Vol. 25 (3) 127–132 31 December 2013

Biokemistri

An International Journal of the Nigerian Society for Experimental Biology

Original Research

In vitro assessment of trypanocidal activity of aqueous extract of *Citrullus lanatus* (Cucurbitaceae) (Thunb) leaf and its effects on the haematological parameters of *Trypanosoma brucei* infected Albino rats

*Abdullahi Abubakar Biu[1], Lawan Bala Buratai[2], *Joshua Luka[1], Mohammed Konto[1], Jude Onwuatogwu[2] and Nelson Ogenyi Agada[2].

[1]Department of Veterinary Microbiology and Parasitology, Faculty of Veterinary Medicine, University of Maiduguri, P. O. Box 8136, Maiduguri, Nigeria. [2]Department of Biochemistry, Faculty of Science, University of Maiduguri,P. O. Box 8136, Maiduguri, Nigeria.

*Corresponding author: Joshua Luka. E-mail: joshuadiriki@yahoo.com; Tel.: +2348030407578

Received: 23 July 2013: Accepted: 17 December 2013

ABSTRACT: The *in vitro* antitrypanosomal activity of *Citrullus lanatus* leaf aqueous extract and its effects on blood parameters of *Trypanasoma brucei* infected albino rats was investigated in this study. The plant extract showed *in vitro* activity against *T. brucei* at a minimum concentration of 0.0875 mg/ml. There was a positive correlation between extract concentration and parasitaemia clearance, with administration of the extract at 10 mg/ml concentration resulting in zero parasitaemia count. Thirty albino rats divided into six groups (A-F) of five rats each were used for the haematological study. Graded extract doses of 200, 400 and 600 mg/kg were administered orally to groups A, B, and C respectively following an establishment of parasitaemia of 4×10^6 two days post infection. Group D (Veriben®–treated) was given a single dose of 3.5 mg/kg diminazine aceturate (Veriben®) intramuscularly. Group E (Untreated) was not treated with any trypanocide but given 10 ml/kg of distilled water orally, while group F (Uninfected) were not infected with the parasites and did not receive any treatments. There was a significant ($p<0.0001$) decrease in mean packed cell volume (PCV) and red blood cell (RBC) counts of the infected and untreated group (E), when compared with the uninfected (F), and the infected but treated groups (A-D). The mean haemoglobin concentration was significantly ($p<0.0001$) higher in untreated uninfected and Veriben®–treated albino rats compared with those treated with the extracts (A–C) and the untreated infected rats (E). White blood cell (WBC) counts increased significantly ($p<0.0001$) in the untreated infected group (E) compared with the uninfected (F), extract-treated (A–C) and Veriben®–treated (D) groups. In conclusion, our investigation shows that the aqueous extract of *C. lanatus* leaf is toxic to *T. brucei in vitro*. However, *in vivo* studies are needed to demonstrate that it has any beneficial value in clearing parasites from infected animals.

KEYWORDS: *Citrullus lanatus*, Aqueous extract, Trypanocidal activity, Haematological effects, Albino rats.

INTRODUCTION

The disease trypanosomosis in man and animals is caused by the protozoa species, *Trypanosoma*, biologically transmitted through the bite of tsetsefly, and also mechanically by *Tabanus* and *Stomoxys* as intermittent blood feeders (Ngulde *et al.*, 2013). It is reported to cause anaemia, weight loss, decreased milk yield, abortion and mortality in affected animals (Onyeyili and Egwu, 1995). About 300,000 new cases of African trypanosomosis are reported annually in some 36 developing African countries south of the Sahara (Chretien and Smoak, 2005; Tijani *et al.*, 2009).

Chemotherapy and chemoprophylaxis using orthodox drugs such as diminazine aceturate and isomethamidium chloride, still remains the most effective methods of controlling the

disease (Peni *et al*., 2012). However, these trypanocides are expensive, toxic and have the tendency to elicit drug resistance (Legros *et al*., 2002; Wurochekke *et al*., 2004). Unfortunately there is limited progres in the development of new antitrypanosomal drugs in recent decades (Onyeyili and Egwu, 1995). In addition, the lack of available vaccine coupled with the emergence of resistance (Anene *et al*., 2006) to the commonly available antitrypanosomes are among myriad of factors necessitating the need for development of a new drug from locally available plant material that will be safe, effective and affordable.

Several plants have been used traditionally in Nigeria for the treatment of trypanosomiasis. The potential antitrypanosomal activities of some of these have been evaluated scientifically (Umar *et al*., 2009; Alli *et al*., 2011; Atawodi *et al*., 2011).

The plant *Citrullus lanatus* with origin from Southern Africa belongs to the family Curcubitaceae, and is distributed throughout different parts of Africa, with its fruit called 'Pepo' used as a source of food (Sultan *et al.,* 2010). The plant is known to contain bioactive compounds such as cucurbitacin, triterpenes, sterols and alkaloids (Yuan *et al*., 2006). It has been widely used in traditional herbal medicine as an energy source to cleanse and purify the kidney and bladder, lower high blood pressure, prevent erectile dysfunction, acts as antioxidant, and used to treat hepatomegaly and jaundice (Yativ *et al*., 2010).

In view of the reported use of *C. lanatus* in the management of several conditions such as those that lead to haemopoietic crisis (Chiej, 1984; Mandel *et al*., 2005), and as part of a wider screen of its potential medicinal value, this study was designed to investigate its potential use in the treatment or management of trypanosomiasis. We started by carrying out a preliminary study on the *in vitro* trypanocidal activity of the aqueous extract of *C. lanatus* leaf with the aim of establishing a basis for future investigation of the plant extract's in vivo activity. In order to assess its safety, we determined the effects of the aqueous extract on haematological parameters of *Trypanosoma brucei* infected albino rats.

MATERIALS AND METHODS

Plant collection and identification

Fresh and matured leaves of *Citrullus lanatus* were obtained from a commercial farm at Yanakri, Borno State, Nigeria and was identified and authenticated by a botanist in the Department of Biological Sciences, University of Maiduguri. A voucher specimen has been deposited at the herbarium of the Department for referencing.

Plant preparation and extraction

The leaves were rinsed in clean tap water to remove dirt and later dried for one week under shade to avoid solar leaching. Dried leaves were ground into fine powder using pestle and mortar and then passed through a 2-mm sieve. The dried, powdered and sieved sample (205 g) was extracted using a Soxhlet extractor (Quick fit, England) for eight hours at 60 ^{0}C in 700 ml of water. The extract was concentrated on an aluminum tray, placed in oven and maintained overnight at 60 ^{0}C to dry. The dried sample was obtained and later stored at room temperature (27 ^{o}C) until used.

Trypanosoma brucei

Trypanasoma brucei samples were obtained from the National Institute for Trypanasomiaisis Research (NITR) Vom, Plateau State, and identified based on morphology and negative blood inhibition and infectivity test (BIIT). They were stabilized by serial passages in donor albino rats.

Experimental animals and treatment groups

Thirty albino rats of both sexes, weighing between 90 and 160 g were used in this study. They were kept in plastic cages and allowed to acclimatize to the laboratory condition of ambient temperature 27^{o}C±1, relative humidity of 85±1%, photoperiods of 12 hours natural light and 12 hours dark for two weeks before commencing the experiment. All albino rats were fed commercially pelleted feeds (Vital Feeds®, Jos, Nigeria PLC) and given clean drinking water *ad libitum*. Handling of experimental rats was done according to the International guiding principles for biomedical research involving animals (C.I.O.M.S., 1985).

The animals used for the haematological study were randomly divided into six groups (A-F) of five rats. Parasitaemia was established in groups A-E 2 days post inoculation with 4×10^6 *Trypanosoma brucei*/ml of infected blood in phosphate buffered saline. Graded extract doses of 200, 400 and 600 mg/kg were administered orally to groups A, B, and C respectively following an establishment of parasitaemia of 4×10^6 two days post infection. Group D (Veriben®–treated) was given a single dose of 3.5 mg/kg diminazine aceturate (Veriben®) intramuscularly. Group E (Untreated) was not treated with any trypanocide but given 10 ml/kg of distilled water orally, while group F (Uninfected) were not infected with the parasites and did not receive any treatments.

Determination of haematological parameters

Blood for haematological studies was obtained from the tail vein on alternate days post treatment into EDTA-containing sample bottles. Differential leucocyte counts were made from blood smears stained with Giemsa (Hewitt, 1984), packed cell volume (PCV) was determined by microhaematocrit method (Murray *et al*., 1983). Haemoglobin concentration was measured by Sahlis method (Brown, 1976), and white blood cell and red blood cell counts were determined using the haemocytometer counting chamber (Brown, 1976).

In vitro assessment of trypanocidal activities of extracts

Serial dilution of aqueous extract of *C. lanatus* leaf were prepared in phosphate buffered saline to give concentrations of 0, 0.875, 1.75, 2.5, 5, 10, 20, and 40 mg/kg. Samples of each concentration were prepared into test tubes of 8 replicates each. Blood samples were taken from the tail of a *Trypanosoma brucei* infected albino rat and two drops were diluted in glucose solution (4 x 10^6 parasites/mm^3). The diluted blood samples were added to each of the test tubes and incubated at 37 ^{0}C for 2 hours. Neubauer's chamber was used to count the number of parasites per field and antitrypanosomal activities were determined based on cessation of motility. The percentage inhibition calculated using the expression described by Atawodi and Ogunbusola (2009).

$$\% \text{ inhibition} = \frac{P_C - P_e}{P_C} \times 100$$

P_C (parasite count in control untreated)

P_e (parasite count in extract-treated)

Statistical analysis

Haematological Data: Haematological parameter data were expressed as mean ± standard deviation (SD) and subjected to analysis of variance (ANOVA) to define the extent of variation, and 'p' values equal to or less than 0.0001 were considered significant (GraphPad Instat, 2000).

***In vitro* data:** Data were presented as mean ± SD at the various time intervals with variations among the means and % inhibition analyzed using student's t-test at 5% confidence interval (GraphPad Instat, 2000).

RESULTS

Figure 1 shows the *in vitro* efficacy of increasing concentrations of the aqueous extract *Citrullus lanatus* leaf against *Trypanosoma brucei* after 2 hours incubation. Extract doses of 0.875, 1.75, 2.5 and 5.0 mg/ml had parasite counts (10^6/mm^3) of 1.73±0.43, 1.51±0.39, 1.07±0.66 and 0.91±0.52 respectively, which were all significantly lower than the 2.23±0.11 parasite count observed for the control (0 mg/ml) ($p < 0.05$). Extract concentration of 10 mg/ml and above resulted in 100% growth inhibition or parasite clearance. As a whole, the results show that the extracts had a concentration-dependent growth inhibition effect on *Trypanosoma brucei in vitro.*

Table 1 shows the effect of oral administration of aqueous extract *Citrullus lanatus* leaf on the haematological parameters of rats infected with *Trypanosoma brucei*. The results show a significant ($p<0.0001$) decrease in the mean packed cell volume and red blood cell counts of infected untreated group (E) when compared with the animals in the uninfected group (F) and the treated groups (A-D). The mean haemoglobin concentration was higher in animals in the uninfected (F) and Veriben®-treated (D) groups compared with those in the extract-treated (A-C) and infected untreated (E) groups. The whole white blood cell count however was significantly higher ($p<0.0001$) among animals in the infected untreated group (E) when compared with the uninfected ones (F) and the treated groups (A-D). Interestingly, there was no correlation between the hematological parameters and the dose of the extracts administered.

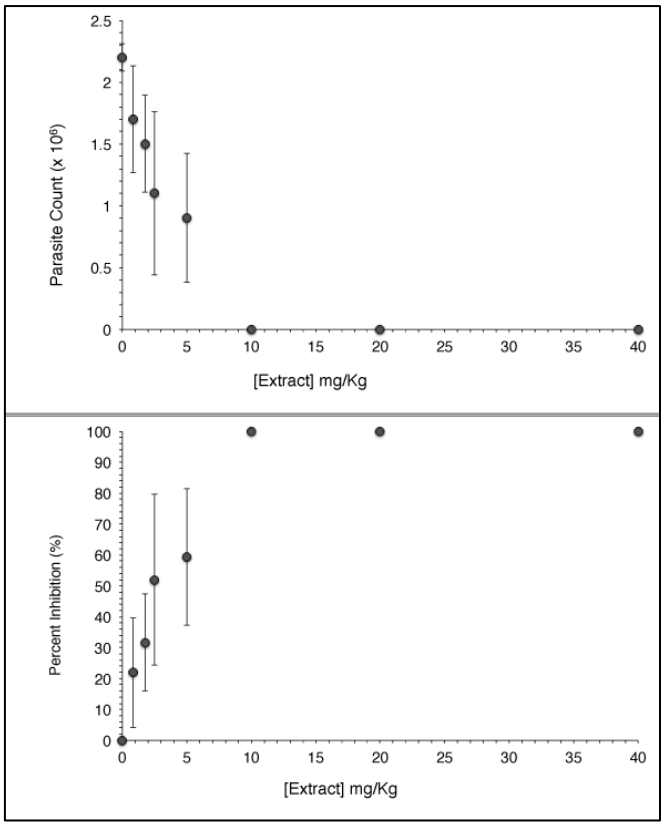

Figure 1: *In vitro* efficacy of aqueous extract of *Citrullus lanatus* leaf on *Trypanosoma brucei.* The parasite count data shown in **A** (upper panel) is used in determining the percent inhibition shown in **B** (lower panel).

Table 2 shows the effect of oral administration of aqueous extract *Citrullus lanatus* leaf on differential leucocytes count of rats infected with *Trypanosoma brucei*. The mean scores of lymphocytes and neutrophils were significantly ($p<0.0001$) lower in rats that were infected but untreated when compared with those not infected (F), and those infected but treated with the extracts (A-C) or with Veriben® (D). In contrast, the monocytes, eosinophils and basophils scores were significantly ($p<0.0001$) higher in rats that were infected but untreated when compared with those not infected (F), and those infected but treated with the extracts (A-C) or with Veriben® (D).

Table 1: Effect of aqueous extract of *Citrullus lanatus* leaf on haematological parameters of albino rats infected with *Trypanasoma brucei*. Values are expressed as Mean ± SD, and means with different superscripts in a column are extremely significant (p<0.0001).

Groups	PCV (%)	Hb (g/dl)	RBC ($\times10^3mm^3$)	WBC ($\times10^3mm^3$)
A (200 mg)	35.79 ± 4.89^b	10.61 ± 0.82^b	3.54 ± 0.64^b	12475 ± 448.4^b
B (400 mg)	33.93 ± 7.61^b	10.56 ± 0.99^b	3.80 ± 0.78^b	12646 ± 1186.4^b
C (600 mg)	35.43 ± 4.62^b	10.48 ± 1.49^b	4.08 ± 0.84^b	13332 ± 1134.7^b
D (Veriben 3.5 mg)	47.43 ± 4.86^a	12.56 ± 1.43^a	5.06 ± 0.91^b	1240 ± 747.3^b
E (Untreated)	32.79 ± 4.98^c	10.21 ± 0.74^b	2.95 ± 0.22^c	14586 ± 586.2^c
F (Uninfected)	47.29 ± 3.12^a	12.59 ± 1.36^a	6.03 ± 0.28^a	10550 ± 326.4^a

Table 2: Effect of aqueous extract of *Citrullus lanatus* leaf on differential leucocytes count of albino rats infected with *Trypanasoma brucei*. Values are expressed as Mean ± SD, and means with different superscripts in a column are extremely significant (p<0.0001).

Groups	Neutrophils	Monocytes	Eosinophils	Basophils	Lymphocytes
A (200 mg)	27.29 ± 1.9^b	10.21 ± 1.8^b	8.36 ± 1.3^b	2.64 ± 1.3^b	51.50 ± 3.8^a
B (400 mg)	28.36 ± 2.2^b	9.54 ± 1.7^b	8.36 ± 1.2^b	2.86 ± 1.2^b	50.80 ± 3.4^a
C (600 mg)	29.57 ± 2.5^b	10.14 ± 1.6^b	8.21 ± 1.2^b	2.50 ± 1.5^b	49.36 ± 3.4^a
D (Veriben 3.5 mg)	30.93 ± 2.7^b	8.43 ± 1.4^a	7.14 ± 1.3^a	2.07 ± 1.9^b	51.36 ± 4.4^a
E (Untreated)	24.86 ± 3.4^c	13.29 ± 2.3^c	10.79 ± 2.2^c	5.00 ± 1.4^c	46.07 ± 4.12^c
F (Uninfected)	35.57 ± 1.7^a	7.07 ± 1.7^a	6.14 ± 1.0^a	0.07 ± 0.3^a	51.57 ± 2.3^a

DISCUSSION

In vitro trypanocidal activities of *C. lanatus* leaf extracts

The trypanocidal activity of *Citrullus lanatus* observed in this study conforms earlier reports that plant extracts could contain potent trypanocidal constituents (Igweh and Onabanjo 1989, Atawodi *et al*., 2003). The mechanism by which natural products exhibit their trypanocidal activity could be by interfering with the redox balance of the parasite, by acting either on the respiratory chain or on the cellular defenses against oxidative stress (Sepulveda-Boza and Cassels, 1996). *Citrullus lanatus* contains active components such as *Cucurbitacin* that are capable of generating radicals that may cause peroxidative damage to cellular targets that are sensitive to alterations in redox balance. It is also known that some agents act by binding with the kinetoplast DNA of the parasite (Sultan *et al*., 2010; Alli *et al*., 2011). Other studies have linked the trypanocidal or trypanostatic efficacy of plant products with their bioactive components such as flavonoids, saponins, tannins and alkaloids (Haruna *et al*., 2013).

Citrullus lanatus contains bioactive compounds such as cucurbitacin, triterpenes, sterols and alkaloids (Yuan *et al*., 2006). Specific phytochemical and mechanistic studies are required to pinpoint the active phytocomponents responsible for the trypanocidal activities and to characterise the cellular targets that they act upon. In vivo demonstration of the activity of these extracts in parasite clearance would lend weight to recent reports on the use of plants in the traditional management of trypanosomiasis have been reported recently (Atawodi *et al*., 2002).

Effect of *C. lanatus* leaf extracts on haematological parameters of T. brucei infected infected rats

The observation of significant decrease in mean packed cell volume and red blood cell counts in infected untreated rats may be indicative of haemolytic anemia associated with *T. brucei* infections. However, haemoglobin levels were higher in infected, Veriben®-treated animals. A similar but less pronounced increase in haemoglobin levels were seen in infected, extract-treated rats. The simple explanation for this

is that the antitrypanosomal effect of the treatments had some beneficial effects on the red blood cell count of the infected animals. There was also a significant decrease in the mean scores of lymphocytes, neutrophils and basophils in infected untreated rats, compared with uninfected, extract-treated and Veriben®-treated groups. There was increased white blood cell count in extract-treated, Untreated and Veriben®-treated groups. The haemopoietic system has been reported as one of the most sensitive targets of toxic plant compounds and serves as an index of the physiological and pathological status in man and animals (Diallo *et al*., 2010). The increased white blood cell count is consistent with normal body response to infection. The white blood cells constitute the primary defense mechanism against pathogen infections. Eosinophils are potent inflammatory cells, basophils are immune-modulatory cells, and monocytes are macrophages or scavenger cells that remove debris from areas of tissue destruction and engulf cellular remnants, antibody coated cells and foreign bodies (Buratai *et al*., 2011). Among important clinical responses, these haematological parameters provide an excellent basis for assessing the nature of disease, extent of tissue and organ damage, response of defense mechanism of the patient, diagnosis of possible type of anaemia and as an index to characterize health status of animals (Albers *et al.,* 1990). Hence, the beneficial effect of the aqueous extract of *C. lanatus* leaf on these parameters in rats infected with *T. brucei* indicates that the plant could be further explored for possible use in the management of trypanosomiasis.

Conclusion

Our investigation shows that the aqueous extract of *C. lanatus* leaf is toxic to *T. brucei in vitro*. However, *in vivo* studies are in progress to investigate whether products of *C. lanatus* have any activities in clearing parasites from the blood of infected animals. Chemical characteriation of the active phytocomponents responsible for such activities will provide a foundation for developing the extracts into products that can be used for more rigorous testing of its value in the management or control of trypanosomiasis.

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