In vitro antitrypanosomal potential of chloroform leaf extract of *Punica granatum* L. on *Trypanosoma brucei brucei* and *Trypanosoma evansi*

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Abstract

The plant Pomegranate (*Punica granatum* Linn.) selected for this study is native to the region of Eurasia. The objective of this study was to evaluate the antitrypanosomal potential of the plant against *Trypanosoma brucei brucei* (*T.b. brucei*) and *Trypanosoma evansi* (*T. evansi*). Similarly, the parasites used for this study have two entirely different modes of transmission that is Cyclical Transmission (*T.b. brucei*) and Mechanical Transmission (*T. evansi*). The chloroform extract of *Punica granatum* (*P. granatum*) was analysed *in vitro* for trypanocidal activity against *T.b. brucei* and *T. evansi* at concentrations of 100 mg/mL, 50 mg/mL, 25 mg/mL, 12.5 mg/mL and 6.25 mg/mL. The chloroform extracts of *P. granatum* had trypanocidal activity against *T. evansi* and was inactive against *T.b. brucei*. These findings suggest that the mode of transmission may have an effect on the parasite-drug reaction and the possible use of the chloroform extract of *P. granatum* in the management of trypanosomiasis due to *T. evansi* which may require further elucidation.

Introduction

Trypanosomosis is one of the major obstacles for livestock production in Africa,[1] and continues to cause morbidity and mortality on a large scale in the world.[2] The trypanosomes had been first reported to occur in trouts (Valentine, 1841) and frogs (Gluge, 1842),[3] but it was not until 1881 that Griffith Evans found trypanosomes in the blood of horses and camels with a wasting disease called *Surra* and suggested that the parasites might be the cause of this disease.[4] In 1895, the Scottish Pathologist and Microbiologist David Bruce (1855-1931) discovered *Trypanosoma brucei* as the cause of cattle *nagana* (cattle Trypanosomiasis).[5]

Surra is widespread in different parts of the world and poses a major constraint to camel productivity.[6] *Surra* is endemic and its geographical distribution is continuous from the northern part of Africa through the Middle East to South-East Asia.[7] The Parasite is transmitted through mechanical transmission by *Tabanid striatus* flies, similar to the transmission of parasites in animals.[8]

African animal trypanosomiasis was initially thought to be transmitted mechanically by tsetse flies. However, it was the German military surgeon Friedrich Karl Kleine (1869-1951) who showed in 1909 the cyclical transmission of *T.b. brucei* in tsetse flies.[5] *T. b. brucei*, the causative agent of *nagana,* is closely related to *Trypanosoma brucei rhodesiense* (East to South of Africa) and *Trypanosoma brucei gambiense* (West and Central Africa) which cause human African trypanosomiasis, or sleeping sickness.[3] The distribution of trypanosomiasis in Africa corresponds to the range of tsetse flies and comprises currently an area of 8 million km^2 between 14 degrees North and 20 degrees South latitude.[5] It is difficult to estimate the overall burden of African trypanosomiasis because of under diagnosis in the most heavily infected countries. However, it is believed to be in the vicinity of 100,000 new cases per year, with between ⅓ and ½ of cases remaining undetected and untreated.[9,10]

However, the efficiency of the drugs available is limited by a number of features, which include increasing parasite resistance[11], treatment failures, undesirable toxicity,[12,13] unavailability, logistics of administration, long period of treatment, and high cost.[14] In the pursuit for novel trypanocides, an extensive variety of medicinal plants have been nominated for antitrypanosomal activity and reasonable quantity of them has been stated to have noteworthy antitrypanosomal activity.[9,15,16] Several secondary plant metabolites including alkaloids, tannins and anthraquinones have been designated with trypanocidal activity.[17] Plants have been reported to be the foundation of traditional treatment for various types of disorders.[6,15,18,19] Nevertheless, there have been lately numerous reports on the assessment of the antitrypanosomal properties of plant extracts and plant derivatives.[2,18,20-24]

Punica granatum L., commonly recognized as the pomegranate, belongs to the family Punicaceae which comprises only one genus and two species, the other one, little-known, being *P. protopunica* (Balf.) peculiar to the island of Socotra. The pomegranate is another extensively exploited fruits for food, juice, flavour, fragrance and colour. The fruit was used in countless ways as it is today and was featured in Egyptian mythology and art, acclaimed in the Old Testament of the Bible and in the Babylonian Talmud, and it was carried by desert caravans for the sake of its thirst-quenching juice.[25]

The plant has been used in several research studies for its properties such as antibacterial,[26,27] anticancer,[28-30] antidiarrhoeal,[31] antifungal,[22] antihelminthic,[30,32,33] antimalarial,[34-36], andantioxidant properties.[37] It is suggested that there is a significant link between cancer and trypanosomiasis chemotherapies.[9,38]

Materials and Methods

Sample preparation and extraction

The fresh leaves of *Punica granatum* were collected from Area BZ of Main Campus of Ahmadu Bello University, in Samaru, Zaria. They were authenticated at the Herbarium, Department of Biological Science, Ahmadu Bello University, Zaria; was given a voucher no. 1917. The leaves were air-dried at Room Temperature; then, were subjected to powdering which was then being subjected to the Soxhlet extrac-

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tion method (also known as hot percolation) with Chloroform solvent. The extracted analytes was concentrated by distilling off the excess solvent.[39]

Phytochemical screening

Standard protocols to identify the constituents were carried out to test for the presence of alkaloids, flavonoids, glycosides, resins, saponins and tannins.[39]

Preparation of extract dosages

To produce a stock solution of 100 mg/mL for the extract, weighed 1 gram (1000 mg) of the extract which were solubilised in 1 mL of dimethylsulfoxide (DMSO) solution and made up to 10 mLin Dextrose saline. Serial dilutions were made for the 50, 25, 12.5 and 6.25 mg/ml concentrations of the extracts. All subsequent dilutions were made in Dextrose saline and were freshly prepared.[40]

Laboratory animals

Wister strain albino rats were used for the *in vitro* analysis were obtained and kept in the animal house of National Research Institute for Chemical Technology, Basawa, Zaria. The animals were kept under well–ventilated conditions, fed on standard Feeds (Excel Feeds PLC.) throughout the course of the experiment and had access to clean and fresh water *ad-libitum.* The experimental animals were handled in accordance with: i) Good Laboratory Practices for Quality Practices for Regulated Non-Clinical Research and Development (World Health Organization)[41]; ii) CPCSEA Guidelines for Laboratory Animal Facility [the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA)][42]; iii) Animal Use and Care Policy in the Research Policy [Ahmadu Bello University Committee on Animal Use and Care (ABUCAUC)].[43]

This experimental work had the approval of the Ahmadu Bello University Committee on Animal Use and Care (ABUCAUC) (Approval No. ABUCAUC/2012/MICROB/APP/001).

The experimental animals were screened for any ailment at the Faculty of Veterinary Medicine's Parasitology Laboratory at the commencement of the experiment using the laboratory's recommended procedure and were cleared of any ailment.

Test organisms

T. b. brucei and *T. evansi* was obtained from National Research Institute for Chemical Technology (NARICT), Basawa, Zaria. The parasites were maintained in the laboratory by continuous passage in rats. Blood from the tail was used as estimation of parasitaemia.[17]

In vitro test for trypanocidal activity

The trypanosome parasitaemia was determined by the use of wet mount, according to the *Wet and Thick Blood Film* method,[44] and microscopic evaluation at 400× magnification using the *Rapid Matching* method.[45] Assessment of the *in vitro* trypanocidal activity was performed in duplicate in 96 round bottom well microtitre plates. The infected rat to undergo euthanasia must have attained a blood parasitaemia of log 8.4or higher.[45] Euthanatized animal's blood was dissolved in heparin (1 mL of heparin/10 mL of blood) and was mixed with glucose (0.1 gm of glucose/10 mL of blood). Then, have aseptically been using a clean micropipette to transfer the blood (50 µL) to a clean, sterile micro-titre plate into a multiple number of well. To the well containing blood, same volume of the drug/extracts (*i.e.* 50 µL) was added of different concentrations respectively. The negative control blood was mixed with dextrose saline. The plates were incubated at room temperature.[17,40] For reference, positive tests were also performed with the standard recommended concentrations of *Diminazine aceturate* (*Sequene*, PI Drugs and Pharmaceuticals Ltd, India, and *Diminor plus* Changzhou Animal Health Products Co. Ltd, China) - a commercially available trypanocidal drug.

Results

The results in the chloroform extraction were found a Dark Green Residue and Percentage yield obtained was 11.80 (%W/W). The phytochemical screening results are shown in Table 1. The phytoconstituents of *P. granatum* include Carbohydrates and Glycoside. The *in vitro* analysis results for the extract are shown in Figure 1 and 2. The chloroform extract did not have any activity on the *T.b. brucei* even at 100 mg/mL the parasite remained viable

Table 1. Phytochemical screening of chloroform extract of *Punica granatum* L.

Constituents	Chloroform extract
Alkaloids	-
Carbohydrates	+
Tannins	-
Saponin	-
Flavonoids	-
Terpenes	-
Glycosides	+
Cardiac glycosides	+/-
Anthraquinone	-

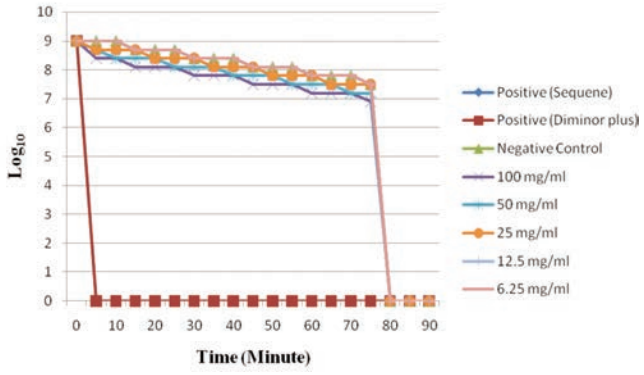

Figure 1. Chloroform Extract of *Punica granatum L.* against *Trypanosoma brucei brucei.*

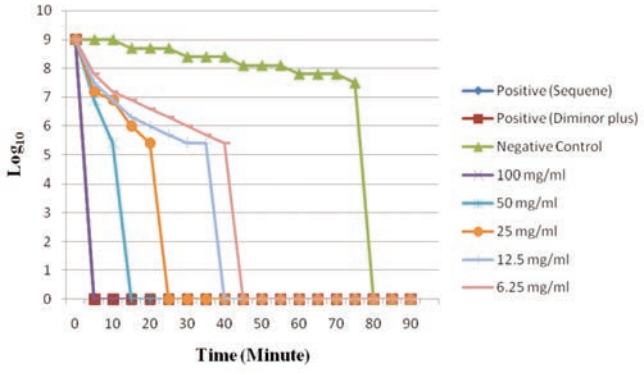

Figure 2. Chloroform Extract of *Punica granatum L.* against *Trypanosoma evansi.*

though the red blood cells were lysed, the parasite motility cleared in the negative control. However, the chloroform extract had antitrypanosomal activity on *T. evansi* although between 6.25-100 mg/mL, the red blood cells were still intact and the parasite motility increased with a reduction in concentration. After 50 minutes, the motility of the parasite was eliminated even though in the negative control the parasite motility continued for up to 80 minutes. The positive control involving two commercial trypanocidal drugs which were prepared to standard specifications cleared the parasite in less than 5 minutes (about 3 minutes) but it lysed the red cell.

Discussion and Conclusions

The result of the present study showed that the plant had activity against *T. evansi* and was inactive against *T.b. brucei* may be due to the absence of alkaloids and flavonoids in the crude extract.[46,47] This finding suggests that the mode of transmission may have an effect on the parasite-drug interaction. It is thus possible for any plants that did not show activity to a species of the parasite, thus can have activity against other species of the parasites.[1,40] Moreover, the result suggestive and similar with that reports that some plants had promising activity against trypanosomes.[2,15,20,48,49] The morphology of the blood cells was maintained while that of the parasites was affected when compared to the control that still had very active parasites. The mechanism by which the extracts eliminate/immobilize the parasites is not immediately known at this stage of the work.[50]

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