously (Reed and Muench, 1938). Three different concentrations of each plant extract or isolated compounds were assayed.

*Statistics.*

The concentration of the test solution reducing cell viability by 50% (mean cytotoxic concentration,  $CC_{50}$ ) and the concentration reducing viral-induced cytopathic effect by 50% (mean effective concentration,  $EC_{50}$ ) were calculated by regression analysis using dose-response curves (not shown) generated from the experimental data. A selectivity index (SI) was calculated for each extract and virus, by dividing the  $CC_{50}$  by the  $EC_{50}$  value.  $CC_{50}$  and  $EC_{50}$  values were compared using ANOVA, followed by Tukey's multiple mean comparison tests.  $EC_{50}$  values from samples that inhibited both viruses were compared using the unpaired Student's t test. All comparisons were performed using the Statistica 6.1 software. Values of  $p < 0.05$  were considered indicative of statistical differences.

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**RESULTS AND DISCUSSION**

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In order to assess the cytotoxic effects of the tansy extracts, a colorimetric MTT assay was performed after incubating Vero cell cultures with increasing concentrations of the plant samples (Mosmann 1983). The  $CC_{50}$  values obtained (Table 1) correlated well with morphological changes such as cell rounding, presence of cytoplasmic inclusions and loss of monolayer confluence recorded by microscopic examination of treated cultures. These changes became evident from 250  $\mu\text{g/mL}$  of the crude extract, petroleum ether and ethyl acetate fractions; or from 100 or 500  $\mu\text{g/mL}$  for the chloroform fraction or isolated 3,5-DCQA respectively. The water and butanol extracts were innocuous to Vero cells since no morphological alterations were observed during the course of the experiment (data not shown). At the highest concentrations used in antiviral assays for all samples, the mean cell viabilities recorded were close to 100%.

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3 The analyses of crude extracts from aerial parts of tansy grown in Britain showed  
4 antiviral activity against HSV-1, in agreement with previously published data of tansy  
5 of Brazilian origin (Onozato *et al.* 2009). In the present report we have found that this  
6 antiviral effect could be also extended to an HSV-2 clinical isolate (Table 1).  
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11 In order to investigate the nature of the active antiviral principles of tansy, five fractions  
12 obtained from the crude extract using solvents of ascending polarity (Juan-Badaturuge  
13 *et al.* 2009) were also tested. As shown in Table 1, the petroleum ether and ethyl acetate  
14 extracts were active against both herpes virus types with good selectivity indexes. The  
15 water and butanol fractions did not show anti-herpetic effects up to 150µg/mL.  
16 However it should be noted that, due to their low cytotoxicity in mammalian cells  
17 (CC<sub>50</sub>>500 µg/mL), antiviral activity at higher concentrations cannot be ruled out.  
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26 Three antioxidant compounds, namely 3,5-DCQA, axillarin and luteolin, previously  
27 isolated from the ethyl acetate fraction were also included in antiviral analyses.  
28 Interestingly, the strongest inhibition (Table 1) was recorded for 3,5-DCQA which was  
29 isolated as a major constituent of the plant (Juan-Badaturuge *et al.* 2009). Other  
30 constituents such as axillarin, showed anti HSV-2 activity with a good selectivity index  
31 (Table 1) while luteolin appeared to be toxic to Vero cells and was unable to block  
32 HSV-2 replication at non-toxic concentrations (Table 1). The chloroform fraction  
33 showed severe cytotoxic effects at low concentrations and its antiviral activity could not  
34 be further analyzed.  
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43 Recently, a germacrane type sesquiterpene lactone (namely parthenolide) was reported  
44 to be one of the major anti HSV-1 active principles in *T. vulgare* (Onozato *et al.* 2009).  
45 In the present study, we assessed the cytotoxicity of parthenolide together with its  
46 putative antiviral activity against HSV-1 and HSV-2 clinical isolates. This compound  
47 showed the highest toxicity (25.2 µg/mL) among all tested samples although the CC<sub>50</sub>  
48 value obtained in our study was about 4-times the previously reported (CC<sub>50</sub>=5.5  
49 µg/mL) (Onozato *et al.* 2009). Surprisingly, our data revealed that purified parthenolide  
50 did not inhibit the virus-induced CPE (Table 1) up to a concentration 1.5-fold lower  
51 than the CC<sub>50</sub> value (SI<1.5). In order to further investigate the correlation between  
52 parthenolide and antiviral/cytotoxic effects, we measured its relative concentration in  
53 crude tansy extracts and fractions using HPLC. The analyses gave the following  
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3 parthenolide yields: chloroform (2.7% w/w vs the tansy extract), petroleum ether (1.88%), ethyl  
4 acetate (0.49%), crude aerial parts (0.33%). Parthenolide could not be detected in n-butanol,  
5 water or crude rhizome extracts. Considering that the antiviral effect of the tansy rhizome  
6 extract was similar to that of the aerial parts, and that rhizome extract did not contain  
7 parthenolide it is reasonable to conclude that the observed anti-HSV activity should be  
8 due to some other compound.  
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16 Whether the active principles in tansy extracts acted at an intracellular stage of viral  
17 replication or directly inactivate HSV virions was investigated through a series of  
18 specific experiments including time-of-addition, adsorption inhibition and virucidal  
19 assays. In these analyses we investigated the effects of ethyl acetate, petroleum ether  
20 and rhizome extracts as well as purified parthenolide and 3,5-DCQA. The studies on  
21 reduction of virus-induced lysis plaques as a function of the time of addition showed a  
22 very high HSV-1 inhibition (close to 100%) when the ethyl acetate, petroleum ether  
23 fraction or 3,5-DCQA were added to cultured cells, up to 6 hpi (Figure 1A). These data  
24 suggested that the active compounds acted on late processes of the viral replication  
25 cycle. However, some differences were observed using HSV-2. In the case of ethyl  
26 acetate extract, a slight drop in HSV-2 inhibition was recorded when this extract was  
27 added beyond 1 hpi, suggesting that some immediate-early or early event in HSV-2  
28 replication cycle was the main target of active principle(s) in this sample (Figure 1B).  
29 Some differences were also observed for the rhizome extract, because the reduction of  
30 HSV-2 virus-induced plaques dropped to 50% (Figure 1B), instead of completely losing  
31 its effect as observed for HSV-1 when this sample was added at 2 hpi. Parthenolide did  
32 not inhibit HSV-1 or HSV-2 in this assay and virus plaques were observed in all cases  
33 in a similar number to those in control wells, no matter the time of addition of this  
34 compound.  
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51 The ability of *T. vulgare* extracts to inhibit the attachment of HSV particles to Vero  
52 cells was investigated using adsorption inhibition experiments. For this purpose the cell  
53 monolayers were pre-incubated with HSV-1 (Figure 2A) or HSV-2 virions (Figure 2B)  
54 in the presence of the tansy extracts or purified compounds at 4°C. The number of  
55 virus-induced lysis plaques dramatically decreased when adsorption was performed in  
56 the presence of ethyl acetate, petroleum ether extracts or purified 3,5-DCQA (Figure 2).  
57 Adsorption inhibition ranged from 66.2% to 100% at concentrations between 18.7  
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$\mu\text{g/mL}$  and  $150 \mu\text{g/mL}$ . No inhibition was observed when parthenolide was used below its  $\text{CC}_{50}$ , at  $5 \mu\text{g/mL}$  or  $15 \mu\text{g/mL}$  (Figure 2). The rhizome extract also inhibited HSV-1 and HSV-2 adsorption by 85.5 and 70.1%, respectively using  $500 \mu\text{g/mL}$  of this extract during virus adsorption (data not shown).

Considering that adsorption inhibition might be due to virion inactivation, we further carried out virucidal assays as described in Materials and Methods. The data indicated that the ethyl acetate extract did not significantly decreased HSV-1 nor HSV-2 infectivity (Figure 3) whereas the petroleum ether extract ( $\geq 500 \mu\text{g/mL}$ ) showed a virucidal index greater than 2  $\log_{10}$  units, corresponding to the inactivation of more than 99% virions in suspension (Hu & Hsiung 1989). At  $1 \text{ mg/mL}$  the petroleum ether extract completely inactivated HSV particles (undetectable residual infectious titre). In contrast, mild virucidal effects were recorded for 3,5-DCQA against both herpesviruses at the highest assayed concentration ( $1 \text{ mg/mL}$ ), which is consistent with the observed inhibition beyond 6 hpi in the time-of-addition assay (see Figure 1). Neither the rhizome extract nor parthenolide inactivated virions at any tested concentration. Since the rhizome extract was able to inhibit HSV propagation when added to cell cultures before or at the time of infection (Figure 1), it could be suggested that its active constituents could interfere with virus adsorption perhaps by interacting with the cell surface receptors of HSV virions.

A relevant finding of the present study is the observation that the previously described antiviral principle of tansy, parthenolide, showed no anti herpesvirus activity in our study model, neither against an intracellular viral replication event nor as an extracellular virucidal agent. On the other hand, our data pointed out to 3,5-DCQA as a promising antiviral molecule, in agreement to previous studies where HIV-1 integrase inhibition (McDougall *et al.* 1998) and antiherpetic effects were reported (Shi *et al.* 2007). In addition, other minor constituents of the plant, such as axillarin, observed in the present study are likely to contribute to the antiherpetic effect of tansy. Although the precise molecular targets for tansy extract and active principles remain to be elucidated, the observed virucidal properties suggest their potential use in topical formulations to treat the widespread dermatological lesions caused by HSV. Further research is required to identify additional as yet unknown antiviral principles, in particular from the petroleum ether fractions and the underground rhizome extracts of tansy.

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For Peer Review

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**Figure captions.**

**Figure 1.** Effect of *T. vulgare* extracts and compounds on HSV-1 (A) and HSV-2 (B) lysis plaques counts, when added to cell cultures at different times before, at or after infection. ○ ethyl acetate extract, ◇ petroleum ether extract, ■ rhizome extract, ● 3,5-DCQA, ▲ parthenolide. Data are presented as the mean of two independent experiments and their standard deviation values.

**Figure 2.** Effect of *T. vulgare* extracts and compounds on HSV-1 (A) and HSV-2 (B) adsorption to Vero cells. ○ ethyl acetate extract, ◇ petroleum ether extract, ● 3,5-DCQA, ▲ parthenolide. Data are presented as the mean of two independent experiments and their standard deviation values.

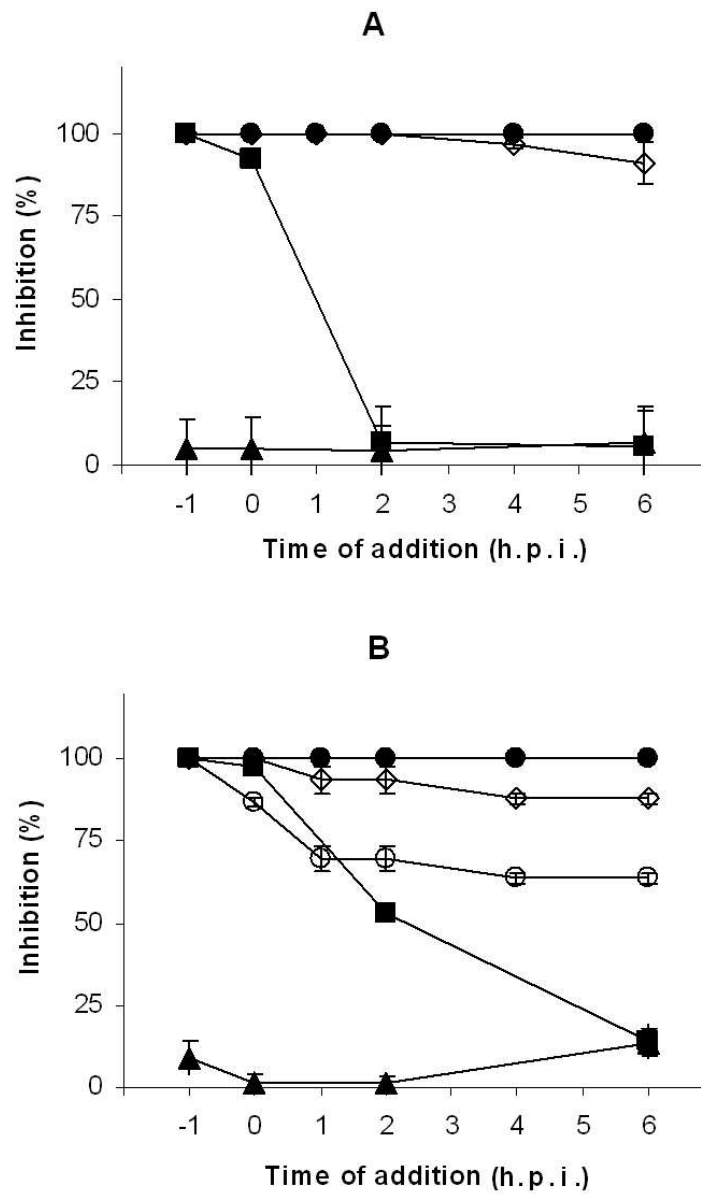
**Figure 3.** Virucidal activity of *T. vulgare* extracts and purified compounds against HSV-1 (A) and HSV-2 (B).



**Table 1.** Cytotoxicity and antiviral activity of *T. vulgare* extracts and purified compounds. Data are shown as the mean values and standard deviations from two independent experiments.

Extract/ fraction	Cytotoxicity	Antiviral activity			
	CC <sub>50</sub> (µg/mL)	HSV-1		HSV-2	
		CE <sub>50</sub> (µg/mL)	SI	CE <sub>50</sub> (µg/mL)	SI
<b>Crude aerial part</b>	515.2 ± 10.3	327.0 ± 48.7	1.57	>400	<1.3
<b>Petroleum ether</b>	235.7 ± 2.316	69.9 ± 7.69	<b>3.37</b>	61.16 ± 0.92	<b>3.85</b>
<b>Chloroform</b>	56.55 ± 8.451	>50	<1.1	>50	<1.1
<b>Ethyl acetate</b>	194.2 ± 2.908	95.7 ± 2.81	<b>2.03</b>	59.4 ± 0.13	<b>3.27</b>
<b>Butanol</b>	>500	>150	ND	>150	ND
<b>Water</b>	>500	>150	ND	>150	ND
<b>Rhizome</b>	1089.4 ± 71.2	295.8 ± 63.5	<b>3.68</b>	500.0 <sup>a</sup>	<b>2.18</b>
<b>3,5-DCQA</b>	409.5 ± 10.61	31.1 ± 3.92	<b>13.2</b>	46.99 ± 2.19	<b>8.71</b>
<b>Axillarin</b>	209.5 <sup>a</sup>	NA	-	42.7 ± 3.63	<b>4.91</b>
<b>Luteolin</b>	67.92 ± 10.34	NA	-	>50	<1.3
<b>Parthenolide</b>	25.17 ± 0.58	>16	<1.5	>16	<1.5
<b>Acyclovir</b>	>1000	0.943 ± 0.229	>1060	0.977 ± 0.051	>1023

<sup>a</sup>: value from an individual experiment. ND: not determined. NA: not analyzed.



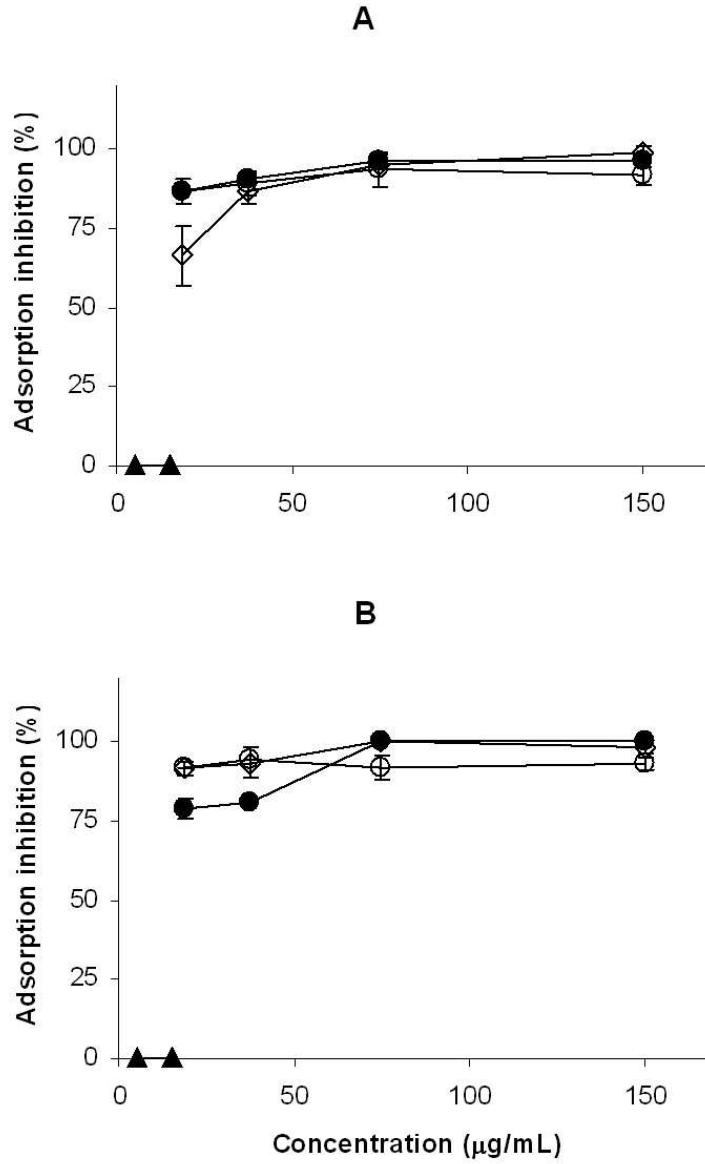


Figure 2  
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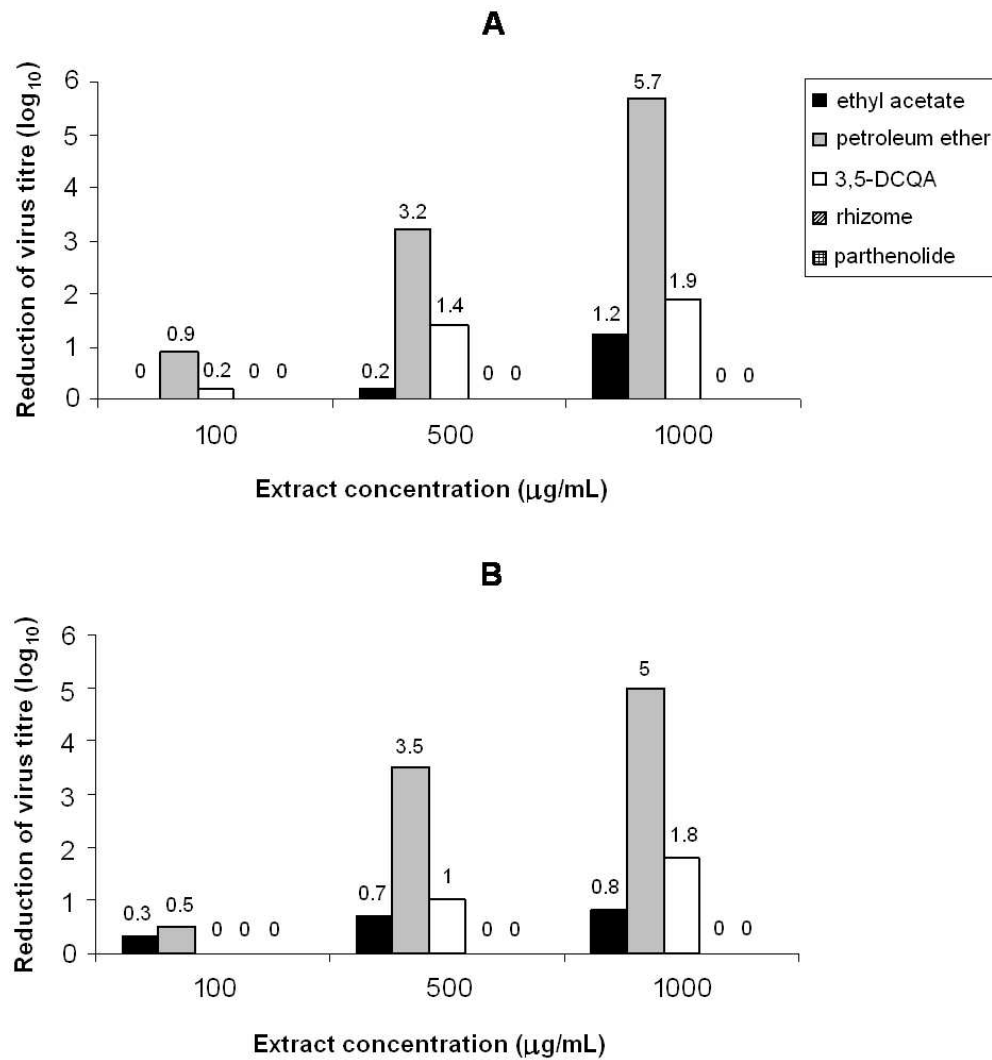


Figure 3  
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