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Antiplasmodial Activity of the Root Extracts of *Cochlospermum tinctorium* in Mice Experimentally Infected with Clinical Isolates of *Plasmodium berghei berghei* (NK 65)

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Authors' contributions

This work was carried out in collaboration between all authors. Authors ROA and EK were involved in the research design. Author ROA carried out the laboratory work and analysis of result. Authors EK and ISN were involved in co-editing the manuscript. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: To determine *in vivo* the antiplasmodial activity of two chromatographic fractions obtained from *Cochlospermum tinctorium* A. Rich root bark extract against *Plasmodium berghei berghei* in mice.
Methodology: Two chromatographic fractions were obtained from the crude methanolic extract of *C. tinctorium* root bark using petroleum ether (Fraction 1) and ethanol (Fraction 2) which were tested to determine their antiplasmodial activity in Swiss Albino mice infected with *P. berghei berghei*. Fraction 1 was administered at dose levels of 50, 100 and 200mg/kg/day, while fraction 2 was administered at dose levels of 25, 50 and 100 mg/kg/day. Chloroquine at 5 mg/kg per day was used as positive control and 0.2 ml normal saline was applied per day as sham.
Results: Fraction 1had a percentage antiplasmodial activity of 80.67%, 64.14% and 69.71% for the dose level of 50, 100 and 200 mg/kg respectively. Data shows that fraction 1 treatment was not dose dependent as the lowest dosage of 50 mg/kg/day produced the highest percentage antiplasmodial activity of 80.67%, while 5 mg/kg chloroquine gave

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100% cure. The effects of all treatments were significantly different ($p<0.05$). Fraction 2 produced a dose dependent antiplasmodial activity of 62.42%, 65.70% and 98.17% in infected mice treated with 25, 50 and 100 mg/kg/day of the fraction respectively in direct proportion. Only the 100 mg/kg fraction antiplasmodial activity was not significantly different ($p>0.05$) from that of 5 mg/kg chloroquine. The Median survival time post infection for infected mice treated with sham was 11 days, for infected mice treated with 50, 100 and 200mg/kg of fraction 1, the median survival time was 15.5, 15.5 and 19 days respectively, the log-rank (Mantle-Cox) test value of $p<0.05$ showed that survival curves are significantly different. The median survival time post infection for mice treated with 0.2ml normal saline per day was 11days, for mice treated with 25, 50 and 100mg/kg of fraction 2 the median survival time post infection was 19, 18.5 and 18 days respectively, Log-rank (Mantle-Cox) test value of $p<0.05$ indicates that survival curves are significantly different. The high antiplasmodial activity of 100 mg/kg fraction 1 was countered by its low survival time indicating a probable increase in toxicity to the mice with increase in dosage. The mean Packed Cell Volume (PCV) for infected mice treated with 50, 100 and 200 mg/kg fraction 1, the mean PCV was 36.83%, 32.42% and 37.96% respectively, while infected mice treated with25, 50 and 100mg/kg the mean PCV was 40.22%, 27.50% and 43.04% respectively. Since the mean PCV for infected mice treated with sham was 29.50%and 43.40% for 5mg/kg chloroquine treated mice, both fractions of *C. tinctorium* possess anaemia ameliorating property. However, only that of 25 and 100 mg/kg fraction 2 were the same as that of 5 mg/kg chloroquine ($p>0.05$).There was however no significant difference ($p>0.05$) in the mean percentage antiplasmodial activity of fraction 2 (65.26%) which was higher than that of fraction 1 (62.90%).

Conclusion: *C. tinctorium* root bark extractpossess antimalarial and anaemia ameliorating properties which validates its use in traditional medicine for the treatment of malaria.

Keywords: Cochlospermum tinctorium; antiplasmodial activity; Plasmodium berghei berghei; plant extract; anaemia.

1. INTRODUCTION

Malaria is one of the world's most deadly diseases. Even though it is highly preventable and treatable, it causes approximately 660,000 deaths yearly with nine out of ten deaths occurring in sub-Saharan Africa, and 85% of malaria related deaths in children under five years of age [1].

Plant extracts are still widely used in the treatment of malaria and other ailment, in Africa and other continents [2]. In Africa, up to 80% of the population still use traditional medicine for primary health care. In Ghana, Mali, Nigeria and Zambia, the first line of treatment for 60% of children with high fever resulting from Malaria is the use of herbal medicines at homes [3]. Verification of the efficacy of folk medicine has led to the isolation of hitherto excellent antimalarial, quinine from *Cinchona* spp and Artemisinin (and their derivatives) from *Artemisia annua* [4,5,6]. In the absence of vaccines and due to the widespread resistance to many antimalarial in current use, new chemotherapies are urgently needed to help in the treatment, prevention and control of malaria. The most promising strategy is to strive to discover new chemically diverse entities directed towards novel biological targets.

Cochlospermum tinctorium A. Rich (*Cochlospermaceae*) is a widely used medicinal plant in many West African countries [7] in Nigeria, Senegal, Mali, Southern Sudan, Uganda, Togo, Burkina Faso among others. It is a subshrub, grows up to 80 cm tall with woody subterranean rootstock producing annual shoots. Leaves are alternate and palmate. The leaves and shoots are possibly toxic because it is said that cattle will not graze the plant even in times of food shortage [8].

C. tinctorium also known as Rawaya in Northern Nigeria is used for the treatment of acute malaria attack. The objective of this study was to evaluate the antiplasmodial activity of *C. tinctorium* root bark extract to validate or dispute the claim of its use in traditional medicine as an antimalarial.

2. MATERIALS AND METHODS

2.1 Collection and Identification of Plant Material

Cochlospermum tinctorium roots were collected from Basawa, Sabon Gari Local Government area of Kaduna State, Nigeria in October 2008. The plant was identified with voucher number 1391 in the herbarium unit of the Department of Biological Sciences, Ahmadu Bello University Zaria, Nigeria.

2.1.1 Processing of plant material

The roots of *C. tinctorium* were debarked, washed and dried at room temperature and then pulverised using wooden mortar and pestle. 1kg of the homogenate was extracted in methanol. The methanol soluble portion was concentrated under pressure in a rotary evaporator and stored in a desiccator.

2.1.2 Column chromatography

20g of silica gel was soaked in 150ml methanol and packed in a column (19 x 3.6 cm). 8 g of the crude methanolic extract was loaded on silica gel and sequentially eluted with petroleum ether/methanol (1:1) and ethanol/methanol (1:1). The recovered extracts from the column was evaporated to dryness on a water bath at 35ºC and weighed. The recovered petroleum ether fraction was regarded as fraction 1 while that of the ethanolic fraction was fraction 2.

2.2 Animals

Fifty Swiss albino mice of both sexes weighing between 18 and 25g were used in the study. The animals were obtained from the animal house and left for a week to acclimatize in the laboratory. The animal house used during the study was well ventilated and maintained at room temperature. The animals were feed with standard livestock feed (Vital Feed® grower mash) and sufficient water was provided throughout the period of study.

2.3 Parasites

Plasmodium berghei berghei strain NK 65 (chloroquine sensitive) was obtained from the National Institute of Medical research (NIMR) Yaba, Lagos, Nigeria. A standard inoculum of $1x10^7$ parasitised erythrocytes in volumes of 0.2ml was used to infect a donor mouse intraperitoneally.

2.4 *In vivo* Evaluation of the Antimalarial Properties of Recovered Fractions of *C. tinctorium* Root Bark Extract against *Plasmodium berghei berghei* in Mice

2.4.1 Inoculation of mice with *plasmodium berghei berghei*

Fifty (50) Swiss albino mice of both sexes weighing between 18-25g in ten batches of five mice per group fed on a standard diet were used for the study. A donor mouse infected with *Plasmodium berghei berghei* (parasitaemia of about 20-30%) was anaesthetised with chloroform and blood collected by cardiac puncture with a sterile disposable needle and syringe. The blood was diluted with normal saline in such a way that 0.2ml of blood contained approximately 1×10^7 infected red blood cells. Each mouse received 0.2ml of blood intra-peritoneally [9].

2.4.2 Treatment of *plasmodium*-infected mice (curative test or rane test)

The procedure of Ryley and Peters [10] was used. In this case, the challenged mice were left for 72 hours after root infection intraperitoneally with standard inoculums of 1×10^7 *P. berghei berghei* before commencement of the treatment. Twenty five mice divided into five groups of five mice each in two batches were used for the study. In the first batch, group 1 was administered 0.2 ml normal saline as the sham, group 2 was administered 5mg/kg chloroquine as the positive control, groups 3 to 5 were treated respectively with 50,100and 200mg/kg of fraction 1 respectively. In the second batch, group 1 was administered 0.2ml normal saline as the negative control, group 2 was administered 5mg/kg chloroquine as the positive control, groups 3 to 5 were treated with 25, 50 and 100 mg/kg of fraction 2respectively. The reduced dosage of fraction 2 was due to the small quantity recovered at the end of the column extraction. The administration was done daily for four consecutive days. On day five, two drops of blood was taken from each mouse via caudal vein and transferred on to a clean slide, a thin film was prepared and stained with geimsa stain after which the number of parasites was determined microscopically by observing the slides using an oil immersion microscope at a magnification of x1000 for the presence of *Plasmodium berghei berghei*-infected red blood cells (RBCs). 10 fields were viewed and the number of infected RBCs in the 10 fields were added up and divided by 10 to give the average number of parasites in a field (average parasitemia).

Percentage average antiplasmodial activity was evaluated using the methods of [11] as follows:

$$\% \text{ Average antiplasmodial activity} = \frac{A-B}{A} \times 100$$

Where A = Average parasitemia in the negative control and B = Average parasitemia in the test group.

After day six, the animals were fed *ad-libitum* and observed for 28 days during which mortality was noted and used to determine the median survival time post infection.

2.5 Packed Cell Volume (PCV)

Part of the blood sample taken for each treatment on the fifth day were put into heparinised capillary tubes, one end of each tube containing the blood sample was sealed by heating

with flame from a Bunsen burner and was placed in a microheamatocrit centrifuge set at 15,000 revolutions per minute (RPM) for 5 minutes. The percentage PCV was read using a microhaematocrit reader [12].

2.6 Data Analysis

One-way ANOVA with Dunnetts and Bonferroni's post test was performed to test the significance of difference between the treatment groups, T-test was used to compare between treatment with fractions 1 and 2, survival analysis was performed to determine whether a treatment changed survival and survival curves were compared using both Log rank and Gehan-wilcoxon. Significance was determined at $p<0.05$ using Graph pad prism version 5.02 for windows from Graph pad software Inc. (www.graphpad.com).

3. RESULTS AND DISCUSSION

3.1 Yield of Plant Material from Extraction

The total yield was 47.97g crude methanolic extract, while the recovered extract after column chromatography was 2.491g and 0.559g for the fraction 1 and 2 respectively.

3.2 Antiplasmodial Activity of *C. tinctorium* Root Bark Extract

3.2.1 Antiplasmodial activity of fraction 1

The antiplasmodial activity of fraction 1 from *C. tinctorium* root bark extract had 80.67%, 64.14% and 69.71% antiplasmodial activities for the 50, 100 and 200mg/kg/day doses respectively Fig. 1. However, the antiplasmodial activity of fraction 1 was not dose dependent as the highest suppression of parasites was observed in the lowest dosage of 50mg/kg Fig. 1. Chloroquine at the standard treatment dose of 5mg/kg/day exhibited 100% cure of the infected mice with marked polychromatic cells Plate 1. Bonferroni's multiple comparison tests showed the difference of sham versus Chloroquine and fraction 1 doses; 5mg/kg Chloroquine versus fraction 1 doses and 50 mg/kg versus 100mg/kg fraction 1 were significantly different ($p<0.05$), while the difference between 100mg/kg treatment versus 200mg/kg treatment was not statistically different (at $p>0.05$). This implies that none of the doses of fraction 1 could cure *P. berghei berghei* infection in mice although they all had antiplasmodial activity.

3.2.2 Antiplasmodial activity of fraction 2 from *C. tinctorium* Root Bark Extract

Fraction 2 doses showed a significant ($P<0.05$) antiplasmodial activity of 62.42%, 65.70% and 98.17% for 25, 50 and 100mg/kg respectively. The antiplasmodial activity was observed to be dose dependent in a direct proportional relationship Fig. 2. Chloroquine at 5mg/kg per day exhibited 100% cure of *P. berghei berghei*-infected mice but with marked polychromatic cells Plate 2. Bonferroni's multiple comparism test revealed the difference between the sham versus chloroquine and fraction 2 doses; 5mg/kg Chloroquine versus 25 and 50mg/kg treatment; 25mg/kg versus 50mg/kg and 50mg/kg versus 100mg/kg treatment were significantly different ($P<0.05$), while the difference between 5mg/kg Chloroquine versus 100mg/kg treatment and 25mg/kg versus 50mg/kg fraction 2 treatments were not significantly different ($P>0.05$). This implies that only the 100 mg/kg dose of fraction 2 had the capacity to cure *P. berghei berghei*-infected mice as did 5 mg/kg chloroquine.

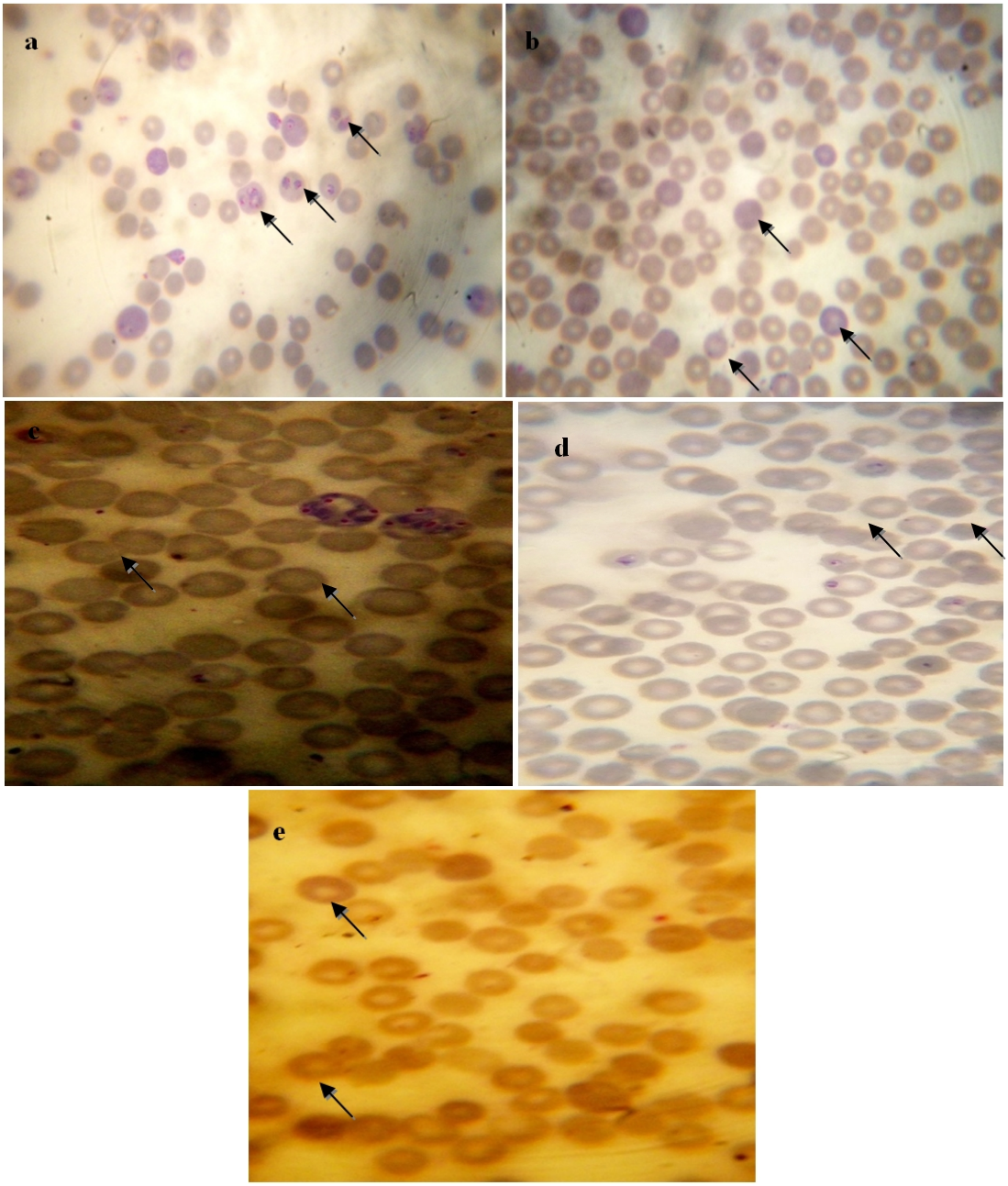

Plate 1. Photomicrographs of blood smears of *P. berghei berghei*-infected mice treated with fraction 1: (a) sham with heavily parasitised RBCs, (b) 5 mg/kg chloroquine RBCs cleared of infection but with marked polychromatic cells, (c-e) 50, 100 and 200 mg/kg fraction 1 respectively with RBCs showing parasites

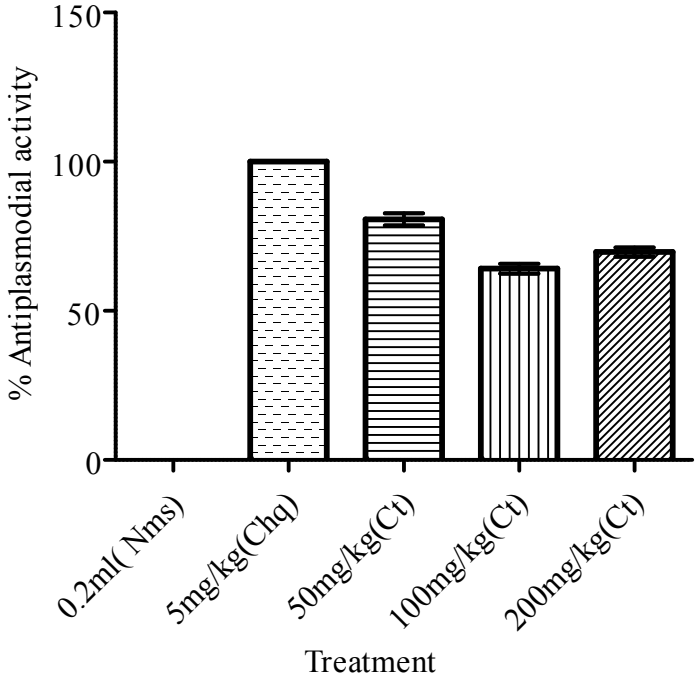

Fig. 1. Percentage antiplasmodial activity for mice treated fraction 1, with chloroquine (Chq) as positive control and Normal saline (Nms) as sham

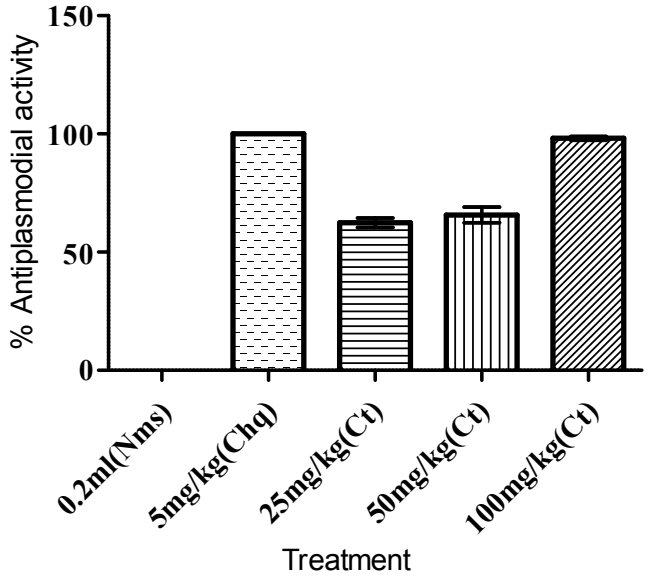

Fig. 2. Percentage antiplasmodial activity for *P. berghei berghei*-infected mice treated with doses of fraction2, with chloroquine (Chq) as positive control and Normal saline (Nms) as sham

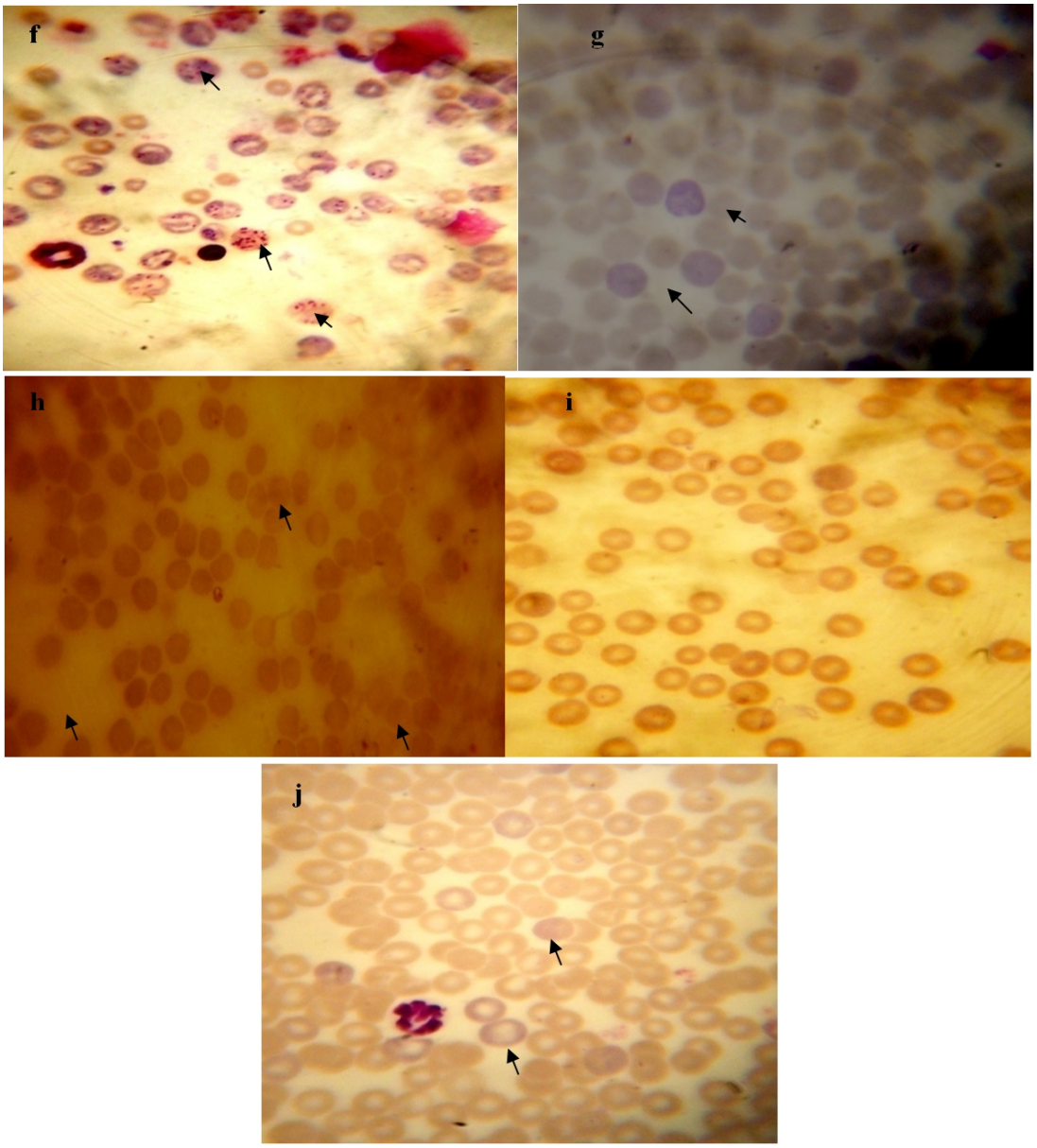

Plate 2. Photomicrographs of blood smears of *P. berghei berghei*-infected mice treated with fraction 2: (f) sham with heavily parasitised RBCs, (g) 5 mg/kg chloroquine RBCs cleared of infection but with marked polychromasia, (h-j) 50, 100 and 200 mg/kg fraction 2 respectively with RBCs cleared of *Plasmodium* parasites with polychromatic cells

3.3 Comparison of the Average Antiplasmodial Activities of Fraction 1 and Fraction 2 Treatments

The mean percentage antiplasmodial activities of the three treatments each of fractions 1 and 2 showed that fraction 2 had a slightly better antiplasmodial activity (65.26%) than that

of fraction 1 (62.90%) although the dosages of fraction 2 were half those of fraction 1.This difference was however not significant (t=0.3300, p=0.6085) See Fig. 3.

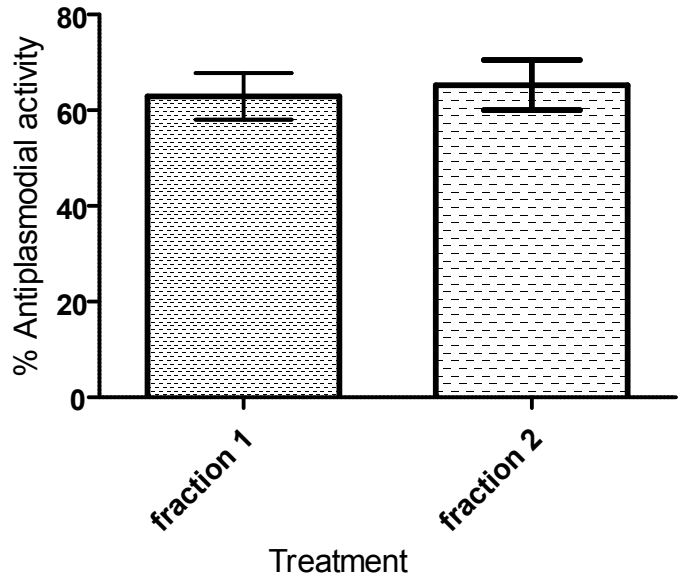

Fig. 3. Comparison of mean percentage antiplasmodial activity for *P. berghei berghei* infected mice treated with fraction 1 and fraction 2

3.4 Percentage Packed Cell Volume (PCV) for Mice Treated with Fraction 1 and Fraction 2

3.4.1 PCV of mice treated with fraction 1

The mean PCV of the sham treated mice was 29.50% and 43.40% for 5mg/kg chloroquine treated mice. For 50, 100 and 200mg/kg treatments of fraction 1, the mean PCV were 36.83%, 32.42% and 37.96% respectively Fig. 4. The PCV for 50 and 100 mg/kg treatment were higher than at treatment with 200mg/kg, PCV values were not dose dependent. However, all the treatment had a higher PCV value than the negative control (29.50%). This suggests fraction 1 hadanaemia ameliorating property.

3.4.2 Percentage PCV for mice treated with fraction 2

The mean percentage PCV for the sham treated mice was 29.50%, Chloroquine at 5mg/kg had a mean PCV of 43.40%, while 25, 50 and 100 mg/kg of fraction 2 produced a mean PCVs of 40.22, 27.50 and 43.04% respectively Fig 5. The PCV value for 25 and 100mg/kg treatments were higher than at treatments of 50mg/kg which fell below the sham treated mice. The PCV for the 25 and 100mg/kg treatments were the same with that of the chloroquine treated mice ($p<0.05$). The mean PCV values were also not dose dependent. This suggests that fraction 2also hadanaemia ameliorating property that was higher than that of fraction 1.

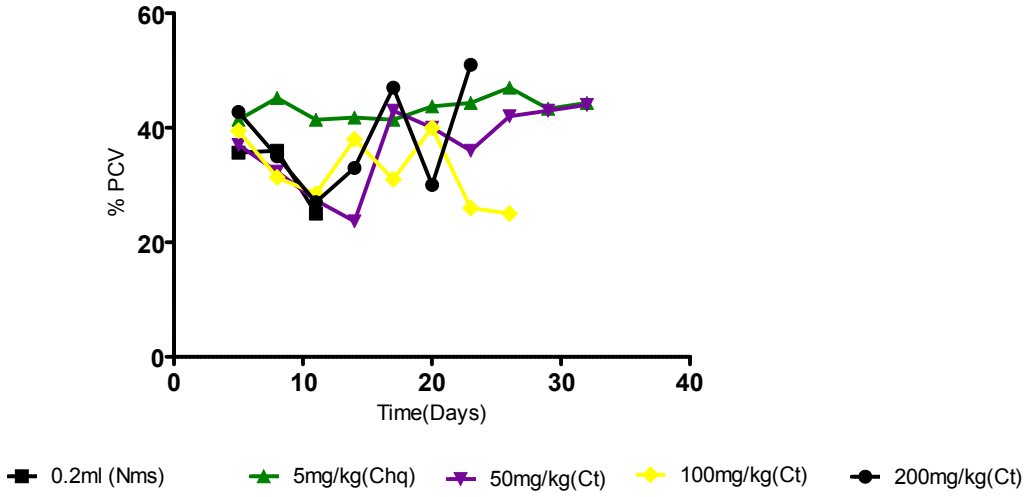

Fig. 4. PCV profile for mice treated with dosages of fraction 1

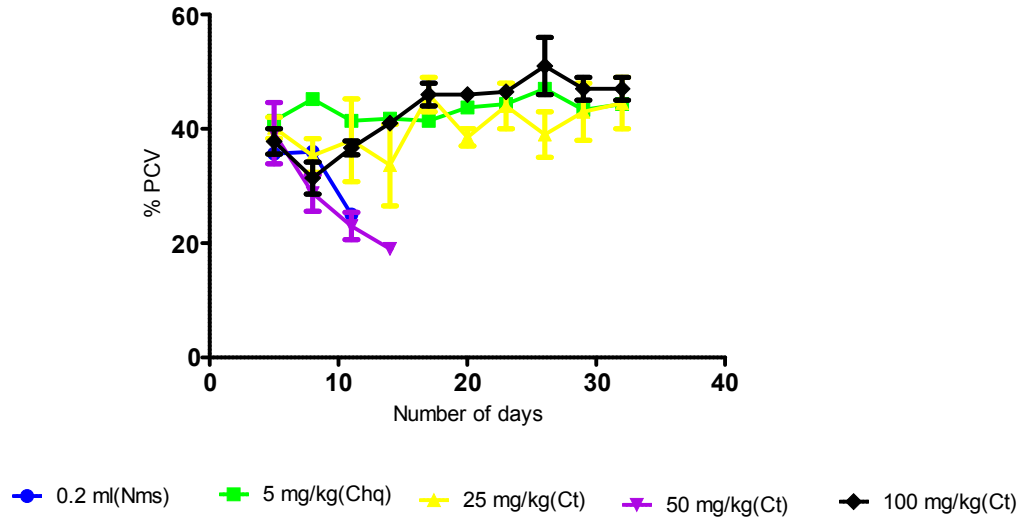

Fig. 5. Packed Cell volume (PCV) profile for mice treated with fraction 2

3.5 Survival Time of Mice Treated with *C. tinctorium* Root Bark Extract

3.5.1 Survival rates of mice treated with fraction 1 of *C. tinctorium* root bark extract

Survival curve plots of percent death against time (number of days post treatment), Graph pad prism calculates the survival time using product limit (Kaplan- Meier method), because the graph begins at zero its show the fraction still alive. Mice treated with shamhad a survival time of 11 days at 100% mortality. Mice treated with 5 mg/kg chloroquine had a survival time of 36 days at 40% death, mice treated with 50 mg/kg at 100% death had a survival time of 19 days, mice treated with 100mg/kg at 100% death, had a survival time of 30 days and mice treated with 200mg/kg at 80% death had survival

time of 27 days Fig. 6. This shows that there was a direct relationship between dose of fraction 1 and survival time. The log-rank (Mantle-Cox) test value at p=0.0064 showed that the survival curves are significantly different. However, the log-rank test for trend with P value =0.8028 showed that there was no linear trend between the column order and the median survival. Median survival was the time at which the survival rate equals 50%, the median survival for 0.2ml normal saline treatment was 11days, for mice treated with 5mg/kg chloroquine the survival exceeds 50% at the longest time point, therefore median survival could not be computed. Mice treated with 50, 100 and 200mg/kg of fraction 1 had median survival of 15.5, 15.5 and 19 days respectively. Multiple comparison tests were used to compare two treatment groups at a time. At a significance level of 0.05 and K=10 which is the number of comparisons made, the Bonferroni corrected threshold was 0.005. There was only one significant difference observed when the treatment with 5mg/kg chloroquine was compared with 50 mg/kg treatment of fraction 1 with a p value of 0.0040, which is less than the Bonferroni corrected threshold (0.005). However, for all other comparisons there was no significant difference.

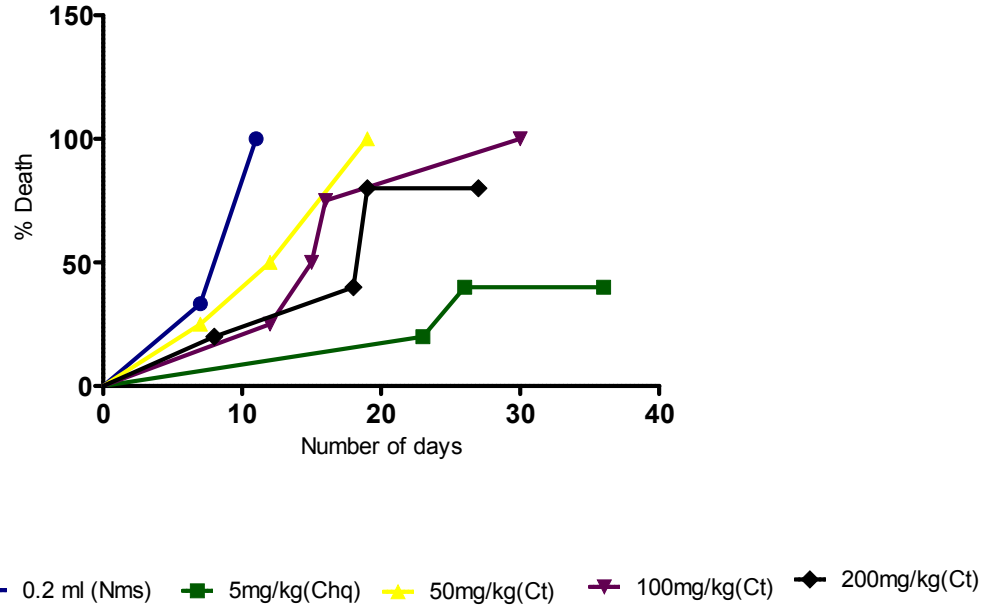

Fig. 6. Survival proportions of mice treated with fraction 1 from *C. tinctorium* root bark Extract

3.5.2 Survival time of mice treated with fraction 2 of *C. tinctorium* root bark extract

Mice treated with sham at 100% mortality had a survival time of 11days, mice treated with 5 mg/kg chloroquine at 40% mortality had a survival time was 36 days, mice treated with 25 mg/kg at 60% death, had a survival time of 19 days, mice treated with 50 mg/kg at 100% death, had a survival time of 18.5 days, mice treated with 100mg/kg at 100% death, had a survival time of 18 days Fig. 7. The log-rank (Mantle-Cox) test value at p=0.0009, shows survival curves were significantly different. However, looking at the log-rank test for trend with p value 0.7419 showed that there was no linear trend between the column order and the median survival. The median survival for the sham treated mice was 11 days, for mice treated with 5mg/kg chloroquine the survival exceeded 50% at the longest time point.

Therefore, the median survival could not be computed, for mice treated with 25, 50 and 100mg/kg fraction 2, the median survival were 19, 18.5 and 18 days respectively. At a level of significance of 0.05 and K=10 which is the number of comparisons made, the Bonferroni corrected threshold was 0.005. On comparison of all treatment groups using the Log-rank (Mantel-cox) test, there was only one significant difference on comparison of 5mg/kg chloroquine with the treatment with 50mg/kg dose of fraction 2. The p value was 0.0027 which was lower than the Bonferroni corrected threshold at 0.005.

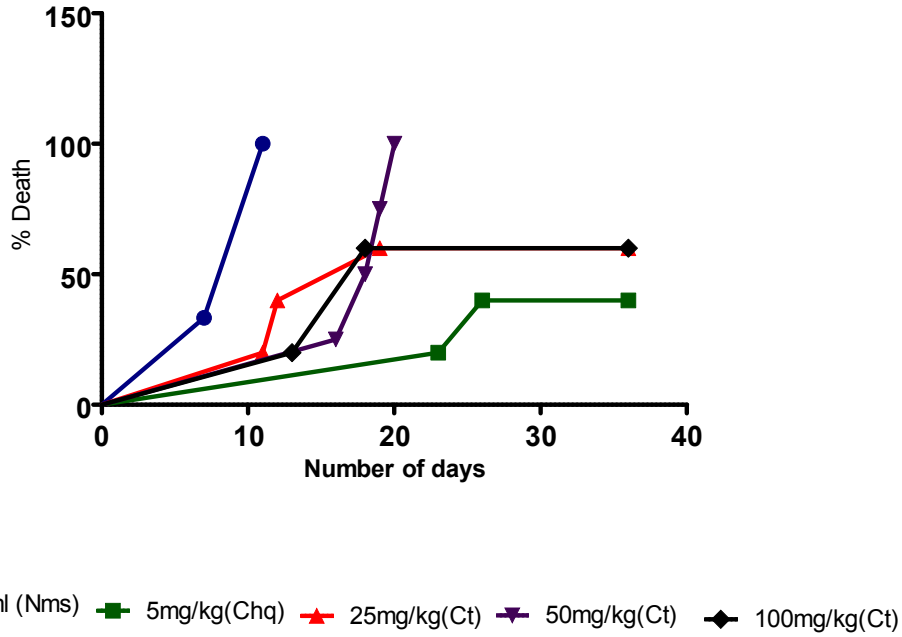

Fig. 7. Survival proportions of mice treated with fraction 2 from *C. tinctorium* root bark extract

4. CONCLUSION

Phytoconstituents can vary based on several factors such as climate, habitat, soil, nutrients, time of harvest, stress and physiological age of the plants [13]. Selection of an appropriate solvent for extraction when the chemical nature of the potential phytoconstituents is unknown can be a tedious task. It was observed in this study that the type of solvent used for extraction played a major role in its antimalarial activity of *C. tinctorium* root bark extract. Furthermore, antagonistic substances present can result to failure in detecting individually active compounds and result in obtaining essentially negative result. Many plants are known to accumulate large quantities of inorganic constituents notable among which are selenium, nitrates, copper etc. The predominant action of any of these in a plant extract (containing organic compounds with potential biological activity) could result in activities being undetected. The climate, habitat and time of collection of plant material were taken into consideration during the collection of root samples of *C. tinctorium*. The two fractions used in this study were obtained using different extraction solvents. Although fraction 2 had greater antiplasmodial property than fraction 1, the yield of fraction 2 was however too low for both fractions to be tested using the same dose. The lowest dosage of 50 mg/kg of fraction 1

produced a better antiplasmodial effect than the higher doses implies that there may be inhibitory substances in this fraction which prevent better antiplasmodial activity with an increase in dosage. It is therefore suggested that lower doses of fraction 1 could have better antiplasmodial activity in the treatment of malaria. The survival time for mice treated with fraction 1 showed as the dosage was increased from 50 mg/kg to 200mg/kg the median survival time of mice increased from 15.5 days to 19 days, there was however no difference in the median survival time when the dosage was increased from 50mg/kg to 100mg/kg. However, the lowest dosage of 50mg/kg/day had the highest percentage cure of 80.67% and a median survival time of 15.5 days while the highest dosage of 200mg/kg/day had the longest median survival time of 19 days. The short median survival time observed in the lowest dosage even though it had the highest percentage cure of 80.67% may be as a result of stress due to handling of animals or that inhibitory substances that affected antiplasmodial activity of the plant were present in the extract.

The 100 mg/kg dose of fraction 2 had similar antiplasmodial activity to 5 mg/kg chloroquine only that it did not cure the infection and the survival time of the infected mice was significantly lower than the chloroquine treated mice. The highest dosage of 100mg/kg/day had the highest percentage antiplasmodial activity of 98.17% as well as the shortest median survival time of 18 days. This may have been as a result increased toxicity of the fraction as the dosage used increased.

ANOVA revealed that there was significant difference among all test doses as well as the control groups at ($p<0.05$). On comparison of fraction 2 with the test drug chloroquine using Bonferronipost test, there was no significant difference between the chloroquine and the 50mg/kg treatment, which implies that the 100 mg/Kg of fraction 2 was as effective as the control group. The antiplasmodial activity of this plant is in agreement with previous finding where an ethanolic extract of the roots of *C. tinctorium* showed antiplasmodial activity; however this study was carried out *In-vitro* using chloroquine-sensitive (3D7) and -resistant (Dd2) *Plasmodium falciparum* strain [14].

The low mean PCV value in the mice treated with 50mg/kg of fraction 2 root bark may be as a result of the fact that the mice may have suffered from stress due to handling as it was also observed the group survived for a short period of time, this is in agreement with the findings of [15], who stated that in experiments involving test animals suffering from a severe disease the amount of disturbance by handling has to be taken into account, if standardization is to be achieved.

The result of the present work therefore reveals that fractions from *C. tinctorium*root bark possess antimalarial and anaemia ameliorating properties. This study therefore validates the use of this plant in traditional medicine.

CONSENT

Not applicable.

ETHICAL APPROVAL

All authors hereby declare that "principles of laboratory animal care" (nih publication no. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

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COMPETING INTERESTS

The authors declare that there is no competing interest on the manuscript.

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