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RESEARCH ARTICLE

ANTI-PLASMODIAL ACTIVITY AND TOXICITY OF SELECTED CRUDE PLANT EXTRACTS FROM KENYA, AGAINST *PLASMODIUM BERGHEI* IN BALB/C MICE

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ABSTRACT

This study was conducted to evaluate the toxicity *and* activity of *Rubia cordifolia, Harrizonia abyssinica, Leucas Calistochys Olive* and *Sanchus schwein furthii against Plasmodium berghei*. Approximately $1x10^5$-$1x10^6$ of parasitized erythrocytes with *Plasmodium berghei,* were inoculated into 7 week old naïve BALB/c mice. On the fourth day, these mice were treated with four plant extracts dispensed using the following dosage; 25mg/kg of body weight, 50mg/kg of body weight, and 100mg/kg of body weight twice daily for 4 days. The negative control group received equal dosage of normal saline. Results showed that *Rubia cordifolia* and *Sanchus schwein furthii* had higher anti-plasmodial activity against *P. berghei* parasites with values of 82.4 % (p = 0.001) and 78.6% (p = 0.003). All the mice treated with the above extracts survived up to day 15 just as the controls. The percentage parasitaemia reduction in mice treated with extracts of *Harrizonia abyssinica* and *Leucas Calistochys* also showed values of 65.1% (p=0.011) and 59.1% (p=0.04) respectively. LD_{50} doses of <10mg/kg were observed. *Harrizonia abyssinica* and *Leucas Calistochys Olive* had moderate parasite suppressive effects with LD_{50} doses ranging between 10mg/kg and 100mg/kg. The aqueous extracts of *Rubia cordifolia,* and *Sanchus schwein furthii* were more efficacious with highest parasitaemia suppression on *Plasmodium berghei*

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INTRODUCTION

One of the most impressive developments in the history of medicine is the emergence of the Peruvian (Cinchona) bark (Rubiaceae) coupled with its pharmacologically active substance - quinine. Quinine is a classic anti-malarial drug found in the bark of a tree that is native to South America (Coleman *et al.,* 2004). Studies on treatment for uncomplicated *falciparum* malaria were until recently, limited to chloroquine, for treating chloroquine sensitive cases of malaria, and to mefloquine, sulfadoxine/ pyrimethamine (SP) in chloroquine resistant cases (Williams *et al.,* 2004). Chloroquine has been the first line drug used for the treatment of uncomplicated malaria, over the last forty years in Kenya (WHO, 2000). Rampant drug resistance developed against chloroquine leading to policy shift replacing it with sulfadoxine/pyrimethamine. Since then the resistance to malaria has been exponentially increasing in Kenya as well its neighboring countries (Mengesha and Makonnen, 1998). However, the introduction of ACT treatment brought relieve that did not last long as resistance to Artemisinin based drugs has been reported in South East Asia. This serious situation required rapid and radical search for new anti-malarial. One of the areas in search for new anti-malarial is the traditionally claimed medicinal plants from the African flora (Whitefield, 1995). In developing countries, conventional drugs or formal health systems may not be available or affordable to most of the rural populations.

Although up to 80% of the African population use plant remedies, in the management of diseases including malaria. There is lack of scientific validation to confirm anti-malarial potential of used herbal medicines. Medicinal plants have been the focus of many anti-infective drugs and alternative sources of anti-malarial agents in various parts of the world including Kenya (Lee, 2002; Attiso, 1983). This study was conducted to evaluate the toxicity *and* anti-plasmodial activity of extracts of R*ubia cordifolia, Harrizonia abyssinica, Leucas Calistochys Olive* and *Sanchus schwein furthii.*

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MATERIALS AND METHODS

Preparation of crude plant extracts

Stems, floral and foliar parts of *R. cordifolia, H. abyssinica, L. Calistochys Olive* and *S. schwein furthii* were collected from Kuria district, Kenya. Botanical identification was carried out with the help of taxonomists from the National Museums of Kenya. All the collected parts of the plants were left to dry completely under a shade for one month and then transported to the laboratory where they were left to dry further under room temperature. The sample extraction procedure was carried out as described by Harborne (1994). Briefly, cold sequential extraction was carried out on plant material with analar grade organic solvents of increasing polarity. Six hundred millilitres of n-hexane were added to 300g of the shred specimen and flasks placed on a shaker and soaked for 48 hours. The residue was filtered using a Buchner funnel under vacuum until the sample dried. The sample was soaked further with 600 ml of n-hexane for 24 h until the filtrate remained clear. The filtrate was then concentrated under vacuum by rotary evaporation at 30-35°C. The concentrate was later transferred to a sample bottle and dried under vacuum using a rotary evaporator; the weight of the dry extract was recorded and stored at 4°C until required for bioassay.

Plasmodium berghei cultures in BALB/c mice

Plasmodium berghei (ANKA) used for the study was obtained from Institute of Primate Research IPR), Karen, Kenya. The parasite was maintained in healthy mice throughout the duration of the study. Laboratory bred 7 week old naive male and female BALB/c mice with an average weight of 26.5 grams were used to propagate the parasites. They were maintained in an animal care facility at the Institute of Primate Research, Karen being fed on a commercial diet (LabDiet, PMI Nutrition International, MO, and USA) and water. Before the experiments commenced, the mice were randomly picked and grouped into 6 cages of four mice each and labeled using visible yellow dye for identification.

Infection of Mice, treatment and Tissue processing for histopathological studies

Seven week old laboratory bred naive male and female BALB/c mice with an average weight of 25 grams were used for this experiment. Before the experiments commenced, the mice were randomly picked and grouped into 6 cages of four mice each and labeled using visible yellow dye for easy identification. In each cage the four mice, were each treated with different concentration of herbal extract for dose levels (1000 mg/kg, 500 mg/kg and 200 mg/kg, 100 mg/kg), of four extracts namely, *R. cordifolia, H. abyssinica, L. Calistochys Olive* and *S. schwein furthii.* One group was not treated (positive control) and another group was treated with *Artemisia annua* herbal extracts. The treatment procedure was done by holding the mouse by the scruff, wiping the lower abdomen with a cotton swab soaked in 70% ethanol. The mice were observed for 14 days for signs such as changes in physical appearance, appetite, fur, weight loss, behavioral change, and death. After 14 days the remaining mice were sacrificed by decapitation and their tissues including; brains, livers, spleens and kidneys carefully dissected out and fixed in boun's liquid, embedded in paraffin wax and cut into 3 to 5µm thick sections and then stained with safranin. The slides obtained were used for processing the photomicrographs using a digital camera. The histology revealed in the various organs of the mice treated with each extract was compared with those from the organs of the control mice which had been given distilled water. The slides showing the cyto-histpathological complications of the liver, spleen, kidney and brain were preserved for further studies.

In vivo parasite assays of aqueous and methanol herbal extract

Thirty experimental naïve BALB/c mice seven week-old males and females weighing an average of 26.5grammes were inoculated intra-venously with approximately $1x10^5$-$1x10^6$ parasitized erythrocytes in volumes of 0.2 ml innoculum (David, *et al.,* 2004; Peter and Anatoli*,* 1998). The inoculated mice were then randomly picked and grouped into 6 cages of four mice each and labeled using a visible dye for identification. These mice were maintained in an animal care. On the third day, the parasitaemia levels were determined using Giemsa stain method and ranged between 1.2-5.3%. Four-day suppressive test method described by Peters *et al.,* (1974) was used for anti-malarial screening, and the determination of percent inhibition of parasitaemia and mortality. Briefly, four infected mice were each treated with the following different concentrations of extract: *R. cordifolia, H. abyssinica, L. calistochys Olive* and *S. schwein furthii* at a dosage of 0.5 ml of four different concentrations (100mg/kg, 50mg/kg, 25mg/kg, and 0mg/kg body weight) once daily before 9.00AM for four days. The dosage was innoculated intra-peritonially using Guage 27 syringes. The fifth group received the same amount of standard anti-malarial (*Artemisia annua*) which acted as the positive control while the sixth group received the same amount of physiological saline. Clinical parameters such as weight and parasitaemia were monitored for 14 days. All the experiments were carried out in a calm laboratory setting that had ambient illuminations and a temperature (20-22^{0}C) that is close to those in the animal sanctuary (Peter and Anatoli*,* 1998).

Screening for Parasitaemia

In vivo studies for anti-plasmodial activity of the test drugs was assessed by monitoring mouse survival and parasitaemia level for over 14 day's period. Parasitaemia was assessed by microscopic examination of Giemsa stained thin blood smears prepared from mouse –tail blood. Thin smears of blood films were obtained from the peripheral blood on the tail from each mouse on day four after infection (David *et al.* 2004; WHO, 1998). Slides were fixed with methanol and stained with Giemsa at pH 7.2 then observed using a compound microscope. The daily behavior was recorded including; eating habits, fluffy fur, urine colour, weight loss, behavioral change and shivering. Percentage of suppression was calculated by using the following formula (Peter *et al.,* 1998; David *et al.* 2004).

% Suppression=Parasitaemia in negative control-Parasitemia in study group / Parasitemia in negative control

Ψ: Parasitaemia suppression was calculated as S = 100[(A-B)/A]
S = Suppression
A = Mean Parasitaemia Negative control B = Parasitaemia in the test group
Æ: Percentage parasitaemia presented is a mean of three independent experiments.

Ethical considerations

Handling of animals was done in accordance to the Guide of the Care and Use of the Laboratory Animals, Animal Resource Institute of Primate Research, NMK.

RESULTS

Mortality and behavior of BALB/c mice treated with aqueous extracts

Mice inoculated with 1000 and 500mg/kg body weight of the extract did not survive beyond 24 hrs. They shivered, developed bulged eyes, raised fur and lost appetite. By day 7 all mice administered with 500mg/kg body weight of crude extract had died except for those inoculated with *L. calistochys Olive*. 50% of mice administered with *L. calistochys* Olive survived by day seven and continued till day 14. All the mice administered with 200 and 100 mg/kg body weight survived until day 7. At this point only mice in the control group and those administered with *A. annua* and *R. cordifolia***,** showed normal behavior. At the end of the experiment only mice in the negative control group were surviving at 1000mg/kg body (Table 1).

Table 1. Mortality and exhibited behavior of BALB/c mice treated with aqueous extracts

Herbal extracts (Drug)	Mortality (n/N)* 100	Exhibited Behavior change
Rubia cordifolia	0/4	No sign of abnormal
Harrizonia abyssinica	2/4	Oliguria
Leucas Calistochys Olive	4/4	Oliguria, weight fluffy fur, anaemia,
Sanchus schweinfurthii	0/4	Fluffy fur.
Artemisia annua L.	0/4	Normal behavoiur.
Control (Negative)	4/4	Fever, agility, fluffy fur, weight loss.

*Mortality is defined as n/N, where n is the number of dead mice and N is the number of mice in each group

Suppression and mortality of *BALB/c mice* infected with *Plasmodium berghei*

All mice developed patent parasitaemia following inoculation with *P. berghei* (Tables 2 and 3). Extracts of *S.s schwein furthii* displayed highest parasitaemia suppression at 2.31% which corresponded to 60.17% suppression. In this group, all the mice survived up to day 15 just as the positive control. This also corresponded to 70% similarity in activity when compared with *Artemisia annua* (positive control). There was a significant difference in anti-plasmodial activity of *S. schweinfurthii* extracts when compared with the control ($p = 0.003$). Aqueous extracts of *H.abyssinica* displayed 47.93% inhibition of parasite growth over the same period of time. Suppressive activity of *R. cordifolia,* and *L. calistochys Olive* were similar at 29.83% compared to negative control. As expected most successful inhibition of parasitaemia was observed in the positive control *A. annua,* at 88.97%. Analysis of results of dosage 100mg/kg/day showed that *R. cordifolia* and *S. schwein furthii* had higher percentage parasitaemia suppression with values of 82.4 % ($p = 0.001$) and 78.6% ($p = 0.003$). Percentage parasitaemia suppression in mice treated with extracts of *H. abyssinica* and *L. Calistochys* also showed values of 65.1% ($p = 0.011$) and 59.1% ($P = 0.04$). Aqueous extracts of *R. cordifolia,* and *S. schwein furthii* with LD_{50} doses of <10mg/kg showed a significant difference on parasitaemia suppression as compared to *H. abyssinica* and *L. Calistochys Olive* that had moderate suppression, with LD_{50} doses ranging between 10mg/kg and 100mg/kg.

Table 2. Mean parasitaemia suppression in BALB/c mice infected with *Plasmodium berghei*

Herbal extracts LD_{50}	Dosage tested mg/kg/day	Final Parasitaemia (%)	Suppression (%)
Leucas calistochys Olive	100	4.07Æ	29.83Ψ
Sanchus schweinfurthii	100	2.31	60.15
Rubia cordifolia	100	4.07	29.83
Harrizonia abyssinica	100	3.02	47.93
Artemisia annua L	100	0.64	88.97
Control (Negative)	0	4.88	0

Table 3. Percentage parasitaemia suppression and mortality of BALB/c mice infected with *Plasmodium berghei*

Herbal extracts	Dosage tested mg/kg/day	Day 14 % Parasitaemia suppression	Day 14 (p.i) survivals (%)	Mortality* (n/N)
Leucas	100	82.4±3	100	0/4
calistochys	50	77.5±4	100	0/4
Olive	25	43.3±3	100	0/4
Sanchus	100	78.6±3	100	0/4
schwein	50	67.9±3	100	0/4
furthii	25	45.8±2	100	0/4
Rubia	100	59.1±4	50	2/4
cordifolia	50	55.7±3	0	4/4
	25	29.8±3	0	4/4
Harrizonia	100	65.2±4	75	1/4
abyssinica	50	49.0±3	50	2/4
	25	22.1±4	100	0/4
Artemisia annua	100	100	100	0/4
Control (Negative)	100	0	0	4/4

*Mortality is defined as n/N, where n is the number of dead mice and
N is the number of mice in each group (4). LD_{50} Dose inhibiting 50% growth of parasites.Parasitaemia suppression was calculated as:
S= 100[(A-B)/A]
S = Suppression A = Mean Parasitaemia in Negative control
B = Parasitaemia in the test group

Survivorship of mice following treatment with aqueous extracts

Results on survivorship show that all mice treated with *A.annua* survived for 9 days. However, 75% of mice in the

negative control group died by day 4 post-treatment (Figure 1). All mice treated with *L. calistochys Olive* extracts survived until 7th day of post-treatment and there were no survivors thereafter. Mice treated with *R. cordifolia* extracts, 100% of them survived until day seven post-treatment, 25% till day eight post-treatment. 100% of mice treated with *S.schweinfurthii* extracts survived until day six post-treatment and only 25% survived to day nine post-treatment. 100% of mice treated with extracts from *H. abyssinica* survived until day three post –treatment, 75% survived to day five post-treatment and 25% survived till day eight. *H. abyssinica,* was least protective herb since only 25% of the mice were surviving by day 5 post treatment as compared to 100% percent of mice treated with herbal extracts from *S. schweinfurthii* and *L. calistochys Olive* survived by day 6 post treatment. All mice (100%) treated with *S. schweinfurthii* survived by day 7 post treatment and 25% survived until the end of the experiment in day 9.

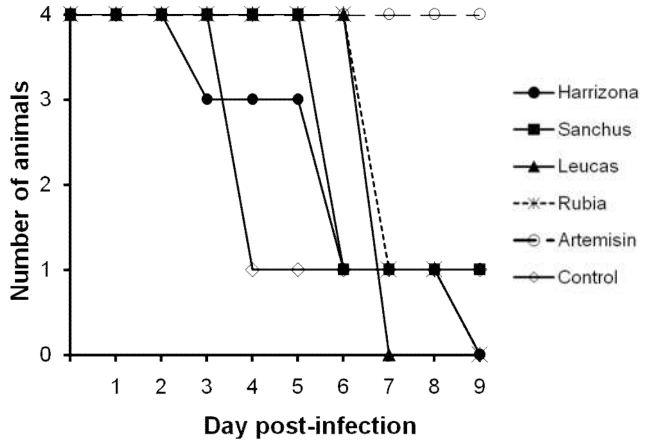

Figure 1. Mean survivorship of *Plasmodium berghei* ANKA infected mice inchemotherapeutic regimen with plant extracts

In vivo anti-plasmodial activity and parasite inhibition of herbal medicines

Plasmodium berghei infected mice treated with the extracts of *R. cordifolla, H. abyssinica, L. calistachys Olive* and*S. S. furthii, showed* parasitemia significant change from those mice in the control group. In untreated mice, the parasite count increased from day to day until the death of the mice. Mice treated with extracts had longer survival correspondent with significant suppression in mice either on day 4 or 7 post infection, whereas all mice of untreated control died between days 4-10. 100% of mice treated with *R. cordifolia* and *S. schweinfurthii* and 50% of mice treated with *H. abyssinica* and *L. Calistochys* extracts survived up to day 15 and 21 far beyond the survival of the controls. There was significant reduction in parasitemia by the aqueous extract of *R cordifolla, H abyssinica, L calistachys Olive* and *S schweinfurthiion* day 4. The test extracts of *R. cordifolla, H. abyssinica, L. calistachys Olive* and *S. Schwein furthii* roots and aerial parts significantly prevented weight loss at some dose levels compared to the controls, the increase in body weight was not consistent with increasing dose of the extracts.

Toxicity and survivorship of experimental mice treated with extracts

Mice treated with extracts *S.schweinfurthii, R. cordifolia,*and *L. calistochys Olive,*100mg/kg body weight survived until day five post treatment. Mice treated with *S. schweinfurthii*, 100% of them survived by day seven post treatment and 25% survived until the end of the experiment in day nine. *H. abyssinica* was least protective herb since only 25% of the animals were surviving by day 5 post treatment as compared to 100% of mice treated with herbal extracts from *S. schweinfurthii* and *L. calistochys Olive* which survived by day 6 post treatment.

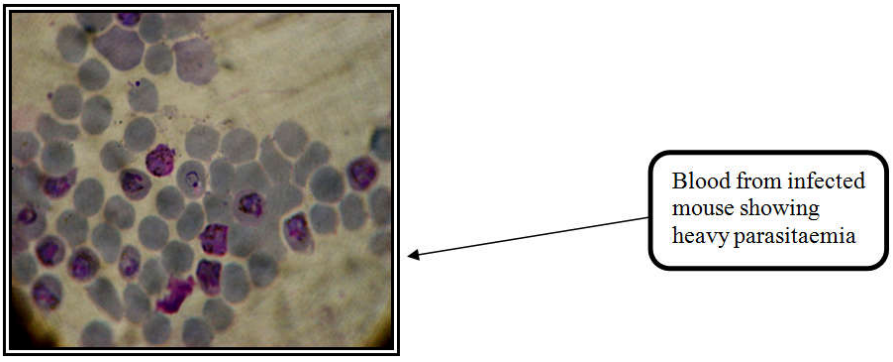

Plate 1a. Parasitized red blood cells (reticulocytes) from BALB/c mouse infected with *Plasmodium berghei*

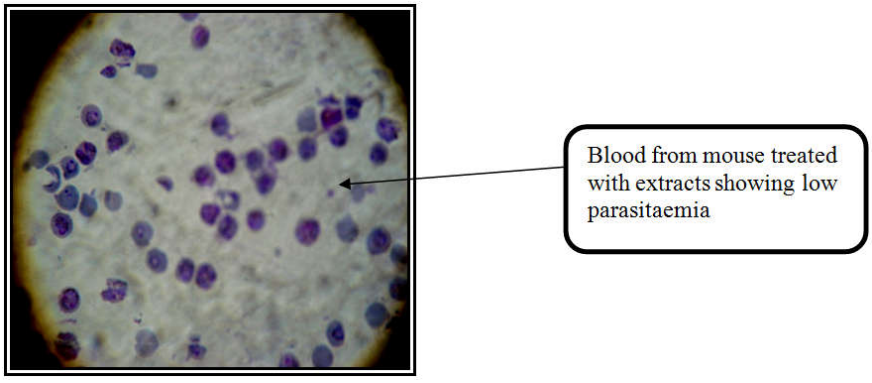

Plate 1b. Reticulocytes of BALB/c mice infected with *Plasmodium berghei* on treatment with *S. schweinfurthii* extracts

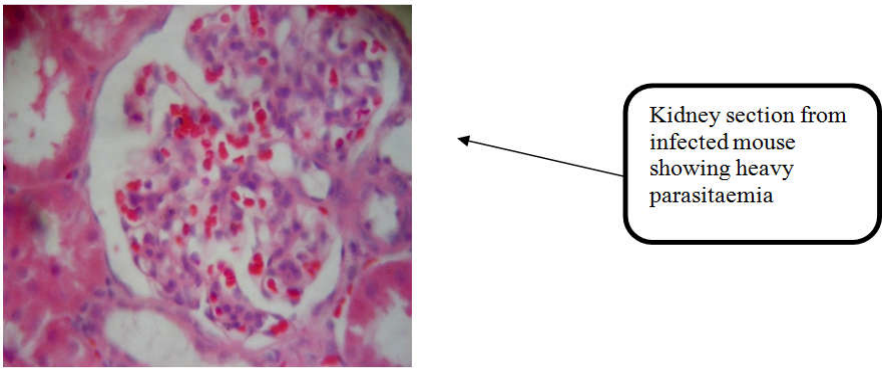

Plate 2a. Cyto-histopathological sections of kidney infected with*Plasmodium berghei* showing high parasitaemia

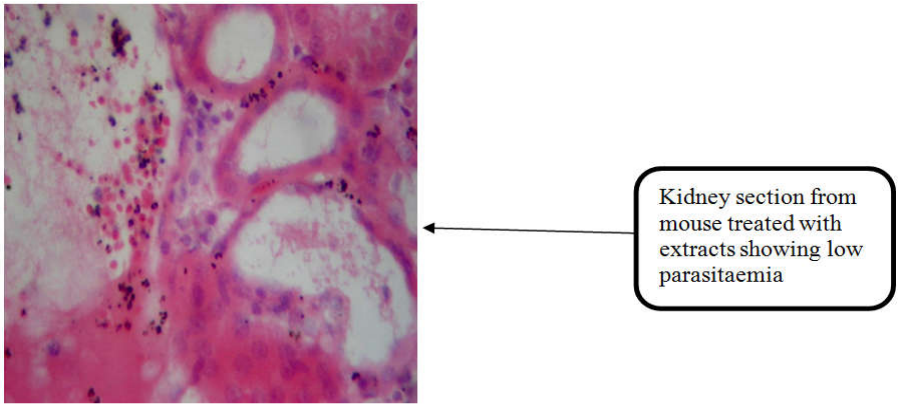

Plate 2b. Cyto-histopathological sections of kidney from mice*on* treatment with extract *S. schweinfurthii* showing reduced parasitaemia

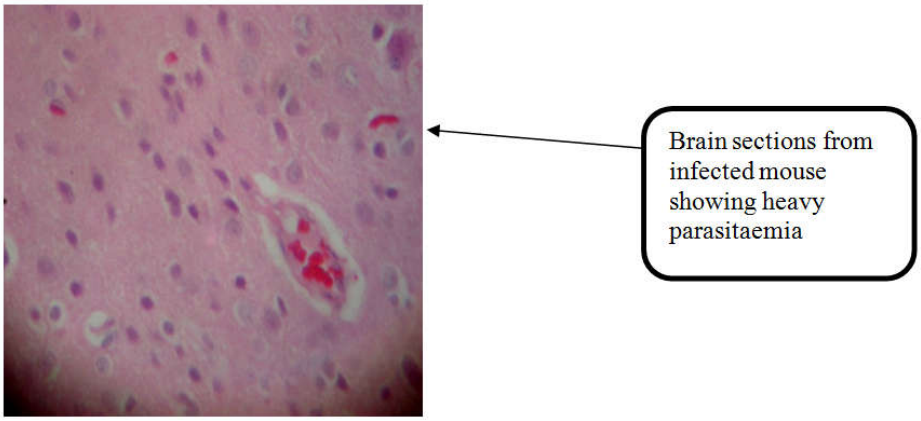

Plate 3a. Cyto-histopathological section of brain tissue from infected mice showing heavy parasites of *P. berghei*

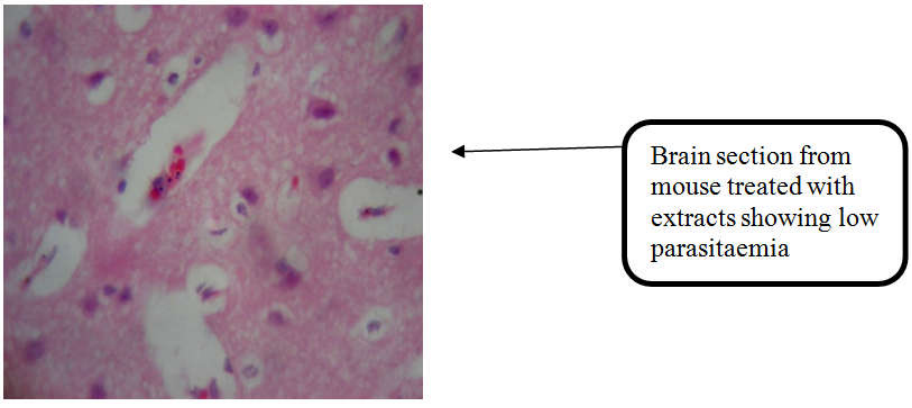

Plate 3b. Cyto-histopathological section of brain tissue from mice on treatment with *S. Schwein furthii*s showing reduced parasitaemia

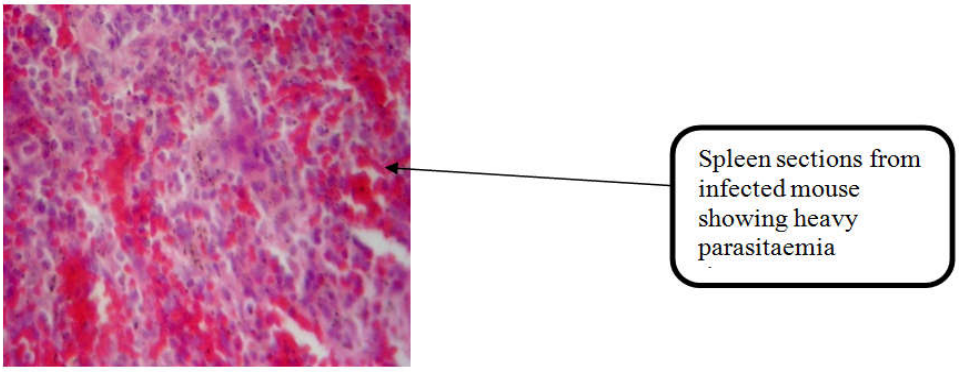

Plate 4a. Cyto-histopathological section of spleen infected mice showing heavy parasite of *P. berghei*

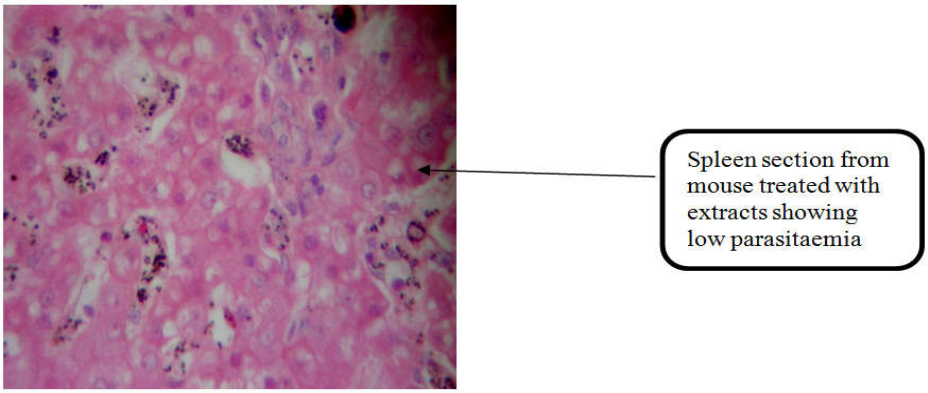

Plate 4b. Cyto-histopathological section of spleen from mice on treatment with *Sanchus schwein furthii*s showing reduced parasitaemia

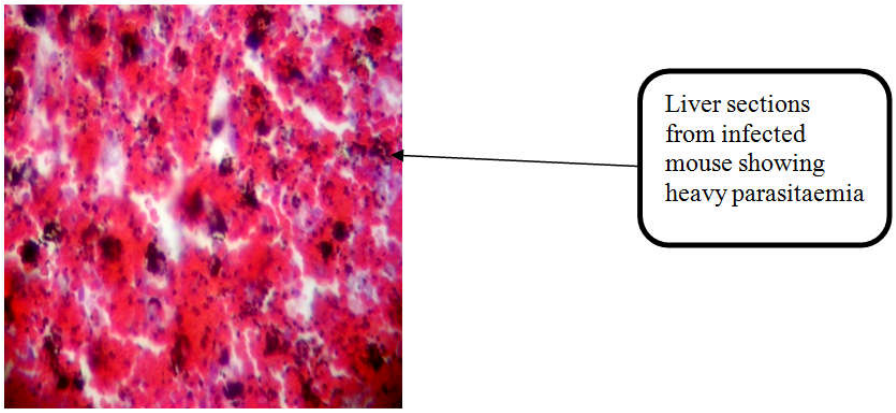

Plate 5a. Cyto-histopathological section of liver from infected mice showing heavy parasite of *P. berghei*

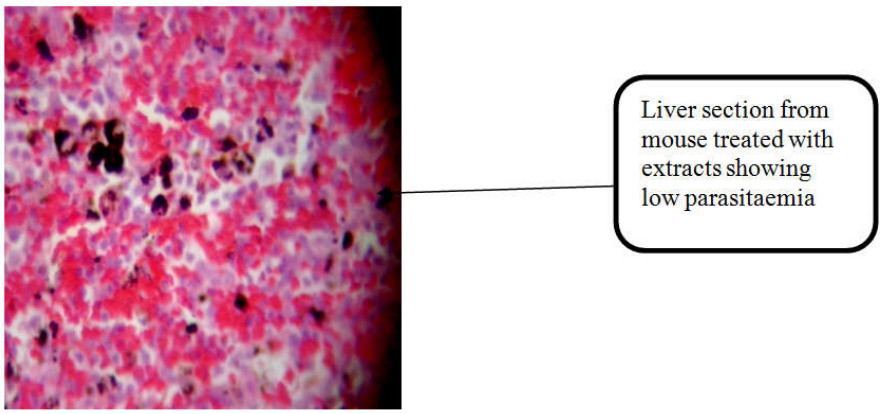

Plate 5b. Cyto-histopathological section of liver from mice on treatment with *Sanchus schwein furthii*s showing reduced parasitaemia

DISCUSSION

Mortality and exhibited behavior of BALB/c mice treated with aqueous extracts of *S. schwein furthii, R. cordifolia, H. abyssinica,* and *L. calistochys Olive*

Results indicate that mice inoculated with 500mg/kg body weight of the extract did not survive beyond 24 hours. This results show that *L. calistochys Olive* was the least toxic while *Sanchus schwein furthii,* and *Rubia cordifolia* were the most toxic having killed all the mice at 100mg/kg body weight. In addition mice administered with *Sanchus schwein furthii* showed signs of severe anaemia. This significant suppression of parasitemia by the aqueous extract of *R. cordifolla, H, abyssinica, L. calistachys Olive* and *S. schwein furthii on* day 4 is in agreement with that shown for a water extract employed against four different malaria schizont strains *In vitro* and observed anti-malarial activity (Oketch – Rabah, 2003; Peter & Anatoli, 1998; David, *et al.,* 2004).

The results obtained from the study showed that aqueous extracts were more active *in vivo* with BALB/c mice as compared to methanolic extracts and these findings are in agreement with those obtained by Muthaura *et al.,* (2007) which showed that the aqueous extracts gave higher efficacy *in vivo* Assays as compared to methanolic extracts. Similar results on parasitaemia reduction with aqueous extracts as compared to methanolic extracts have been obtained with *Toddalia asiatica,* (root bark 71%), *Maytenus senegalensis* (49%) in studies conducted in Kenya by Muregi *et al.,* 2007 and on *Croton mubango* herb studies conducted in Democratic republic of Congo (Mesia *et al.,* 2005).

The finding on parasitaemia suppression are in agreement with other studies conducted using a standard anti-malarial drug on mice infected with *P. berghei* where it suppressed parasitaemia to non-detectable levels (Kiseko *et al.,* 2000). However, although the results clearly indicated that the test extracts of *R.cordifolla, H. abyssinica, L. calistachys Olive* and *S. schwein furthii* roots and aerial parts significantly prevented weight loss at some dose levels compared to the controls, the increase in body weight was not consistent with increasing dose of the extracts.

Observations made during the study showed that in untreated mice, the parasite count increased from day to day until the death of the animal. Similar results have been observed in previous studies by Ayodele, 1979 who reported that the parasite count increased and the hematocrit packed cell volume (PCV) decreased markedly from day to day until the death of the animal. In general, if the lethal dose (LD_{50}) of the test substance is three times more than the minimum effective dose (MED), the substance is considered a good candidate for further studies.

Mice treated with extracts had longer survival correspondent with significant suppression in mice either on day 4 or 7 post infection, whereas all mice of untreated control died between days 4-10. 100% of mice treated with *R.cordifolia* and *S. schwein furthii* and 50% of mice treated with *H. abyssinica* and *L. Calistochys* extracts survived up to day 15 and 21 far beyond the survival of the controls. This observation corresponds with the report on studies conducted by Ancolio *et al.,* 2002 on traditional antimalarial plants in Mali and Sao Tome.

In the recent study, intra-peritoneal administration of BALB/c mice, produced dose dependent multiple organ toxicities including the kidneys, liver, lungs and brain. Toxicity studies have been conducted using herbs like *Croton mubango* and *Nauclea pobeguinii* in Democratic Republic of Congo (Mesia *et. al.,* 2005) and have shown similar outcomes as those observed with the four herbal medicines in this study. The LD_{50} of the extracts in conjunction with photomicrographs of histopathological stained tissues gave a good picture of the toxic characteristics of the four herbal plants. Histological sections of the kidney of related mice showed features of consistent with renal epithelial injury from toxins.

Many herbal preparations have been found to exhibit renal tubular necrosis showing extensive interstitial fibrosis and severe tubular loss most prominent in the outer cortex Mengs *et al.* (1982) showed that aristolochic acid was nephrotoxic in female Wister mice which rapidly developed necrosis and renal failure. Results obtained by Muthaura, 2007 on safety and toxicity of 10 herbal extracts indicate that some herbals which showed a high anti-plasmodial activity also indicated toxicity in high doses. This is the trend shown by the four herbal extracts *R. cordifolia, S. schwein furthii, H. abyssinica* and *L Calistochys* tested for toxicity using BALB/c mice in this study.

The aqueous extracts of *R cordifolia,* and *S.schwein furthii* were more efficacious with highest parasitaemia suppression on *P.berghei* infected BALB/c mice in low LD_{50} doses of <10mg/kg/day. Histopathological results from tissues harvested from liver, spleen, kidney and brain (Plates 1-5) showed a great and observable difference between mice treated with *S. schwein furthii*s and untreated mice. This shows that administered herbal extracts were efficacious and safe in *P. berghei* though the herbs were toxic at higher dosages.

Survivorship curves of mice following treatment with aqueous extracts from *S. schwein furthii, R. cordifolia, H. abyssinica* and *L. calistochys olive*

Results on survivorship demonstrated that tissue toxicity of ethno-medicines occurs when consumed in large quantities in mice. Mice treated with extracts *S schwein furthii, Rubia cordifolia,* and *Ls calistochys Olive,* 100mg/kg body weight survived until day five post –treatment. Mice treated with *Sanchus schwein furthii* 100% survived by day seven post treatment and 25% survived until the end of the experiment in day nine. This is an indication that the herbs are less toxic as compared *H abyssinica,* was least protective herb since only 25% of the animals were surviving by day 5 post treatment as compared to 100% percent of mice treated with herbal extracts from *S schwein furthii* and *Leucas calistochys Olive* survived by day 6.

Conclusion

Considering the potential toxicity of *Rubia cordifolia,* and *Sanchus schwein furthii* herbal practitioners should be educated on this important finding especially when they

recommend this herbs as part of a complex regime. Herbal practitioners and the community should be educated on this findings especially when to recommend herbal plant as part of a complex regime in the long term management of chronic illnesses. All herbs used for treatment and chemoprophylaxis should undergo toxicity testing.

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