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Phytochemical composition of *Annona senegalensis* leaf and its antioxidant activity during *Trypanosoma brucei brucei* induced oxidative stress in mice

Titilayo O. Johnson[1*], Ahmed Olatunde[2] and Lazarus Nwachukwu[1]

[1]*Department of Biochemistry, University of Jos, Nigeria*
[2]*Department of Biochemistry, Abubakar Tafawa Balewa University, Nigeria*

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Abstract
The quantitative phytochemical analysis of *Annona senegalensis* leaf was carried out and the effects of its ethanolic extract on some haematological and biochemical parameters of oxidative stress in *Trypanosoma brucei brucei* infected mice were determined. Treatment of *T. b brucei*-infected mice with 50, 100 and 150 mg/kg body weight of ethanolic extract of *A. senegalensis* leaf, which commenced on the first day parasites were sighted in the blood of rats was done daily for seven days. Parasitaemia levels of mice were observed daily. Haemoglobin (Hb) concentration, packed cell volume (PCV), lipid peroxidation; catalase (CAT) and superoxide dismutase (SOD) activities were determined after the seventh day treatment. *A. senegalensis* leaf was found to contain 34% tannins, 20% flavonoid, 7.9% alkaloids, 7.42% saponins and 3.2% steroids. Treatment with 50, 100 and 150 mg/kg of ethanolic leaf extract of *Annona senegalensis* reduced the parasitaemia of infected mice by 70%, 87.86% and 88.57% respectively by day 9 post infection. The values of haemoglobin concentration and packed cell volume, as well as SOD and CAT activities were significantly increased ($p<0.05$) and lipid peroxidation significantly reduced ($p<0.05$) in *T. b. brucei*-infected mice treated with 50, 100 and 150 mg/kg the ethanolic extract compared with the infected untreated ones. The high percentage of tannins and flavonoids contained in *A. senegalensis* leaf as observed in this study could have played a significant role in the antitrypanosomal and antioxidant activity displayed by the extract and this might be useful in the fight against sleeping sickness.

Keywords: *Annona senegalensis*, *Trypanosoma brucei*, oxidative stress, iron chelation, phytochemical analysis

INTRODUCTION

Human African trypanosomiasis (HAT) or sleeping sickness a vector borne neuroinflammatory disease caused by protozoan of the species *Trypanosoma brucei* has been a burden for life quality and economy in 36 sub-Saharan African countries [1]. In the year 2014, approximately 3800 cases of disease were reported and the lives of millions of people were equally threatened [2]. However, a great decline in the number of new cases was recorded in 2015 and the disease has been targeted for elimination as a public health problem by 2020 [3].

Upon invasion of the host, trypanosomes produce numerous changes in the cellular and biochemical constituents of blood and other tissues [4,5,]. Oxidative stress, defined as the imbalance between oxidants and antioxidants, in favor of the

* Corresponding author. *E-mail*: bchresearchers@gmail.com *Tel*: +234 (0) 8060775453
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former, which potentially leads to damage, has been implicated in trypanosomiasis. Indication of systemic oxidative stress, manifesting as increased lipid peroxidation [6], increased production of free radicals [7], and reduced systemic antioxidants [8] has been presented to support the assertion that the pathogenesis of trypanosomiasis involves oxidative stress, at least in part. The disease is characterized by increased red blood cell destruction, which results in anaemia as well as tissue damage [9-11]. The mechanism of aneamia in trypanosomiasis is greatly associated with the generation of free radicals and superoxides following lipid peroxidation. These oxidative products generally attack the cellular integrity of host cells during trypanosomiasis [12-14]. These changes together with the need by the host to destroy the parasite are presumably responsible for the fatal consequences of African sleeping sickness.

The damaging effects of reactive oxygen species, such as singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals and peroxynitrite, could however be ameliorated by natural antioxidants. Plant and its products are rich sources of phytochemicals that have been found to possess a variety of biological activities including antioxidant potential [15].

Annona senegalensis Pers (Annonaceae) commonly known as "Wild Custard Apple" is a shrub or small tree widely distributed in Africa [16,17]. The plant decoction is used in the treatment of sleeping sickness in Northern Nigeria and scientific investigations have revealed the antimicrobial, antimalarial, antitrypanosomal, antioxidant and free radical scavenging activity of *A. senegalensis* leaf extracts [17-21].

This study was therefore aimed at investigating the possible beneficial role of ethanolic extract of *A. senegalensis* in reducing the extent of oxidative stress during *T. b. brucei* infection in mice.

EXPERIMENTAL

Parasite. Bloodstream forms of *Trypanosoma brucei brucei* (*T. b. brucei*) was obtained from the Nigerian Institute of Trypanosomiasis Research, Vom. Nigeria.

Experimental mice. Albino mice of both sexes weighing between 19-35 g were obtained from the animal unit of the Department of Pharmacology, University of Jos, Nigeria. The mice were inoculated intraperitoneally with 0.1 ml of inoculum containing about 10^3 trypanosomes/ml of infected blood in normal saline.

In vivo trypanocidal activity of ethanolic extract of *A. senegalensis* leaf. Experimental mice were randomly distributed into six groups of four mice each. Group A, B, and C mice were uninfected untreated, uninfected treated and infected untreated respectively. Group D, E and F rats were infected but treated with 50 mg/kg, 100 mg/kg and 150 mg/kg body weight of ethanolic extract of *A. senegalensis* leaf respectively. Oral administration of extracts to the infected rats, which commenced on the first day parasites were sighted in the blood of mice, was done daily for seven days. Parasitaemia levels of mice were observed daily under Light microscope at x100 magnification.

Plant material. Fresh leaves of *A. senegalensis* were obtained from Federal College of Forestry, Jos, Plateau State, Nigeria and were authenticated by a Botanist at the Herbarium of the Department of Botany, University of Jos, Nigeria.

Preparation of extracts. The leaves of *A. senegalensis* were washed and shade dried to a constant weight. The dried leaves were then pulverized using an electric blender. 60 g of the powdered leaf was added to a beaker containing 500 ml of 70% ethanol and allowed to stand for 24 hours. The setup was filtered using a Whatman No. 1 filter paper. The filtrate was concentrated to dryness at 60^0

C using a water bath until the ethanol portion was removed. The extract was then reconstituted in dimethyl sulfoxide (DMSO) to give the required doses of 50, 100 and 150 mg/kg body weight.

Phytochemical screening. The qualitative phytochemical screening of the plant was carried out using the methods described by Sofowora [22], Trease and Evans [23] and Harborne [24].

Quantitative determination of phytochemical composition of *A. senegalensis* leaf.

*Alkaloid***.** The concentration of alkaloid in the leaf of *A. senegalensis* was carried out using the alkaline precipitation gravimetric method described by the Harbone [24]. 5 g of the powdered sample was soaked in 20 ml of 10% ethanolic acetic acid. The mixture was made to stand for four (4) hours at room temperature. Thereafter, the mixture was filtered through Whatman filter paper (No. 42). The filtrate was concentrated by evaporation over a steam bath to ¼ of the original volume. To precipitate the alkaloid, concentrated ammonia solution was added in drops to the extract until it was in excess. The resulting alkaloid precipitate was recovered by filtration using previously weighed filter paper. After filtration, the precipitate was washed with 9% ammonia solution and dried in an oven at 60°C for 30 minutes, cooled in a desiccator and reweighed. The process was repeated two more times and the average was taken. The weight of alkaloid was determined by the differences and expressed as a percentage of weight of sample analysed as shown below.

$$\%\ Alkaloid = \frac{W2-W1}{\text{Weight of sample}}\ x\ \frac{100}{1}$$

Where: W1 = weight of filter paper,
W2 = weight of filter paper + alkaloid precipitate

Flavonoid. The flavonoid content was determined by the gravimetric method described by Harbone [24]. 50 ml of water and 2ml HCl were added to 5 g of the powdered sample in a conical flask. The solution was allowed to boil for 20 minutes, cooled and filtered through Whatman filter paper (No 42). 10ml of ethyl acetate extract which contained flavonoid was recovered and dried in an oven at 60 °C. It was cooled in a desiccator and weighed. The quantity of flavonoid was determined using the formula:

$$\%\ Flavonoid = \frac{W2-W1}{\text{Weight of sample}}\ x\ \frac{100}{1}$$

Where: - W1= Weight of empty filter paper
W2= Weight of paper + Flavonoid extract

Saponins. The saponin content of the sample was determined by double extraction gravimetric method [24]. 5 g of the powered sample was mixed with 50 ml of 20% aqueous ethanol solution in a flask. The mixture was heated with periodic agitation in water bath for 90 minutes at 55ºC; it was then filtered through Whatman filter paper (No. 42). The residue was extracted with 50 ml of 20% ethanol and both extracts were poured together and the combined extract was reduced to about 40 ml at 90ºC and transferred to a separating funnel where 40 ml of diethyl ether was added and shaken vigorously. Separation was by partition during which the ether layer was discarded and the aqueous layer reserved. Re-extraction by partitioning was done repeatedly until the aqueous layer became clear in colour. The saponins were extracted, with 60 ml of normal butanol. The combined extracts were washed with 5% aqueous sodium chloride (NaCl) solution and evaporated to dryness in a pre-weighed evaporation dish. It was dried at 60ºC in the oven and reweighed after cooling in a desiccator. The process was repeated two more times to obtain an average. Saponin content was calculated as a percentage of the original sample as follows:-

$$\%\ Saponin = \frac{W2-W1}{\text{Weight of sample}}\ x\ \frac{100}{1}$$

Where W1 = weight of evaporating dish
W2 =weight of dish + sample

Tannin. The tannin content of the leaves of the plants was determined using the Folin Dennis spectrophotometric method described by Pearson [25]. The powered sample (2 g) was mixed with 50 ml of distilled water and shaken for 30 minutes in the shaker. The mixture was filtered and the filtrate used for the experiment. 5 ml of the filtrate was measured into 50 ml volume flask and diluted with 3 ml of distilled water. Similarly 5 ml of standard tanuric acid solution and 5 ml of distilled was added separately. 1 ml of Folin-Dennis reagent was added to each of the flask followed by 2.5 ml of saturated sodium carbonate solution. The content of each flask was made up to mark and incubated for 90 minutes at room temperature. The absorbance of the developed colour was measured at 760 nm wavelength with the reagent blank at zero. The process was repeated two more times to get an average. The tannin content was calculated as shown below:-

$$\% \, tannin = \frac{100}{w} \, x \, \frac{AY}{AS} \, x \, \frac{C}{100} \, x \, \frac{VF}{VA} \, x \, D$$

Where: W= weight of sample analysed
AY=Absorbance of test sample
AS= Absorbance of standard solution
C= Concentration of standard in mg/ml
VA= volume of filtrate analysed
D= Dilution factor where applicable

Steroid. The steroid content of the leaves of the plants was determined using the method described by Harborne [24]. 5 g of the powdered sample was hydrolysed by boiling in 50 ml hydrochloric acid solution for about 30 minutes. It was filtered using Whatman filter paper (No. 42). The filtrate was transferred to a separating funnel. Equal volume of ethyl acetate was added to it, mixed well and allowed separation into two layers. The ethyl acetate layer (extract) was recovered while the aqueous layer was discarded. The extract was dried at 100°C for 5 minutes in a steam bath. It was then heated with concentrated amyl alcohol to extract the steroid. The mixture becomes turbid and a reweighed Whatman filter paper (No. 42) was used to filter the mixture properly. The dry extract was then cooled in a desiccator and reweighed. The process was repeated two more times and an average was obtained. The concentration of steroid was determined and expressed as a percentage as follows:-

$$\% \, Steroid = \frac{W2 - W1}{\text{Weight of sample}} \, x \, \frac{100}{1}$$

Where, W1= weight of filter paper.
W2 = weight of filter paper + steroid

Collection of blood sample. Blood was collected from the jugular veins of the animals into sample bottles with and without anticoagulant. The blood with anticoagulant was kept at 4^0C until required for haematological studies. Clear serum was obtained from the other blood sample by centrifugation of the clear part of blood after clotting at 3,000 g for 15min and kept frozen until required.

Determination of haematological indices. Packed cell volume (PCV) and haemoglobin (Hb) values were determined using Mindray Haematology Analyzer (Mindray BC-2300), a product of Guangzhou Medical Equipment Co., Ltd, China.

Biochemical analysis. The activity of superoxide dismutase (SOD) was determined as described by Misra and Fridovich [26], Catalase [27] activity was determined using the method described by Beers and Sizer [27] and the level of malondialdehyde (MDA) was determined using the procedure described by Reily and Aust [28].

Statistical analysis. The data were expressed as Mean ± Standard Error of Mean (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA) and Duncan multiple range test at 5% level of confidence ($p<0.05$). All statistical analyses were performed using GraphPad InStat.

RESULTS

Trypanocidal activity. Figures 1 and 2 show the effects of ethanolic extract of *A.*

senegalensis leaf on the parasitemia of *T. brucei* infected mice. Treatment with 50 mg/kg, 100 mg/kg and 150 mg/kg of ethanolic leaf extract of *Annona senegalensis* significantly reduced the parasitemia of infected mice by 70%, 87.86% and 88.57% respectively.

Phytochemical analysis. Phytochemical screening of ethanolic extract of *A. senegalensis* leaf revealed the presence of alkaloids, flavonoids, tannins, saponins, cardiac glycosides, balsams, carbohydrates, terpenes and steroids (Table 1). Quantitative phytochemical analysis revealed that *A. senegalensis* leaf is composed of 34% tannins, 3.2% steroids, 20% flavonoid, 7.9% alkaloids, and 7.42% saponins (Table 2).

Haematological and biochemical parameters. As shown on table 3, the values of haemoglobin concentration and packed cell volume; as well as serum superoxide dismutase and catalase activities were significantly increased ($p<0.05$) in infected mice treated with 50 mg/kg, 100 mg/kg and 150 mg/kg of ethanolic extract of *A. senegalensis* leaf compared with the infected untreated ones. The various doses of the extract also brought about a significant reduction ($p<0.05$) in the concentration of malondialdehyde in the serum of infected mice.

Table 1: Phytochemical contents of *A. senegalensis* leaf

Phytochemicals	*A. senegalensis* leaf
Alkaloids	+
Flavonoids	++
Tannins	++
Saponins	+
Cardiac glycosides	+
Terpenes and steroids	+
Balsams	+
Anthraquinones	-
Phlobatannins	-
Carbohydrates	+

Key= + present; - absent.

Table 2: Quantitative phytochemical Composition of *A. senegalensis* leaf

Phytochemicals	*A. senegalensis* leaf (%)
Tannins	34
Flavonoids	20
Alkaloids	7.9
Saponins	7.42
Steroids	3.2

Key: %= percentage composition

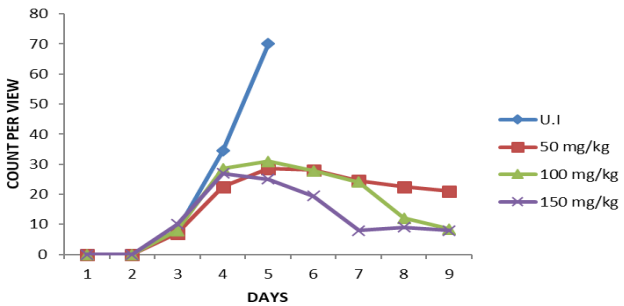

Fig. 1: Parasite count of *T. brucei* infected mice treated with 50, 100 and 150 mg/kg ethanolic extract of *A. senegalensis* leaf as the infection progressed until death.

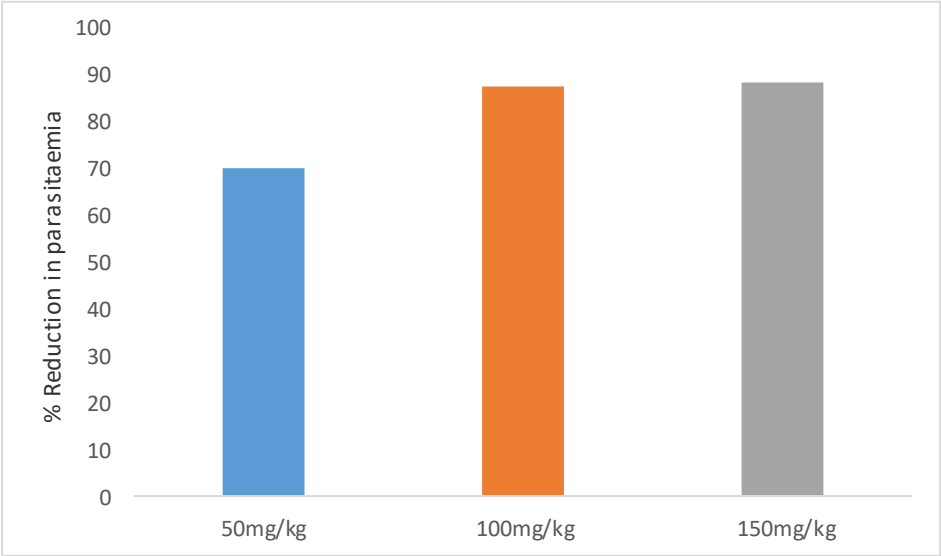

Fig. 2: Percentage (%) reduction in parasitaemia of *T. brucei* infected mice with 50, 100 and 150 mg/kg ethanolic extract of *A. senegalensis* leaf.

Table 3: Effect of ethanolic extract of *A. senegalensis* leaf on some haematological and biochemical parameters of oxidative stress in *T. brucei brucei* infected mice.

	Uninfected untreated	Infected untreated	Infected treated with 50mg/kg	Infected treated with 100mg/kg	Infected treated with 150mg/kg
HB (g/dl)	9.3 ± 0.83^{a}	5.73 ± 0.89^{b}	7.08 ± 0.97^{c}	8.08 ± 0.74^{d}	9.20 ± 0.08^{ad}
PCV (%)	28.00 ± 2.45^{a}	17.40 ± 0.43^{b}	21.25 ± 2.99^{c}	24.25 ± 2.22^{d}	27.63 ± 0.26^{ad}
SOD (x 10^{-3}) (µmol/min)	4 ± 0.10^{a}	0.4 ± 0.10^{b}	0.7 ± 0.10^{c}	0.9 ± 0.10^{d}	1.2 ± 0.20^{e}
CAT (µmol/min)	4.10 ± 0.79^{a}	1.67 ± 0.06^{b}	1.99 ± 0.08^{c}	2.36 ± 0.24^{d}	3.65 ± 0.67^{a}
MDA (µmol/g)	4.55 ± 1.08^{a}	10.34 ± 0.34^{b}	8.00 ± 1.20^{c}	7.03 ± 1.22^{c}	5.81 ± 0.48^{ac}

Hb (Haemoglobin concentration), PCV (Packed Cell Volume), SOD (Superoxide dismutase activity), CAT (Catalase activity), MDA (Malondialdehyde). Values are mean ± SEM of 4 replicates. Values with different superscripts are significantly different ($p<0.05$).

DISCUSSION

The detection of alkaloids, flavonoids, tannins, saponins, cardiac glycosides, balsams, carbohydrates, terpenes and steroids in the ethanolic extract of *A. senegalensis leaf* in this study, is in line with our previous report on the phytochemical constituents and antimicrobial activity of the leaf [21]. It also agrees with the work of Theophine *et al*. [29]. These phytochemical compounds have been shown to play significant roles in the pharmacological activities of medicinal plants. Quantitative phytochemical analysis of the leaf, which revealed 34% tannins, 20% flavonoid, 7.9% alkaloids, 7.42% saponins and 3.2% steroids, has provided a clue to the mechanism of antitrypanosomal and antioxidant action of the plant. The high percentage of tannins and flavonoids in *A. senegalensis leaf* could have played a significant role in the activities displayed by the extract. Flavonoids and tannins have been reported to possess iron-chelating properties [30]. Iron-chelation have been reported in our previous study to inhibit proliferation of trypanosomes through inhibition of the enzyme ribonucleotide reductase (RR) – a unique enzyme responsible for reducing

ribonucleotides to their corresponding deoxyribonucleotides, which are the building blocks required for DNA replication and repair. [31,32,33]. Apart from inhibiting proliferation of parasites, removal of excess iron through chelation was also suggested to prevent iron mediated injury to cells thereby reducing the pathology of anaemia and tissue damage associated with the infection. Iron chelation was also reported to regulate NO production thereby limiting its destructive effects on cells [11]. Apart from being iron chelators, tannins and flavonoids have been reported to possess antioxidant and free radical scavenging activities [34-36,].

The high antitrypanosomal activity displayed by *A. senegalensis* leaf in this study agrees with the report of Ogbadoyi *et al.* [17]. They observed that crude and partially purified aqueous extracts of the *Annona senegalensis leaf*, at a dose of 200 mg/kg body weight per day completely cured experimental *Trypanosoma brucei brucei* infection in mice. Kabiru *et al.* [37] also found that 400 mg/kg body weight of hexane extract and 300 mg/kg body weight of aqueous extract of the stem bark of *Annona senegalensis* cleared parasites from circulation after three weeks of treatment.

The significant reduction in PCV and Hb levels of the infected untreated mice is an indication of anaemia, which is greatly associated with the generation of free radicals and superoxides. Reduction in RBCs, PCV and Hb concentration have been shown in previous studies to correlate with increased level of serum iron [11,38-40]. Accumulation of "free" iron is injurious to the animal because it can catalyse the formation of hydroxyl radical from hydrogen peroxide [41]. The hydroxyl radical is highly reactive, and attack lipids, protein and nucleic acids [42]. This leads to oxidative stress in which the level of the oxidants such as the hydroxyl radical is greater than the endogenous antioxidants such as catalase (CAT) and superoxide dismutase (SOD). This might be one of the reasons for the significantly low levels of catalase and superoxide dismutase activities and the significantly high lipid peroxidation observed in the serum of infected untreated mice in this study. The ability of ethanolic extract of *A. senegalensis* leaf to ameliorate this condition is most likely due to the plants antioxidant activity contributed by the high presence of tannins and flavonoids.

In conclusion, quantitative phytochemical analysis showed that *A. senegalensis* leaf contains 34% tannins, 20% flavonoid, 7.9% alkaloids, 7.42% saponins and 3.2% steroids. Ethanolic extract of the leaf displayed high antitrypanosomal activity and ameliorated some haematological and biochemical parameters of oxidative stress during *Trypanosoma brucei brucei* infection in mice. The high percentage of tannins and flavonoids in *A. senegalensis leaf* could have played a significant role in the activities displayed by the extract. This important property of *A. senegalensis leaf* could be exploited in the fight against African sleeping sickness.

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