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▣ BRIEF COMMUNICATION

In Vitro Trypanocidal Activity of Macela (*Achyrocline satureioides*) Extracts against *Trypanosoma evansi*

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Abstract: The aim of this study was to verify the trypanocidal effectiveness of aqueous, methanolic, and ethanolic extracts of *Achyrocline satureioides* against *Trypanosoma evansi* in vitro. *A. satureioides* extracts, known as macela, were used on trypomastigotes at different concentrations (1, 5, 10, 50, 100, 500, and 1,000 µg/ml) and exposure times (0, 1, 3, 6, and 9 hr). A dose-dependent effect was observed when the 3 extracts were tested. The concentrations of 1, 5, and 10 µg/ml were not able to kill trypomastigotes until 3 hr after exposure, and the highest concentrations (500 and 1,000 µg/ml) were able to kill all trypomastigotes after 1 hr. When the time of exposure was increased up to 9 hr, the concentrations at 50 and 100 µg/ml were 100% effective to 3 extracts. The chemical analysis of the extracts revealed the presence of flavonoids, a trypanocidal compound already described. Based on the results, we can conclude that the *A. satureioides* extracts exhibit trypanocidal effects.

Key words: *Achyrocline satureioides*, *Trypanosoma evansi*, in vitro effect, flavonoid

Achyrocline satureioides is an annual aromatic plant originally from South America [1], belonging to the Asteraceae family, popularly known as "*marcela*" or "*macela*", used in human medicine in both Central and South America throughout the years [2]. Its inflorescence is commonly used in the form of tea because of its digestive, anti-inflammatory, antispasmodic, antidiabetic, and repellent effects [1-4]. Investigations on its chemical composition showed that the extract obtained from inflorescences is rich in flavonoids, mainly quercetin and luteolin, compounds known for their antioxidant effects [5]. The anticancer properties of quercetin and luteolin were also described, and researchers say that they are promising biomolecules for the development of new anticancer drugs in the future [6]. There are some studies regarding its antiparasitic activity of the aqueous extract against Giardia lamblia in vivo [7], and the ethanolic extract with larvicidal activity against mosquitoes of the species *Aedes fluviatilis* [8].

Trypanosoma evansi is a protozoan flagellate, the etiological agent of the disease known as "*surra*" or "*mal das cadeiras*" in horses [9]. This protozoan has wide geographical distribution, being found parasitizing various species of domestic and wild animals causing several pathological findings [9]. Patterns of this disease vary from acute epidemics with high case-fatality rates to subclinical and/or chronic disease in endemic animal populations. It is a problem of great economic importance due to the death of sick animals and high treatment costs [9-11].

Therapy for trypanosomiasis is based on chemotherapy with suramin, diminazene aceturate, quinapyramine, melarsoprol, homidium chloride, and isometamidium chloride, drugs known for their high toxicity in some cases (neurotoxic, hepatotoxic, and nephrotoxic), even at therapeutic doses [12]. However, some cases of parasite resistance to these drugs have been reported [10,11], as well as cases of inefficiency of diminazene aceturate, the only marketed drug in Brazil to treat animal with trypanosomosis. The inefficiency and recurrence of the disease after treatment have been related to the impossibil-

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ity of the drug to pass through the blood-brain barrier in a sufficient dose creating the possibility for trypanosomes to escape treatment during the systemic phase of the drug [13]. As a result of the toxicity of antiprotozoal associated with the inefficiency of some drugs, arises the need to find alternative drugs against *T. evansi*. Therefore, the aim of this study was to assess the in vitro trypanocidal effectiveness of aqueous, methanolic, and ethanolic extracts of *A. satureioides* against *T. evansi*, a plant with well known therapeutic properties.

A. satureioides was purchased from *Farmácia e Laboratório Homeopático Cruz Vermelha* (Santa Maria, RS, Brazil). The aqueous, ethanolic, and methanolic extracts were prepared by decoction in an open system [14]. High performance liquid chromatography (HPLC-DAD) was performed with a Shimadzu Prominence Auto Sampler (SIL-20A) HPLC system (Shimadzu, Kyoto, Japan) equipped with Shimadzu LC-20AT reciprocating pumps connected to a DGU 20A5 degasser with a CBM 20A integrator, SPD-M20A diode array detector and LC solution 1.22 SP1 software [15]. The extracts were analyzed at a concentration of 20 mg/ml. Stock solutions of standards used as references were prepared in the HPLC mobile phase at a concentration range of 0.030-0.250 mg/ml catechin, epicatechin, quercetin, quercitrin, isoquercitrin, kaempferol, and rutin, and 0.045-0.500 mg/ml for gallic, chlorogenic, caffeic, and ellagic acids. The chromatography peaks were confirmed by comparing its retention time with those of reference standards and by DAD spectra (200-600 nm). Limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of the responses and the slope using three independent analytical curves [16].

The trypomastigotes were acquired from blood of a dog naturally infected by *T. evansi* [17], which was maintained cryopreserved in liquid nitrogen under laboratory conditions. In order to obtain the trypanosomes for this experiment, 1 rat was experimentally infected intraperitoneally with blood of an infected dog. When the rat showed high parasitemia (1×10^7 trypanosomes/µl), it was anesthetized with isoflurane and its blood was collected by cardiac puncture and stored in an EDTA tube. For separation of the infected blood in rat, 200 µl of complete culture medium (diluted 1 v/v) stored in microtubes and centrifuged for 10 min at 400 g was used. The supernatant was removed, placed on the culture medium, and the parasites count was performed in a Neubauer chamber [18]. The procedure was approved by the Animal Welfare Committee of Federal University of Santa Maria under no. 65/2012.

The culture medium for *T. evansi* was formulated according to the method of Baltz [19], modified by Baldissera et al. [17]. Once prepared, the culture medium was stored under refrigeration (10℃) until the beginning of the experiment. Thus, 20 ml medium was divided into a test tube with the addition of 1 µl/ml of 50 mM hypoxanthine (dissolved in NaOH 0.1M) and 2 µl/ml of 1.2 mM 2-mercaptoethanol. After this procedure, the culture medium was enriched and taken to a CO_2 incubator (37℃ in 5% CO_2) for 2 hr prior to testing. The culture medium with the parasites was distributed in microtiter plates (270 µl/wells), followed by the addition of 25 µl of aqueous, ethanolic, and methanolic extracts at concentrations of 1, 5, 10, 50, 100, 500, and 1,000 µg/ml. As a control for the test, we used a medium and trypanosomes without any treatment, as well as a known anti-trypanosome drug (diminazene aceturate; 0.5 µg/ml). Water and DMSO were included in bioassays in order to validate the experiment since these two components of the extracts were used as diluents. The tests were performed in triplicate, with parasites count at 1, 3, 6, and 9 hr after the onset of the experiment in Neubauer chambers.

Bioassay results were subjected to analysis of variance (ANOVA) and Tukey's test to verify the accuracy of the data. Values $P < 0.05$ were considered statistically different.

The HPLC profile of *A. satureioides* (ethanolic, methanolic, and aqueous extracts) was also performed showing peaks (Fig. 1). Among the many compounds found in the extract, rutin, and quercetrin were in greater quantities. Fig. 1 shows the presence and amount of phenolic compounds and flavonoids in the constitution of different *A. satureioides* extracts.

In the present study, it was found that the diluents (DMSO and water) did not affect parasite viability and therefore they could be used as diluents without any interference in the results. That is, no significant difference was found in the control (no extract) compared to the control with water and 1% DMSO, used to dilute the extracts ($P > 0.05$). The diminazene aceturate in therapeutic dose was able to kill all trypanosomes after 6 hr of exposure (Fig. 2). A dose-dependent effect of trypanocidal effect was observed for aqueous, ethanolic, and methanolic extract at concentrations of 1,000, 500, 100, and 50 µg/ml and at different periods tested when compared to the control group (Fig. 2A-C). The 2 highest concentrations of aqueous, ethanolic, and methanolic extracts evaluated were able to kill all parasites after 1 hr of exposure. Three hours after the beginning of the test it was possible to identify a reduction of 78.8%, 45.8%, and 94.4% for the aqueous, ethanolic, and methanolic extracts,

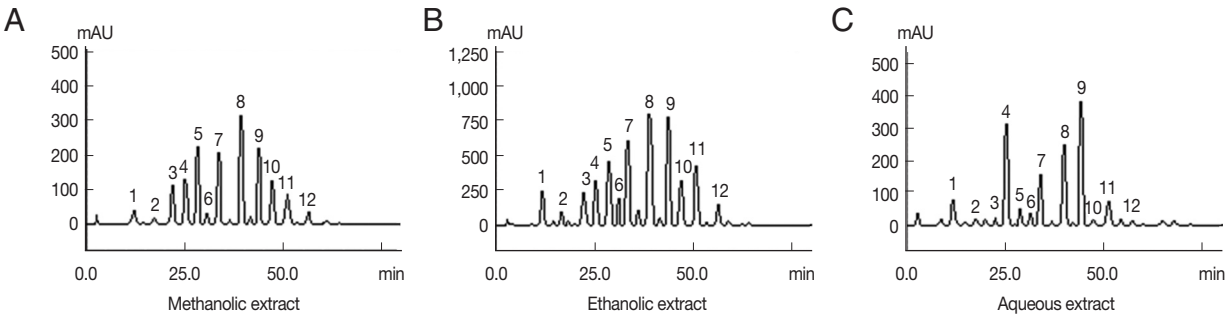

Fig. 1. High-performance liquid chromatography profile of phenolic and flavonoid compounds of *Achyrocline satureioides*, in the methanolic (A), ethanolic (B), and aqueous extract (C). Gallic acid (peak 1), catechin (peak 2), chlorogenic acid (peak 3), caffeic acid (peak 4), caffeic acid derivative (peak 5), epicatechin (peak 6), ellagic acid (peak 7), rutin (peak 8), quercitrin (peak 9), isoquercitrin (peak 10), quercetin (peak 11), and kaempferol (peak 12).

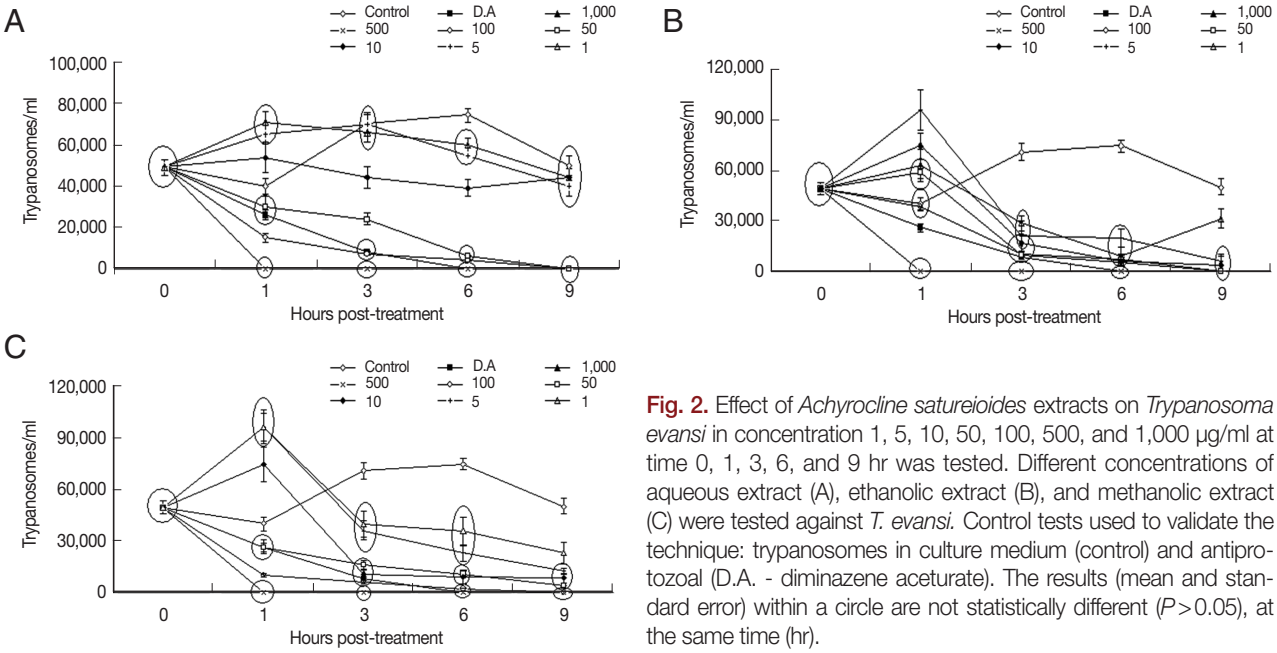

Fig. 2. Effect of *Achyrocline satureioides* extracts on *Trypanosoma evansi* in concentration 1, 5, 10, 50, 100, 500, and 1,000 μg/ml at time 0, 1, 3, 6, and 9 hr was tested. Different concentrations of aqueous extract (A), ethanolic extract (B), and methanolic extract (C) were tested against *T. evansi.* Control tests used to validate the technique: trypanosomes in culture medium (control) and antiprotozoal (D.A. - diminazene aceturate). The results (mean and standard error) within a circle are not statistically different ($P>0.05$), at the same time (hr).

respectively, in the number of live trypanosomes compared to the control group in this period when 100 μg/ml was used. For the concentration of 50 μg/ml, it was possible to identify a reduction of 28.8%, 17.0%, and 62.7% for the aqueous, ethanolic, and methanolic extracts, respectively, in the number of live trypanosomes when comparing to the control group in this period. Six hours after the beginning of the test, it was possible to observe a reduction of 90.3%, 87.0%, and 99.7% for the aqueous, ethanolic, and methanolic extracts, respectively, in the number of live trypanosomes compared to the control group in this period in the concentration of when 100 μg/ml was used. For the concentration of 50 μg/ml, it was possible to observe a reduction of 67.7%, 86.1%, and 91.9% for the aqueous, ethanolic, and methanolic extracts, respectively, in the number of live trypanosomes compared to the control group in this same period. After 9 hr from the beginning of the assay, it was not observed any live trypanosomes in the concentrations of 100 and 50 μg/ml, differently of what was observed in lower concentrations and control groups, keeping the living parasites.

In the present study, *A. satureioides* extracts showed trypanocidal effects against *T. evansi*. This plant has been cited by many authors because of its natural and active principles with various therapeutic properties [7,20,21], and it has been the subject of intensive scientific research using models in vivo and in vitro, which provided experimental evidence that plant extracts may have anti-inflammatory, antioxidant, and antiparasitic properties [20]. These previous studies have shown

that the caffeic acid and derivatives have antimicrobial and antiprotozoal activities [7,22]. In the current study, a dose-dependent trypanocidal effect of *A. satureioides* extracts against *T. evansi* was observed, i.e., in the concentrations of 500 and 1,000 µg/ml there was no longer trypomastigotes of *T. evansi* 1 hr after incubation.

The chromatography analyses showed that in 3 types of extraction, the major compounds found were flavonoids, but difference between the amounts of compounds in each extract is due to the choice of solvent extraction according to researchers [14]. Many authors have identified several types of flavonoids as trypanocidal principles of plant extracts [23-25]. These compounds have different mechanisms to cause death of trypanosomes, such as oxidative stress, autophagy, and osmotic pressure [26]. The other mechanism was the trypanocidal activity of plant compounds related by Mittra et al. [27] involving mitochondria, interfering with cell respiration of the parasite as described. Furthermore, flavonoid compounds induce the loss of both maxicircles and minicircles, resulting in the formation of dyskinetoplastid cells. The loss of mitochondrial DNA causes alteration in mitochondrial structure and consequently decreases in mitochondrial ATP production.

We concluded that aqueous, ethanolic, and methanolic extracts of *A. satureioides* have trypanocidal activities against *T. evansi* in vitro. The flavonoid compounds have shown promising trypanocidal activities/properties, and these results suggest that these extracts, once proven their in vivo action, may be an alternative in single treatment, or in association with others drugs.

CONFLICT OF INTEREST

We have no conflict of interest related to this work.

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