

Senna occidentalis (L.) Link root extract inhibits Plasmodium growth in vitro and in mice

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Abstract

Background: *Senna occidentalis* (L.) Link has been used worldwide in traditional treatment of many diseases and conditions including snakebite. In Kenya, a decoction from the plant roots taken orally, is used as a cure for malaria. Leaf extracts from the plant have been shown to possess antiplasmodial activity, *in vitro*. However, the curative potency of the plant root against established malaria infection is yet to be scientifically validated, *in vivo*. On the other hand, there are reports on variation in bioactivity of extracts obtained from this plant species, depending on the plant part used and place of origin among other factors. In this study, we demonstrated the antiplasmodial potency of *Senna occidentalis* roots extract *in vitro*, and in mice.

Methods: Methanol, ethyl acetate, chloroform, hexane and water extracts of *S. occidentalis* root were tested for *in vitro* antiplasmodial activity against *Plasmodium falciparum*, strain 3D7. Cytotoxicity of the most active solvent extracts was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and the curative potency in *Plasmodium berghei* infected mice evaluated by Rane's test.

Results: All the solvent extracts showed suppressive activity against *P. falciparum*, *in vitro*, in a concentration dependent manner. The suppression was also dependent on polarity of solvents used with the methanolic extract (most polar) being 10-fold more active than hexane extract (least polar). The extracts showed no toxicity to Vero cells at the tested concentrations and significantly suppressed *P. berghei* parasitemia in Rane's test. Similarly, the extracts prolonged the survival time the infected animals significantly.

Conclusions: *Senna occidentalis* (L.) Link root extract suppresses malaria parasites growth *in vitro* and in BALB/c mice. The plant root, therefore, is a potential source of antimalarial principles. This study has scientifically validated the ethnomedical usage of *S. occidentalis* roots in management of malaria among some Kenyan communities.

Background

Malaria remains a public health challenge particularly in Sub-Saharan Africa (1, 2). There were an estimated 241 million malaria cases and 627,000 deaths globally in 2020 (3). Malaria control has proven to be very effective when vector control is combined with prompt treatment. However, drug resistance to the insecticides used in vector control and antimalarial drugs pose a significant challenge to malaria control programs (1, 4–7).

To address the emerging problem of drug resistance, the World Health Organization (WHO) recommends combining artemisinin and its derivatives with other antimalarial drugs in combination therapy (2, 8, 9). This approach has proven to be effective in the treatment of *P. falciparum* malaria in various parts of the world. However, *Plasmodium* malaria resistance to current first line drugs, including artemisinin, has been

reported (2, 3, 9–12). As such, priority should be given to the discovery and development of antimalarial drugs with novel and distinct mechanisms of action.

Medicinal plants are a rich source of untapped antimalarial activities (1, 2, 4, 13). This is supported by the successful discovery of Artemisinin, Quinine and their derivatives as effective antimalarial drugs (14, 15). *Senna occidentalis* (L.) Link is a widely used plant in traditional medicine around the world (16). This plant is commonly used to treat a variety of parasitic infections and other conditions like snake and insect bites (16–19). In Kenya, the plant root decoction is taken orally three times a day for three to four days by the Luhya, Digo and Duruma communities to treat malaria (20–22). Previous studies have demonstrated the antiplasmodial activity of leaf extracts of *S. occidentalis in vitro* (16, 23). This current study sought to investigate the antimalarial potency of the plant root extract against *P. falciparum*, *in vitro* and *P. berghei*, *in vivo*.

Methods

Ethical clearance

Approval to conduct this study was granted by the Institute of Primate Research (IPR) Institutional Scientific and Ethics Review Committee (ISERC/02/18). The care and handling of experimental mice was done according to the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines. The Institute of Primate Research facility is accredited by the National Commission for Science, Technology and Innovation (NACOSTI) and Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International.

Plant Collection, Extraction And Processing

Senna occidentalis roots were collected from Migori county (0.9366° S, 34.4198° E), Kenya in the months of August and September. This plant was identified by a taxonomist and a voucher specimen (38/81) deposited at the National Museums of Kenya's East African Herbarium for future reference. Further, the plant name was verified with <http://www.theplantlist.org> on 10/05/2022.

The collected plant roots were air dried in the shade, ground into powder and stored in airtight plastic containers at 4°C until extraction.

Hexane, chloroform, ethyl acetate, methanol and distilled water were used for extraction by maceration. The aqueous decoction was included to mimic the traditional method of preparing the antimalarial therapy as used by the locals. The filtrates were concentrated by rotary vaporization (BÜCHI R-200 rotary evaporator) for organic solvents and lyophilization (NANBEI freeze dryer: NBJ-10-1, Zhengzhou, China) for aqueous solutions. Upon drying, the extracts were stored in sealed sample bottles at 4°C until required for use.

Plant secondary metabolites are excellent indicators of their bioactivity potential (24). As such, standard procedures were used to screen the extracts for the presence of saponins, tannins, alkaloids, flavonoids and sterols (25) in order to predict the bioactivity of *S. occidentalis* root.

Plasmodium falciparum propagation and extracts preparation

Senna occidentalis root extracts were tested for antiplasmodial efficacy, *in vitro*, using *P. falciparum*, strain 3D7 obtained from Kenya Medical Research Institute (KEMRI) repository. A modified version of a previously used method (26) was utilized to establish a continuous culture of these malaria parasites. In summary, the parasites were propagated at 37°C in blood group O + red blood cells (RBCs) maintained in RPMI 1640 growth medium (Life technologies Ltd., Paisley, UK) supplemented with 1 molar 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), (Gibco, Life technologies Ltd., Paisley, UK), 1 molar Sodium hydroxide (EMD Millipore corporation, Darmstadt, Germany), 20% D-glucose (PANREAC QUIMICA SA Barcelona, Spain), 200mM L-glutamine (Gibco, Life technologies Ltd., Paisley, UK), 10% human serum (group O+) and a gas mixture of 90% N₂, 5% O₂ and 5% CO₂. The culture was refreshed every 48 hours at 3% haematocrit.

Dilutions of the extract and standard drug were performed as described (27), with minor modifications. To make a stock solution (5mg/ml), each extract was first dissolved in dimethyl sulfoxide (DMSO), vortexed and RPMI 1640 (incomplete medium) added to the required volume. For extract combinations, each respective extract was weighed and blended in a 1:1 ratio. A 0.5mg/ml stock solution of pyrimethamine was also prepared.

In vitro growth inhibition assay

Growth inhibition of *P. falciparum* malaria parasites by *S. occidentalis* root extract was assessed as described (28), with minor modification. A ten-fold dilution was prepared from the stock solutions using complete RPMI 1640 and serially diluted 7-fold across a 96-well cell culture plate. This provided a dose-titration range of 250µg/ml to 1.95µg/ml for the extracts and 25µg/ml to 0.20µg/ml for pyrimethamine. Sorbitol synchronized ring stage parasitized RBCs (1% parasitemia) suspended in complete RPMI at 3% hematocrit was then added to respective wells. Non-infected RBCs as well as DMSO were also included in the assay as controls. The plates were incubated at 37°C. After 72 hours, thin smears were prepared and parasites quantified by light microscopy. Percentage parasitemia suppression was computed and 50% inhibitory concentration (IC₅₀) values determined for each extract (29, 30).

Evaluation Of The Extract For Cytotoxicity

To assess the *in vitro* cytotoxicity of *S. occidentalis* root extract, Vero cell line (sourced from KEMRI) was grown to confluent monolayer in Minimum Essential Medium Eagle (MEM) containing sodium bicarbonate and L-glutamine. The growth medium was supplemented with 1% pen-strep (Sigma), 1%

HEPES (Gibco) and 10% Fetal bovine serum (FBS). The assay was performed as previously described (31, 32).

Experimental Animals And Rodent Malaria Parasites

In this study, male and female inbred BALB/c mice aged 8–9 weeks were used. The rodent facility at IPR provided the animals. They were housed in standard Macron type II cages within 12 hours' dark/light cycle at 23°C. Food and water was provided *ad libitum* according to the IPR's Animal Science Department standard operating procedures.

Plasmodium berghei, strain ANKA (sourced from KEMRI) was retrieved from the IPR repository and maintained in mice (33) for use in this study. This parasite strain was utilized because it causes severe disease in BALB/c mice with clinical characteristics similar to *P. falciparum* infection (34). Despite the phylogenetic distance between rodent and human malaria parasites, *P. berghei* and the human malaria parasites possess conserved genes that have over time allowed their use in Peters' model of antimalarial drug efficacy testing (35).

Mice Infection, Treatment And Monitoring Of Parasitemia And Survivorship

A modification of the method by Ryley and Peters (1970) was used to evaluate the curative potency of the extract against *P. berghei* in mice (36, 37). Mice were inoculated intraperitoneally with 1×10^6 *P. berghei* infected erythrocytes and assigned randomly to 5 experimental groups (5 mice per group). At approximately 5% parasitemia, treatment was started. Group 1 was treated with 1mg/kg pyrimethamine (Sigma – Aldrich Chemie, Steinheim, Germany). Group 2 (placebo group) was administered with 1% DMSO in phosphate buffered saline (vehicle). Groups 3 and 4 were treated with 200mg/kg and 100mg/kg of the methanolic extract, respectively. Group 5 was treated with 200mg/kg of the aqueous extract. The treatment was done orally for 4 consecutive days. The oral route was chosen based on documented ethnomedical usage of the plant (22). The mice were monitored for survival till day 30 post infection. Meanwhile, parasitemia suppression was determined. The administered extract quantity was based on dose recommendations for *in vivo* administration of crude extracts (38) and, previous antimalarial studies regarding *Senna* family (5, 39).

Determination Of Parasite Growth Suppression

The effect of the extract on *P. berghei* growth inhibition in mice was determined as described previously (36). Tail blood was used to prepare thin smears that were fixed and observed under $\times 100$ lens of a light microscope. The number of parasitized red blood cells (pRBCs) was examined per at least 1000 RBCs. Percentage parasitemia and parasite suppression was then computed using the following formulas (40);

$$\% \text{ parasitemia} = \frac{\text{Number of infected RBC} \times 100}{\text{Total number of RBCs}}$$

$$\% \text{ suppression} = \frac{\% \text{ parasitemia in control group} - \% \text{ parasitemia in treatment group} \times 100}{\% \text{ parasitemia in untreated control group}}$$

Determination Of Mean Survival Time

To assess the effect of the extract on survival time of the infected mice, the number of days that each mouse lived from the day of parasite inoculation to death was recorded in a 30 days' period. The following formula was used to determine the mean survival time of each group (41);

$$MST = \frac{\text{The sum of survival time (days) for the mice in a group}}{\text{The total number of mice in the group}}$$

Statistical Analysis

The collected data was recorded as means $\pm$ standard error of the means ($M \pm SEM$). *In vivo* parasite suppression was analyzed through Ordinary One-Way ANOVA followed by Tukey's multiple comparisons test at 95% confidence level ($\alpha = 0.05$). Log rank analysis was used to compare survival time of mice in the different treatment groups. Graph Pad prism (Version 7.00, California, USA) was used for the analyses.

Results

Senna occidentalis root extract possesses secondary plant metabolites

Plants with active phytomedicine properties possess secondary metabolites (Okokon *et al.*, 2017). Methanol, ethyl acetate, chloroform, hexane and aqueous extracts of *S. occidentalis* roots were screened for presence of secondary metabolites. All extracts contained saponins, tannins, flavonoids, alkaloids and sterols. These extracts were subsequently assayed for biological activity against *Plasmodium* spp. especially suppression of malaria parasites growth.

Senna occidentalis roots extract suppresses Plasmodium falciparum growth, in vitro

Methanol, ethyl acetate, chloroform, hexane and aqueous roots extracts of *S. occidentalis* were tested for growth suppression efficacy against *P. falciparum*. The use of solvents with differing polarity would enable evaluation of antiparasmodial efficacy of both polar and nonpolar metabolites of the plant root. The results (Table 1) show that methanolic extract exhibited minimum IC₅₀ value whereas hexane extract exhibited maximum IC₅₀ value. This implied that methanolic extract was more active against the parasites compared to hexane extracts. When the antiparasmodial activity of the other extracts was compared to that of hexane, the efficacy was 10, 8, 4, 2.7 and 1.5-fold higher for methanol, aqueous macerate, ethyl acetate, aqueous decoction and chloroform respectively. This suggests that polar extracts of *S. occidentalis* roots are more active against *P. falciparum*, *in vitro*. Furthermore, these findings demonstrate that both polar and non-polar metabolites of *S. occidentalis* roots are potent against *P. falciparum* *in vitro*.

Senna occidentalis **roots extract is non-toxic to Vero cells**, *in vitro*

Growth inhibitory effects observed in antiparasmodial assays may result from general toxicity of the substances being tested rather than specific activity on the *Plasmodium* parasites (42, 43). The cytotoxicity of methanol and aqueous extracts were assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. These two extracts were selected for cytotoxicity evaluation because they showed greater antiparasmodial activity in the *in vitro* assay and, were as such, candidates for pre-clinical analysis for antiparasmodial activity in animal models. Both the methanolic and aqueous extracts exhibited selectivity indices greater than 10 (Table 1), an indication of high selectivity for malaria parasites (32). This observation implies that *S. occidentalis* root extract is non-toxic to animal cells at the tested concentrations.

Table 1

The 50% inhibitory concentrations, 50% cytotoxicity concentration and selectivity index values of the extracts

Type of extract/Control	IC ₅₀ (µg/ml) on 3D7	CC ₅₀ (µg/ml) on Vero cells	SI
Methanol	1.756	247	140
Aqueous macerate	2.283	1786	781
Methanol + Ethyl acetate	4.209	ND	
Ethyl acetate	4.728	ND	
Aqueous macerate + ethyl acetate)	5.831	ND	
Aqueous decoction	6.831	ND	
Chloroform	12.130	ND	
Methanol + Hexane	14.560	ND	
Aqueous macerate + hexane	17.650	ND	
Hexane	18.470	ND	
Pyrimethamine (positive control)	0.003		
IC ₅₀ (50% inhibitory concentrations); CC ₅₀ (50% cytotoxicity concentration); SI (selectivity index); ND (Not determined).			

Senna occidentalis roots extract suppresses Plasmodium berghei parasitemia in mice

The *P. berghei*-BALB/c mouse model was utilized to assess the antiplasmodial activity of *S. occidentalis* polar extracts *in situ* in an animal system. The effect of *S. occidentalis* extract on *P. berghei* parasitemia suppression was determined by monitoring parasite multiplication in the infected mice upon treatment with the extract. At a dose of 200mg/kg body weight, the extract significantly suppressed *P. berghei* growth ($P < 0.05$) on days 6, 8, 10 and 12, relative to the phosphate buffered saline (placebo) group. The inhibitory efficacy of methanol extract (200mg/kg) and aqueous extract (200mg/kg) was comparable ($P > 0.05$). However, on day 6 post infection, methanolic extract depicted higher activity ($P = 0.03$). The suppressive activity of 100mg/kg methanol extract was also comparable to 200mg/kg aqueous extract ($P > 0.05$). Similarly, 100mg/kg methanolic extract suppressed parasitaemia in mice at equal levels to 200mg/kg extract of the same solvent. This suggests using 100mg/kg methanolic extract as optimum for use in mice. Parasite multiplication increased steadily in the placebo group until day 8 post-infection when it began to lag. This was indicative of reticulocyte depletion resulting in fewer cells available for malaria parasite infection (44, 45). The pyrimethamine control group achieved 100% parasite clearance by day 9 post infection. Overall, these findings show that aqueous and methanolic extracts of *S. occidentalis* roots suppress *P. berghei* propagation, *in vivo* and that the aqueous extract is as effective as the methanolic extract at the tested concentrations. Parasitemic profiles of *P. berghei* in mice treated with

the extract and the controls are shown in Fig. 1. The parasite suppression levels for the different treatment groups are as shown in Table 2.

Table 2
Suppression of *Plasmodium berghei* in mice and survival time of the infected mice upon treatment

Treatment	Parasite suppression in percentage (%)				Mean survival time(Days)
	Day 6	Day 8	Day 10	Day 12	
A	13.11	73.63	47.11	41.74	19.00 ± 1.14
B	15.79	68.35	49.3	38.32	18.8 ± 0.73
C	8.68	59.36	44.87	26.34	17.44 ± 1.75
D	41.37	97.27	100	100	30.00 ± 0.00
E	0	0	0	0	13.00 ± 1.67

A (Methanol extract: 200mg/kg body weight); B (Methanol extract: 100mg/kg body weight); C (Aqueous extract:200mg/kg body weight); D (Pyrimethamine:1mg/kg body weight); E (Phosphate buffered saline)

Senna occidentalis **roots extract enhances the survival time of** Plasmodium berghei **infected mice**

The effect of *S. occidentalis* extract on the survival of *P. berghei* infected mice was assessed by monitoring survival time of the extract-treated animals for 30 days, post- infection. The extract prolonged the survival time of the infected mice in the range of 4 to 6 days, which is approximately 0.3- to 0.5-fold extension of survival time relative to the placebo-treated animals. Treatment with the standard drug, pyrimethamine was associated with 100% survival of the infected mice. When compared to the placebo group, the increase in survival time of the extract treated mice was significant ($P < 0.0001$). The number of days survived by the infected animals post infection is as shown in Table 2 above. Figure 2 (below) represents survivorship curves for the animals in the different treatment groups.

Discussion

We show, in this study, that *S. occidentalis* root extract inhibits malaria parasites growth *in vitro* and in mice. The documented ethnomedical use of the plant root as a cure for malaria in Kenya influenced the selection of the plant (21, 22). This ethnopharmacological approach to drug development has been associated with high success rates of discovering active compounds from natural sources (43). It is also a cost effective and time saving approach compared to mass screening of plants for specific biological activity (46, 47).

To obtain polar and nonpolar root extracts of *S. occidentalis* for evaluation of antimalarial potential, the plants roots powder was extracted with methanol, ethyl acetate, chloroform, hexane and water. Preliminary phytochemical analysis of the extracts revealed the presence of biologically important

secondary metabolites, some of which have been linked to antimalarial activities of plant extracts (24, 37, 48–50). These findings paved way for further analysis of *S. occidentalis* extracts as antimalarials. A bioactivity guided extract fractionation will reveal the actual molecules containing antiplasmodial activity.

In this current study, the extracts were found to be active, *in vitro*, against *P. falciparum*. This malaria parasite species is the foremost contributor to the global burden of malaria (1, 4, 8). Our findings revealed that antiplasmodial activity of *S. occidentalis* root is polarity dependent with extracts from highly polar solvents showing higher activity and vice versa. This suggests that, during extraction, the bioactive principles of the plant roots get localized more in polar solvents than non-polar ones. Extraction efficiency has been shown to be dependent on the solvent of extraction (Zhang et al., 2018). These findings imply that the antimalarial ingredients of the roots extract of *S. occidentalis* are mainly polar.

Toxicity of *S. occidentalis* seems to be influenced by among other factors, the plant part used, selected dose, method of preparation, animal species involved and length of exposure (51–53). In this current study, *S. occidentalis* root extract showed no toxicity to Vero cells and was highly selective of *P. falciparum*. This is suggestive of possible safety of the extracts when used in *in vivo* models of antimalarial efficacy testing.

Drug interaction with malaria parasites is heavily influenced by the host biological system (54, 55). In this current study, *S. occidentalis* root extract was evaluated for antiplasmodial activity *in situ* using the *P. berghei* mouse model of antimalarial drug efficacy testing. In this *in vivo* assay, use of human malaria parasites (*P. falciparum*), just like in the *in vitro* test, would have been more appropriate. However, we did not have access to an animal model for *P. falciparum* antimalarial drug efficacy testing. Alternatively, our laboratory has established protocols for *in vivo* antimalarial drug efficacy testing using *P. berghei* in a mouse model. The structure, life cycle and physiology of *P. berghei* compare with those of the human malaria parasites (56). Accordingly, mouse specific parasite species have been used in Peters' standard test to provide important data in support of antimalarial drug development (57, 58). As such the *P. berghei* mouse model would provide valuable information regarding the *in vivo* antiplasmodial potential of the extract under investigation in the current study. The effect of *S. occidentalis* extract on *P. berghei* parasitemia was determined by monitoring parasite propagation in infected BALB/c mice upon treatment with the extract. The extract significantly suppressed *P. berghei* parasitemia in mice and increased the survival time of the infected animals. The longer survival time of mice treated with the extract corresponded with reduced parasitemia, suggesting that the extract may play a role in reducing the pathologic effects of the infection. A positive correlation between parasite density, disease severity and mortality due to malaria has been described (59, 60). The next step would, therefore, be the analysis of the antimalarial activity of *S. occidentalis* extracts in a non-human primate model for a more complete preclinical testing.

The presence of secondary metabolites in the extract investigated herein may explain the growth suppressive activities of the extract against malaria parasites observed in this study. Plant secondary

metabolites have been shown to induce malaria parasite death through diverse mechanisms. For instance, flavonoids have been shown to possess antioxidant potential and kill *Plasmodium* parasites by chelating nucleic acids of malaria parasites (61, 62). It is also reported elsewhere that antioxidants can inhibit heme polymerization, and that unpolymerized heme is very toxic to *Plasmodium* parasites, hence kills malaria parasites (63). More studies are required to determine the mechanism used by *S. occidentalis* root extract in suppressing *P. falciparum* and *P. berghei* parasites growth.

Conclusions

Overall, our findings suggest that *Senna occidentalis* (L.) Link root extract has remarkable antimalarial activity, *in vitro* and in mice. The plant root, therefore, is a potential source of antimalarial agents. This study has scientifically validated the use of *S. occidentalis* roots by some Kenyan communities as an antimalarial remedy. Isolation and identification of the bioactive entities as well as establishment of the antimalarial mechanism of action of the extract is warranted. Understanding the antimalarial potency of the root extract of this important medicinal plant when used in synergy with conventional antimalarial medicines against which the malaria parasites have developed resistance is also recommended.

Abbreviations

AAALAC: Association for Assessment and Accreditation of Laboratory Animal Care; ARRIVE: Animal Research: Reporting of In Vivo Experiments; ANOVA: Analysis of variance; DMSO: Dimethyl sulfoxide; FBS: Fetal bovine serum; IPR: Institute of Primate Research; ISERC: Institutional Scientific and Ethics Review Committee; KEMRI: Kenya Medical Research Institute; MEM: Minimum Essential Medium Eagle; NACOSTI: National Commission for Science, Technology and Innovation; RBCs: Red blood cells; RPMI: Roswell Park Memorial Institute; WHO: World Health Organization; CC₅₀: 50% cytotoxicity concentration; IC₅₀: 50% inhibitory concentration

Declarations

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Authors contributions

S.M. conducted experiments, formal analysis and wrote the original draft of the manuscript. H.M. performed phytochemical screening. E.K. performed *in vitro* experiments. K.O. supervised the project and edited the manuscript. R.W. supervised the project and edited the manuscript. F.O. contributed in conducting *in vitro* experiments, supervision and manuscript editing. H.O. acquired funding, supervised the project and edited the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets supporting the conclusions of this article are included within the article. The raw data used for analysis can be availed by the corresponding author on request.

Ethics approval

Approval to conduct this study was granted by the Institute of Primate Research (IPR) Institutional Scientific and Ethics Review Committee (ISERC/02/18). The care and handling of experimental mice was done according to the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines. The Institute of Primate Research facility is registered by the National Commission for Science, Technology and Innovation (NACOSTI), Kenya and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International.

Consent for publication

All authors have read and approved the manuscript for submission

Competing interests

There are no competing interests to disclose.

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Figures

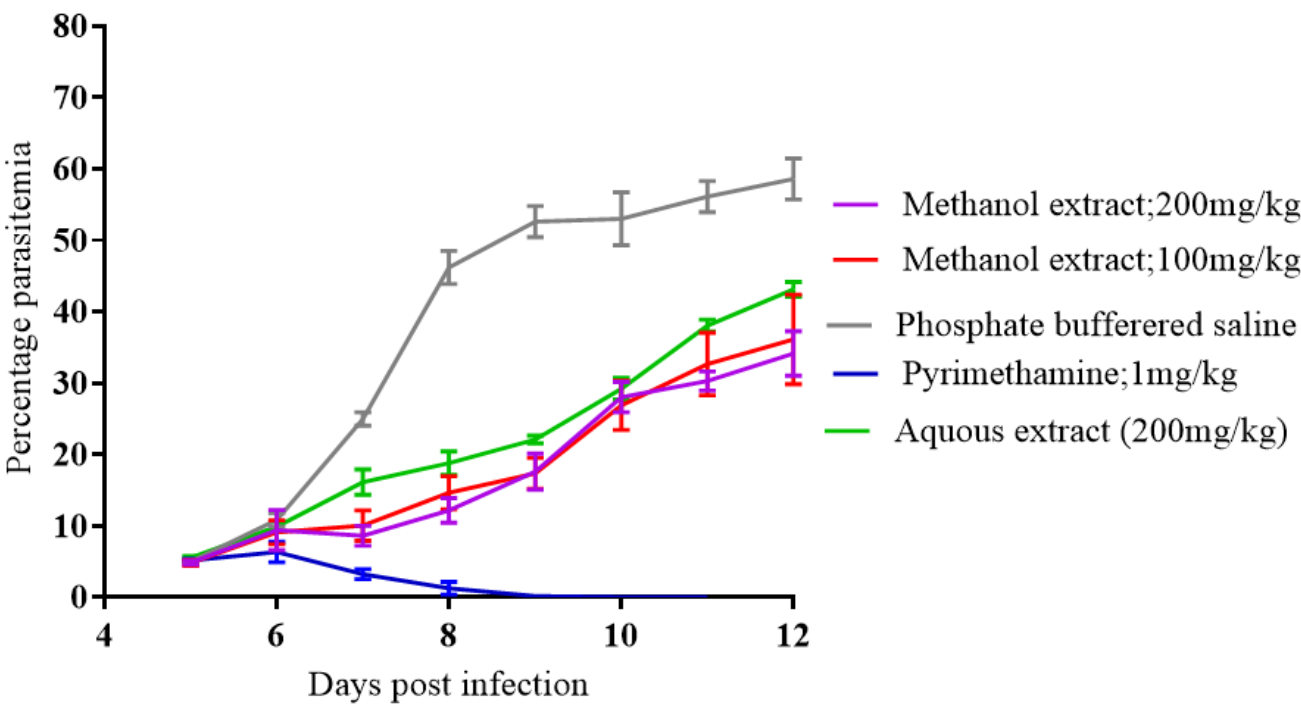


Figure 1

Parasitemic profile of pyrimethamine-sensitive *Plasmodium berghei* 7 days upon extract treatment, in comparison with controls

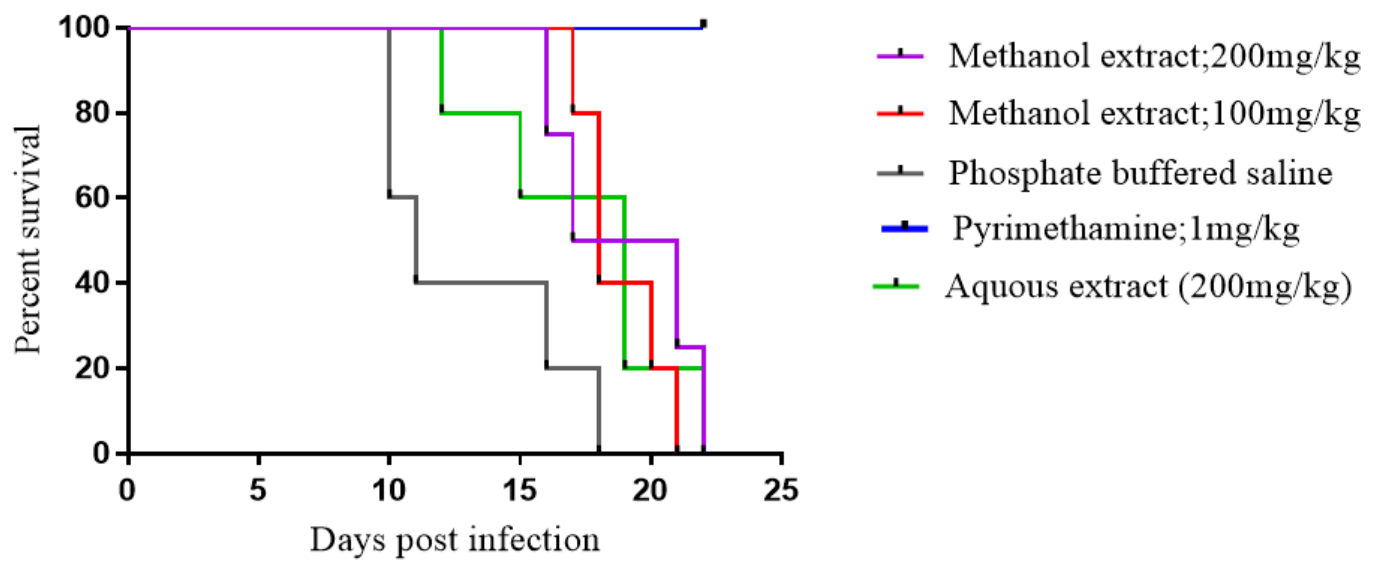


Figure 2

Survivorship curves of *Plasmodium berghei* infected upon treatment with the extracts