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Phytochemical Analysis and Anti-trypanosomal Efficacy of the Methanol Leaf Extract of *Jatropha curcas*

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ABSTRACT: The efficacy of methanol leaf extract of *Jatropha curcas* was investigated through *in-vitro* assay against *Trypanosoma brucei-brucei* in 96 well micro-titer plates. Maceration method was used to obtain the leaf extract using absolute methanol. The extract was screened for phytochemicals and tested at various concentrations; 10 mg/ml, 5 mg/ml, 2.5 mg/ml, 1.25 mg/ml, 0.625 mg/ml, 0.3125 mg/ml, and 0.15625 mg/ml. The phytochemicals detected were alkaloids, saponins, flavonoids, tannins, resins, and terpenes. The result of the antiparasitic activity showed that methanol extract was effective against the *T. b. bruicei* parasite. Although, the extract trypanocidal activity is time and concentration dependent, the student t-test showed there were significant difference ($p > 0.05$) between the extract and drug associated with the trypanosomes mortalities after 6 hours incubation period. In conclusion, *Jatropha curcas* is likely to contain promising chemical compounds which can be utilized as an effective plant treatment against *T. brucei - brucei* parasite. Isolation of the effective secondary metabolites is recommended.

Keywords: Antitrypanosomal, Efficacy, *Jatropha curcas, T. brucei-bruicei*

Introduction

African trypanosomiasis is a parasitic disease caused by a protozoan of the genus *Trypanosoma*. *Trypanosoma vivax*, *Trypanosoma congolense* and to a lesser extent *Trypanosoma brucei* are the main species responsible for African Animal Trypanosomosis (AAT) called nagana in West Africa while *T. b. rhodesiense* and *T. b. gambiense* cause sleeping sickness (Human African Trypanosomiasis, HAT). Surra and Dourine are caused by other trypanosome species *T. evansi* and *T. equiperdum* respectively. The disease is transmitted by a bite of the vector - tsetse fly (Glossina species*)* (Steverding. 2008; Brun *et al*., 2011, Steverding, 2008). Human African Trypanosomiasis is endemic in 36 sub-Saharan African countries, in areas where tsetse flies are found. The public health importance of Human African Trypanosomiasis is underestimated, but the disease causes severe social disruption in many rural areas. The importance of trypanosomiasis to human and livestock health in Africa was recognized early in the period of European colonial expansion in Africa (Ford, 1971). Various historical accounts of the early work on epidemiology, the control measures, and the social and economic consequences of trypanosomiasis are available (Duggan 1970; Ford 1971).
The current chemotherapy of Human African Trypanosomiasis (HAT) count on only six drugs (suramin, pentamidine, melarsoprol, eflornithine, arsobal, and mel B), five of which were developed more than 30 years ago. Others such as homidium, isometamidium, and diminazene aceturate are used in animal infections. Some of the challenges encountered with the aforementioned drugs include: high cost, adverse effect, need parenteral administration and treatment failure (parasites increasing resistance) (Fairlamb 2003). However, a tireless effort being made by WHO, private partners, and local governments to eliminate HAT is yielding significant success.

Plants produce a great diversity of substances that could be active in many fields of medicine. Natural products from plants are proven templates for new drug development.

Medicinal plants are plants that have in one or more of its organ, active substances that can be used as therapeutic agents or as precursors for the manufacture of synthetic drugs (WHO 1998). About 50000 species of higher plants have been used medicinally. This represents by far the biggest use of the natural world in terms of the number of species (Idu, 2010). Several reviews on medicinal plants used in the treatment of trypanosomiasis had been published (Ogungbe, *et al*, 2009, Atawodi *et al.,* 2009). It is estimated that 66%–85% of the World's population depends directly on plants as medicine and search for drugs derived from plants has accelerated in recent years (Newmann *et al.,* 2013).

Jatropha curcas is a plant species with undeniable prospects and potential in drug discovery. Traditionally, *Jatropha curcas* has been proved to have promising effects for the treatment of bacterial diseases, fungal infections or febrile diseases, muscle pain, or jaundice (Staubmann *et al.,* 1999, Igbinosa *et al.,* 2009).However, the use of methanol leaf extract of *Jatropha curcas,* against trypanosomiasis has not been investigated and documented before. Hence, the present study was carried out to investigate the in vitro antitrypanosomal effect of the methanol leaf extract of *Jatropha curcas* against *Trypanosoma brucei - brucei*.

Materials and methods

Sample collection: The fresh leaf sample of *J. curcas* was collected from a garden at Kwanar Maishayi Unguwar Sunusi, Kaduna, Nigeria. The sample was identified and authenticated by a taxonomist in the Herbarium Unit of the Department of Botany, Ahmadu Bello University Zaria, Kaduna State Nigeria. The voucher number assigned to the sample of the leaf of Jatropha Curcas is ABU099025.

Sample preparation and extraction: The leaf sample of *J. curcas* was dried in a hot air oven at 60ºC (to avoid heat destruction of the active leaf components). The dried leaf sample was grounded with laboratory mortar and pestle to powder. The powdered material was weighed using an electronic weighing balance. Eighty-seven grams (87g) of the powder of *J. curcas* was transferred into a conical flask, it was macerated (extracted) for 48hrs using 600 ml of absolute methanol in the ratio (1:3). The methanol extract was filtered and placed in a water bath to dry. The residues obtained were transferred into a pre-weighed sample container and stored at room temperature until required for use.

Phytochemical analysis: Detection of various active phytochemical analyses was carried out on the methanol leaf extract of *J. curcas* using the standard procedure as described by Sofowora, (1993). The phytonutrients determined in the leaf extract include Alkaloids with (picric solution), saponin with (water), Steroid and Terpenes with (chloroform, Sulphuric acid H_2SO_4 and acetic anhydride). Other phytochemical constituents include Flavonoids with (sodium hydroxide NaOH and lead acetate), resins with (Ethanol and 1% hydrochloric Acid) and Tannins with (distilled water and ferric chloride reagent). Colour changes were observed and recorded respectively (Sofowora 1993, Trease *et al.,* 2002).

Test for Trypanosoma brucei-brucei: Cryopreserved stabilate of *T. brucei-brucei* was obtained from the Cryobank preserved at the Department of Vector and Parasitology Study of the Nigerian Institute for Trypanosomiasis Research, Kaduna, Nigeria. The stabilate was warmed at 37°C and trypanosomes were screened for viability by examining wet smears prepared from the stabilate in the light microscope at x400 magnification. The presence of motile trypanosomes was taken as indication of trypanosome viability (Bulus and Addau, 2013).

Donor animals: Two albino Wistar rats were utilized as donor animals. Each rat was intraperitoneally inoculated with blood suspension (inoculum) prepared from the thawed stabilate. The inoculum was prepared by the addition of normal saline to a small amount of the blood from the stabilate until the trypanosome count was 2 per microscopic field. The rats were inoculated with 0.2 ml of the prepared blood suspension. Parasitemia in the inoculated rats was monitored three days after inoculation to determine the establishment of active infection. Subsequently, the level of parasitemia was determined daily until parasite count was about 10^9 per milliliter of blood. Following infection with the trypanosomes, at which point the donor rats were sacrificed (Bulus and Addau, 2013).

Determination of parasitemia: The trypanosome count in the infected rats was estimated by the rapid matching method of Herbert and Lumsden (1976). A drop of whole blood collected by tail snip or cardiac puncture was placed on a clean, grease free glass slide and a cover glass placed over it and the blood spread into a thin circular film. The slide was then placed in the light microscope and examined at x400 magnification. The distribution of trypanosomes among the red blood cells (RBCs) was matched against the Lumsden's chart and the approximate number of trypanosomes per milliliter of blood estimated (Bulus and Addau, 2013).

Collection of parasitized blood: Blood containing trypanosomes was collected into a 5 ml syringe from the donor rats by the cardiac puncture technique after chloroform anesthesia. The blood was dispensed into ethylene diamine tetra acetate (EDTA) sample container, and gently mixed together to prevent clotting of the blood (Bulus and Addau, 2013).
Medium and supplementation of medium: RPMI 1640 medium (Caisson Laboratory, USA) containing L-glutamine and sodium carbonate was used for the *in vitro* assay. The media were further supplemented with gentamycin (40 µg/L), 10% (v/v) heat inactivated goat serum, and 1% (w/v) glucose (Bulus and Addau, 2013).
Reconstitution of the plant extract and reference drug: Solutions of the extract and drug (Diminazene Diaceturate) were reconstituted in the supplemented medium. Initially, 100 mg/ml stock solutions of the respective extract and standard drug were prepared. Subsequently, the respective stock solutions were serially diluted to yield extracts with concentrations ranging from 10 mg/ml to 0.3125 mg/ml (Bulus and Addau, 2013).
In vitro assay of the crude extract (drug incubation survival assay): The Drug Incubation Survival Test (DIST) was used for this assay. 100 µl of the reconstituted solutions of each of the extracts, as well as the reference drug, were separately dispensed in duplicates into wells of a 96-well microtiter plate. 20 µl of the blood suspension containing *T. b. brucei* was added to each of these wells, and gently mixed together. Control wells containing only 100µl supplemented medium and 20 µl blood suspension were also included. The microplate was placed in a desiccator containing about 5% carbon dioxide and maintained at 37°C in an incubator. Wet smears were prepared from each of these wells, twenty (20) hours post-incubation. Each smear was examined in the light microscope (X400 magnification) and the counts of motile trypanosomes were taken over three fields of view, a total of nine observations per concentration of drug/extract. Similarly, trypanosome counts were also taken for smears prepared from the control wells. A reduction in the number of motile trypanosomes compared to the control wells was taken as an index for *in vitro* activity (Bulus and Addau, 2013).

Results

Phytochemical cconstituents: The methanol extraction yielded 7.65g of extract with a dark green color and gummy texture. The results of the phytochemical analysis indicate the presence of the secondary metabolites in the extracts. From Table 1, methanol extract of leaf of *J. curcas* shows presence of tannins, saponins, steroids, resin, alkaloids and flavonoids.

Table 1: Phytochemical constituent of *Jatropha curcas*

Phytochemical	Methanol Extract
Alkaloids	+
Saponins	+
Flavonoids	+
Steroids and Terpenes	+
Tannins	+
Resins	+

Key: + (present)

In vitro assay: Effect of graded concentrations of the extract on mean trypanosome count: The mean count of motile trypanosomes after six hours of incubation in graded concentrations of the methanol leaf extract and drug (Diminazine Diaceturate) is shown in Table 2. The results show that there was gradual decline in the number of surviving trypanosomes with increasing concentrations of the extract, and the decrease in the mean count was statistically significant ($p < 0.05$). In the negative control, the mean count of surviving trypanosomes was 25.8 ± 0.86 trypanosomes per field, while at 0.15625 mg/ml the mean count was 16.5 ± 0.96 trypanosomes per field. The mean counts at 0.3125, 0.625, 1.25, 2.5, and 5.0 mg/ml concentrations of the extract were 15.5 ± 0.56, 10.5 ± 0.56, 8.83 ± 0.54, 6.17 ± 0.40, and 2.33 ± 0.42 trypanosomes per field, respectively. Motile trypanosomes were not detected at 10 mg/ml concentrations of the extract. From the results obtained the minimum trypanocidal concentration of the extract *in vitro* was 10 mg/ml after six hours of incubation.
The results of the mean numbers of surviving trypanosomes six hours post-incubation in graded concentrations of the trypanocidal drug, Diminazine Diaceturate indicated a decrease in the mean count of surviving trypanosomes with increasing concentrations of the drug. At 0.15625 and 0.3125mg/ml concentrations of the drug, the mean number of surviving trypanosomes was 7.33 ± 0.61 and 6.50 ± 0.43 trypanosomes per field, respectively. Motile trypanosomes were not detected at drug concentrations between 1.25 mg/ml and 10 mg/ml. The minimum trypanocidal concentration of Diminazine diaceturate, six hours post-incubation, was 0.625mg/ml.

Table 2: Mean number of surviving trypanosomes after six hours of incubation

Concentration (mg/ml)	Mean Trypanosome Count/field	
	Jatropha curcas	**Diminazine**
0.0 (Control)	25.8±0.86^{f}	24.8±1.28^{c}
0.15625	16.5±0.96^{e}	7.33±0.61^{b}
0.3125	15.5±0.56^{e}	6.50±0.43^{b}
0.625	10.5±0.56^{d}	0.00±0.00^{a}
1.25	8.83±0.54^{cd}	0.00±0.00^{a}
2.5	6.17±0.40^{c}	0.00±0.00^{a}
5.0	2.33±0.42^{b}	0.00±0.00^{a}
10	0.00±0.00^{a}	0.00±0.00^{a}
p-value	< 0.0001	< 0.0001

Values are given as mean ± standard error of the mean. In each column, mean values with the same letters have no statistically significant difference ($p < 0.05$)

Percentage mortality and median lethal concentration (LC_{50}): The percentage mortality in trypanosome populations incubated in graded concentrations of the extract, universal drug as well as the extrapolated median lethal concentration of the extract are shown in Table 3. The trend in percentage mortality parallels that observed in the mean number of surviving trypanosomes, with increasing mortality associated with an increase in concentrations of either the extract or drug. Student *t*-test revealed that there were significant differences ($p > 0.05$) between extract and drug-associated trypanosome mortalities with significantly ($p > 0.05$) higher percentage mortality recorded with the standard drug than with the extract at a given concentration, except at 10 mg/ml concentration where the extract exhibited comparable activity as the drug (Table 2).

Also shown in Table 3 are the median lethal concentrations (LC_{50}) of the extract and the drug. After six hours of incubation *in vitro*, the LC_{50} values of the extract and drug were 0.57 ± 0.05 and 0.10 ± 0.02 mg/ml, respectively.

Table 3: Percentage mortality in trypanosome population and median lethal concentration (LC_{50}) after six hours of incubation

Concentration (mg/ml)	Percentage Mortality (%)	
	Jatropha curcas	**Diminazine**
0.0 (Control)	-	-
0.15625	28.2±3.31^{a}	68.3±6.23^{b}
0.3125	32.7±13.3^{a}	71.6±6.16^{b}
0.625	54.6±5.48^{a}	100±0.00^{b}
1.25	61.4±8.11^{a}	100±0.00^{b}
2.5	73.4±3.49^{a}	100±0.00^{b}
5.0	89.9±4.67^{a}	100±0.00^{b}
10	100±0.00^{a}	100±0.00^{a}
LC_{50} (mg/ml)	**0.57±0.05^{b}**	**0.10±0.02^{a}**

Values are given as mean ± standard error of the mean. In each row, mean values with the same letters have no statistically significant difference ($p < 0.05$)

Discussion

Phytochemicals are compounds that occur naturally in plants that are known to be biologically active and therefore aid the antiparasitic activities of *J. curcas*. These secondary metabolites exert antiparasitic activity through different mechanisms. Tannins have been found to form irreversible complexes with proline-rich protein (Shimada, 2006) resulting in the inhibition of cellular protein synthesis. Parekh and Chanda (2007) reported that tannins are known to react with proteins to provide the typical tanning effect which is important for the treatment of inflamed or ulcerated tissues. Herbs that have tannins as their main components are astringent and are used for treating intestinal disorders such as diarrhea and dysentery (Dharmananda, 2003). These

observations, therefore, support the use of *J. curcas* in herbal cure remedies. Li and Wang (2003) reviewed the biological activities of tannins and observed that tannins have anticancer activity and can be used in cancer prevention, thus suggesting that *J. curcas* has potential as a source of important bioactive molecules for the treatment and prevention of cancer. The presence of tannins in *J. curcas* supports the traditional medicinal use of this plant in the treatment of different ailments. Another secondary metabolite compound observed in the leaf extract of *J. curcas* was alkaloid. One of the most common biological properties of alkaloids is their toxicity against cells of foreign organisms. These activities have been widely studied for their potential use in the elimination and reduction of human cancer cell lines (Nobori *et al*., 1994). Alkaloids which are one of the largest groups of phytochemicals in plants have amazing effects on animals and this has led to the development of powerful pain killer medications (Kam and Liew, 2002). Just *et al*. (1998) revealed the inhibitory effect of saponins on inflamed cells. Saponin was found to be present in *J. curcas* leaf extract and has supported the usefulness of this plant in managing inflammation. Steroidal compounds present in *J. curcas* leaf extract are of importance and interest due to their relationship with various anabolic hormones including sex hormones (Okwu, 2001). Flavonoids, another constituent of *J. curcas* leaf extracts exhibited a wide range of biological activities like antiparasitic, anti-inflammatory, analgesic, anti-allergic, cytostatic, and antioxidant properties (Hodek, *et al*., 2002). Different parts of *J. curcas* contain the toxic alkaloids; curcin and phorbal ester which prevent animals from feeding on it. The therapeutic utility of plants has been associated with the activity of bioactive principles, called secondary metabolites, which were observed in the methanol leaf extract of *Jatropha curcas* screened. (Oluchukwu *et al.,* 2013)

A decrease in the number of surviving trypanosomes after six hours of incubation with the increased concentration of *Jatropha curcas* leaf extract to the highest concentration (10 mg/ml) reveals that the extract affects the parasite (*Trypanosoma brucei-brucei*). It acted by reducing their surviving rate until they are absent in the highest concentration of 10 mg/ml. The percentage mortality and median lethal concentration (LC_{50}) of trypanosomes, there is an absence of motality in the extract and standard drug at 10 mg/ml concentration within six hours of incubation. This present research agrees with the earlier report on *in vitro* trypanocidal effect of methanol extract of some Nigerian savannah plants (Atawodi *et al.,* 2003) and another report on indigenous knowledge system for treatment of trypanosomiasis in Kaduna state of Nigeria (Atawodi *et al.,* 2002).

Conclusion

This study revealed the presence of various phytochemicals which were discovered by qualitative screening. The methanol leaf extract of *J. curcas* was found to have antitrypanosomal effect at higher concentrations of the by eliminating the parasite.

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