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An investigation into the antimalarial effect of methanolic extract of *Paullinia pinnata* leaves in *plasmodium berghei* infected mice and course of infection

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Abstract

Aim: The aim of this study was to investigate the antimalarial activity of methanolic leaves extract of *Paullinia pinnata* on chloroquine- sensitive *Plasmodium berghei* NK 65 infected mice.

Methodology: The curative study was conducted in thirty- six Wistar albino mice of both sexes which were divided into six groups of six animals each. The animals were infected with *P.berghei* NK 65. Group I was the negative control and received the vehicle (10% DMSO). Group II received no treatment. Groups III and IV were the positive controls and received chloroquine (CQ) (10mg/kg) and artesunate (4 mg/kg)-amodiaquine (10mg/kg) combination (ACT) respectively. Groups V and VI received 100mg/kg and 200mg/kg doses of the extract respectively. Administration was done orally once for three or four days for the standard drugs or the extract/ vehicle respectively. The percentage parasitaemia, packed cell volume (PCV), body weight and death was monitored on days 0, 1, 2, 3, 4 and 11 (7 day post administration). The study of the course of infection of *P.berghei* was monitored in eighteen Wistar albino mice of both sexes which were similarly grouped, infected and treated for 3 days. Group A received the vehicle (distilled water) only. Group B was treated with CQ (10 mg/kg) and Group C with ACT. The percentage parasitaemia and death was monitored from day 0 to day 30 (27 day post administration).

Results: In the curative study, the extract suppressed parasitaemia at both doses on day 4. The group treated with 200mg/kg dose showed a higher percentage chemosuppression though not significant. The course of infection study revealed that recrudescence occurred on day 8 in the CQ treated group which lasted until day 23 after which the recrudescence was lost without re- treatment. A similar result was observed in the ACT group.

Conclusion: The methanolic leaves extract of *Paullinia pinnata* has weak anti-malarial property. Chloroquine -sensitive *P.berghei* NK65 loses credibility and needs to be revalidated biannually.

Key words: *Paullinia pinnata, artemisinin combination therapy, chloroquine- sensitive Plasmodium berghei NK 65, recrudescence, percentage chemosuppression, packed cell volume*

Résumé

But: Le but de cette étude était d'investiguer l'activité antipaludique des extraits méthanoïques de feuilles de Paullinia pinnata sur des souris infectées par le Plasmodium berghei NK 65 et sensibles à la chloroquine.

Méthodologie: L'étude curative a été menée parmi trente-six souris Wistar albinos des deux sexes qui ont été divisées en six groupes de six animaux chaque. Les animaux ont été infectés par P. berghei NK 65. Groupe I étais le contrôle négatif et reçu le véhicule (10% de DMSO). Groupe II n'a reçu aucun traitement. Groupes III et IV ont été les contrôles positifs et ont reçu de la chloroquine (CQ) (10 mg / kg) et la combinaison (ACT) artésunate (4 mg / kg) – amodia-quine (10 mg / kg) respectivement. Groupes V et VI ont reçu des doses de 100 mg / kg et 200 mg / kg de l'extrait respectivement. L'administration a été fait oralement une fois pour trois ou quatre jours pour les médicaments standard ou l'extrait / véhicule respectivement. Le pourcentage de parasitémie, le volume d'hématocrite (PCV), le poids corporel et la mort a été suivie aux jours 0, 1, 2, 3, 4 et 11 (7 jours après administration). L'étude du cours d'infection de P. berghei a été surveillée dans dix-huit souris Wistar albinos des deux sexes qui ont été regroupées de façon similaire, infectés et traités pendant 3 jours. Le groupe A a reçu le véhicule (eau distillée) seulement. Le groupe B a été traité avec le CQ (10 mg / kg) et le groupe C avec ACT. Le pourcentage de parasitémie et de la mort a été suivi du jour 0 au jour 30 (27 jours post-administration).

Résultats: Dans l'étude curative, l'extrait a supprimé la parasitémie dans les deux doses le

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jour 4. Le groupe traité avec la dose de 200 mg / kg a montré un pourcentage plus élevé de chimio-suppression mais non significative. L'étude sur le cours d'infection a révélé que la recrudescence est survenue le jour 8 dans le groupe traité avec CQ qui a duré jusqu'au jour 23, après quoi la recrudescence a été perdue sans retraitement. Un résultat similaire a été observé dans le groupe d'ACT.

Conclusion: L'extrait méthanoïque de feuilles de Paullinia pinnata a une faible propriété anti-paludéenne. Chloroquine -sensible P.berghei NK65 perd de sa crédibilité et doit être revalidé de manière parsemestre.

Mots clés: *Paullinia pinnata, thérapie combinée à base d'artémisinine, Plasmodium berghei NK 65chloroquine-sensible, recrudescence, pourcentage chimio-suppressif, volume hématocrite.*

Introduction

Malaria affects 3.3 billion people, or half of the world's population, in106 countries and Territories. The World Health Organisation (WHO) estimates that 216 million cases of malaria occurred in 2010, 81% of which are in Africa [1].Malaria is a major public health problem in Nigeria where it accounts for more cases and deaths than any other country in the world. There are an estimated 100 million malaria cases with over 300,000 deaths per year in Nigeria. This compares with 215,000 deaths per year in Nigeria from HIV/AIDS [1].Chloroquine is one of the drugs used for the treatment of malaria but in recent times the malaria parasite has developed resistance to this drug. Hence the current use of Artemisinin combination therapy (ACT) for the treatment of malaria. The high cost of ACTs makes them unaffordable to the teeming masses. They also have undesirable side effects prompting many people to use herbs [2]. There is therefore a need to ascertain the antimalarial activity of these herbs.

Paullinia pinnata(Linn.) (PP) is a subwoody climber found in West Africa and in various parts of tropical Africa except the driest regions. The leaves are taken along with other herbs for the treatment of several diseases including malaria [3, 4]. The leaves have been shown to contain flavonoids, saponins, alkaloids, cardiac glycosides and tannins [5-7].

Investigations carried out on the therapeutic effects of the leaves of PP reported anti- inflammatory and analgesic activities[6], antidiarrhoeal property [8],antioxidant activity *in- vitro* [9, 10] and anti- malarial activity *in- vivo* [11]. Previous studies have shown that a safe dose for administration of the methanol extract of the leaves is 200mg/kg body weight [7].

The aim of this study was to investigate the anti- malarial activities of the methanol extract of the leaves of PP by employing the curative test using chloroquine-sensitive *Plasmodium berghei* in wistar albino mice. This would serve as a preliminary study to further investigations.

Materials and methods

The administered doses of the methanol extract of the leaves of *Paullinia pinnata* in this study was based on a previous report [7].

Animal experiment ethical review:

The methods for the preparation of the animals, group size and mode of administration were in compliance with International scientific standard requirements :-Chandel and Bagai [12].

Reagents and Drugs:

Giemsa's stain (Sure Chem Products, England), Methanol (Analar, England), Dimethyl sulfoxide (DMSO) (Analar, England), Immersion oil (with refractive index 1.5, PanScanXtra, U.K.), amodiaquine (Sigma, St.Louis MO U.S.A.), chloroquine (Sigma, St.Louis MO U.S.A.) and artesunate (Swiss pharma Ltd, Lagos, Nigeria)

Plant Material:

The leaves of PP were collected from the Forestry Research Institute of Nigeria (FRIN), Ibadan, Nigeria. The plant was authenticated at the same Institute and given the specimen voucher number FHI 106555.

Extraction and preparation of plant materials:

The leaves were air-dried, milled and extracted by cold maceration in absolute methanol initially for a period of 6 days. The solvent was filtered and the marc was re- soaked in absolute methanol for 24hours. This was repeated 3 times and the recovered solvent was pooled and concentrated using a rotary evaporator (Heidolph HB, Germany) and a vacuum oven (Gallenhamp, England) at a temperature of 40- 42°C.

Preparation of animals for curative and course of infection studies:

Thirty- six Wistar albino mice of both sexes weighing between 20- 30g were used for the

curative study and eighteen Wistar albino mice of both sexes weighing between 20- 30g were used for the course of infection study. They were obtained from the Animal house of the Institute for Advanced Medical Research and Training (IAMRAT), College of Medicine, University of Ibadan, Ibadan, Nigeria and kept there. Feed (Capfeed, Oyo, Nigeria) and laboratory water were given *ad libitum* before and throughout the period of the experiment with a 12 hour light/ dark cycle.

Curative study:
A modified Rane's method as reported by Ryley and Peters was used [13]. The animals were infected with *Plasmodium berghei* NK 65 and then divided into six groups of six mice each. Parasitaemia level was determined at 72 hours after infection in blood films which were fixed in methanol, stained with 10% Giemsa's stain and observed under the binocular microscope (Olympus, Japan). The day parasitaemia was established was taken as Day 0. Group I was the negative control and received the vehicle (10% DMSO) only. Group II received no treatment. Group III received chloroquine (CQ) (10mg/kg) only. Group IV received artesunate (4 mg/kg) - amodiaquine (10mg/kg) combination (ACT). Groups III and IV were the positive control groups. Groups V and VI received 100mg/kg and 200mg/kg doses of the methanol extract of PP respectively. Administration was done orally once for three or four days for the standard drugs or the extract/ vehicle respectively. The body weight, percentage parasitaemia (% parasitaemia), Packed Cell Volume (PCV) and death were monitored daily throughout the period of administration and on 7th day post-administration (Day 11). The percentage survival (% survival) for each of the groups and percentage chemo- suppression for each treatment were calculated.

Determination of Packed Cell Volume:
Blood was collected from the tail vein into heparinised capillary tube and centrifuged using a microheamatocrit centrifuge (Hawksley, England) for 6mins. Using a ruler, the level of packed cell was measured, divided by the level of the whole blood and the result was multiplied by 100. The readings were taken on day 0, 1, 2, 3, 4 and 11.

Determination of Percentage Parasitaemia:
Blood smears were made from the tail vein of each mouse, fixed with methanol, stained with 10% Giemsa's stain and examined under the binocular microscope using the x100 objective lens under a drop of immersion oil in order to assess the activity of the drug/extract on the parasite. Percentage parasitaemia was calculated by using the following equation:
(no. of infected red blood cells/ total no. of red blood cells) x 100

Determination of Percentage Chemosuppression:
Percentage Chemosuppression was determined by using the following equation:
(Average parasitaemia in control group - Average parasitaemia in treatment group/ Average parasitaemia in control group) x 100.

Determination of Percentage Survival:
(no. of animals that survived in a day /total no. of animals in the group) x 100

Course of Infection Study:
The animals were infected with *Plasmodium berghei* NK 65 and divided into three groups of six mice each. Percentage parasitaemia after 72 hours was determined and subsequently daily until parasitaemia was established and this was taken as day 0. On day 0, Group A received the vehicle (distilled water) only. Group B was treated with CQ (10 mg/kg) while Group C was treated with ACT. Administration was conducted orally once for three days. The percentage parasitaemia and survival rate was monitored from day 0 to day 30 (27 day post administration).

Parasite:
The chloroquine- sensitive *Plasmodium berghei berghei* NK 65 was obtained from the Institute for Advanced Medical Research and Training (IAMRAT), University of Ibadan, Ibadan, Nigeria. The parasites were kept alive by continuous intra- peritoneal passage in mice every four days and these constituted the donor mice.

Inoculation:
A standard inoculum of 1×10^7 of parasitized erythrocytes from a donor mouse, which was prepared by taking 0.2ml of blood of the donor mouse in normal saline, was used to infect the experimental animals intra-peritoneally.

Statistical Analysis: One- way analysis of variance was carried out. Values with $p < 0.05$ were taken to be significant. The mean and

standard error of mean was determined where necessary and was stated in the results as appropriate.

Results

A gradual loss of weight, which was not statistically significant ($p < 0.05$), was observed in the groups treated with the extract by day 11 while the ACT group showed a gradual increase in weight. The untreated and control groups also presented a non- statistically significant ($p < 0.05$) weight loss on day 11. (Table 1). A reduction in PCV was observed in the control and untreated groups (Figure 1). CQ and the ACT groups showed a rise in the PCV from the 2nd day of administration while the group treated with 100 mg/kg and 200mg/kg body weight doses of the extract did not show a significant increase on day 4.With the exception of the ACT group, all the other groups showed a decrease in the PCV on the 11th day.

A reduction in PCV was observed in the control and untreated groups (Figure 1). CQ and the ACT groups showed a rise in the PCV from the 2nd day of administration while the group treated with 100mg/kg and 200mg/kg body weight doses of the extract did not show a significant increase on day 4. With the exception of the ACT group, all the other groups showed a decrease in the PCV in the 11th day.

Table 1: Weight of Wistar albino mice in the control and treatment groups over the period of study

	WEIGHT (g)					
Day	Control	Untreated	CQ	ACT	100mg/kg	200mg/kg
0	24.05±1.23	26.48±1.28	25.97±0.59	25.55±1.44	23.70±0.42	25.07±0.76
1	24.32±1.01	27.13±1.39	25.17±0.81	26.44±1.52	22.22±0.54	25.32±1.21
2	23.60±2.17	26.65±1.39	24.75±1.17	26.52±1.57	21.25±0.69	24.68±1.25
3	23.12±2.24	26.03±1.56	24.00±1.49	26.82±1.77	20.57±0.91	24.43±1.21
4	22.78±0.85	26.68±0.78	25.08±2.66	27.84±1.85	19.90±1.28	23.80±1.10
11	14.90±3.20	22.50±3.50	24.83±3.59	28.82±1.60	14.90±1.24	16.68±0.46

Note: n =6

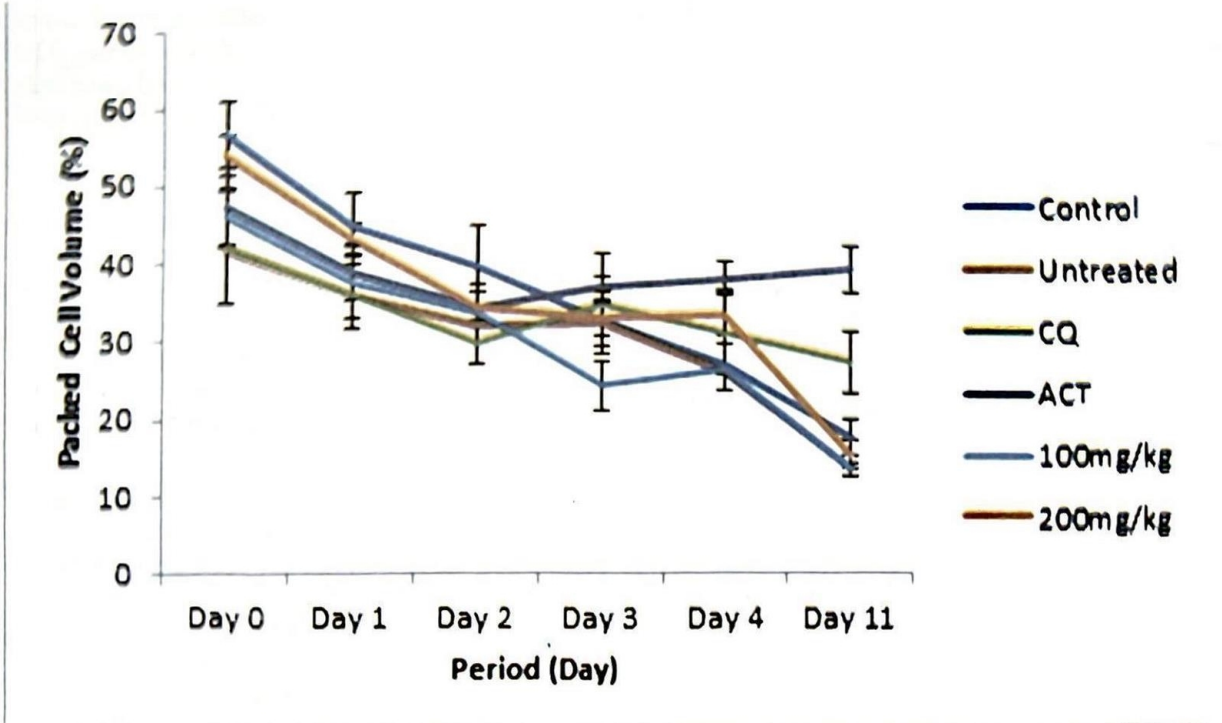

Fig. 1: A plot of the mean values of the Packet Cell Volume in the various groups over the period of study

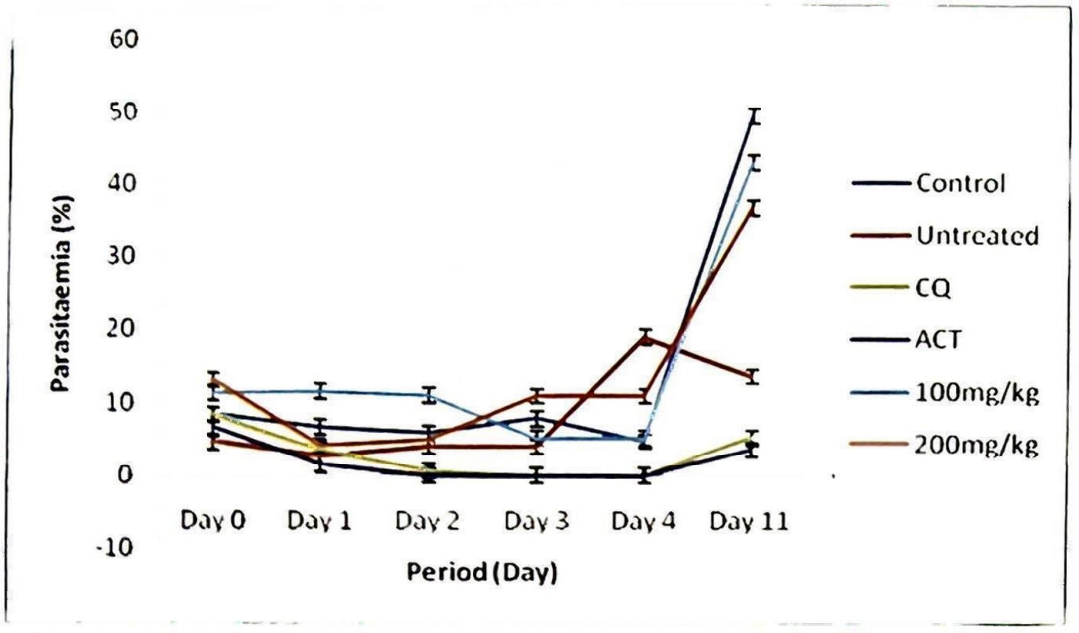

Fig. 2: Percentage parasitaemia for the various treatments and the control

The percentage parasitaemia reduced gradually in the groups treated with the CQ and ACT with a total removal of the parasites by the 4th day of administration. However, recrudescence occurred in groups III and IV by Day 11 and this let to its investigation in the course of infection study. The groups treated with 100mg/kg and 200mg/kg doses of the extract did not show a significant decrease in parasitaemia on day 4 and on day 11 there was an increase in the level of parasitaemia (Figure 2).

Table 2 shows that none of the animals died during the period of administration. However, the survival rate of the animals post administration is in the following order: ACT > 100mg/kg dose of PP > CQ > 200mg/kg dose of PP > control and untreated groups.

In table 3, PP had no chemosuppressive activity at both doses on day 4. By day 11, the percentage chemosuppressive activity were 13% and 26% at the 100 mg/kg and 200 mg/kg doses of the extract respectively.

Table 2: Percentage survival of the animals over the duration of the experiment

Day	Control	Untreated	CQ	ACT	100mg/kg	200mg/kg
1	100%	100%	100%	100%	100%	100%
2	100%	100%	100%	100%	100%	100%
3	100%	100%	100%	100%	100%	100%
4	100%	100%	100%	100%	100%	100%
11	40%	40%	66.67%	100%	80%	66.67%

Note: n = 6

Table 3: Percentage chemosuppression of *P.pinnata* extract compared to the standard drugs

Day	CQ	ACT	100mg/kg	200mg/kg
1	53%	75%	0%	39%
2	90%	99%	0%	18%
3	100%	100%	34%	0%
4	100%	100%	0%	0%
11	90%	93%	13%	26%

Note: n = 6

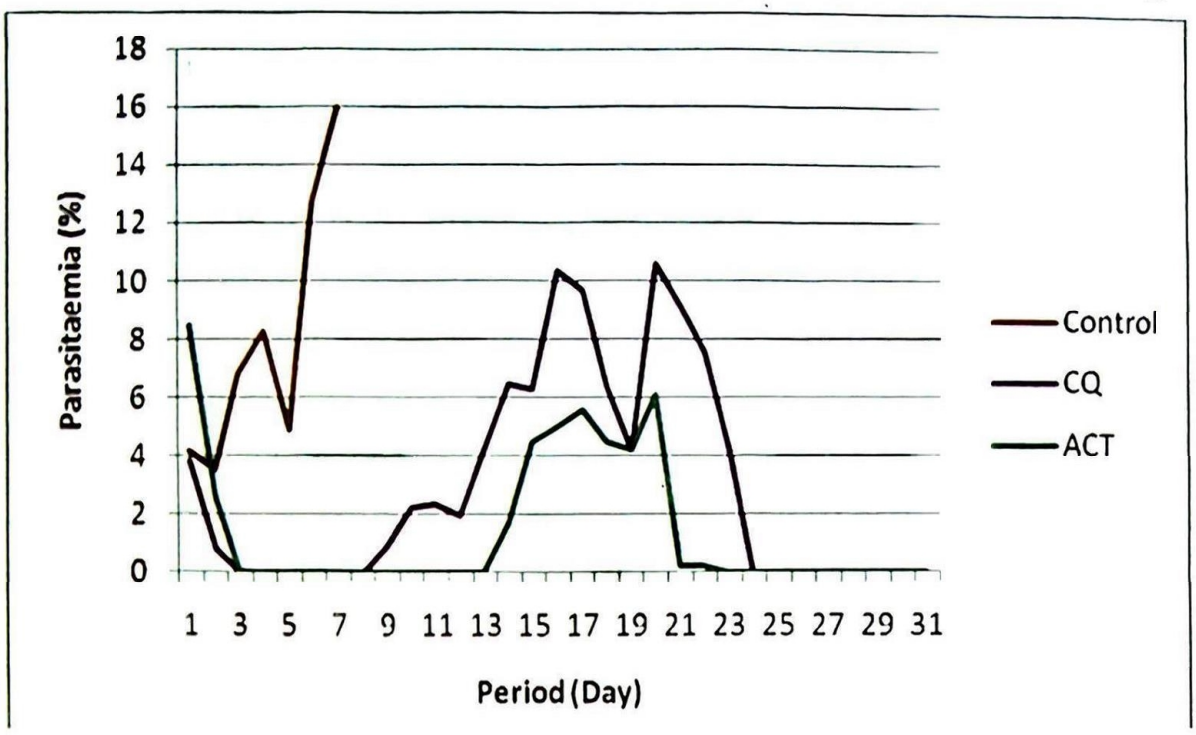

Fig. 3: A plot of the values for percentage parasitaemia in the course of infection study

The course of infection study showed that percentage parasitaemia increased steadily in the control group (Figure 3). The chloroquine treated group showed a decrease in percentage parasitaemia to 0% by day 3 before recrudescence occurred on day 8 which lasted until day 23 after which there was loss of parasitaemia without re- treatment till the end of the study for the animals that survived the recrudescence. The ACT group also showed recrudescence on day 13 up until day 22 followed by a loss of parasitaemia without re- treatment in the animals that survived the recrudescence till the end of the study.

Figure 4 reveals that the survival rate is highest in the ACT group followed by the CQ group . The control group showed the least survival rate

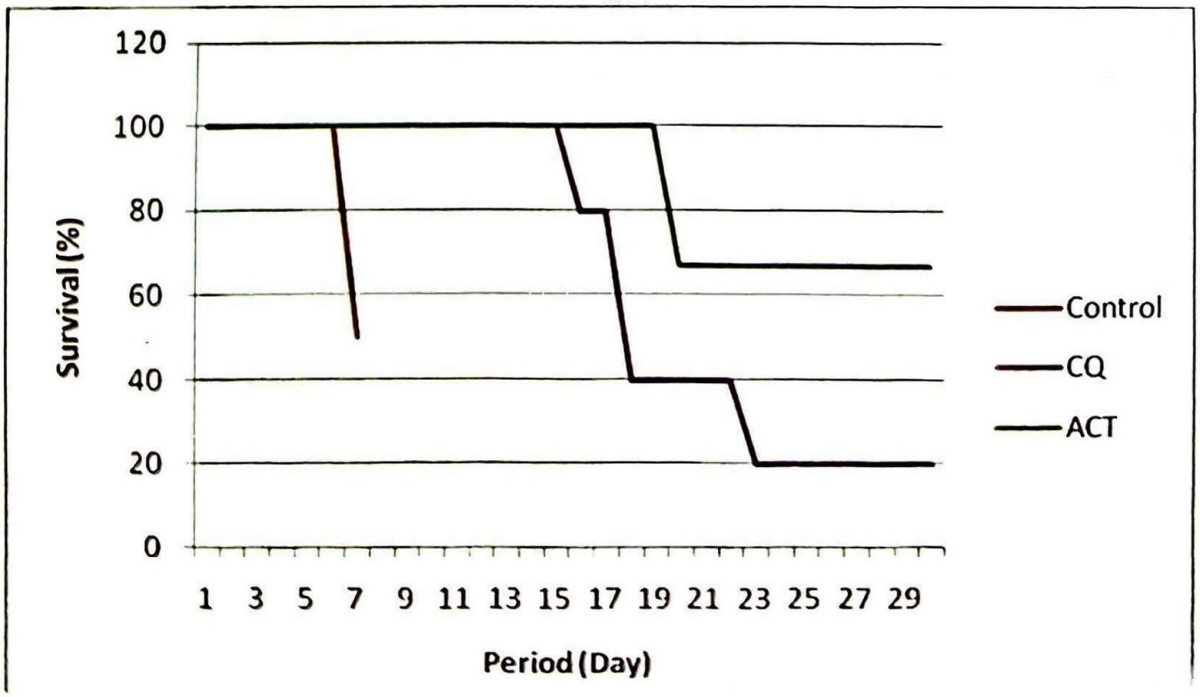

Fig. 4: Survival rate in the course of infection study

Discussion

The results showed that the methanolic leaves extract of *Paullinia pinnata* does not have significant curative capacity on malaria. Also the course of infection study showed that chloroquine-sensitive *Plasmodium berghei* NK65 can lose credibility.

Malaria causes the excessive destruction of red blood cells during the parasites life cycle resulting in anaemia. Anaemia is a hematological disorder caused by a decrease in the production of red blood cells, a bone marrow failure or by an increased destruction of red cells. It is a predominant symptom of malaria in which there is decreased erythropoiesis due to the malarial infection. There is the parasitization of red cells by the malaria parasite which leads to shortened survival or death of erythrocytes [14, 15].As a result of this destruction, PCV and hemoglobin values are reduced when measured in this disease condition. As expected, the PCV for the untreated group decreased throughout the period of study. The groups treated with chloroquine and ACT showed a gradual decrease until Day 2 after which the PCV began to rise steadily but dropped after Day 3 in the chloroquine treated group while the group treated with ACT continued to show a steady rise. The groups treated with 100 mg/kg and 200 mg/kg doses of the extract did not show a significant increase in the PCV on day 4 while there was a decrease by the 11th day. This shows that the extract was not effective at the 100 mg/kg and 200 mg/kg doses to arrest the destruction of the red blood cells by the parasites after the period of administration.

Percentage parasitaemia is a measure of the level of parasites in the blood. Figure 2 shows that the extract at 100 mg/kg and 200 mg/kg doses had mild effects in reducing the percentage parasitaemia by day 4 and by day 11 there was an increase in the level of parasitaemia. This implies that the extract does not have the ability to eliminate the parasites and therefore can not effectively ameliorate the disease condition. By day 4, artesunate- amodiaquine combination and chloroquine removed the parasites from the blood but recrudescence occurred by day 11. This was investigated in the course of infection study. The survival rate of the animals in the control compared with that of the treatment groups showed that the extract is not toxic to the animals at the doses administered and the death observed are likely due to the effect of the parasite.

The percentage chemosuppressive activity of the extract was higher in day 11 at 200mg/kg dose than at the 100mg/kgdose but was not significant. The results generated in this study do not compliment the findings of Majc *et al* [11]. This may be due to the difference in the geographic location of where the plant materials were collected.

Plasmodium berghei is one of the four *Plasmodium* species that have been described in African murine rodents. It is a practical model organism in the laboratory for the experimental study of human malaria[16]. *P.berghei* is of two main types: NK 65 and ANKA.NK 65 is of two strains: chloroquine –resistant strain and chloroquine- sensitive strain. Chloroquine-sensitive NK 65 is sensitive to chloroquine and recrudescence does not occur when treated with chloroquine. The recrudescence which was observed in the chloroquine and ACT treated groups of the curative study was therefore investigated in the course of infection study. Treatment of the chloroquine and the ACT groups showed a total loss of parasitaemia by the third day of administration. However recrudescence occurred at Day7 and Day 13 for the chloroquine and ACT groups respectively. The parasitaemia cleared without re-treatment in the animals that survived the recrudescence with the ACT group showing the greatest survival rate (66.67%). This shows that the parasite has lost its credibility.

Conclusion

In conclusion, the methanolic leaves extract of *Paullinia pinnata* possesses a weak curative effect on malaria. Chloroquine –sensitive *Plasmodium berghei* NK 65 loses its credibility if kept for long in passage and therefore should be revalidated biannually.

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