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# *In vitro* and *in vivo* trypanocidal action of aescin and aescin liposomes against *Trypanosoma evansi* in experimental mice

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PEER REVIEW

**Peer reviewer**

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**Comments**

The present study has addressed an important aspect of trypanosomosis research by evaluating the effectiveness of a potential adjuvant to trypanosomosis treatment in animals, aescin and aescin liposomes. Undoubtedly, the rearch results will add value to the current knowledge in this field.

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ABSTRACT

**Objective:** To verify the trypanocidal effectiveness of aescin and aescin liposomes against *Trypanosoma evansi in vitro* and *in vivo*.

**Methods:** Aescin and aescin liposomes were used *in vitro* on trypomastigotes at different concentrations (0.5%, 1.0% and 2.0%) and exposure times (0, 1, 3, 6 and 9 h). *In vivo* tests were performed using mice as the experimental model. *Trypanosome evansi* infected mice were treated with aescin and aescin liposomes with doses of 60 and 100 mg/kg during 4 d.

**Results:** The three concentrations tested in free form and nanoencapsulated showed trypanocidal activity *in vitro*, completely eliminating the parasites in small concentration after 6 h of assay. Animals treated with aescin (100 mg/kg) and aescin liposomes (100 mg/kg) showed increase in longevity, however without curative effect.

**Conclusions:** Active compounds present in natural products, such as aescin, may potentiate the treatment of trypanosomosis when used in association with other trypanocidal drugs.

KEYWORDS

Surra, Nanotechnology, *Aesculus hippocastanum*, *Trypanosome*

## 1. Introduction

*Trypanosoma evansi* (*T. evansi*) is the etiologic agent of a disease known as *Mal das cadeiras* or surra in horses[1]. Surra is an important disease in a wide geographic region (Africa, Asia, and South and Central America), and infects mainly camels, cattle, horses, buffalos and some wild animals[2-4]. The parasite is transmitted by infected blood through haematophagous insects such as tabanid flies[5,6]. The disease is endemic in some regions (Mato Grosso, Pantanal in Brazil,) and in other regions it occurs in outbreaks, hindering the prophylaxis and control.

The treatment of this disease in Brazil is based on diminazene aceturate (DA), but this drug is ineffective for umpteen animals[7,8]. Most of the drugs used for the treatment of the disease do not provide total elimination of the infection and are associated with recurrence and mortality[8]. In many cases, DA treatment may not be effective, leading to recurrent parasitemia[8-10], as well as hepatotoxicity, and nephrotoxicity[1,11]. As a result, researchers have tested natural products such as oils of copaiba, andiroba, aroeira, tea tree[12-14], and propolis extract[15].

Aescin is the predominant active constituent of *Aesculus hippocastanum* seed extract, which is a mixture of triterpene, saponins, consisting of A, B, C and D aescin[16]. Research has been focused on plant secondary compounds, such as saponins,

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for the control of parasite in sheep[17,18]. According to Carrasco and Vidrio aescin has been shown to be effective in the treatment of inflammatory conditions[19]. Actually, some studies have indicated that aescin is also a potential anticancer agent[20,21].

The field of nanoparticle synthesis, assembly, and application in biology is a fast growing area of nanotechnology and nanomedicine[22]. Through the development of materials that exhibit novel optical, chemical, and electrical properties at the nanometer-sized scale, it is hoped that it will be possible improve therapies for disease[23]. In this context, it can be highlighted the liposomes. Liposomes are spherical self-closed structures, composed of curved lipid bilayers, that encapsulate both hydrophilic and lipophilic substances[24]. The simplicity of production, their biocompatibility, low toxicity, size and similar composition to cells make them a revolutionary tool in biomedical domain. Based on the needs for curative therapy of *T. evansi* and on the properties of aescin and the antiparasitic properties of saponins mentioned above, the present study analyzed for the first time the *in vitro* and *in vivo* activity of aescin and aescin liposomes against *T. evansi*.

## 2. Materials and methods

### 2.1. T. evansi isolate

This study was set up in two consecutive experiments (*in vitro* and *in vivo*). The same *T. evansi* isolate was used in both experiments[10]. Two rats (R1 and R2) were infected intraperitoneally with trypomastigotes contaminated blood kept cryopreserved in liquid nitrogen. This procedure was performed to obtain a large amount of viable parasites for *in vitro* tests (R1), and to infect the experimental groups (R2).

### 2.2. Aescin and aescin liposomes

Aescin was purchased from (Sigma-Aldrich®, St. Louis, USA). Aescin liposomes were prepared with 0.5% aescin using a proprietary method from Inventiva®. Particle size and polydispersion index were evaluated by dynamic light scattering. Zeta potential was evaluated using electrophoretic mobility technique. The samples were diluted in Milli-Q water (500 ) and the assays were performed using Zeta Sizer Nanoseries, Malvern. The pH was assessed by direct use of Digimed potentiometer according to Da Silva[25].

### 2.3. In vitro tests

The culture medium for *T. evansi* was adapted from Baltz as previously published by Baldissera[13,26]. The trypomastigotes were acquired from the infection of one rat (R1) with a *T. evansi* isolate. Five days post-infection rat showed high parasitemia ($7.5\times10^6$ trypanosomes/μL). The rat was was anesthetized with isoflurane for blood collection by cardiac puncture, and blood was stored in ethylene diamine tetraacetic acid tubes. For trypanosomes separation, each 200 μL blood was diluted in complete culture medium (200 μL), stored in microcentrifuge tubes and centrifuged for 10 min at 1 550 r/min. The supernatant was removed and resuspended in culture medium and the number of parasites was counted in a Neubauer chamber.

The culture medium with the parasites was distributed in microtiter plates (270 μL/well), followed by the addition of 25 μL of aescin (diluted in culture medium) at concentrations of 0.5%, 1.0% and 2.0%. The aescin liposomes also were used at concentrations of 0.5%, 1.0% and 2.0 %. A positive control (DA at a dilution of 0.5%) was also used, at the same volume (25 μL). The tests were performed in duplicates and the parasites were counted at 1, 3, 6 and 9 h after the onset of the experiment in Neubauer chambers. The microtiter plates were placed in a 5% $CO_2$ incubator at 37 °C according to Baltz[26].

### 2.4. In vivo test

#### 2.4.1. Animal model

Forty-two, female, 60-day-old-mice weighing an average of (23.0±0.7) g were used as the experimental model. They were kept in cages with six females each, housed on a light/dark cycle of 12 h, in an experimental room with controlled temperature (23±1) ℃ and humidity 70%. They were fed with commercial feed, and water *ad libitum*. All animals were subjected to a period of 10 d for adaptation.

#### 2.4.2. Experimental design and parasitemia estimation

The mice were divided into seven groups (A to G). Group A consisted of uninfected mice and untreated (negative control); Group B consisted of infected mice and untreated (positive control); Group C was composed of animals infected and treated with aescin 60 mg/kg; Group D was composed of animals infected and treated with aescin 100 mg/kg; Group E was composed of animals infected and treated with aescin liposomes 60 mg/kg; Group F was composed of animals infected and treated with aescin liposomes 100 mg/kg; Group G was composed of animals infected and treated with DA. Infected animals were inoculated intraperitoneally with 0.05 mL of blood from one rat containing $1.1\times10^6$ trypanosomes.

The DA was administered in a single dose of 7.0 mg/kg, intraperitoneally injection, and 1 h after infection of the animals. Aescin and aescin liposomes were administered orally for 4 d, starting the 1 h following infection.

The evolution of parasitemia and the effect of the treatment were daily monitored through blood smear. Each slide was prepared with fresh blood collected from the tail vein, stained by the panoptic method, and visualized at a magnification of 1000× according to Da Silva[27].

#### 2.4.3. Treatment efficacy

Treatment efficacy was determined by the number of mice that did not show clinical signs of *T. evansi* infection after treatment. Prepatent period, longevity and animal mortality were also observed.

### 2.5. Statistical analysis

Data from *in vitro* were analyzed by analysis of variance for repeated measures and comparison of concentrations tested for aescin. Data of the prepatent period and longevity were submitted for analysis of variance according to Duncan test ($P<0.05$).

## 3. Results

### 3.1. Aescin liposomes

The prepared suspension was evaluated regarding to their physical-chemical properties. The particle size was (223±6) nm and the

polydispersion index was (0.211±0.059) with a zeta potential of (-18.1±1.7) mV.

### 3.2. In vitro test

The results showed a trypanocidal effect of aescin and aescin liposomes on *T. evansi* directly proportional to the concentration used (Figure 1). After 1 h, there were no living trypomastigotes in 1.0% and 2.0% concentrations. A reduction of live trypomastigotes was observed at the concentration of 0.5% when compared with the control group. After 3 h of the assay, there were no living trypomastigotes in 0.5% concentration and DA treatments (Figure 1A).

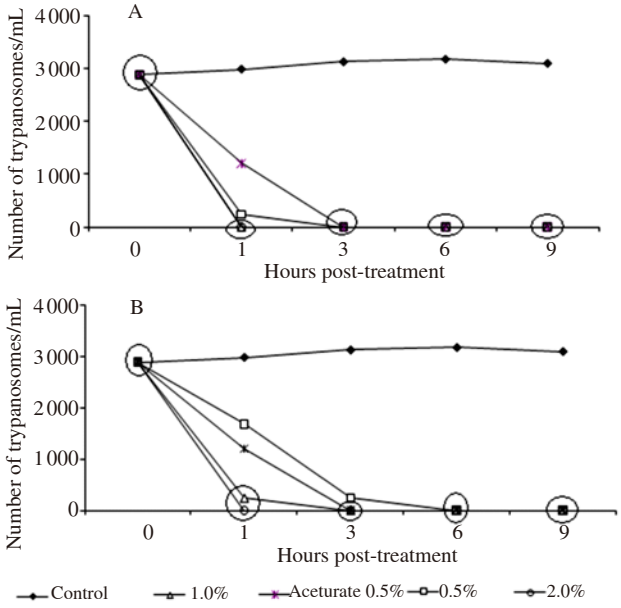


**Figure 1.** *In vitro*, trypanocidal activity in concentrations 0.5%, 1.0% and 2.0% of aescin (A) and aescin liposomes (B) against *T. evansi* when compared to the control (not-treated) and diminazene aceturate (anti-protozoa drug).

The results within a circle are not statistically different ($P$>0.05), at the same time (h).

The aescin liposomes results are shown in Figure 1B. After 1 h, a reduction of live trypomastigotes was observed at the concentrations of 0.5%, 1.0% and 2.0%, respectively, when compared with the control group. After 3 h of the assay, there were no living trypomastigotes in 2.0% and 1.0% concentration and DA, and a reduction of live trypomastigotes at the concentration of 0.5%. After 6 h of the assay, there were no living trypomastigotes forms in 0.5% concentration. To the contrary, in control tests (not using drugs), the parasites were all alive (Figure 1), which validates our experiment.

### 3.3. In vivo test

There were no differences between groups regarding the prepatent period (Table 1). Longevity of the group A was exactly represented by the days that the experiment lasted (10 d). Longevity in the groups B, C, D, E, F and G were 4.0, 5.1, 11.5, 6.0, 10.5 and 40 days, respectively. The groups C, D, E and F had no curative efficacy, but the groups D, E and F increased longevity compared to the group B. The animals treated with DA showed negative blood smear during the 40 days of the period of our study.

**Table 1**

*In vivo* test-mean and standard deviation of the prepatent period, longevity and mortality using treatment with aescin, aescin liposomes and DA in mice experimentally infected by *T. evansi*.

| Groups ($n$=6) | Treatment[#] | Prepatent period (d) | Longevity (d) | Mortality ($n$) |
|---|---|---|---|---|
| A | Negative control | - | 40.00±0.00[a] | 0/6 |
| B | Positive control | 1.16±0.40[a] | 4.00±0.63[d] | 6/6 |
| C | Aescin (60 mg/kg) | 1.83±0.75[a] | 5.10±0.98[cd] | 6/6 |
| D | Aescin (100 mg/kg) | 2.00±0.89[a] | 11.50±1.01[b] | 6/6 |
| E | Aescin liposomes (60 mg/kg) | 2.11±0.75[a] | 6.00±1.22[c] | 6/6 |
| F | Aescin liposomes (100 mg/kg) | 1.66±1.03[a] | 10.50±1.04[b] | 6/6 |
| G | DA (7.0 mg/kg) | 0.00±0.00[a] | 40.00±0.00[a] | 0/6 |

Means followed by same letter in the same column do not differ significantly in the Duncan test; The experiment lasted 40 d post-infection; [#]: Survival animals in the end of experiment, showed negative PCR and blood smears.

## 4. Discussion

This study observed a trypanocidal action *in vitro* and *in vivo* of aescin and aescin liposomes. The results showed a dose-dependent trypanocidal effect of aescin and aescin liposomes against *T. evansi* trypomastigotes *in vitro*. The aescin had apparently a faster trypanocidal effect *in vitro* than the aescin liposome. This was expected, since nanotechnology process usually provides a more slow and gradual properties of release. Saponins have been cited by researchers because of their active substances with various therapeutic properties and have a range of biological and pharmacological properties such as antimicrobial, anti–inflammatory and anti-cancer[28]. *In vivo* and *in vitro*, studies have shown the effect of saponins against protozoa in the rumen of lambs[29,30]. There are also as reports the saponins extracts from *Maesa balansae*, *Careya arborea* and *Astragalus oleifolius* having antileishmaninal activity[31].

Based on these previous promising *in vitro* results, we have designed an *in vivo* experiment using mice infected by *T. evansi* as a model. However, the therapeutic protocol used with aescin had no curative effect for all the groups, but in a group's treated with aescin and aescin liposomes (Groups D, E and F), an increase in longevity of animals was observed. According to Kedzierski[32], β-aescin, the major compound found in *Aesculus hippocastanum*, showed moderate active on the intracellular amastigote stage of *Leishmania infantum* and highest active on the extracellular promastigote stage. The accurate mechanism the active of β-aescin is uncertain, but saponins show anti-leishmaninal activity through the induction of apoptosis or programmed cell death in the parasite[33,34].

Biological compounds with trypanocidal activity incorporated into liposomes are described since 1987. Liposomes containing stearylamine rapidly killed epimastigotes, amastigotes and trypomastigotes forms of *Trypanosome cruzi in vitro*[35]. Van de Ven also demonstrate *in vitro* that β-aescin nanoparticles showed leishmanial activity[36]. This study also looked for action against of *T. evansi*, a protozoon of the same family of *Trypanosome cruzi* and *Leishmania* sp. The best therapeutic response observed with use of liposomes is mainly the ability to modify the surface of target cells and tissues, and the absorption peak or phagocytic mechanisms and the ability to overcome intracellular barriers and to massively deliver trypanocidal drugs into an extremely small volume[37].

The utilization of liposomes is advantageous because these nanoparticles are very similar to the cell membranes interacting more closely and with greater efficiency with cells and tissues[38].

In addition, liposomes are nontoxic, biodegradable and can be produced easily and quickly on a large scale. Finally, they can be administered by intravenous, ocular, pulmonary, or dermal route[24]. It is also important to emphasize the great advantage of overcoming physicochemical properties of encapsulated drugs (such as water solubility or membrane), thus improving the pharmacodynamics (therapeutic effect potentiation), pharmacokinetics (absorption control and tissue distribution) and their toxicological effects (reduction of local and systemic toxicity)[39]. Barratt also showed that some liposomes may lead to dose reduction, decreasing the frequency used without loss of effectiveness, reducing costs of therapy and the risk of toxicity, thus demonstrating that the strategy to modify the biodistribution profile of the drug is more effective than the free drug use[40].

Based on the result it was concluded that pure aescin pure and liposome aescin has trypanocidal action against *T. evansi* in culture medium, to cause induction to apoptosis or programmed cell death in the parasite. Nevertheless, the treatment had no curative efficacy in infected mice with *T. evansi*, however, it demonstrated increased longevity of animals.

## Conflict of interest statement

We declare that we have no conflict of interest.

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## Comments

### *Background*

Surra, a disease caused by *T. evansi,* is of economic importance and affects a wide range of domestic animals such as horses, cattles, camels, buffalos as well as wild animals. The currently available drugs are usually expensive and toxic, and their effectiveness in most cases has been curtailed by the development of drug resistance. Thus, the present research evaluated the trypanocidal action of aescin and aescin liposomes against *T. evansi* as potential treatments for trypanosomosis.

### *Research frontiers*

Development of new trypanocidal drugs is based on rational drug design, synthesis or natural product screening, but also taking advantage of compounds already in use against other animal or human pathogens for the development of new drugs. The current state of knowledge of parasite biochemistry and the sequencing of the parasite genome has favoured the development of new chemotherapeutic approaches based on newly validated biochemical targets and the present research is within these bounds.

### *Related reports*

Aescin is a mixture of saponins that has been shown to have anti-inflammatory, vasoconstrictor and vasoprotective properties. Research has demonstrated the bioactivity and therapeutic effects of saponins such as aescin. Saponins can complex with cholesterol to form pores in the cell membrane bilayers. Their modes of action can augment the cytotoxicity of toxins against cells.

### *Innovations and breakthroughs*

In the present study, the authors have demonstrated the trypanocidal effects of aescin and aescin liposomes on *T. evansi*, a disease of economic importance.

### *Applications*

The saponins present in aescin can potentiate the treatment of trypanosomosis when used with other trypanocidl drugs. This may also alleviate the potential for drug resistance.

### *Peer review*

The present study has addressed an important aspect of trypanosomosis research by evaluating the effectiveness of a potential adjuvant to trypanosomosis treatment in animals, aescin and aescin liposomes. Undoubtedly, the rearch results will add value to the current knowledge in this field.

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