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ANTITRYPANOSOMAL POTENTIALS OF ETHANOLIC LEAF EXTRACTS OF *PUNICA GRANATUM* AGAINST *TRYPANOSOMA BRUCEI BRUCEI* INFECTION

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ABSTRACT
The current treatment regimens based on chemotherapy for Human African Trypanosomiasis and Animal Trypanosomosis are limited, and are not ideal as they are often associated with severe side effects. The emergence of drug resistant parasites presents an additional and major problem. The urgent need for the development of new, cheap, safe, and easy-to-administer compounds for the treatment of these infectious diseases prompted this study. Three doses (20mg/kg, 40mg/kg,80mg/kg) of ethanolic extracts of the leaves of Punica granatum were screened for trypanocidal activity against Trypanosoma brucei bruce in Balb Strain Albino mice. Parasitaemia and disappearance of clinical signs were used as parameters to monitor the efficacy of the extracts using the rapid matching method. Packed cell volume and weights of the mice were determined before and after treatment with extracts. Packed cell volume and Weight improved significantly ($p<0.05$) in the group which was admnistered 40 mg/kg ethanol extract when compared to the negative group. The LD_{50} value calculated for intra-peritoneal route of administration was 90mg/kg. The findings are indicative of the trypanocidal potential of Punica granatum in the management of trypanosomosis.

Keywords: Trypanosomosis, Trypanosoma brucei brucei, Trypanocidal activity, Punica granatum, Ethanol

INTRODUCTION

Medically important trypanosomes cause trypanosomiasis also known as sleeping sickness in man while trypanosomes of veterinary importance cause nagana in animals Drug efficacy is limited by several factors such as increasing parasite resistance, drug toxicity unavailability, route of administration, period of treatment, and high cost of drug. Therefore, there is a need to search for cheaper, more effective, easily available and less toxic chemotherapeutic agents for combating trypanosomiasis. The use of herbal preparations for the treatment of the disease still holds a strong potential in that some ethnomedicinal plants have been demonstrated to contain potent trypanocide ((Nok *et al.*, 1993; Atawodi, 2005).

In the search for new trypanocides, many medicinal plants have been screened for antitrypanosomal activity and quite a number of them have been reported to have significant antitrypanosomal activity(Ogbadoyi *et al.*, 2007).. Several reports of phytochemicals with trypanocidal effects have been reported (Bodley and Shapiro, 1995; Freiburghaus *et al.*, 1996,1997, 1998; Atawodi *et al.*, 2003). The plant *Punica granatum* L., Pomegranate belongs to the family Punicaceae. The pomegranate tree is native to the region ranging from Iran to the Himalayas in northern India and has been cultivated since ancient times throughout the Mediterranean regions of Asia, Africa and Europe (Morton; Pomegranate, 1987). The recent research studies showed that *P. granatum* has antibacterial activity and anti-fungal properties (Duman *et al.*, 2009). However, the plant is yet to be scientifically evaluated for efficacy against African trypanosomes. This study reports the antitrypanosomal activity of the ethanolic extract of the plant *Punica granatum* in experimental *Trypanosoma brucei brucei* infection of Balb strain albino mice.

MATERIALS AND METHODS

Plant collection and uthentication

The fresh leaves of *Punica granatum* were collected from Area BZ of Main Campus of Ahmadu Bello University, in Samaru, Zaria, Nigeria. They were authenticated at the Herbarium of Biological Sciences Department of the Ahmadu Bello University, Zaria, Nigeria and voucher specimen has been deposited in the Department for reference purpose. *Punica granatum* l. Voucher no. 1917

Plant extract preparation

The leaves were air-dried at room temperature and ground to powder. Extraction of the powdered plant samples was done exhaustively with ethanol using a Soxhlet extractor. Each extracted analyte was concentrated by distilling off the solvent and then evaporated to dryness on a water bath. The extract obtained without the solvent was weighed. The percentage yield was calculated in terms of air dried weight of the plant material as:

Percentage yield = Amount of extract obtained / Amount of initial sample X 100

Experimental Animals

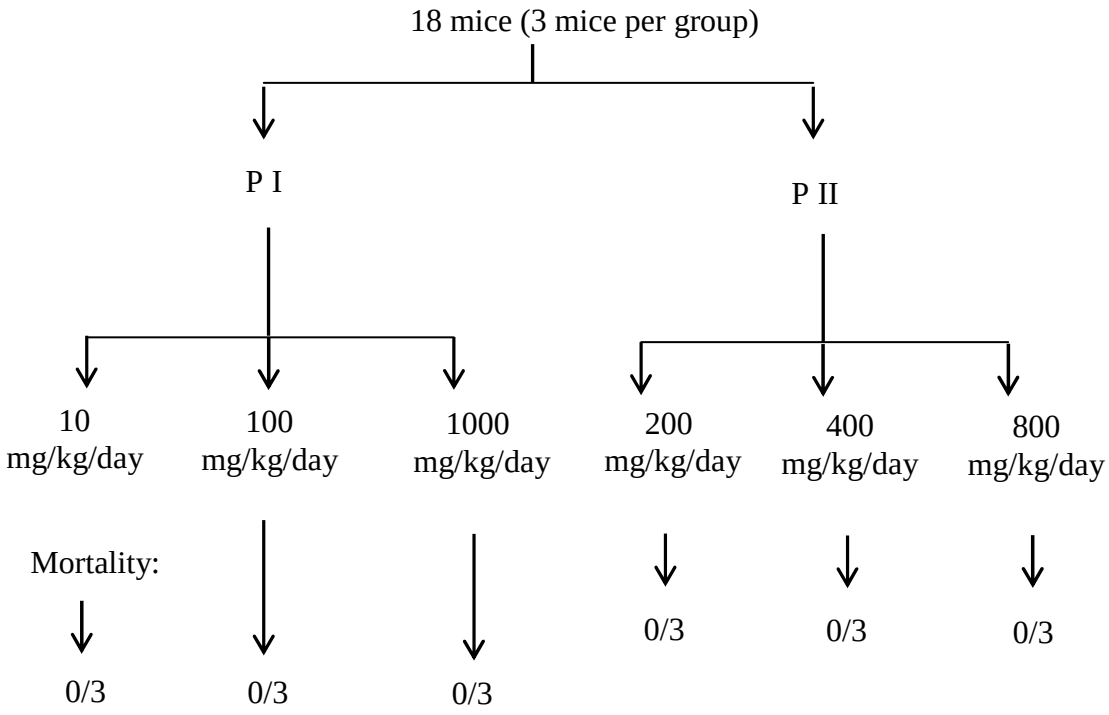
Balb strain albino mice were used for *in vivo* analysis. They were obtained and kept in the animal house of National Research Institute for Chemical Technology, Basawa, Zaria,Nigeria . They comprised albino rats of mixed sexes (5 - 8 weeks old), weighing 15 to 30 g. The animals were kept under well–ventilated plastic cages and fed on standard feeds (Excel Feeds Plc.) throughout the experiment. The animals had access to water *ad libitum.*

Trypanosomes and Determination of Parasitemia

Trypanosoma brucei brucei was obtained from National Research Institute for Chemical Technology (NARICT), Basawa, Zaria.Nigeria. The parasite was maintained in the laboratory by continuous passage in rats. Blood obtained from the tail was used for the estimation of parasitemia by preparing a wet mount (Chappuis *et al.,* 2005) and microscopic evaluation using the 'Rapid Matching' method of Herbert and Lumsden (1976). The standard drug used in this experimental work plan was diminazine aceturate. Packed Cell Volume (PCV) and weight were investigated at the Department of Parasitology and Entomology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria , Nigeria

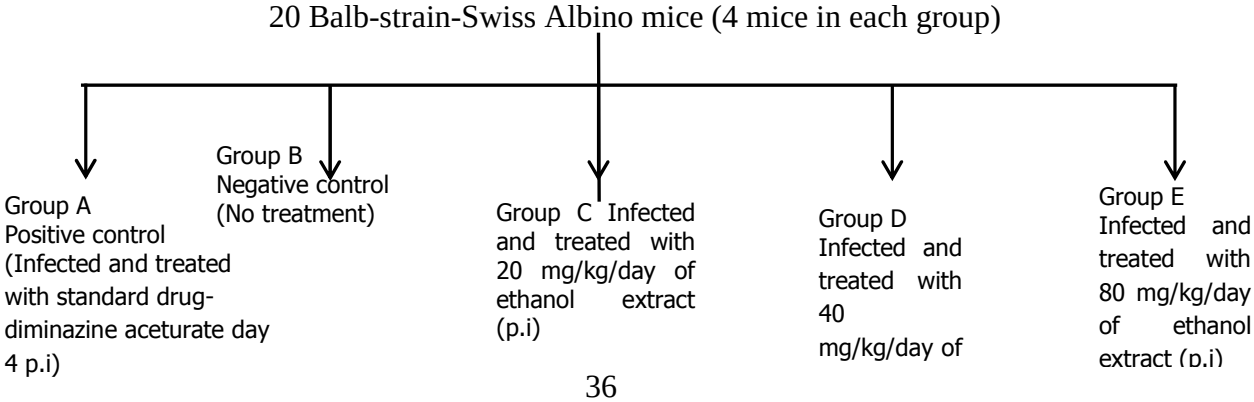
Evaluation of the Median Lethal Dose (LD_{50})

The Lethal Dose (LD_{50}) for each extract was determined by Lorke (1983).



LD_{50} = 900 mg/kg, P I = Phase I
P II = Phase II

***In vivo* antitrypanosomal activity of plant extracts**



The mice were divided into 5 groups, each comprising of 4 mice. The mice were inoculated intraperitoneally each with 0.2 - 0.5 ml of the parasite diluted from blood with a parasitaemia level (**>/=** log 8.4) twice a day (9.00 am and 4.30 pm) for 3 days . All treatments were initiated 4 days post infection (Adamu *et al.*, 2009). The freshly prepared ethanolic leaf extracts were administered at the doses of 20 mg/kg/day, 40 mg/kg/day and 80 mg/kg/day. Mice were checked daily using the 'Rapid Matching' method for estimating the host's parasitemia (Tasdemir *et al.*, 2006). Packed cell volume (PCV) was measured daily and was determined by a micro-method, using a Hawskley microhaematocrit centrifuge (Adamu *et al.*, 2009). For the assessment, the animals were checked daily for parasitemia for 15 days. Packed Cell Volume (PCV) and weight of mice were determined. The day of the death of the animals were also determined (Tasdemir *et al.*, 2006; Adamu *et al.*, 2009; and Ogoti *et al.*, 2009).All treatments were initiated day 4 post infection.

Statistical Analysis

The data obtained from the study were summarized as Mean ± Standard Deviation using Microsoft Excel of the Microsoft Office Professional Plus 2010. Further, Data Analysis was performed using Statistical Package for Social Science (SPSS), version 16.0. Statistical methods employed included descriptive statistics, paired sample t-test, One-way Anova and Tukey-HSD was used for post hoc comparisons. P values less than 0.05 were considered significant.

RESULTS

Lethal Dose (LD_{50})t

The Lethal dose **(**LD_{50}) of the ethanol extract was 90 mg/Kg.

Parasitaemia level and Survival Time of experimental animals

Figure 1 shows *In vivo* antitrypanosomal activity of Ethanol extracts of *Punica granatum* against *Trypanosoma brucei brucei*.

Weight of experimental animals

Figure 2 shows the mean weight values of mice infected with *T. brucei brucei* and subsequently treated at three doses of ethanol extract of *Punica granatum* and diminazine aceturate. Pre-infection mean weight values (g) of the mice were: Positive control (16.80±2.62), negative control (15.53±1.34), E80mg/kg(17.03±4.16)E40 mg/kg(16.13±1.68) and E20 mg/kg(17.05±2.49) respectively. However, on day 3, the mean weight values increased. Positive control (23.10±3.81), negative control (20.73±1.70), E80 mg/kg (22.3±5.57), E40 mg/kg (21.63±1.76) and E20 mg/kg (22.93±3.77) respectively. Mean weight of mice in the E40 mg/kg group improved significantly ($p = 0.010$; $p < 0.05$) when compared to the negative group (fig.4)..

Packed cell volume (PCV) of infected mice

Figure 3 shows the mean PCV values of mice infected with *T. brucei brucei* and subsequently treated with three doses of the ethanolic leaf extract of *Punica granatum*. The mean PCV values (%) pre-infection for groups were: Positive control (50.5±2.38), negative control (48.75±3.40), E8mg/kg (48.00±5.60), E40 mg/kg(46.25±3.30) and E20 mg/kg(53.25±2.63) respectively. However, on day 5, the mean PCV values decreased: Positive control (41.50±3.11), negative control (41.00±4.32), E80 mg/kg (44.50±4.20), E40 mg/kg (37.50±1.73) and E20 mg/kg (42.50±5.74) respectively. However, the PCV of the E40 mg/kg group improved significantly ($p = 0.003$; $p < 0.05$) when compared with the other groups (Figure 4).

The survival time (days) of the mice showed that the negative control had a mean survival time of 5.75 ± 0.5 days as against 15 ± 0.0 days for the positive control. The administration of various doses of the ethanol extract of *P. granatum* L. showed the following survival times for the mice at the doses stated: 80mg/kg (6 ± 0.82 days); 40 mg/kg (6.25 ± 0.50 days); and 20 mg/kg (6.5 ± 0.58 days).Parasite clearance was achieved on day 8 post infection (day 4 post treatment) with the positive control and remained so until the end of the observation period of 15 days. The ethanol leaf extract showed the following survival times for the mice at the doses stated: E80 mg/kg (6 ± 0.82 days); E40 mg/kg (6.25 ± 0.50 days); and E20 mg/kg (6.5 ± 0.58 days).This shows that the dose of 20 mg/kg was the most effective in prolonging the survival of the infected mice (6.5 ± 0.58 days) when compared to the to the negative control (5.75 ± 0.5 days). The mean survival times in the infected and ethanol treated mice differed significantly ($p < 0.05$) when compared with the negative control (Figure 4).

Parasitaemia was first detected in all the infected groups on day 2 post-infection. Parasite counts thereafter rose sharply in a similar manner in all the infected groups by day 4 post-infection. The group treated with diminazine aceturate (Positive control), showed a sharp reduction in parasites count from day 5 - 6 post-infection (1 - 2 days post treatment), with complete parasite clearence from the blood on day 6 -8 post infection (2 - 4 days post treatment). There was no relapse recorded in this group during the study. Table 1 shows the comparison of parameters among the experimental groups. Weight and PCV of the treated mice improved significantly.

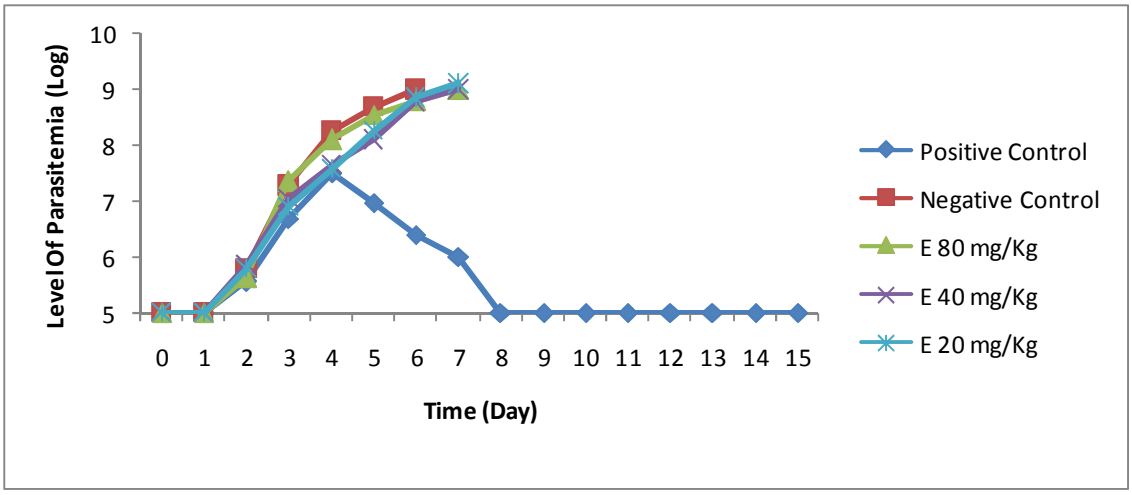


Figure 1: *In vivo* antitrypanosomal activity of Ethanol extracts of *Punica granatum* against *Trypanosoma brucei brucei*

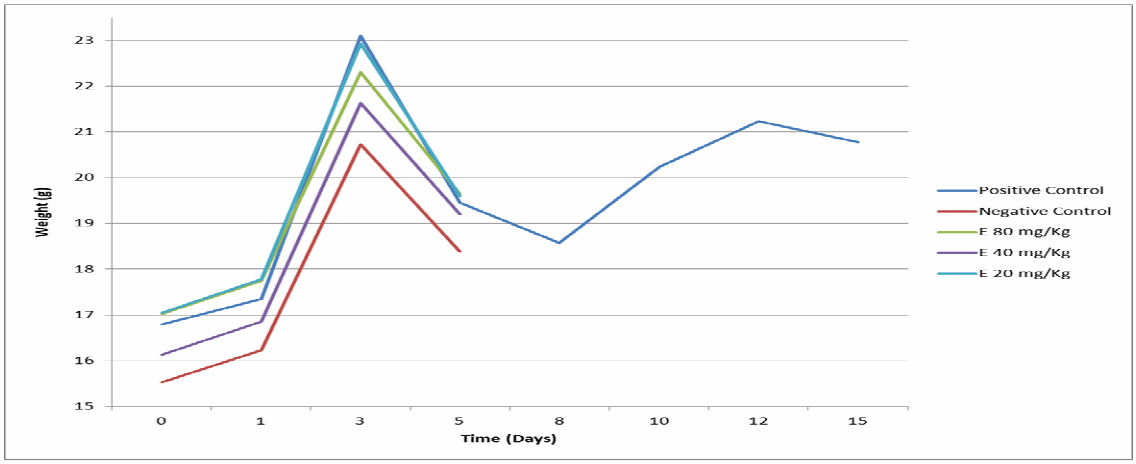


Figure 2. Mean weight of infected mice treated with ethanol extracts of *Punica granatum*

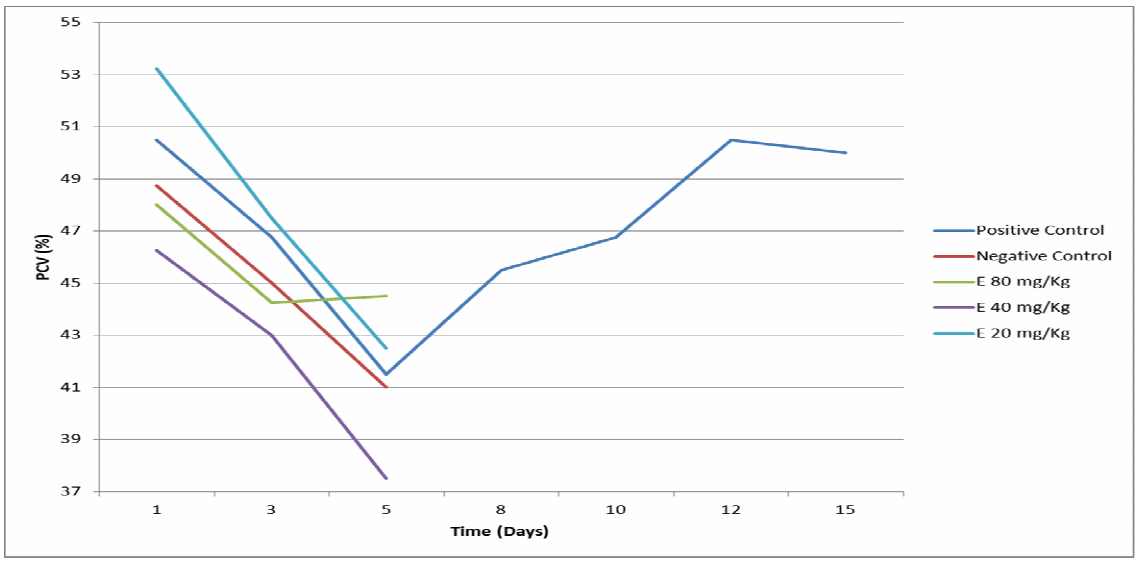


Figure 3: PCV of infected mice treated with three doses of ethanol extracts of *Punica granatum*

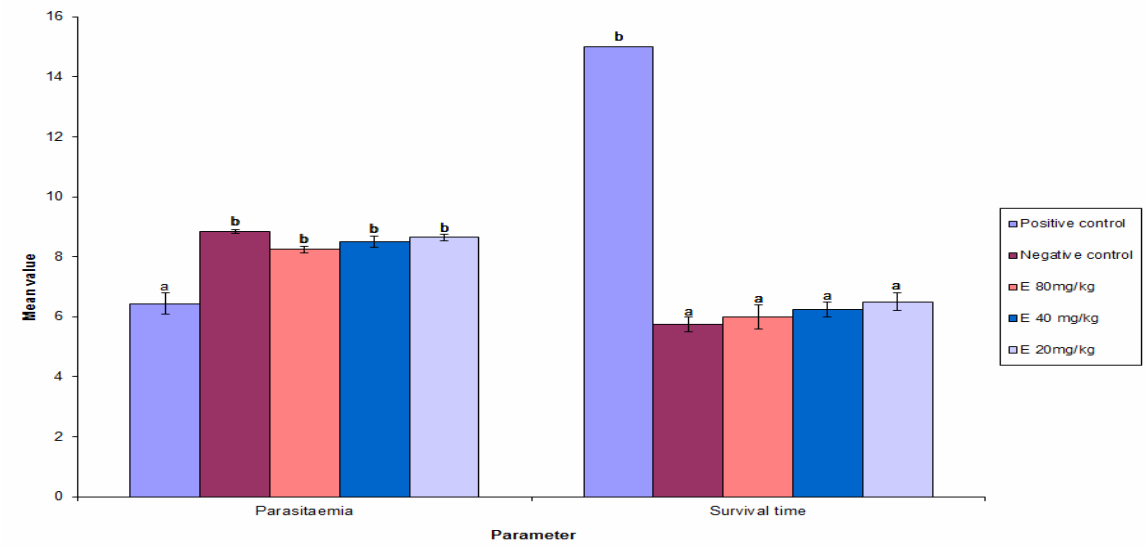


Figure 4: Comparison of parasitaemia and survival times among the experimental groups of mice.

Table 1. Comparison of parameters among the experimental groups of mice

Group	Parasitaemia	Weight (Kg)	Packed Cell Volume (%)	Survival time (days)
Positive control	6.44 ± 0.36^{a}	20.05 ± 1.30^{a}	46.75 ± 1.28^{a}	15.00 ± 0.00^{c}
Negative control	8.85 ± 0.06^{d}	18.38 ± 0.62^{a}	41.00 ± 2.16^{a}	5.75 ± 0.25^{a}
E 80mg/kg	8.24 ± 0.11^{cd}	19.65 ± 2.19^{a}	44.50 ± 2.10^{a}	6.00 ± 0.41^{a}
E 40 mg/kg	8.50 ± 0.20^{cd}	$19.20 \pm 0.91^{a*}$	$37.50 \pm 0.87^{a*}$	6.25 ± 0.25^{ab}
E 20mg/kg	8.64 ± 0.11^{cd}	19.60 ± 1.41^{a}	42.50 ± 2.87^{a}	$6.50 \pm 0.29^{ab*}$

= significant difference at α = 0.05

$^{a, b, c, d}$: represent different significant homogenous subsets in the increasing order of the mean value.

- Groups in the different homogenous subsets differ significantly from each other while groups in the same homogenous subset do not differ significantly.

DISCUSSION

The consistent suppression of parasitaemia combined with prolonged survival time of mice as shown in *in vivo* results may be linked to the ability of the extract (20mg/kg) to inhibit glycolysis which is the major source of energy for blood stream *Trypanosoma brucei*.

Death observed in the negative control groups resulting from massive parasitaemia may be due to haemolytic anaemia caused by *Trypanosoma brucei brucei* infections.Increased red blood cell destruction resulting in anaemia as well as tissue damage is brought about by *Trypanosoma brucei* infection (Ogbadoyi *et al*., 1999; Umar *et al*., 2000).

There was no outstanding *in vivo* activity of the extracts and may be attributed to degradation or metabolisation of the active principle through various metabolic processes in the host animal, or to the toxicity of high levels of the crude extract required for therapeutic efficacy. The high level of parasitaemia in the infected mice could also be a factor.

Trypanosome infection may cause anaemia as a result of massive erythrophagocytosis by an expanded and active mononuclear phagocytic system of the host (Igbokwe and Nwosu, 1997).The observed trypanostatic effect of the ethanolic leaf extract of *Punica granatum* was accompanied by corresponding increase in PCV. Anaemia is the most outstanding clinical and laboratory feature of African trypanosomiasis (Suliman and Fieldman, 1989;Umar *et al*., 2000). Anemia as indicated by PCV level is known to worsen with increasing parasitaemia (Ogbadoyi *et al*., 1999; Sulaiman and Adeyemi. 2010). The prolongation of lives of treated animals may therefore also be associated with the ability of this extract to improve the PCV possibly by reducing the parasite load or inactivating the toxic metabolites produced by trypanosomes.

Haematological results obtained in this study agree with earlier studies (Anosa, 1988; Igbokwe *et al*., 1994; Ekanem *et al*., 2008). The low PCV observed in the infected group may be as a result of acute haemolysis due to increasing parasitaemia.

Increased susceptibility of red blood cell membrane to oxidative damage as a result of reduced glutathione depletion on the surface of the red blood cell may be caused by infection with trypanosomes (Igbokwe *et al*., 1997; Akanji *et al*., 2009).

Many natural products exhibit their trypanocidal activity by acting either on the respiratory chain or on the cellular defenses against oxidative stress through interference with the redox balance of the parasites. It is known that natural products possess structures capable of releasing radicals that may cause peroxidative damage to enzymes that are very sensitive to alterations in redox balance (Sepulveda-Boza and Cassels, 1996;Akanji *et al*., 2009).

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