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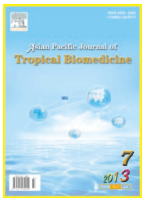

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Saponins-rich fraction of *Calotropis procera* leaves elicit no antitrypanosomal activity in a rat model

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PEER REVIEW

Peer reviewer

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Comments

The paper is highly relevant in terms of use of herbal medicines for treatment of diseases. Materials and methods are well designed. Findings are interesting and interpreted scientifically in discussion section.
Details on Page 572

ABSTRACT

Objective: To examine the *in vitro* and *in vivo* anti-*Trypanosoma evansi* (*T. evansi*) activity of saponins-rich fraction of *Calotropis procera* (cpsf) leaves as well as the effect of the fraction on the parasite-induced anemia. **Methods:** A 60-minutes time course experiment was conducted with various concentrations of the fraction using a 96-well microtiter plate technique, and subsequently used to treat experimentally *T. evansi* infected rats at 100 and 200 mg/kg body weight. Index of anemia was analyzed in all animals during the experiment. **Results:** The cpsf did not demonstrate an *in vitro* antitrypanosomal activity. Further, the cpsf treatments did not significantly ($P>0.05$) keep the parasites lower than the infected untreated groups. At the end of the experiment, all *T. evansi* infected rats developed anemia whose severity was not significantly ($P>0.05$) ameliorated by the cpsf treatment. **Conclusions:** It was concluded that saponins derived from *Calotropis procera* leaves could not elicit *in vitro* and *in vivo* activities against *T. evansi*.

KEYWORDS

Anemia, Asclepiadaceae, *Calotropis procera*, saponins, *Trypanosoma evansi*

1. Introduction

Animal trypanosomiasis is still a major factor retarding the growth of the livestock industry in Africa. One of the important pathogenic agents of the disease in animals is *Trypanosoma evansi* (*T. evansi*), the causative agent of *Surra* that is highly fatal to a number of domesticated mammals such as camels, horses and water buffaloes among others[1]. Since the adaptation of the parasite to mechanical transmission by blood sucking insects (tabanids), the disease has spread beyond its original distribution in sub-Saharan Africa and is now also present in South America, North Africa and large parts of Asia[1]. Prevalence of the disease is strongly dependent on control measures, which are often neglected during periods of political instability leading to resurgence and on the other hand, the hope for vaccine development against the infection is still elusive[2,3]. Thus, in the absence of either effective vector control or vaccine, chemotherapy remains the only available option. Unfortunately however, the chemotherapeutic approach is beset with several problems, which include a limited repertoire of compounds, cost, drug resistance and toxicity to a protracted treatment protocol[4].

Traditional herbal medicines have been used throughout the world for the treatment of various diseases and their influence in drug discovery is impressive because a number of clinically active drugs are derived from these natural products or have a natural product pharmacophore[5]. Interestingly, a number of ethnopharmacological studies revealed that hundreds of tropical plants contain potent trypanocidal agents[6-9]. This makes a recourse to plants as sources of antitrypanosomal agents an appealing alternative. However, in order to produce effective trypanocides, potential group(s) of phytochemical(s) need to be identified but at present, very little information exists on the group(s) of phytochemical(s) responsible for the observed antitrypanosomal activity of most tropical plants.

Calotropis procera (*C. procera*) (milkweed) is a wild-growing tropical plant belonging to the family Asclepiadaceae and is commonly used in the traditional treatment of epilepsy,

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inflammation, microbial and protozoan infections[10]. Plants of the Asclepiadaceae family are characteristically known to be rich in cardinolides and saponin glycosides[11]. Saponins chemically consist of fat-soluble nucleus (aglycone) that is either a triterpenoid (C-30) or steroid (C-27) attached with one or more sugar side chains (glycone) at different carbon sites of the aglycone. They have characteristic surface active properties and form foamy solutions in water[11]. In our continued interest to search for novel active trypanocidal compounds from medicinal plants, we investigated the *in vitro* and *in vivo* antitrypanosomal activity of a partially purified saponins- rich fraction of *C. procera* with a view to determine their antitrypanosomal potentials, or otherwise.

2. Materials and methods

2.1. Plant material and preparation of saponins-rich fraction

The leaves of *C. procera* was collected from the Samaru campus of Ahmadu Bello University Zaria (ABUZ), Nigeria and the species was identified at the herbarium unit of Biological Sciences Department of the same university. The voucher herbarium specimen was deposited with number 900219. The leaves were removed and shade-dried for two weeks to a constant weight. The dried leaves were pounded to fine powder with mortar and pestle, and then stored in dry containers. The saponins-rich fraction was prepared as described by Aliyu *et al*[11]. Briefly, the powdered dried plant material (500 g) was defatted with petroleum ether and exhaustively extracted with 2.5 L of methanol (maceration) for a week. The extract was filtered using Whatman filter paper (No. 2) and concentrated on a Büchi rotary evaporator at 40 °C under reduced pressure to yield a residue (83.5 g) referred to as crude methanol extract. The methanol crude (50 g) was suspended in 1 L of water saturated with *n*-butanol in a separatory funnel. The *n*-butanol portion was separated and collected from the aqueous portion. Subsequently, diethyl ether (200 mL) was added to the *n*-butanol portion to precipitate the saponin (15.37 g) which was considered as the saponins-rich fraction (cpsf).

2.2. Experimental animals and trypanosome parasites

The protocol employed met the rules and regulations governing handling of laboratory animals as stipulated by the animal research ethics committee of ABUZ and the guidelines of the Good Laboratory Practice regulations of World Health Organization were also duly followed. Apparently healthy wistar rats weighing 130-165 g were obtained from the animal house of National Research Institute for Chemical Research Technology, Zaria. The animals were maintained in polycarbonated laboratory cages [(25±2) °C, 12-h light/dark cycle] and fed on a commercial rat chow (ECWA Feeds, Jos, Nigeria) with drinking water *ad libitum*. The *T. evansi* parasite (Kano strain) used for the study was obtained from the Protozoology laboratory, Department of Veterinary Parasitology and Entomology, Faculty of Veterinary Medicine, ABUZ, Nigeria.

2.3. In vitro screening of cpsf against T. evansi

Different concentrations of the cpsf ranging from 5 to 20 mg/mL were prepared. The *in vitro* antitrypanosomal activity was assessed in triplicates in 96-well microtiter plates. In the wells of the microtiter plates, aliquots of 20 μL of each extract concentration were incubated with 40 μL of the infected blood (about 10^9 parasites per mL of blood), achieving effective cpsf concentrations of 6.66, 3.33 and 1.67 mg/mL in the reaction mixtures. The fraction was replaced with phosphate buffered saline glucose and 6.66 mg/mL of a standard trypanocidal drug diminazine aceturate (DA) for control and reference tests respectively. Parasite count was then monitored under a microscope at ×400 magnification. The percentage of motile parasites was counted at 5 min intervals for 1 h. Cessation or drop in motility of the parasites in cpsf- and berenil-treated blood compared to that of parasite-loaded control blood without the fraction was taken as a measure of antitrypanosomal activity[12].

2.4. In vivo anti- T. evansi activity of cpsf

Thirty rats were randomly divided into 6 groups of 5 rats each. Rats in four of the groups were each infected by intraperitoneal injection of about 10^6 *T. evansi* per 100 g bw while rats in the remaining two groups were uninfected. The level of parasitemia was monitored daily as described by Herbert and Lumsden[13]. On day 4 post infection (pi) when parasitemia approximately reached 10^8 trypanosomes/mL of blood, a pair of infected groups was orally treated with 100 mg/kg bw of cpsf and DA whereas one infected group was treated with 200 mg/kg bw of cpsf and the remaining group of infected rats was left untreated (infected control). One group of uninfected rats was also treated 200 mg/kg bw of cpsf (fraction control) and the remaining one group was left untreated (normal control). All treatments were given daily from day 4 pi to the end of the experiment on day 14 pi. The pre-infection and terminal packed cell volumes (PCV) of all the rats were determined using the microheamatocrit method at Days 0 and 14 pi .

2.5. Statistical analysis

All data are presented as the mean±SD of triplicates determination. Data were analyzed by using a statistical software package (SPSS for Windows, version 18, IBM Corporation, NY, USA) using Tukey's-HSD multiple range *post-hoc* test. Values were considered significantly different at $P<0.05$.

3. Results

The cpsf was initially tested for *in vitro* activity against the bloodstream form of *T. evansi* but the fraction was not effective in reducing the *T. evansi* motility (Figure 1A) because there was no difference in the number of motile parasites between the parasite-loaded control blood and the cpsf-treated blood samples. The parasitemia profiles (Figure 1B) to demonstrate the effects of oral treatments of different doses of the cpsf indicated that the trypanosomes were first detected, in the bloodstream of all infected groups on day 4 pi, but the cpsf treatment did not suppress the multiplication of the parasites. However, diminazine aceturate significantly ($P<0.05$) lowered the number of parasites than what was recorded in the infected untreated group.

There were no significant ($P>0.05$) differences in the initial PCV of all groups of rats (Figure 2) but all infected groups developed anemia as the infection progressed. This is indicated by the significant ($P<0.05$) drops in the final PCV of all infected groups. However, the T.evansi-induced anemia was not significantly (P 0.05) ameliorated by all the cpsf treatments.

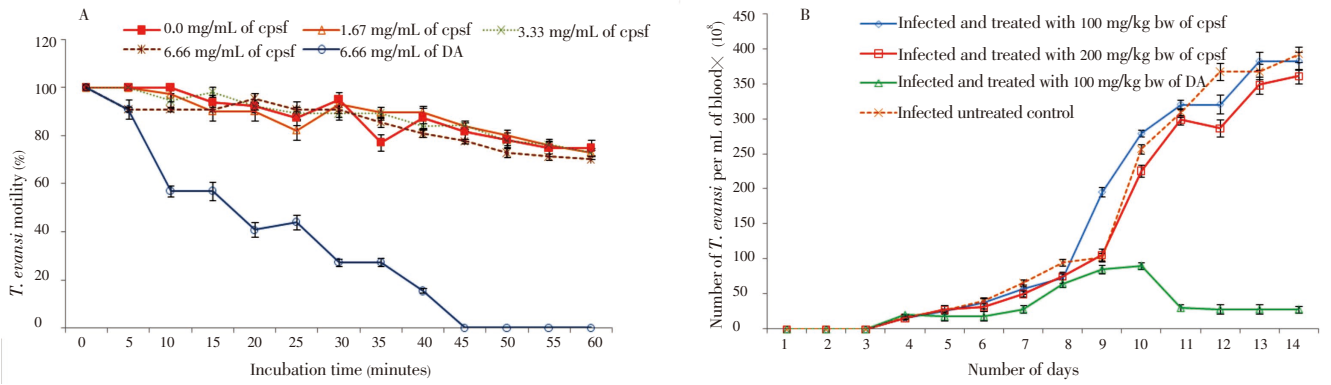

Figure 1. *In vitro* (A) and *in vivo* (B) anti-*Trypanosoma evansi* activities of saponins-rich fraction of *C. procera*.
Cpsf and DA means saponins-rich fraction of *C. procera* and diminazine aceturate respectively.

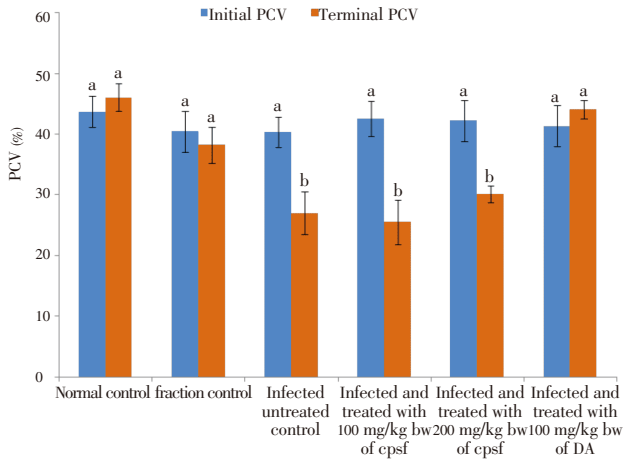

Figure 2. Effects of different doses of cpsf on the PCV levels of *T. evansi* infected rats.
Values with different letters over the bars for a given group are significantly different from each other (Tukey's-HSD multiple range post hoc test, $P<0.05$). Cpsf and DA means saponins-rich fraction of *C. procera* and diminazine aceturate respectively.

4. Discussion

Identification of some group(s) of phytochemical(s) with trypanocidal potentials is a serious concern to scientific community and pharmaceutical industries, as it would help to establish the usefulness, or otherwise, of such phytochemicals as chemical leads for the development of newer generation of trypanocides. Parasite motility is a relatively reliable indicator of viability of most zooflagellate parasites[14]. Cessation or drop in motility of trypanosomes therefore serves as a measure of antitrypanosomal potential of an agent when compared to the control. In the present study, the cpsf did not affect the *T. evansi* motility. This insensitivity to cpsf by the parasites could indicate that the fraction neither cross the trypanosome membrane to alter the dynamics of an obligatory parasite process nor interfere with the function of any cell surface protein. However, a phytochemical with high *in vitro* activity may show no *in vivo* antitrypanosomal activity and vice versa, due to xenobiotic metabolism that may convert active therapeutic molecules to inactive ones, therefore, we further tested cpsf for *in vivo* activity so that a definite statement can be made on its antitrypanosomal effects.

Various classes of phytochemicals such as hydrozable tannins, flavonoids, sesquiterpene lactones, alkaloids, diamidines and lipophylic amines have been reported to possess antitripanosomal activity[14,15]. However, saponins derived from *C. procera* in this study did not mediate the killings of *T. evansi* using *in vitro* and *in vivo* models but at the same time, did not completely exclude the trypanocidal potentials of saponins from other plant species. This is because geographical location, sample collection time and solvent system used are known to affect the phytochemistry of plant materials. It is also possible that the observed resistance to cpsf is restricted to *T. evansi* and not to other trypanosome species because the biochemistry and pathophysiology of *T. evansi* are distinct from others[16,17]. Indeed, species dependent factors have been shown to affect the sensitivity of trypanosomes to different phytochemicals[8].

Anemia is a consistent feature of trypanosome infections[18], caused by, among other factors, oxidative damage to erythrocyte membrane components[6,19]. Thus, the presence and severity of this pathological alteration is a good indicator of the disease status and its control is an integral part of the disease management. Our study demonstrated that the cpsf did not ameliorate the trypanosome-induced anemia which could be linked to the failure of the fraction to inhibit the survival of the parasites *in vivo* since the severity of the trypanosome-induced anemia was linearly linked to the degree of parasitemia[6]. Moreover, the cpsf lowered the PCV levels, though insignificantly ($P>0.05$), in uninfected animals and this may not be unconnected to the hemolytic activity of some saponins previously reported[20,21]. This could further imply that a subtle difference exists between the mechanisms of interaction of cpsf to the membrane components of erythrocytes compared to those of *T. evansi*.

In conclusion, data from this study suggest that saponins-rich fraction of *C. procera* leaves did not possess *in vitro* and *in vivo* activity but could not exclude the antitrypanosomal potential of other members of the saponin group from other plant species. Our future work will investigate the antitrypanosomal potentials of some specific saponins using different species of the parasite.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

We are grateful to the authorities of Ahmadu Bello University, Zaria for funding and providing the facilities for this research.

Comments

Background

Human African trypanosomiasis or sleeping sickness is a widespread tropical disease that can be fatal if not treated. Multi-drug resistance of human pathogenic organisms to synthetic medicines enforced to use plants derived medicine to cure many diseases including trypanosomiasis and this practice is even encouraged by WHO. The control of this diseases by natural products is increasingly becoming more popular and appropriate for use in developing countries.

Research frontiers

The non-anti-trypanosomal effects of saponins-rich fraction of *C. procera* leaves as well as the effect of the fraction on the parasite-induced anemia was explored. This may pave ways for further investigation of other fractions for similar activity.

Related reports

Related works were well reported in both the methods and results.

Innovations and breakthroughs

This article has established the non-anti-trypanasomal activity of saponin-rich fraction of *C. procera* which was not earlier published. This gives the possibility of testing other fractions for possible effect. Furthermore, the study will assist other researchers to exclude this fraction in their search for novel trypanocidal agents.

Applications

This article is very significant in searching for active agents that could be used to treat many trypanosome infections. Further work should be carried out on the effect of this fraction on other causative protozoa, like *T. congolense* and *T. brucei*.

Peer review

The paper is highly relevant in terms of use of herbal medicines for treatment of diseases. Materials and methods are well designed. Findings are interesting and interpreted scientifically in discussion section.

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