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Original Article

Potent inhibition of *Western Equine Encephalitis* virus by a fraction rich in flavonoids and phenolic acids obtained from *Achyrocline satureioides*

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ABSTRACT

Achyrocline satureioides (Lam.) DC. Asteraceae, 'marcela del campo', possess several pharmacological properties. Previously we reported antiviral activity of an aqueous extract of *A. satureioides* against an alphavirus, *Western Equine Encephalitis* virus. Alphaviruses are highly virulent pathogens which cause encephalitis in humans and equines. There are no effective antiviral to treat its infections. The aim of this study was to evaluate *in vitro* cytotoxic and antiviral activities against *Western Equine Encephalitis* virus of five water extract chromatographic fractions from *A. satureioides* and identify the main compounds of the bioactive fraction. Also, it was to assess *in vivo* cytogenotoxic ability of the active fraction. Cytotoxicity studies revealed low toxicity of the most of fractions in Vero and in equine peripheral blood mononuclear cells. Antiviral studies showed that the water crude extract – Sephadex LH 20 – fraction 3 MeOH–H_2O (Fraction 3) was active against *Western Equine Encephalitis* virus with Effective Concentration 50% = 5 μg/ml. Selectivity Indices were 126.0 on Vero and 133.6 on peripheral blood mononuclear cells, four times higher than aqueous extract selectivity index. Regarding the mechanism of action we demonstrated that F3 exerted its action in intracellular replication stages. Further, fraction 3 showed important virucidal action. Fraction 3 contains, in order of highest to lowest: chlorogenic acid, luteolin, 5,7,8-trimethoxyflavone, 3-*O*-methylquercetin and caffeic acid. Fraction 3 did not induce *in vivo* toxic nor mutagenic effect. Therefore, it is safe its application as antiviral potential. Further studies of antiviral activity *in vivo* will be developed using a murine model.

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Introduction

Medicinal plants are a relevant source of biologically active substances. *Achyrocline satureioides* (Lam.) DC. is a medicinal plant commonly known as 'marcela del campo' which belongs to family Asteraceae, is native to America and also grows up in Europe and Africa. This plant is widely used in folk medicine. It is popularly consumed in infusion, decoction, macerated, and as syrup (García et al., 1990; Alonso Paz et al., 1992) for the treatment of digestive and respiratory problems, as well as for viral infections (Filot Da Silva and Langeloh, 1994; Instituto Nacional de Investigacion Agropecuaria, 2004; Taylor, 2005). Several pharmacological properties, such as anti-inflammatory, antioxidant, hypocholestorolemic, immunomodulatory, antimicrobial, antitumoral and antiviral have been scientifically confirmed (Ruffa et al., 2002; Bettega et al., 2004; De Souza et al., 2007; Ferraro et al., 2008; González and Marioli, 2010; Espiña et al., 2012; Barioni et al., 2013; Casero et al., 2015). In previous studies we demonstrated absence of cytogenotoxicity *in vitro* and *in vivo* of cold aqueous extract of *A. satureoides* at high concentrations (Sabini et al., 2013). In other studies, we reported strong antiviral activity of this crude aqueous extract against *Western Equine Encephalitis* virus (Sabini et al., 2012).

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Western Equine Encephalitis virus (WEEV) is a member of alphaviruses (Togaviridae family), which are a group of enveloped viruses with a positive sense, single-stranded RNA genome (Contigiani, 1996) which generates encephalitis in humans and equines. The encephalitic alphaviruses also include *Eastern* and *Venezuelan Equine Encephalitis* viruses (EEEV and VEEV). This complex of virus is arboviruses, arthropod-borne viruses, and they cause febrile illness and encephalitis. They are also the most important emerging pathogens and that have caused many epidemics in recent decades (Weaver and Reisen, 2010). Alphaviruses infect neurons resulting in CNS inflammation and neuronal destruction (Steele and Twenhafel, 2010). These highly virulent pathogens can cause severe disease in humans with high lethality, as well as long-term neurological sequelae in most survivors (Raveh et al., 2013). In infants and children this disease is even more serious and is often associated with seizures (Griffin, 2001). Moreover, this illness has economic importance because it causes significant morbidity and mortality in humans and animals. Besides, there are currently no effective antiviral drugs for alphavirus infections (Steiner et al., 2010). These viruses have been classified as potential agents of biological weapons that could endanger public safety (Zacks and Paessler, 2010). Therefore, it is a public health priority to develop antiviral agents for treatment of alphaviruses infection.

On the other hand, it has been shown that several flavonoids and phenolic acids have antiviral activity against viruses' spectrum (Orhan et al., 2009; Andres et al., 2009; Ozçelik et al., 2011; Ikeda et al., 2011). Therefore, vegetal fractions rich in flavonoids and phenolic acids obtained of *A. satureioides* could be active against *WEEV*.

The aim of the present study is to evaluate *in vitro* cytotoxic ability and antiviral action of five water extract chromatographic fractions obtained from *A. satureioides* against *Western Equine Encephalitis* virus and identifies the main compounds of the bioactive fraction by HPLC–ESI-MS/MS. Other aim is to evaluate *in vivo* cytogenotoxic ability of the active fraction, in order to consider its future application as antiviral in the treatment of viral diseases caused by alphaviruses.

Material and methods

Plant material

Achyrocline satureioides (Lam.) D.C., Asteraceae, plants were collected manually from Villa Jorcoricó, located in southern Córdoba hills (32°41′S; 64°43′W; 800 m.a.s.l.) in March 2010. 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).

Obtention of water extract chromatographic fractions of Achyrocline satureioides

Aerial vegetal parts (leaves, stems and blooms) were mechanically grinded using a knife mill (Retsch K.G. 5657Haan West-Germany) with a mesh no. 5 (sieve opening 4 mm) and were submitted to extraction with cold water (4 °C) (15 g of dried and pulverized material per 700 ml of water) for 2 days. This suspension was identified as cold aqueous extract (CAE). The suspension was filtered and lyophilized. The dried extract (1 g) was resuspended in water and was submitted to fractionation on Sephadex LH20 column (2.5 cm × 20 cm) eluted successively with CH_3OH (100%) to CH_3OH:H_2O (9:1). Five fractions were collected from CAE (fractions 1–5), which were subjected to drying in rotary evaporator.

Cell culture and virus

Bioassays were performed on Vero cells (ATCC CCL-81) grown in Eagle's minimal essential medium (MEM; Gibco, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Natocor, Argentina), L-glutamine (30 μg/ml) and gentamicin (50 μg/ml) (both from Sigma–Aldrich, Italy). Cell cultures were maintained at 37 °C with 5% CO_2 in a 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 Rockefeller mice. Viral stocks were stored at −70 °C.

Virus titration

Virus was titrated by quantification plaques-forming unit (PFU) method for arbovirus (Early et al., 1967). Cell monolayers grown in 24-well culture plates (Cellstar, Greiner Bio-One, Germany) were infected with 100 μl of ten-fold serial dilutions of virus per well, in duplicate and were incubated for 1 h at 37 °C. After that, residual virus was removed. Cells were washed with PBS, and overlaid with MEM-0.5% UltraPure Agarose (Invitrogen, USA) and further incubated for 96 h at 37 °C. After incubation, cell monolayers were fixed with 10% formalin (Cicarelli, Argentina) and further stained with 1% crystal violet solution.

Cytotoxicity assays

Cytotoxicity on Vero cells

For cytotoxicity assays, the cells (2×10^5 cells/ml) were cultured in 96-well culture plates. After incubation for 24 h at 37 °C, cells were exposed to increasing concentrations of F1, F2, F4, F5 (0–200 μg/ml) and F3 (0–1000 μg/ml). Assays were carried out in triplicate. Monolayers incubated only with maintenance medium (MM: MEM with 2% of FBS) were used as controls of cellular viability. The cytotoxic concentration of fraction which reduced the viable cell number by 50% (CC_{50}) was determined by neutral red uptake (NRU) assay.

After treatment of cells with water extract chromatographic fractions (Fs) for 96 h, the microplates were incubated with NR solution for 3 h at 37 °C and finally, with an extraction solution (49% distilled water:50% ethanol:1% acetic acid) for 15 min in a shaker. The absorbance was read at 540 nm on a multiwell spectrophotometer (Bio-Tek, ELx800, USA) (Rajbhandari et al., 2001; Seth et al., 2004). Percentage survival fraction was calculated considering optical density (OD) of treated cultures *versus* controls.

Isolation of equine peripheral blood mononuclear cells

Peripheral blood was drawn from healthy individuals. PBMC (Peripheral Blood Mononuclear Cells) were isolated from blood samples using Hystopaque-1077 centrifugation (Sigma–Aldrich, St. Louis, USA). From an optimal suspension 1×10^6 cells/ml, cell viability was determined by Trypan blue dye exclusion assay (Mongini and Waldner, 1996). The study was approved by the Comité de Ética de la Investigación Científica (COEDI) with number 73/2012, Universidad Nacional de Río Cuarto.

Cytotoxicity on equine PBMC

The cells (2×10^5 cells/well) in 200 μl of final volume were cultured in sterile 96-well microplates containing RPMI-1640 medium, added with 25 mM Hepes, 2 mM L-glutamine, 5% FBS, 50 mM 2-mercaptoethanol, 100 μg/ml streptomycin and 100 μg/ml penicillin. Cells were exposed to different concentrations of F1, F2, F4, F5 (0–200 μg/ml) and F3 (0–500 μg/ml). Cell cultures with only RPMI-1640 were used as control. The system was incubated at 37 °C with 5% CO_2 and humidified atmosphere

for 24 h. After that time, cell viability was evaluated by Trypan blue dye exclusion using Neubauer chamber for counting of viable cells, as described by Militao et al. (2006). Each experiment was done in triplicate.

Antiviral activity

Monolayers grown in 24-well culture plates (Cellstar, Greiner Bio-One, Germany) were exposed to 100 PFU of WEEV per well for 1 h at 37 °C and treated with one concentration of each F: F1 (50 μg/ml), F2 (20 μg/ml), F3 (50 μg/ml), F4 (50 μg/ml) and F5 (50 μg/ml). Cultures were washed and overlaid with MEM-0.5% UltraPure Agarose (Invitrogen, USA) containing each F at the same concentration which in the adsorption and penetration and incubated for 96 h at 37 °C. After incubation, cell monolayers were fixed with 10% formalin (Cicarelli, Argentina) and then stained with 1% crystal violet solution. Controls of virus, cells and F 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 viral controls to calculate the plaque reduction percentage.

Determination of the mechanism of action of F3

In order to study the antiviral activity of the F3, three experiments were performed by adding the fraction at different times and evaluating the inhibitory action by a plaque reduction assay.

Adsorption and penetration

Monolayers grown in 24-well culture plates (Cellstar, Greiner Bio-One, Germany) were exposed to 100 PFU of WEEV per well for 1 h at 37 °C in the presence of 50 μg/ml of F3. After adsorption, residual inoculum was removed, and MEM-0.5% agarose was added and incubated for 96 h at 37 °C. After incubation, cell monolayers were fixed with 10% formalin and stained with 1% crystal violet solution. Controls of virus, cells and F3 were included in all assays. The number of plaques of treated cells was compared to untreated viral controls to calculate the plaque reduction percentage.

Post-penetration

Cells were infected with 100 PFU of virus per well and incubated for 1 h at 37 °C. Then any unadsorbed virus was removed. Cells were washed with PBS, and then MEM-0.5% agarose containing 50 μg/ml of F3 was added. After incubation, cell monolayers were fixed with 10% formalin and stained with 1% crystal violet solution. Controls of virus, cells and F3 were included in all assays. The number of plaques of treated cells was compared to untreated viral controls to calculate the plaque reduction percentage.

Virucidal activity

To determine the ability of F3 to inactivate directly the virus particles, equal volumes of WEEV (200 PFU/100 μl) and F3 (50 μg/ml) were mixed and incubated for 1 h at 37 °C. Afterwards, each mixture was added to cultures (100 μl per well). It was incubated for 1 h at 37 °C. Then, monolayers were washed and covered with MEM-0.5% agarose. Controls of virus, cells and F3 were included. After incubation for four days at 37 °C, the cells were fixed with 10% formalin and stained with 1% crystal violet solution. The plaque reduction percentage was calculated.

Determination of 50% effective concentration (EC_{50})

Cell monolayers cultured in 24-well microplates were infected with 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 containing increasing concentrations of F3 was added. After four days at 37 °C, the cultures were fixed; stained and viral plaques were counted. The EC_{50} was calculated as the F concentration that reduced the number of PFU to 50% with respect to viral control. EC_{50} and CC_{50} values were estimated by non-linear regression of concentration–response curves generated from the data. Cytotoxicity and antiviral activity results were used to calculate the Selectivity Index (SI) of F3 ($SI = CC_{50}/EC_{50}$).

Genotoxicity assays

Animals and treatment

Male and female Balb/c mice aged 8–12-weeks old, (weighing 20–25 g) were obtained from the Central Bioterio of the Universidad Nacional de Río Cuarto. Animals had access to food and water *ad libitum* and were housed in a temperature controlled environment on a 12 h light/12 h dark cycle throughout the experimental period. All experimental procedures were conducted in accordance with recent legislation. This study was approved with number 73/2012 by Comité de Ética de la Investigación Científica (COEDI), Universidad Nacional de Río Cuarto. Three groups of mice were inoculated by intraperitoneal injection with F3 at concentrations of 3, 6 and 12 mg/kg body weight (b.w.) dissolved in saline solution and 2 control groups were included. The negative control group received saline solution and the positive control group received 30 mg/kg b.w. of cyclophosphamide (Sigma–Aldrich, St. Louis, US). Each treatment group consisted of four animals.

Micronuclei test in mouse bone marrow. The assay was carried out following standard protocols as recommended by Schmid (1975). The animals were sacrificed by cervical dislocation at 24 h post-injection. The bone marrow samples of femoral bone were obtained with FBS, were homogenized, centrifuged, and plated on slides which were fixed by soft flutter. Then, the slides were stained with May-Grunwald–Giemsa. To establish genotoxic capacity of F3, the frequency of micronuclei in 1000 polychromatic erythrocytes per slide was determined. To detect possible cytotoxic effects, the ratio of polychromatic erythrocyte/normochromatic erythrocyte (PCE/NCE) in 1000 polychromatic erythrocyte was calculated. The slides were scored using a light microscope at a 1000× magnification. Average number of micronucleated polychromatic erythrocytes (MNPCE) in individual mice was used as the experimental unit, with variability based on differences among animals within the same group.

Single-cell gel electrophoresis (comet assay). After 24 h of treatment with F3, peripheral blood was drawn from the tail vein of mice in heparinized tubes to perform the comet assay. Comet assay (CA) was carried out following a method described by Singh et al. (1988) with slight modifications. All determinations were performed by quadruplicate. A 50 μl aliquot of blood was mixed with 100 μl 0.75% low melting point agarose at 37 °C. Immediately 75 μl was spread onto two microscope slides per concentration pre-coated with 0.75% normal melting point agarose. The slides were coverslipped and allowed to gel at 4 °C for 20 min. The coverslips were gently removed and 75 μl of 0.75% low melting point agarose at 37 °C was added. Again, the slides were coverslipped and allowed to gel at 4 °C for 20 min. The coverslips were removed and the slides were immersed in cold, freshly prepared lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris (pH 10), supplemented with 1% Triton X-100 and 10% DMSO (Merck)). The slides, which were protected from light, were allowed to stand at 4 °C for 1 h. They were placed in a gel box, and left in high pH (>13) electrophoresis buffer (300 mM NaOH, 1 mM EDTA, prepared from a stock solution of 10 N NaOH and 200 mM EDTA) at 4 °C for 20 min before

electrophoresis to allow the DNA to unwind. Electrophoresis was carried out in ice bath (4 °C) for 20 min at 250 mA and 30 V (0.722 V/cm). The slides were submerged in neutralization buffer (0.4 M Tris–HCl, pH 7.5) for 15 min, dried at room temperature and fixed in absolute ethanol for 10 min. The slides were dried and stored overnight or longer before staining. For staining the slides were briefly rinsed in distilled water, covered with 25 μl 1× ethidium bromide staining solution prepared from a 200 μg/ml 10× stock solution, and coverslipped. The material was evaluated immediately at 400× magnification using fluorescence microscope (Axiophot, Carl Zeiss, Germany) attached to the image-analysis system (Powershot G6, 7.1 megapixels, Canon INC, Japan with software AxioVision Release 4.6.3, Carl Zeiss, Germany), with 515–560 nm excitation filter and a 590 nm barrier filter. From each treatment, images from 100 "nucleoids" were captured with a camera attached to the fluorescent microscope and linked to the CometScore® 1.5 software. Highly damaged cells were not included in the scoring (clouds were not analyzed). Tail moment (TM) was used to estimate DNA damage (arbitrary units).

Statistical analysis

Statistical analysis was performed using GraphPad Prism program, version 5.00.288 (San Diego, USA, 2007). The CC_{50} and EC_{50} were calculated from concentration–effect plots by non-linear regression analysis (*Boltzmann sigmoidal*). The results account for the mean ± standard error of the mean values of three different experiments. Results obtained by micronucleus assays were submitted to a *oneway analysis of variance* (ANOVA) and the *Tukey's multiple comparison* test. Comet assay results were submitted to *Kruskal Wallis* test and *Dunns multiple comparison* as *a posteriori* test were used in all the experiments. The Spearman statistical test was used to examine possible dose–response effects. In all cases, the level of significance was established at $p < 0.001$.

Identification and quantification of polyphenol and flavonoid derivatives in F3 by HPLC–ESI-MS/MS

Sample preparation

A solution of F3 (2.25 mg/ml) was prepared to carry out its qualitative and quantitative analysis by HPLC–ESI-MS/MS. MeOH–HPLC grade (Merck) was used in all samples, which were filtered through a Millipore membrane (0.45 μm) before HPLC analysis.

HPLC–ESI-MS/MS instruments and chromatographic conditions

An Agilent Series 1200 LC System (Agilent, USA) coupled to a MicrOTOF Q II (Bruker Daltonics, USA) was used for HPLC–ESI-MS/MS analysis. The HPLC system consisted in a micro vacuum degasser, binary pumps, an autosampler (40 μl sample loop), a thermostated column compartment, and a diode array detector. The mass spectrometer equipped with electrospray ion source and qTOF analyzer, was used in MS and MS/MS mode for the structural analysis of phenolics and flavonoids.

HPLC analysis were performed on a thermostated (40 °C) Hypersil 5 column C18 (30 × 4.6 mm, Phenomenex) at a 0.4 ml/min flow rate using MeOH–formic acid 0.16 M (53:47) as mobile phase (De Souza et al., 2002). The injection volume was 40 μl.

ESI-MS detection was performed in negative ion mode with mass acquisition between 100 and 1500 Da. Nitrogen was used as drying and nebulizer gas (7 l/min and 3.5 bar, respectively), and 180 °C for drying temperature. For MS/MS experiments fragmentation was achieved by using Auto MS^2 option. DAD analyses were carried out in the range between 200 and 700 nm.

Calibration standard samples were prepared by appropriate dilutions with MeOH from the stock solutions and filtered on Millipore membrane before use. MS analysis was used for quantification of the compounds with specific calibration curve. When reference compounds were not available, the calibration of structurally related substance was used. Compounds concentrations were calculated in triplicate and reported as means ± standard deviation in each case.

Results

Obtention of water extract chromatographic fractions of Achyrocline satureioides

Five water extract chromatographic fractions were obtained from CAE of *A. satureioides*. After drying them, 202.4 mg of F1, 68.6 mg of F2, 39.9 mg of F3, 48.6 mg of F4 and 277.3 mg of F5 were obtained. It indicated a yield of 20.24%, 6.86%, 3.99%, 4.86% and 27.73% for F1, F2, F3, F4 and F5, respectively.

Cytotoxicity assays

First, five water extract chromatographic fractions of *A. satureioides* were evaluated in their cytotoxic capacity for then to evaluate of their antiviral action. Cytotoxicity on Vero cells was determined by neutral red uptake (NRU) assay and, equine PBMC viability was determined by trypan blue dye exclusion method (TB). Cytotoxic concentrations 50% (CC_{50}) of water extract chromatographic fractions are presented in Table 1. F1, F4 and F5 indicated CC_{50} values above 200 μg/ml in both type of cells. The viability percent at 200 μg/ml was greater than 80% for these fractions. F2 was the most toxic on Vero cells with CC_{50} value of 35 μg/ml. However, it was not toxic in equine PBMCs whose CC_{50} was higher than 200 μg/ml. On the other hand, F3 indicated CC_{50} values of 630 μg/ml on Vero cells and 668 μg/ml on equine PBMC (Figs. 1 and 2). The effect of F3 on viability of Vero cells and equine PBMCs showed a dose-dependent decrease in the number of viable cells.

Antiviral activity of five water extract chromatographic fractions

Antiviral studies indicated that F1, F2 and F4 did not inhibit to WEEV. The percent of inhibition were lower than 30%. While, F5 and F3 indicated 45% and 100% of inhibition, respectively (Fig. 3). F3 was the most effective against WEEV. Thus, it was submitted at antiviral mechanism studies.

Determination of the mechanism of action of F3

To elucidate the mechanism of action of F3, WEEV was treated during adsorption and penetration and post-adsorption and penetration in separate trials. WEEV was not inhibited in adsorption and penetration stages, indicating 15% of inhibition. While it was inhibited in 100% when it was treated post-adsorption and

Table 1
Cytotoxicity of F1, F2, F3, F4 and F5 of *Achyrocline satureioides* on Vero cells and equine PBMCs determined by neutral red uptake (NRU) and trypan blue exclusion (TB) assays, respectively.

Fraction	Cytotoxicity	
	Vero cells by NRU (CC_{50} μg/ml)	Equine PBMCs by TB (CC_{50} μg/ml)
F1	>200	>200
F2	35	>200
F3	630	668
F4	>200	>200
F5	>200	>200

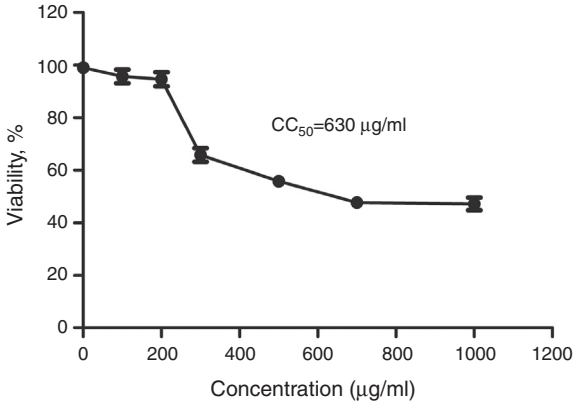

Fig. 1. Viability of Vero cells exposed to different concentrations of fraction 3 (F3) of *Achyrocline satureioides* for 96 h. The results are presented as percentage (mean ± SD). Cell viability was evaluated by neutral red uptake (NRU) assay.

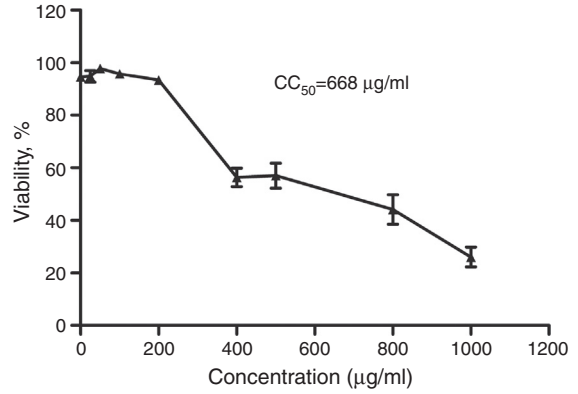

Fig. 2. Viability of equine PBMCs from healthy individuals exposed to different concentrations of fraction 3 (F3) of *Achyrocline satureioides* for 24 h. The results are presented as percentage (mean ± SD). Cell viability was evaluated by trypan blue dye exclusion (TB) method.

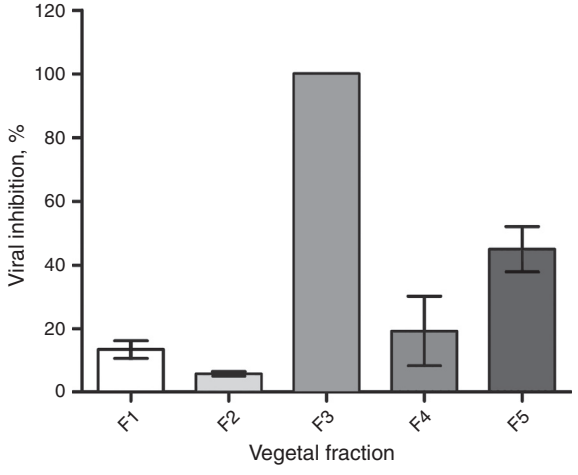

Fig. 3. Percent of inhibition of *Western Equine Encephalitis* virus (Ag 80-646) treated with five water extract chromatographic fractions obtained from *Achyrocline satureioides.*

penetration with 50 µg/ml of F3. Therefore, the fraction exerts its bioactivity in stages intracellular of the replication. Moreover, the virucidal action indicated a significant inactivation (60%) of WEEV when it was treated with 50 µg/ml of F3.

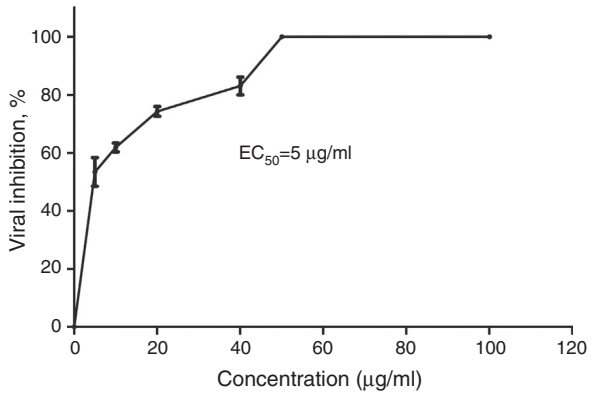

Fig. 4. Determination of effective concentration 50% (EC_{50}) of F3 of *Achyrocline satureioides* against *Western Equine Encephalitis Virus*. *Note*: Vero cell monolayers were infected with about 100 PFU per well, and incubated for 1 h at 37 °C. MEM-0.5% agarose with increasing concentrations (5–100 µg/ml) of F3 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.

Determination of 50% effective concentration (EC_{50})

Cell cultures treated with concentrations from 5 to 100 µg/ml of F3 was used to construct the dose–response curve which allowed to determine the EC_{50} value, see Fig. 4.

Table 2 summarizes the results of EC_{50}, CC_{50} and Selectivity Indices (SI) determined on Vero cells and equine lymphocytes. These values (SI) were 126.0 and 133.6 on Vero and PBMC, respectively. The SI of F3 is four times higher than the SI of cold aqueous extract (CAE) of *A. satureioides* which was 32 by NRU.

Genotoxicity assays

Micronuclei test in mouse bone marrow

The results of cytogenotoxic ability of F3 are shown in Table 3. In this study, the negative control group demonstrated low MNPCE values, as expected, and the positive control group's MNPCE frequency was significantly higher ($p < 0.001$), confirming the sensitivity of the test. The F3 genotoxicity analysis, for all doses tested (3, 6, and 12 mg/kg b.w.), indicated no significant increase in the MNPCE frequency at 24 h when compared with the negative control group ($p < 0.001$). The positive control which indicated a genotoxicity index of 31.75 (±2.21) showed significant difference with all treatments ($p < 0.001$).

Regarding F3 toxicity, statistical analysis of the PCE/NCE ratio revealed no significant differences between any of the treatments *versus* the negative control. Therefore, F3 of *A. satureioides* does not have cytogenotoxic effects.

Single-cell gel electrophoresis (comet assay)

The results of comet assay analyzed in mice's blood are shown in Fig. 5. Based on the tail moment results, positive control revealed significant difference with negative control ($p < 0.001$). All the treatments with F3 of *A. satureioides* did not show significant difference with negative control group while it did show statistically significant difference with mitomycin C (positive control group).

HPLC–ESI-MS/MS analysis of fraction 3 (F3) of A. satureioides

The chemical evaluation of F3 obtained from *A. satureioides*, was oriented toward the search for the active principles reported previously in the vegetal species (De Souza et al., 2002; Polydoro et al., 2004; Retta et al., 2012). By means of the qualitative

Table 2
Selectivity indices of F3 of *Achyrocline satureioides* against WEEV.

Fraction	CC_{50} (μg/ml)		EC_{50} (μg/ml)	SI (CC_{50}/EC_{50})	
	Vero cells	Equine PBMCs	against WEEV	Vero cells	Equine PBMCs
F3	630	668	5	126.0	133.6

Table 3
Frequency of micronucleated polychromatic erythrocytes (MNPCE) and polychromatic erythrocytes/normochromatic erythrocytes (PCE/NCE) ratio (TI) in mice bone marrow cells treated with different doses of F3 of *Achyrocline satureioides* for 24 h, and respective controls.

Treatments	Dose (mg/kg)	MNPCE (‰) (mean ± SD) Total	PCE/NCE (TI) (mean ± SD) Total
Negative control (saline solution)	0	7.00 (±0.70)	1.83 (±0.41)
A. satureioides F3	3	7.25 (±0.95)	1.79 (±0.09)
A. satureioides F3	6	7.60 (±1.34)	1.71 (±0.19)
A. satureioides F3	12	6.50 (±1.76)	1.64 (±0.34)
Positive control (cyclophosphamide)	30	31.75 (±2.21)[a]	1.61 (±0.12)

SD, standard deviation; MNPCE, micronucleated polychromatic erythrocytes; PCE, polychromatic erythrocytes; NCE, normochromatic erythrocytes; TI, Toxicity Index. In all cases 2000 polychromatic erythrocytes (PCE) were analyzed.
[a] Significantly different from negative control ($p < 0.001$) (ANOVA Tukey test).

Table 4
Chemical composition of F3 of *Achyrocline satureioides*.

Compound	Parent ion $[M-1]^-$ *m/z*	t_R (min)	% P/P (mg compound/100 mg F3)
Chlorogenic acid	353	6.8	1.360 ± 0.008
Caffeic acid[a]	179	6.3	0.004 ± 0.001
3-*O*-Methylquercetin[b]	315	22.3	0.009 ± 0.002
Quercetin-3-metoxi	331	5.1	nc
5,7,8-Trimethoxyflavone[b]	311	6.6	0.018 ± 0.002
Luteolin	285	21.9	0.537 ± 0.010

[a] 3-*O*-methylquercetin and 5,7,8-trimethoxyflavone expressed in quercetin.
[b] Caffeic acid expressed in chlorogenic acid.
nc = Is below the detection limit.

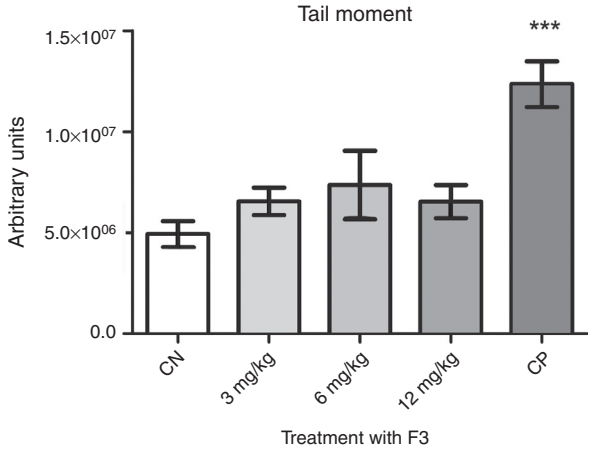

Fig. 5. Comet assay in blood of Balb/c mice inoculated with 3, 6 and 12 mg/kg b.w. of F3 of *Achyrocline satureioides*. Results are expressed as tail moment. Data shown are means of four replicate cells samples ($p < 0.001$; Dunns test).

HPLC-ESI-MS analysis, we were able to identify the presence of different flavonoids and organic acids (Table 4). The amount of each compound in the fraction under study is showed in Table 4 (quantification analysis). Data are expressed as means ± standard deviation (SD) of three separate experiments.

The components detected in the sample were, in order of highest to lowest: chlorogenic acid, luteolin, 5,7,8-trimethoxyflavone, 3-*O*-methylquercetin and caffeic acid.

Discussion

Medicinal plants are a rich source of molecules that may exert different bioactivities. Considering that there is no effective antiviral to treat diseases caused by alphavirus and, moreover, the disadvantages of use synthetic drugs, is very relevant to study medicinal plants as a possible solution to solve this problem.

Cytotoxicity studies revealed that the majority of fractions of *A. satureioides* had low toxicity in both type of cells. F1, F4 and F5 indicated viability percents greater than 80% at 200 μg/ml. F2 showed higher cytotoxic capacity against Vero cells (CC_{50} = 35 μg/ml) than against equine lymphocytes (CC_{50} > 200 μg/ml). Probably, the compounds present in F2 do not affect the integrity of the membranes of lymphocytes at concentrations that generate lysosomal damage in Vero cells.

Given that F3 was active against WEEV its cytotoxic potential was studied at higher concentrations. F3 showed CC_{50} values of 630 and 668 μg/ml on Vero cells and on equine PBMC, respectively. Moreover, the evaluation of F3 against equine lymphocytes allows us to express that this fraction at high concentrations did not affect this type of cells of immune system. This is very important because leucopenia occurs during viremia period of WEEV (Peters and Dalrymple, 1990).

There are few researches which evaluate the antiviral action against alphaviruses and, in particular, against WEEV (Madsen et al., 2014; Raveh et al., 2013; Sindac et al., 2013). There are no antiviral studies employing medicinal plants. However, in a previous paper, we were able to demonstrate the ability of an aqueous extract of *A. satureioides* to inhibit at WEE virus with a great selectivity index (SI = 32) (Sabini et al., 2012). In the present study, we demonstrated that fraction 3 showed to be more potent than the aqueous extract

against this alphavirus. Its SI was four times higher than the SI of aqueous extract. This indicates which fraction could be better for treating alphavirus infections.

Moreover, the SI of F3 was higher (126.0 and 133.6) than the SI of synthetic compounds such as third generation indole-2-carboxamide derivatives whose SI were at or below 14 (Sindac et al., 2013). These results support the use of natural products for treating diseases of viral etiology.

Respect to the mechanism of action, the fraction exerted its bioactivity in stages intracellular of the replication. Further, F3 showed an important virucidal action.

The chemical studies indicated that F3 contains, in order of highest to lowest: chlorogenic acid, luteolin, 5,7,8-trimethoxyflavone, 3-*O*-methylquercetin and caffeic acid. The present study showed that the fraction rich in chlorogenic acid and luteolin (F3) has strong inhibitory activity against *Western Equine Encephalitis* virus.

Some investigations reported antiviral action of the compounds present in F3. However, there is no background about antiviral capacity against WEEV or alphavirus. Thus, Xu et al., 2014 reported the antiviral ability of luteolin against other viruses, *Enterovirus 71* and *Coxsackievirus A16*. In other research it was demonstrated the inhibition of replication of *Enterovirus 71* by chlorogenic acid (Li et al., 2013).

Other researchers reported that *herpes simplex* virus was inhibited by caffeic acid (Ikeda et al., 2011). Also, caffeic acid inhibited the *influenza A* virus multiplication *in vitro* (Utsunomiya et al., 2014). Although caffeic acid is in low proportion in F3 it could be the responsible compound of its bioactivity against WEEV.

Also, it has been demonstrated antiviral capacity 3-*O*-methylquercetin, which was shown to be a potent inhibitor of poliovirus RNA synthesis (Castrillo et al., 1986). Maybe, the compounds present in F3 act alone or in a synergistic combination against WEEV.

On the other hand, the results of genotoxic assessments of F3 of *A. satureioides* by MN (mononucleous) test showed no significant increase the MNPCE frequency at all doses tested (3, 6, and, 12 mg/kg) at 24 h nor exhibited toxic activity by analysis of the PCE/NCE ratio in mouse bone marrow cells. Additionally, the comet assay analyzed in mice's blood indicated absence of genotoxicity in all treatments of F3. Therefore, in the present work, the *in vivo* studies indicated that F3 of *A. satureioides* by both tests of cytogenotoxicity was neither toxic nor genotoxic, and the obtained results support the application of this fraction such as antiviral drug with security. Further relevant studies of antiviral activity *in vivo* will be developed using a murine model.

In conclusion, F3 of *Achyrocline satureioides* showed low cytotoxicity *in vitro* in Vero and in equine lymphocytes cells. It showed great antiviral action *in vitro* against *Western Equine Encephalitis* virus and it could be used as neurotropic alphaviruses inhibitor. Furthermore, the fraction at concentrations tested did not induce *in vivo* toxic nor mutagenic effects. Therefore, its safe its application as antiviral potential.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Authors' contributions

Conceived and designed the experiments: MCS and LS. Performed the experiments: MCS (cytotoxic assays, antiviral activity and micronuclei test), FME and LNC (micronuclei test), FM and DI (comet assay), LC (chemical studies). Analyzed the data: MCS, FM and, SNM. Contributed reagents/materials/analysis tools: JJC, ML, JS, MC. Wrote the paper: MCS.

Conflicts of interest

The authors declare no conflicts of interest.

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