

In Vivo* Anti-Trypanosomal Activity of Ethanolic Leaf Extract of *Carissa Spinarum* (Wild Karanda) in Mice Infected with *Trypanosoma Brucei Brucei Spp.

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ABSTRACT

Carissa Spinarum plant has been implicated as a medicinal plant for joints, muscle, chest pains by the massai people of Kenya, also as an antiviral supplement for HIV treatment in Tanzania. Acute toxicity of the ethanolic leaf extract and minimum inhibition concentration (MIC) using four (4) microorganism (*proteus*, *e.coli*, *staphylococcus aureus spp* & *enterobacter spp*) was evaluated in mice using Lorke's method & serial dilution method respectively. The ethanolic leaf extract was evaluated for *in vivo* anti-trypanosomal activity against federa strain of *Trypanosoma brucei brucei* in albino mice. Four days suppressive, curative effect against established infection and prophylactic models of anti-trypanosomal studies were carried out. The median lethal dose of the extract was determined to be $\geq 100\text{mg/kg}$ body weight. The extract (12.5, 25, 50mg / kg) exerted some dose dependent suppressive effects at the different levels of infections tested, with no significant curative effects recorded. However, further antitrypanosomal property can be explored for the management of *trypanosomiasis*.

Keywords: *Carissa spinarum*, *antitrypanosomal*, *albino mice*, *trypanosome brucei brucei specie*

CONFLICT OF INTEREST

There exist no conflicts of interest as financial contribution towards this research was contributed by all authors.

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Introduction

Trypanosomiasis is one of the most important infectious kinds of livestock and humans of similar and etiology and epidemiology in sub-saharan Africa [18]. Besides death, it causes a heavy economic loss to livestock mainly in Africa, in man, the disease is called Sleeping sickness, while in animal, it's known as Nagana. Sleeping sickness is known to have a prevalence of 300,000 – 500,000 [13] as well as three million death to livestock occurring every year [11]. Despite this prevalence rate, few drugs, namely, melarsoprol, Suramin, pentamidine and efflornithine are available for treatment, which are known to be toxic, old, expensive and not readily available [6]. In addition, resistances to these major drugs as well multiple drug resistant populations have been described for different species of the parasite [1]. Relapses of unknown etiology have also been reported for melarsoprol in recent epidemics. Hence, there is urgent need to seek for new sources of therapeutic agents [9].

With the above development, recent approaches to alternative therapeutic agents for treatment of trypanosomiasis have focused on plants and other natural products [7], thus in this research investigation, we would focus on assessing the phytochemistry of the plant, *Carissa spinarum* as well as the *in vivo* antitrypanosomal activity of its ethanolic leaf extract. *Carissa Spinarum* also known as the conkerberry or Bush Plum is a large shrub, that belongs to the family of Apocynaceae, it's widely distributed in tropical region and in

India, where it's abundantly occurs, it's commonly known as Wild Karanda due to its close relation with the (*C. carandas*), This shrub is found wild in most parts of India, especially in the dry foothills of the Punjab, the sub-Himalayan tract up to 4,000 feet in the trans-Indus territory and also on the coast of the southern Andamans [14]. The plant an erect thorny shrub, with forked branches, 2-3 metres in height; wood, very hard; bark, light brown to green, can be stripped off longitudinally by hand, exposing the white to light-green wood underneath; thorns, 3.2 cm long, brown to greenish at the base and deep brown towards the tip. Leaves are ovate, 4.5 cm long, 2.5 cm broad, leathery; venation, reticulate pinnate; margin, entire; petiole 3 mm long; leaves exuding white latex, when plucked from the stem.

Materials and Method

The plant was collected from Gombe, Gombe State, which is Northeastern zone of Nigeria and was identified at the Herbarium of Ahmadu Bello University, Samaru – Zaria, which is in the Northwestern zone of Nigeria with voucher Det: U.S Gallah 8/02/2012. All reagents and solvents used were of analytical grade.

Leaf parts of the plant were harvested dried under the shade or in open air in the laboratory. Dried materials were pounded in laboratory mortar into small particles. Fifty grams (50g) of the pounded dried plants materials were weighed and extracted with 3 X 150ml ethanol (70%) and allowed to macerate for 3 days, then filtered to obtain the extract which is then dried under electric fan and stored in a refrigerator at 4°C until required.

Animals

Four (4) weeks old albino mice weighing between 18-20 g obtained from the Animal house of NITR, Kaduna were used for the study, they were housed in plastic cages with saw dust as beddings and given food and water ad libitum. Acclimatized for two (2) weeks before commencement of research.

Phytochemical Screening

The ethanolic leaf extract of *Carissa spinarum* was screened for the presence of secondary metabolites and constituents using conventional protocols for detecting the presence of steroids, alkaloids, lignin and phenols [8]; fatty acids, glycosides, triterpenoids and saponins [5]; tannins, leucoanthocyanins and emodins [19]; reducing sugars [17]; anthraquinones [2], flavonoids [15] and coumarins [16].

Determination of Parasitaemia

Parasitaemia was monitored in blood obtained from the tail, pre-sterilized with methylated spirit. The number of parasites was determined microscopically at X 400 magnification using the "Rapid Matching" method of Herbert and Lumsden [10]. Briefly, the method involves microscopic counting of parasites per field in pure blood or blood appropriately diluted with buffered phosphate saline (PBS, pH 7.2). Logarithm values of these counts obtained by matching with the table of Herbert and Lumsden is converted to antilog to provide absolute number of trypanosomes per ml of blood [3], [4].

Minimum Inhibition Concentration

Minimum Inhibitory Concentration (MIC) involves the lowest concentration of an antimicrobial that can inhibit the visible growth of the microorganism after the overnight incubation. In this case, four (4) common microorganism namely, *e.coli*, enterobacter, staphylococcus aureus spp and proteus (fig ii) were subjected to inhibition properties with the ethanolic leaf extract of *Carissa spinarum* via serial dilution incubation of the extract with each microorganism.

Acute Toxicity Test

Acute toxicity test of *Carissa spinarum* leaf extract was carried out using the modified Lorke's method [12]. The study was carried in two phases, the first phase requires 9 (nine) mice randomized into 3 groups of three mice & each given intraperitoneally 10, 100 & 1000mg/kg body weight of the extract. The mice were observed for signs of toxicity which included but not limited to paw licking, salivation, stretching of the body, weakness, sleep, respiratory stress, coma & death in the first four hours of extract administration and subsequently daily for hours. In the second phase, another fresh set of 9 (nine) mice were randomized into 3 groups of three mice again & administered with 1600, 2900 & 500mg/kg of the extract intraperitoneally based on the result of the first phase, further observation of signs of toxicity & mortality for the first 4 (four) critical hours and daily afterwards. The oral median lethal dose was calculated using the formula:

$LD50 = \sqrt{\text{minimum toxic dose} \times \text{maximum tolerated dose}}$

In Vivo Assay

Following in vitro studies, Mice inoculated with *Trypanosoma brucei brucei* were intraperitoneally treated with 500 mg/kg body weight of the extracts when average parasitaemia was approximately two parasite per field for

therapeutic & zero parasite per field for prophylactic. Preliminary investigation indicated relatively poor efficacy with 100 and 200 mg/kg doses of the extracts. The treatment continued daily with continuous monitoring of parasitaemia for 4 days. After withdrawal of treatment, parasitaemia was also monitored daily until the 5th day and thereafter monitoring was reduced for surviving animals. Three animals were used per treatment group. An infected but untreated mouse was included as a negative control.

RESULTS

Phytochemical Screening

Results obtained from the phytochemical screening of the ethanolic leaf extract of *Carissa spinarum* revealed the presence of Anthraquinones, Emodins, Glycosides, Lignins, Phenols, Reducing sugar, Steroids and Tannins, while Alkaloids, Coumarins, Fatty acids, Flavonoids, Leucoanthocyanins, Saponin and Triterpenoids were absent (fig i).

Behavioral signs of toxicity was observed in all mice administered with various doses and mortality of 2 (two) mice recorded in the first 4 (four) hours at 1000mg of extract / kg body weight & total mortality of all mice after several hours. The median lethal dose LD50 was determined to be ≥ 100 mg / kg body weight.

Serological test consisting of kidney function test was conducted on mice administered with extract at 100mg / kg body weight as post mortem indicated highly inflamed kidneys in mice administered with extract. Results show a high deficiency in potassium at 2.2mmol as against the normal range of 3.4 – 5.3mmol (fig iv).

(fig i) Phytochemical composition of ethanolic leaf extract of *Carissa spinarum*

Phytochemical Components	Inference
Alkaloids	Absent
Anthraquinones	Present
Coumarins	Absent
Emodins	Present
Fatty acids	Absent
Flavonoids	Absent
Glycosides	Present
Leucoanthocyanins	Absent
Lignins	Present
Phenols	Present
Reducing sugars	Present
Saponin	Absent
Steroids	Present
Tannins	Present
Triterpenoids	Absent

(fig ii) MINIMUM INHIBITORY CONCENTRATION (MIC) OF ETHANOLIC LEAF EXTRACTS OF *CARISSA SPINARUM* PLANT.

NO	TEST ORGANISMS	LEAVE EXTRACT (10-1mm) 0.5ML	LEAVE EXTRACT (10-2) 0.5ML	LEAVE EXTRACT (10-3) 0.5ML	CONTROL (ETHANOL) 0.5ML
1	E.COLI	-	-	-	-
2	ENTROBACTER	-	-	-	-
3	PROTEUS	20.5mm	18mm	-	-
4	STAPHYLOCOCCUS AUREUS	-	-	-	-

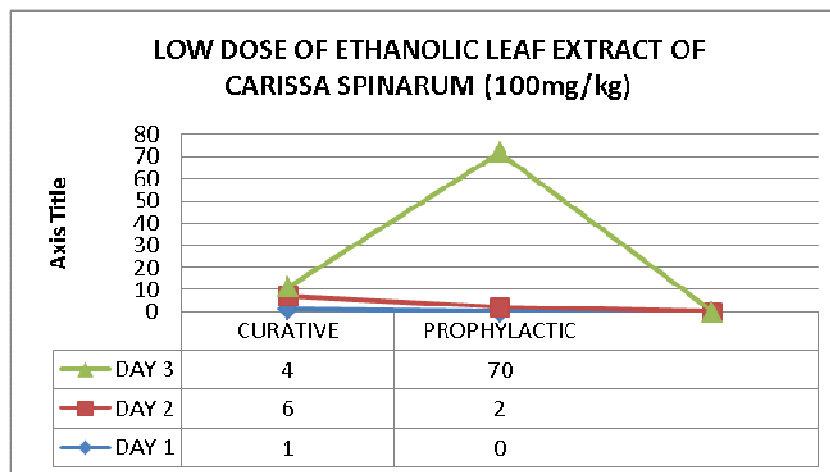
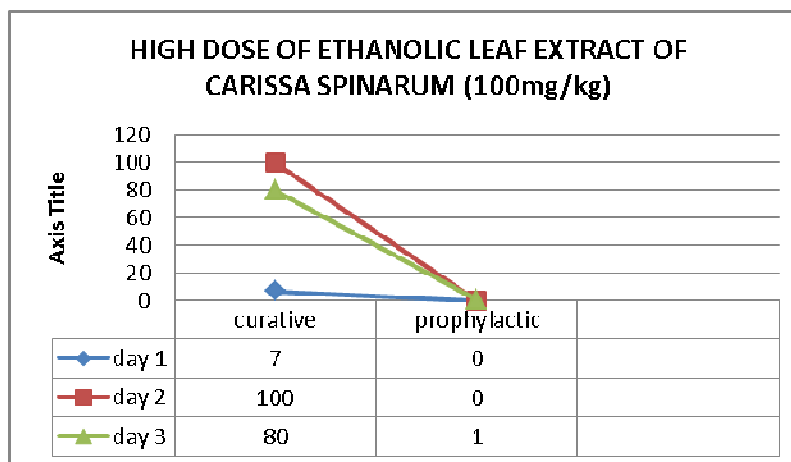
(fig iii) Acute toxicity test for *Carissa spinarum* ethanolic leaf extract in albino mice

Dose (mg/kg)	Total mice	Mortality
10	3	-
100	3	-
1000	3	3
1600	3	3
2900	3	3
5000	3	3

(fig iv) Serological result performed on kidney of mice administered with *Carissa spinarum* ethanolic leaf extract.

Compound	Normal Range (mmol)	Reading (mmol)
Urea	1.7 – 9.1	1.9
Sodium	136 – 148	137
Potassium	3.4 – 5.3	2.2
Chloride	95 – 111	96
Bicarbonate	22 – 32	24
Creatinine	9 – 124	25

(fig v) EFFECTS OF ETHANOLIC LEAF EXTRACT OF *CARISSA SPINARUM* ON PARASITEMIA FOR 3 DAYS



DISCUSSION

That the anti-trypanosomal effect observed under *in vivo* condition following administration of ethanolic leaf extracts of *Carissa spinarum* (fig v) is attributable to the extracts, appears to be confirmed by the death of all members of the control group that were infected with the parasite but left untreated within 7 days of infection, while most survived beyond the 7 days signifying the prophylactic properties of the extract.

The Minimum Inhibition Concentration of the extract against the four microorganism indicate very little inhibition which only occurred with *proteus* spp (fig ii).

The phytochemical analysis indicate the absence of alkaloids & saponins (fig i), which in most cases are positive indicators of non antitrypanosomal activity, however, the presence of other substance like phenol, reducing sugars, Glycosides, steroids, etc may indicate different (fig i). Tannin on the other hand is an antinutrient and may be responsible for the enlarged kidneys observed in the mice from high dosage and thus observed in the low potassium in blood of the mice.

The weakness observed in the mice of different groups with continuous administration of the extracts; even after parasites were eliminated from the blood stream suggest that the extracts may have some cumulative toxic effects at the high dose used. However, put together, these results suggest that *Carissa spinarum* possess significant anti-trypanosomal effect to warrant further detailed studies utilizing bioassay-guided fractionations under varied pharmacological conditions in order to unequivocally establish its therapeutic efficacy.

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