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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

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.

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

INTRODUCTION

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.

MATERIALS AND METHODS

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

GraphPad InStat software (GraphPad, San Diego, USA). The calculated r^2 values of the standard curves were in the range 0.998 to 0.999.

Viruses and cells.

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

Cytotoxicity assay.

Vero cells were seeded into 96-well plates at a density of 2×10^4 cells/well, and incubated at 37 °C in a 5% CO₂ atmosphere for 48-72 h, until 90% or greater confluency of the monolayers was reached. Increasing concentrations of the test extract were added to cells, with a replicate number of six wells per concentration. After a 3-days incubation period, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution was added (final concentration 0.5 mg/mL) and the plates were incubated for another 4 h to allow formazan production. The solid precipitate was dissolved with absolute 2-propanol and the absorbance at 570 nm was measured using a 96-well µQuant Spectrophotometer (Biotek Instruments) at 620 nm. The resulting cell viability was calculated as described previously (Mosmann, 1983).

Antiviral activity assay

Ninety-six-well plates containing confluent cell monolayers were preincubated for 1 h with increasing non-cytotoxic concentrations of the extracts. Six replicate wells were used for each analyzed sample. Afterwards, 10 Tissue Culture Infectious Dose₅₀ (TCID₅₀) of HSV-1 or HSV-2 were added into each well. The plates were incubated at 37 °C in a 5% CO₂ humidified atmosphere and observed daily for cytopathic effect (CPE) using a light microscope. When CPE was observed in all virus control wells, the percentage of wells with CPE was determined for each treatment concentration. Acyclovir (ACV) at concentrations varying from 0.1 to 50 µg/mL served as positive control.

Time-of-addition assay.

The effect on HSV replication of tansy extracts or purified fractions, added to cell cultures at different times before, during or after infection was investigated as described previously (Alvarez *et al.* 2009) with some modifications. The analyzed sample was added to the first series of wells at a fixed non-cytotoxic concentration (100 µg/mL for ethyl acetate and petroleum ether; 500 µg/mL for rhizome extract; 300 µg/mL for DCQA; and 5 µg/mL for parthenolide) and incubated for 1 h. All wells in the plate were then infected with 200 pfu of HSV-1 or HSV-2 and the same concentration of plant sample was added to the second series of wells at the time of infection. After 1 h of adsorption, the medium was removed from each well and fresh medium containing the plant extract was added to the first, the second and the third series of wells corresponding to -1, 0 and 1 h post-infection (hpi). Subsequent additions were performed 2, 4 and 6 hpi. After the last addition of compound the culture medium was replaced by a 1.5% methylcellulose overlay containing an identical concentration of the test extract. The plates were further incubated at 37°C in a 5% CO₂ atmosphere for 96 h, or until the appearance of evident viral lysis plaques in the mock-treated (virus control) wells, observed using a light inverted microscope. The cells were fixed with 500 µL/well 10% formaldehyde solution and stained with 0.5% crystal violet in 20% methanol. For each time of addition the percentage of inhibition (%I) was calculated as follows: $\%I = (1 - \text{number of viral plaques in treated wells} / \text{number of viral plaques in virus control}) \times 100$.

Adsorption inhibition assay.

Twelve-well plates containing confluent Vero cells monolayers were incubated for 1 h at 4°C. Increasing non-cytotoxic concentrations of the test extract were added in duplicate and 200 pfu of HSV-1 or HSV-2 were immediately added. The plates were further incubated at 4°C for 3 h to allow virus adsorption. After this treatment, the medium was removed, the cell monolayers were gently washed with DMEM without bovine serum, 1.5% methylcellulose overlay was added to each well and the plates were finally incubated at 37°C in a 5% CO₂ atmosphere for 96 h or until complete development of viral lysis plaques in mock-treated (virus control) wells. The percentage of inhibition (%I) was calculated as follows: $\%I = (1 - \text{number of viral plaques in treated wells} / \text{number of viral plaques in virus control}) \times 100$.

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.

RESULTS AND DISCUSSION

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%.

The analyses of crude extracts from aerial parts of tansy grown in Britain showed antiviral activity against HSV-1, in agreement with previously published data of tansy of Brazilian origin (Onozato *et al.* 2009). In the present report we have found that this antiviral effect could be also extended to an HSV-2 clinical isolate (Table 1).

In order to investigate the nature of the active antiviral principles of tansy, five fractions obtained from the crude extract using solvents of ascending polarity (Juan-Badaturuge *et al.* 2009) were also tested. As shown in Table 1, the petroleum ether and ethyl acetate extracts were active against both herpes virus types with good selectivity indexes. The water and butanol fractions did not show anti-herpetic effects up to 150 µg/mL. However it should be noted that, due to their low cytotoxicity in mammalian cells ($CC_{50} > 500$ µg/mL), antiviral activity at higher concentrations cannot be ruled out.

Three antioxidant compounds, namely 3,5-DCQA, axillarin and luteolin, previously isolated from the ethyl acetate fraction were also included in antiviral analyses. Interestingly, the strongest inhibition (Table 1) was recorded for 3,5-DCQA which was isolated as a major constituent of the plant (Juan-Badaturuge *et al.* 2009). Other constituents such as axillarin, showed anti HSV-2 activity with a good selectivity index (Table 1) while luteolin appeared to be toxic to Vero cells and was unable to block HSV-2 replication at non-toxic concentrations (Table 1). The chloroform fraction showed severe cytotoxic effects at low concentrations and its antiviral activity could not be further analyzed.

Recently, a germacrane type sesquiterpene lactone (namely parthenolide) was reported to be one of the major anti HSV-1 active principles in *T. vulgare* (Onozato *et al.* 2009). In the present study, we assessed the cytotoxicity of parthenolide together with its putative antiviral activity against HSV-1 and HSV-2 clinical isolates. This compound showed the highest toxicity (25.2 µg/mL) among all tested samples although the CC_{50} value obtained in our study was about 4-times the previously reported ($CC_{50} = 5.5$ µg/mL) (Onozato *et al.* 2009). Surprisingly, our data revealed that purified parthenolide did not inhibit the virus-induced CPE (Table 1) up to a concentration 1.5-fold lower than the CC_{50} value ($SI < 1.5$). In order to further investigate the correlation between parthenolide and antiviral/cytotoxic effects, we measured its relative concentration in crude tansy extracts and fractions using HPLC. The analyses gave the following

parthenolide yields: chloroform (2.7% w/w vs the tansy extract), petroleum ether (1.88%), ethyl acetate (0.49%), crude aerial parts (0.33%). Parthenolide could not be detected in n-butanol, water or crude rhizome extracts. Considering that the antiviral effect of the tansy rhizome extract was similar to that of the aerial parts, and that rhizome extract did not contain parthenolide it is reasonable to conclude that the observed anti-HSV activity should be due to some other compound.

Whether the active principles in tansy extracts acted at an intracellular stage of viral replication or directly inactivate HSV virions was investigated through a series of specific experiments including time-of-addition, adsorption inhibition and virucidal assays. In these analyses we investigated the effects of ethyl acetate, petroleum ether and rhizome extracts as well as purified parthenolide and 3,5-DCQA. The studies on reduction of virus-induced lysis plaques as a function of the time of addition showed a very high HSV-1 inhibition (close to 100%) when the ethyl acetate, petroleum ether fraction or 3,5-DCQA were added to cultured cells, up to 6 hpi (Figure 1A). These data suggested that the active compounds acted on late processes of the viral replication cycle. However, some differences were observed using HSV-2. In the case of ethyl acetate extract, a slight drop in HSV-2 inhibition was recorded when this extract was added beyond 1 hpi, suggesting that some immediate-early or early event in HSV-2 replication cycle was the main target of active principle(s) in this sample (Figure 1B). Some differences were also observed for the rhizome extract, because the reduction of HSV-2 virus-induced plaques dropped to 50% (Figure 1B), instead of completely losing its effect as observed for HSV-1 when this sample was added at 2 hpi. Parthenolide did not inhibit HSV-1 or HSV-2 in this assay and virus plaques were observed in all cases in a similar number to those in control wells, no matter the time of addition of this compound.

The ability of *T. vulgare* extracts to inhibit the attachment of HSV particles to Vero cells was investigated using adsorption inhibition experiments. For this purpose the cell monolayers were pre-incubated with HSV-1 (Figure 2A) or HSV-2 virions (Figure 2B) in the presence of the tansy extracts or purified compounds at 4°C. The number of virus-induced lysis plaques dramatically decreased when adsorption was performed in the presence of ethyl acetate, petroleum ether extracts or purified 3,5-DCQA (Figure 2). Adsorption inhibition ranged from 66.2% to 100% at concentrations between 18.7

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3 $\mu\text{g/mL}$ and $150 \mu\text{g/mL}$. No inhibition was observed when parthenolide was used below
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5 its CC_{50} , at $5 \mu\text{g/mL}$ or $15 \mu\text{g/mL}$ (Figure 2). The rhizome extract also inhibited HSV-1
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7 and HSV-2 adsorption by 85.5 and 70.1%, respectively using $500 \mu\text{g/mL}$ of this extract
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9 during virus adsorption (data not shown).

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

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42 A relevant finding of the present study is the observation that the previously described
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44 antiviral principle of tansy, parthenolide, showed no anti herpesvirus activity in our
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46 study model, neither against an intracellular viral replication event nor as an
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48 extracellular virucidal agent. On the other hand, our data pointed out to 3,5-DCQA as a
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50 promising antiviral molecule, in agreement to previous studies where HIV-1 integrase
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52 inhibition (McDougall *et al.* 1998) and antiherpetic effects were reported (Shi *et al.*
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54 2007). In addition, other minor constituents of the plant, such as axillarin, observed in
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56 the present study are likely to contribute to the antiherpetic effect of tansy. Although the
57
58 precise molecular targets for tansy extract and active principles remain to be elucidated,
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60 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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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 ₅₀ (µg/mL)	HSV-1		HSV-2	
		CE ₅₀ (µg/mL)	SI	CE ₅₀ (µg/mL)	SI
Crude aerial part	515.2 ± 10.3	327.0 ± 48.7	1.57	>400	<1.3
Petroleum ether	235.7 ± 2.316	69.9 ± 7.69	3.37	61.16 ± 0.92	3.85
Chloroform	56.55 ± 8.451	>50	<1.1	>50	<1.1
Ethyl acetate	194.2 ± 2.908	95.7 ± 2.81	2.03	59.4 ± 0.13	3.27
Butanol	>500	>150	ND	>150	ND
Water	>500	>150	ND	>150	ND
Rhizome	1089.4 ± 71.2	295.8 ± 63.5	3.68	500.0 ^a	2.18
3,5-DCQA	409.5 ± 10.61	31.1 ± 3.92	13.2	46.99 ± 2.19	8.71
Axillarin	209.5 ^a	NA	-	42.7 ± 3.63	4.91
Luteolin	67.92 ± 10.34	NA	-	>50	<1.3
Parthenolide	25.17 ± 0.58	>16	<1.5	>16	<1.5
Acyclovir	>1000	0.943 ± 0.229	>1060	0.977 ± 0.051	>1023

^a: value from an individual experiment. ND: not determined. NA: not analyzed.

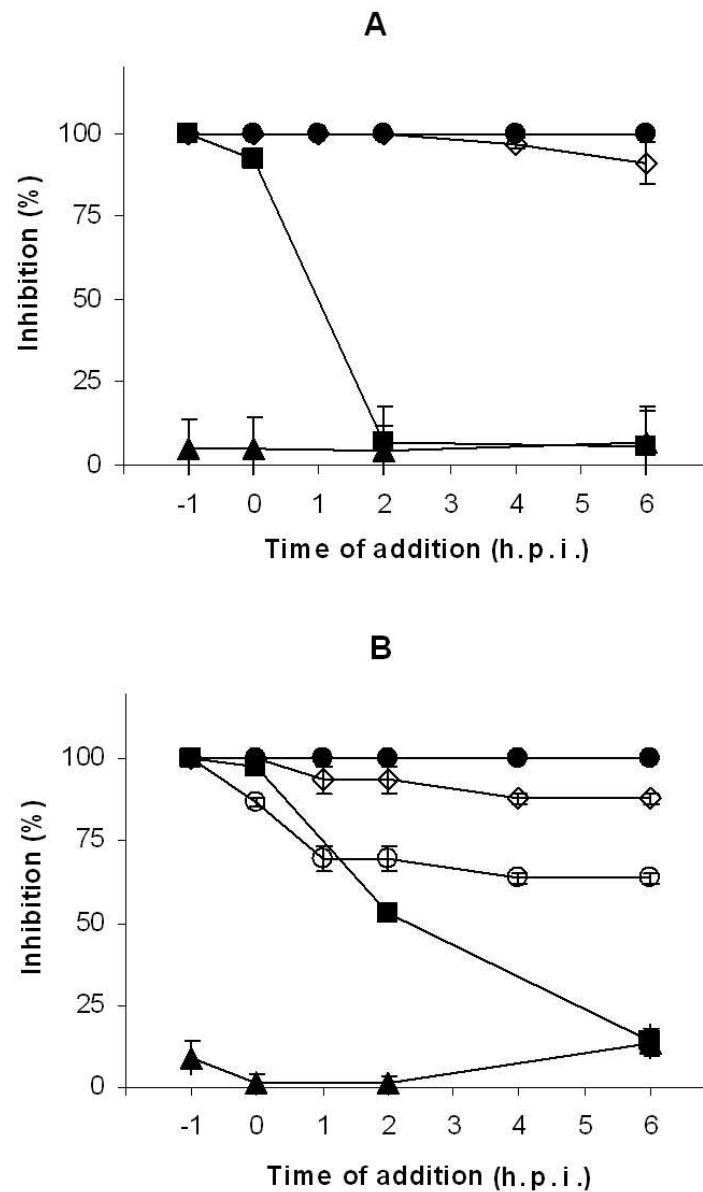


Figure 1
178x295mm (96 x 96 DPI)

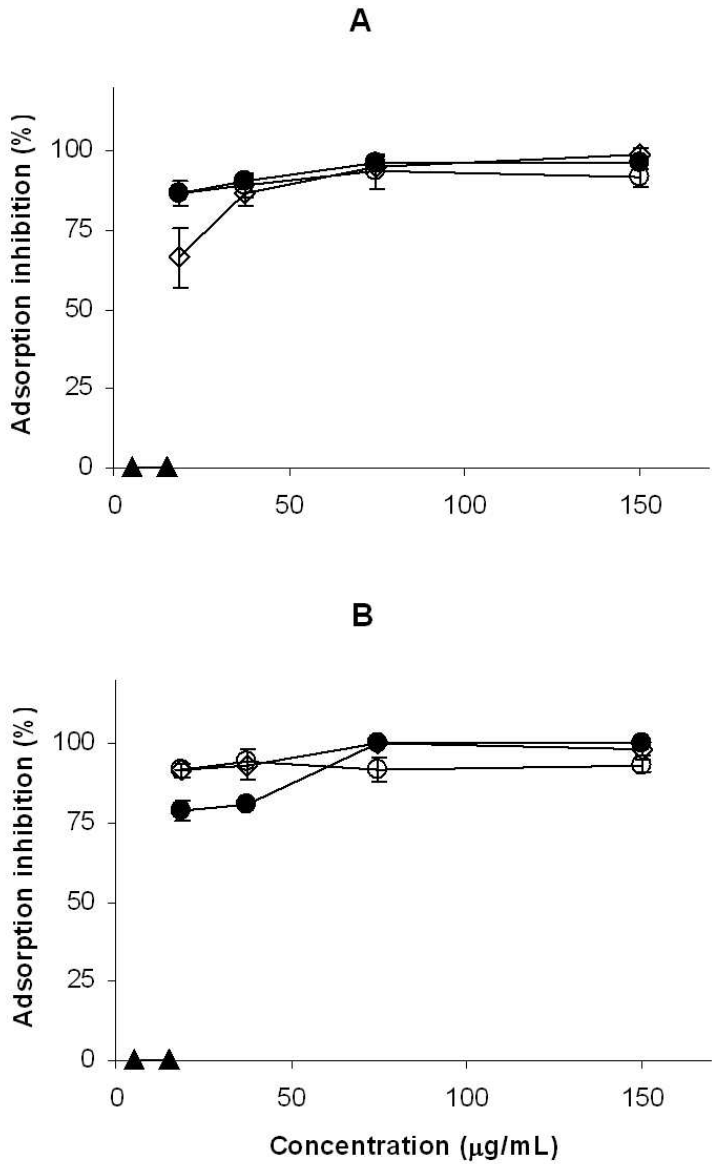


Figure 2
188x302mm (96 x 96 DPI)

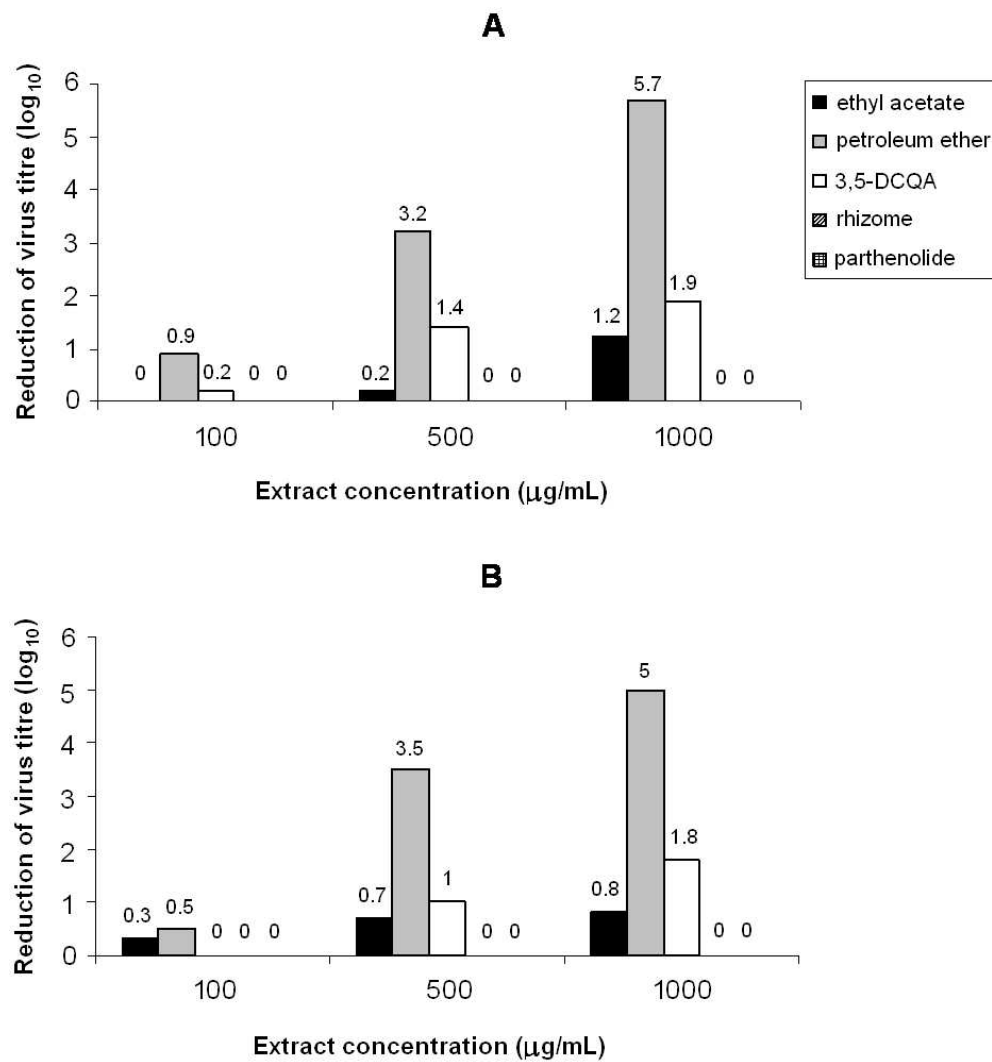


Figure 3
249x263mm (96 x 96 DPI)