**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 when the German military surgeon Friedrich Karl Kleine (1869-1951) who showed in 1909 the cyclical transmission of T. b. brucei in tsefis flies. 4 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 investigated 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.

**Materials and Methods**

**Sample preparation and extraction**

The fresh leaves of Punica granatum L. 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 extract-
tion method (also known as hot percolation) with Chloroform solvent. The extracted analytes was concentrated by distilling off the excess solvent.  

Phytochemical screening

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

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 mL in 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.

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); ii) CPCSEA Guidelines for Laboratory Animal Facility [the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA)]; iii) Animal Use and Care Policy in the Research Policy [Ahmadu Bello University Committee on Animal Use and Care (ABUCAUC)].

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.

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, and microscopic evaluation at 400× magnification using the Rapid Matching method. 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 8.40 or higher. Euthanized 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 microtitre 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. 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. brucei even at 100 mg/mL the parasite remained viable.

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

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Chloroform extract</th>
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<tbody>
<tr>
<td>Alkaloids</td>
<td>-</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>+</td>
</tr>
<tr>
<td>Tannins</td>
<td>-</td>
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<tr>
<td>Saponin</td>
<td>-</td>
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<tr>
<td>Flavonoids</td>
<td>-</td>
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<td>Terpenes</td>
<td>-</td>
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<tr>
<td>Glycosides</td>
<td>+/−</td>
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<tr>
<td>Cardiac glycosides</td>
<td>+/−</td>
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<td>Anthraquinone</td>
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Figures

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 antitypanosomal 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. brucei* may be due to the absence of alkaloids and flavonoids in the crude extract. 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. Moreover, the result suggestive and similar with that reports that some plants had promising activity against trypanosomes. 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/imobilize the parasites is not immediately known at this stage of the work.

### References