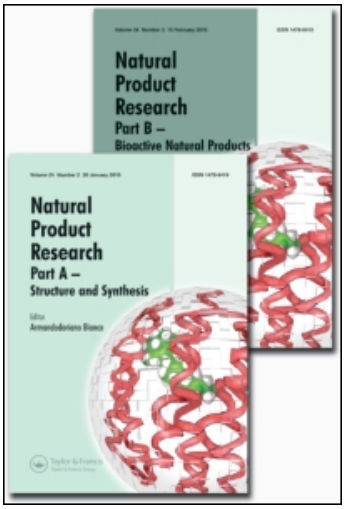


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# Evaluation of antiviral activity of aqueous extracts from *Achyrocline satureioides* against Western equine encephalitis virus

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*Achyrocline satureioides* (Asteraceae) is a medicinal plant traditionally used in Argentina for the treatment of intestinal infections and various digestive disorders. Its infusion is widely utilised for respiratory problems and viral infections. The objective of this study was to investigate cytotoxicity, virucidal and antiviral properties of the cold aqueous extract (CAE) and hot aqueous extract (HAE) of this plant against Western equine encephalitis virus (WEEV). Cytotoxicity in Vero cells was evaluated by maximum non-cytotoxic concentration (MNCC), neutral red (NR) uptake and MTT reduction methods. To study the antiviral activity of aqueous extracts, plaque reduction assay was performed after pre-treatment of host cells, adsorption, penetration and post-penetration of the virus. Extracellular virus inactivation was also analysed by the same method. Extracts showed strong inhibitory activity after virus penetration with selective index values of 32 (NR) and 63.3 (MTT) for the CAE, and 16.2 (NR) and 24.3 (MTT) for the HAE. Both extracts exhibited virucidal action with lower efficacy than their antiviral properties. The present results demonstrate that aqueous extracts of *A. satureioides* are active against WEEV. Further studies are needed in order to identify which compounds could be responsible for this effect, and how they exert antiviral action.

**Keywords:** *Achyrocline satureioides*; antiviral activity; aqueous extracts; WEEV; NR uptake; MTT assay

## 1. Introduction

Many diseases caused by viruses are a problem to treat due to the limited availability of effective antiviral drugs, the toxic effect that they produce on the host cells, as well as the induction of resistance generated in the host following their prolonged use (Pujol, 1995).

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Outbreaks of viral encephalitis are a health issue of increasing importance. Members of the *Alphavirus* genus (Togaviridae), such as Western equine encephalitis (WEE), Eastern equine encephalitis (EEE) and Venezuelan equine encephalitis (VEE) viruses are important aetiologic agents. Western equine encephalitis, a mosquito-borne disease in domestic animals and humans in North, Central and South America, is caused by the WEE virus. WEEV is responsible for large, periodic and extensive epizootics and epidemics of encephalitis in equines and humans (Hu et al., 2008; Johnston & Peters, 1996; Reisen & Monath, 1988). In infants and children the disease is more serious, often associated with seizures.

The WEE complex includes epizootic and enzootic strains. EEE and WEE viruses have caused epizootics in Argentina since the beginning of the last century. During the periods 1972–1973 and 1982–1983, there were epizootics of WEE with sporadic human cases. The latter period had the province of Santa Fe as its epicentre, and the epizootic spread to Viedma, Río Negro province. WEEV was isolated from the mosquito *Ochlerotatus albifasciatus*, which induced 20–50% mortality in horses (Sabattini, Avilés, & Monath, 1998).

Numerous investigations have shown that various medicinal herbs exhibit antiviral effects, either by direct virucidal or prophylactic methods. These results suggest that the mechanism of action could be inactivating the viral proteins or interfering with virus adhesion to the cell. Other data also suggest that they may interfere in the replicative cycle of the pathogen by reducing the viral nucleic acid synthesis (Montanha, Amoros, Boustie, & Girre, 1995). Therefore, there is an increasing need for new antiviral drugs, for which plants are an important source.

The phytogeographic region named 'Monte', included in arid ecosystems, is characterised by many species of plants and herbs with medicinal folk tradition. One of the most relevant species is *Achyrocline satureioides*, which belongs to the family Asteraceae. This plant, commonly known as 'marcela del campo', is native to America and extends throughout the continent, as well as in Europe and Africa. In Argentina, it is often found in sandy and humid soils in the hills of Córdoba, San Luis and Buenos Aires (Tandil) (Instituto Nacional de Investigación Agropecuaria, 2004). Numerous investigations have reported its bioactive properties, such as anti-inflammatory (De Souza, Bassani, & Schapoval, 2007), sedative (Hnatyszyn et al., 2004), hepatoprotective (Kadarian et al., 2002), antioxidant (Arredondo et al., 2004; Polydoro et al., 2004), immunomodulatory and antimicrobial (Calvo, Cariddi, Grosso, Demo, & Maldonado, 2006), antitumoural (Ruffa et al., 2002), antiviral (Bettega, Teixeira, Bassani, Barardi, & Simões, 2004; Zanon, Ceriatti, Rovera, Sabini, & Ramos, 1999) and photoprotective (Morquio, Rivera-Megret, & Dajas, 2005).

The plant is traditionally employed as an antispasmodic in cases of intestinal infections (because of its antibiotic properties) and various digestive disorders. Its infusion is widely utilised for the treatment of respiratory problems, including asthma, bronchitis and upper respiratory tract infections, as well as for viral infections. It has also been used in gynaecological wash preparations, and treatment of cardiovascular diseases (Filot Da Silva & Langeloh, 1994; Instituto Nacional de Investigación Agropecuaria, 2004; Taylor, 2005).

Numerous studies with *A. satureioides* point out its great ethnobotanical potential. However, investigations must be carried out to determine the inhibitory

ability of extracts at non-cytogenotoxic concentrations in the replication cycle of WEE virus.

Therefore, this study was conducted to evaluate the cytotoxicity, virucidal and antiviral properties of aqueous extracts from *A. satureioides* against the WEE virus.

## 2. Results and discussion

### 2.1. *Cytotoxicity*

To discriminate antiviral activities from cytotoxic effects, cytotoxic concentrations that reduced viability of Vero cells by 50% as well as the MNCC were determined.

Cell treatment with CAE at concentrations ranging from 200 to 2000 $\mu g\,mL^{-1}$ showed that 480 $\mu g\,mL^{-1}$ was the MNCC. On the other hand, HAE was assayed at concentrations between 100 and 1400 $\mu g\,mL^{-1}$, leading to a MNCC of 260 $\mu g\,mL^{-1}$ (Table 1).

For the determination of $CC_{50}$ for CAE and HAE, concentrations varying from 100 to 1900 $\mu g\,mL^{-1}$ and from 100 to 1500 $\mu g\,mL^{-1}$ were used, respectively. The values of $CC_{50}$ and $CC_{80}$, which were determined by two methods, are given in Table 1.

The results of both methods indicated that the HAE exhibited more toxicity than the CAE. In addition, NR assay was more sensitive than the MTT method because it showed lower $CC_{50}$ values than those achieved by the latter. It suggests that both extracts affect the phagocytic action at concentrations that are innocuous to the mitochondrial respiratory chain.

Results of cytotoxic studies of the methanolic extract of *A. satureioides* reported by Ruffa et al. (2002) indicated a $CC_{50}$ value of 237 $\mu g\,mL^{-1}$ using the same cell system. A comparative analysis shows that our extracts were less toxic. On the other hand, considering the results of MNCC obtained herein, the CAE of *A. satureioides* was less toxic than the aqueous extracts of *Lithraea molleoides*, *Sebastiania brasiliensis*, *Sebastiania klotzschiana* (all with MNCC > 250 $\mu g\,mL^{-1}$) and *Myrcianthes cisplatensis* (100 $\mu g\,mL^{-1}$), and was similar to *Polygonum punctatum* (>450 $\mu g\,mL^{-1}$), assayed on the same cell line, while HAE toxicity was similar to the three former species, lower than *M. cisplatensis*, but higher than *P. punctatum* (Kott et al., 1999).

Table 1. Cytotoxicity of the CAE and HAE of *A. satureioides* determined by different methods.

| | NR | | MTT | | |
|---|---|---|---|---|---|
| Extracts | $CC_{50}$ ($\mu g\,mL^{-1}$) | $CC_{80}$ ($\mu g\,mL^{-1}$) | $CC_{50}$ ($\mu g\,mL^{-1}$) | $CC_{80}$ ($\mu g\,mL^{-1}$) | MNCC ($\mu g\,mL^{-1}$) |
| CAE | 960 | 290 | >1900 | 418 | 480 |
| HAE | 373 | 170 | 559 | 323 | 260 |

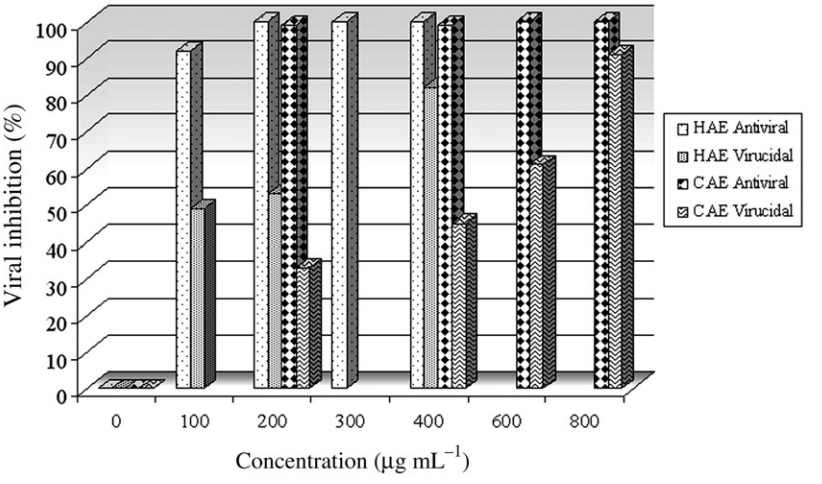


Figure 1. Influence of concentration of the HAE and CAE of *A. satureioides* on virucidal and antiviral activities against WEEV.
Note: Virus suspensions were incubated with different concentrations of extracts for 1 h at 37°C. Immediately, residual infectivity was titrated by plaque reduction assay. Virucidal activity was expressed as the percentage reduction of plaque. For antiviral tests, monolayers infected with virus and incubated for 1 h at 37°C were treated with increasing concentrations of extracts for 96 h. Thereafter the percentage inhibition was determined. Values are averages from three independent experiments.



### 2.2. *Antiviral and virucidal activities*

The inhibition induced by the extracts during viral adsorption and penetration was less than 50% for CAE, and less than 30% for HAE, while in the pre-treated cells the inhibition was less than 40% and 5% for CAE and HAE, respectively (data not shown).

WEEV was totally inhibited by the HAE at concentrations in the range 200–400 $\mu g\,mL^{-1}$ when it was added post-viral penetration, as shown in Figure 1, whereas the virucidal effect of this extract was concentration-dependent, reaching nearly 82%. Similarly, CAE showed excellent antiviral activities at concentrations of 200–800 $\mu g\,mL^{-1}$, while virucidal action was dose-dependent. An important inactivation (>90%) was only observed at the maximum concentration assayed.

As can be seen, the antiviral activity occurred in a stage after viral penetration, thus suggesting selective interference with the replication cycle within the host cell. Further studies are needed in order to determine which step of the multiplicative viral cycle is affected by both polar extracts of this plant.

Additionally, Zanon et al. (1999) reported an important inhibitory effect in the replication of pseudorabies virus (Herpes suis) by the alcoholic extract of *A. satureioides* when the plant sample was added after viral penetration.

Other studies on the mechanism of the antiherpetic activity have demonstrated that the hydroethanolic extract of *A. satureioides* showed no virucidal effects, and did not affect the cell membrane's receptors to which the virus binds. Herpes simplex virus (HSV-1) DNA synthesis was not inhibited. The antiherpetic activity occurred

between the second and the ninth hour of the virus replication cycle, probably indicating a perturbation on the late stages of this cycle (Bettega et al., 2004).

There are no reports on the antiviral activity of medicinal plants against WEEV, but there are reports of strong inhibition of the cytopathic effect induced by this virus on Vero cells by mycelial fractions of *Agaricus blazei* Murill (Sorimachi et al., 2001).

Several investigations have reported inhibitory effects against *Sindbis* virus, another member of *Alphavirus*, with plant extracts from *Melia azedarach* L. (Meliaceae), *Bidens pilosa* (Asteraceae) and *Momordica charantia* (Cucurbitaceae) (Beloin et al., 2005; Waschman, Andrei, Daelli, & Coto, 1984; Yip, Pei, Hudson, & Towers, 1991).



### 2.3. *Determination of $EC_{50}$*

The $EC_{50}$ values found against WEEV were 30 μg mL$^{-1}$ for the CAE and 23 μg mL$^{-1}$ for the HAE (Figure 2). To evaluate the selective antiviral activity *in vitro*, the selectivity index (SI) was determined, as given in Table 2. The SI describes the ratio between the cytotoxic and antiviral activities of a substance. One of the most used criteria to consider antiviral effectiveness is an SI value above 10 (Wyde, Ambrose, Meyer, Zolinski, & Gilbert, 1990). The SI values were 32 (NR) and 63.3 (MTT) for the CAE and 16.2 (NR) and 24.3 (MTT) for the HAE, thus demonstrating that the CAE was more selective as an antiviral agent than HAE. These values are clearly higher than 10, suggesting that both aqueous extracts are effective antivirals.

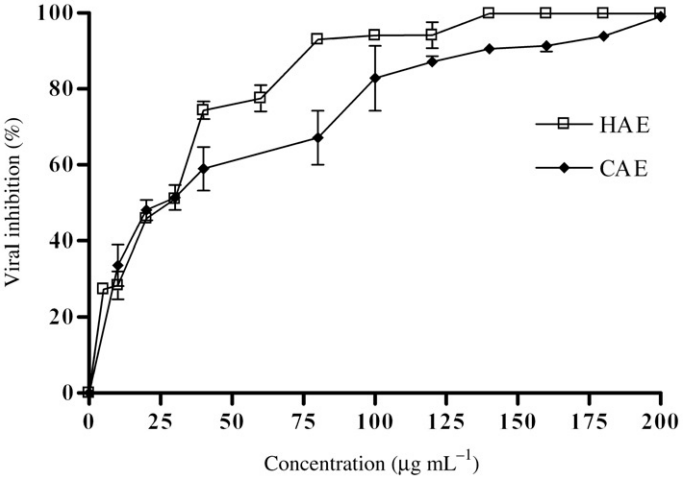


Figure 2. Determination of the $EC_{50}$ of the HAE and CAE from *A. satureioides* against WEEV.

Note: Cell monolayers were infected with about 100 PFU per well, and incubated for 1 h at 37°C. MEM-0.5% agarose with increasing concentrations (5–200 μg mL$^{-1}$) of extract was added. After incubation for four days at 37°C, viral plaques were counted. Thereafter the percentage inhibition was calculated, and the $EC_{50}$ was determined. Data account for the means of three separate experiments.

Table 2. Selectivity index of the CAE and HAE of *A. satureioides* on WEEV.

| | $CC_{50}$ ($\mu g\,mL^{-1}$) | | | SI ($CC_{50}/EC_{50}$) | |
|---|---|---|---|---|---|
| Extracts | NR | MTT | $EC_{50}$ ($\mu g\,mL^{-1}$) | NR | MTT |
| CAE | 960 | >1900 | 30 | $960/30 = 32$ | $>1900/30 \geq 63.3$ |
| HAE | 373 | 559 | 23 | $373/23 = 16.2$ | $559/23 = 24.3$ |

For *A. satureioides*, the occurrence of flavonoids, such as luteolin, quercetin and 3-*O*-methylquercetin, in the aqueous extracts has been reported previously (De Souza, Schapoval, & Bassani, 2002). Other chemical constituents of this species are caffeic, chlorogenic and isochlorogenic acids, pyrone derivatives, kavapyrone, flavonoids, minerals, volatile oil and polysaccharides (Polydoro et al., 2004; Vendruscolo, Rates, & Mentz, 2005).

Other studies revealed that metabolites, such as dicaffeoylquinic acid, present in the aqueous extracts of the flowers of this genus, exhibited antiviral activity *in vitro* against HIV (Abdel-Malek et al., 1996; Robinson et al., 1996) and informed antiherpetic activity of 3,5-dicaffeoylquinic, 1-methoxy-3,5-dicaffeoylquinic and 3-*O*-methylflavones. On the other hand, the inhibitory action of saponins on HSV-1 DNA synthesis has also been reported (Alonso & Desmarchelier, 2006). Therefore, these compounds could be related to the detected antiviral activity of the extracts.



## 3. Experimental

### 3.1. *Plant material*

*Achyrocline satureioides* plants were collected manually from Villa Jorcoricó, southern Córdoba hills (32°41′S; 64°43′W; 800 m sea level) in May 2007. The plant material was identified by Dr Luis Del Vitto, Faculty of Pharmacy and Biochemistry, University of San Luis, San Luis, Argentina. A voucher specimen was deposited in the Herbarium of the University of San Luis (No. 6362).

### 3.2. *Preparation of extracts*

Aerial vegetal parts (leaves, stems and blooms) were submitted to extraction with cold (4°C) and hot water (70°C) sequentially (15 g of dried and pulverised material per 700 mL of water) for two days. The suspensions were filtered and lyophilised. These two solutions were identified as the cold aqueous extract (CAE) and hot aqueous extract (HAE), respectively. Stocks were prepared in phosphate buffered saline (PBS) at a concentration of $100\,mg\,mL^{-1}$ and centrifuged at 10,000 rpm for 30 min. The extracts were stored at −20°C.

### 3.3. *Cell culture and virus*

Bioassays were performed in Vero cells (*Cercopithecus aethiops* green monkey kidney epithelial cell line; ATCC CCL-81) grown in Eagle's minimal essential medium

(EMEM; Gibco, USA) supplemented with 10% (v/v) heat-inactivated foetal calf serum (Natocor, Argentina), glutamine (30 μg $mL^{-1}$) and gentamicin (50 μg $mL^{-1}$) (all from Sigma–Aldrich, Italy). Cell cultures were maintained at 37°C in a 5% (v/v) $CO_2$ humidified atmosphere.

WEEV strain Ag 80-646, an enzootic strain, was isolated in Chaco (Argentina) from *Culex* (*Melanoconion*) *ocossa* mosquitoes (Mitchell et al., 1985). The virus was propagated by intracerebral inoculation in infant mice (*Rockefeller* strain) and titrated by quantification of the plaques-forming unit (PFU) method for arbovirus (Early, Peralta, & Johnson, 1967). Viral stocks were stored at −70°C.

### 3.4. *Cytotoxicity assays*

For cytotoxicity assays, the cells were cultured in 96-well culture plates (Cellstar, Greiner Bio-One, Germany). After incubation for 24 h at 37°C, cells were exposed to increasing concentrations of the extracts. Assays were carried out in triplicate. Monolayers incubated only with EMEM were used as controls for cellular viability.

Maximum non-cytotoxic concentration (MNCC) was determined microscopically by daily observations of morphological cell changes for 72 h (L. Ooi, Wang, Luk, & V. Ooi, 2004). The cytotoxic concentration of the extracts which reduced the viable cell number by 50% ($CC_{50}$) was determined by neutral red (NR) uptake and MTT assays.

After the cells were treated with both extracts for 48 h, the microplates were incubated with NR solution at 37°C for 3 h and finally, with an extraction solution (49% distilled water: 50% ethanol: 1% acetic acid) for 15 min in a shaker. The absorbance was read on a multiwell spectrophotometer (Bio-Tek, ELx800, USA) at 540 nm (Rajbhandari, Wegner, Jülich, Schöpke, & Mentel, 2001; Seth, Yang, Choi, Sabean, & Roberts, 2004).

The $CC_{50}$ was also measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma–Aldrich) method (Mosmann, 1983). Briefly, monolayers treated with extracts for 48 h at 37°C were incubated with MTT solution for 4 h at 37°C. Subsequently, the supernatant was removed and acid-isopropanol (0.04N HCl in isopropanol) was added. After gently shaking for 15 min, the absorbance was read on a multiwell spectrophotometer (Bio-Tek, ELx800) at 570 nm. Percentage survival fraction was calculated considering optical density (OD) of cultures treated versus controls.

### 3.5. *Antiviral activity*

In order to study the antiviral activity of the CAE and HAE, three experiments were performed by adding plant samples at different times and evaluating the inhibitory action by a plaque reduction assay.

#### 3.5.1. *Pre-treatment cells*

Monolayers grown in 24-well culture plates (Cellstar, Greiner Bio-One, Germany) were treated for 1 h at 37°C with different concentrations of the extracts. After

washing with PBS, the cells were exposed to 100 PFU of WEEV per well for 1 h at 37°C. Cultures were washed and overlaid with MEM-0.5% UltraPure Agarose (Invitrogen, USA) and further incubated for 96 h at 37°C.

#### 3.5.2. *Adsorption and penetration*

Cell monolayers were infected with 100 PFU of virus per well in the presence of different concentrations of extracts. After adsorption for 1 h at 37°C, residual inoculum was removed, and MEM-0.5% agarose was added.

#### 3.5.3. *Post-penetration*

Cells were infected with 100 PFU of virus per well, further incubated for 1 h at 37°C, and any unadsorbed virus was removed. Cells were washed with PBS, and then MEM-0.5% agarose with different extract concentrations was added. After incubation, cell monolayers were fixed with 10% formalin (Cicarelli, Argentina) and further stained with 1% crystal violet solution.

Controls of virus, cells and extracts were included in all assays. A positive antiviral control was not included because there are no effective antiviral drugs against WEEV.

The number of plaques of treated cells was compared to untreated controls to calculate the plaque reduction percentage.

### 3.6. *Virucidal test*

To determine the ability of extracts to inactivate the virus particles directly, equal volumes of WEEV (200 PFU 100 $\mu L^{-1}$) and extract (double concentration of those assayed in antiviral experiments) were mixed and incubated for 1 h at 37°C. Afterwards, each mixture was added to cultures (100 µL per well), using three wells for each concentration, and these were incubated for 1 h at 37°C. Then, monolayers were washed and covered with MEM-0.5% agarose. After incubation for four days at 37°C, the cells were fixed with 10% formalin and stained with 1% crystal violet solution.

### 3.7. *Determination of 50% effective concentration*

Cell monolayers cultured in 24-well microplates were infected with about 100 PFU per well, and incubated for 1 h at 37°C. Residual inoculum was removed; cells were washed with PBS and MEM-0.5% agarose was added together with increasing concentrations of the extracts, CAE and HAE. After four days at 37°C, the cultures were fixed, stained and viral plaques were counted. The $EC_{50}$ was calculated as the extract concentration that reduced the number of PFUs to 50% with respect to the viral control.

### 3.8. *Data analysis*

The $CC_{50}$ and $EC_{50}$ were calculated from concentration–effect plots by non-linear regression analysis (Boltzmann sigmoidal origin). The results account for the mean ± standard error of the mean values of three different experiments.

## 4. Conclusion

The results obtained in this study conclude that aqueous extracts of *A. satureioides* exert high antiviral activities against WEEV, and a slight virucidal activity tested *in vitro*.

It can be suggested that the antiviral effect is more likely to occur after the entry of the virus to the host cell, in the subsequent stages of the cell culture replication. Nevertheless, the mechanism of their antiviral action has not yet been identified. Furthermore, additional studies are needed in order to identify which compounds could be responsible for this effect and how they exert antiviral action.



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