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Warburgia ugandensis: A potent *in vivo* phytomedicine against *Plasmodium knowlesi*

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Abstract

Resistance to artemisinin-based therapies by *Plasmodium falciparum* and the entry of *P. knowlesi* as the fifth human malaria parasite present an urgent need for development of safe, affordable and effective therapeutic alternatives. *Warburgia ugandensis*, commonly used in herbal medicine, has displayed remarkable antiplasmodial properties. To be accepted as viable alternatives, herbal medicines must be subjected to the modern rigorous testing and validation procedures as used in convention medicines. This study was designed to determine the efficacy and safety of extracts from *W. ugandensis*. *Plasmodium knowlesi*-infected baboons were treated with an oral dose of 5000 mg/kg-1/birth weight. Changes in parasitaemia, haematology and biochemistry were recorded over a period of 21 days. Data were managed using GraphPad Prism Version 5.00. ANOVA for calculated means between treated and untreated animals (P -value < 0.0001) were separated by Tukeys' Multiple Comparison for significance. Low parasitaemia and increased survivorship were observed in treated animals.

Keywords: Phytomedicine, *Warburgia ugandensis*, *Papio anubis*, *Plasmodium knowlesi*

Introduction

Despite being preventable and treatable, malaria continues to have a devastating impact on people's health and livelihoods around the world particularly in the tropical and subtropical regions, where it disproportionately affects poor and disadvantaged people, who have limited access to health facilities and can barely afford the conventional treatment. Moreover, a robust pipeline of new candidate therapeutic agents are required given the emergence and spread of resistance to current antimalarials. For instance, *P. falciparum* resistance to artemisinin, the current golden treatment, has emerged independently in multiple geographical locations in the Greater Mekong subregion in South-East Asia [1, 2, 3, 4]. The situation is worse along the Cambodia-Thailand border, where *P. falciparum* has become resistant to almost all available antimalarial medicines [3, 5]. Medicinal plants play a key role in the worlds' health care, with about 80% of Africans depending on herbal medicines for treatment of diseases and other ailments. Invariably, most of these herbal products have not been subjected to the rigorous process of scientific validation for efficacy and safety. One such plant that requires urgent scientific validation due its popularity as a herbal medicine in many African communities is *Warburgia ugandensis*. *Warburgia ugandensis* Sprague, a canelaaceae, also known as the East African Greenheart, is a species of evergreen tree native to Africa and a highly valued species within the traditional health systems of the communities where it naturally grows [6, 7]. The species of the genus *Warburgia* are known to be rich in sesquiterpenes [8, 9] which have been shown to possess insect antifeedant, antimicrobial, anticancer, molluscidal and antifungal properties [8, 10, 11, 12, 13]. Medicinally, dried bark of *W. ugandensis* is commonly chewed and the juice swallowed as a remedy for stomachache, constipation, toothache, cough, fever, muscle pains, weak joints and general body pains [12]. The bark, roots or leaves can be boiled in water and the decoction drunk to treat malaria [8]. This study was designed to determine the *in vivo* antiplasmodial activity of methanolic extracts from *W. ugandensis* and the safety of the extract on mammalian tissue using *P. knowlesi* and *P. anubis* as parasite-animal models respectively.

Methods and Material**Chemicals**

All solvents and chemicals used in this study were of analytical grade. All experiments were performed under sterile conditions in a laminar flow. Re-usable glassware was sterilized by auto-claving, with 70% ethanol being used for general sterilization.

Plant materials

Warburgia ugandensis was selected on the basis of its popularity among many Kenyan communities as herbal medicine against malaria and other ailments. The plant materials were collected from Ooloolua Forests, Kajiado County and authenticated at the herbarium, National Museums of Kenya, where voucher specimen were deposited. The stem bark and root barks at the secondary stage of growth were harvested and washed to remove physical impurities; cut into small pieces, air-dried and pulverized. Two hundred grams of ground powder of each was dissolved and macerated in 1000 ml of methanol for 72 hours and filtered. The filtrates were evaporated in vacuo using a rotor evaporator at reduced temperature of 40°C and packaged as 250mg capsules.

Test malaria parasites

The simian malarial parasites, *P. knowlesi* (H strain) were used to test for activity and safety of the extracts in olive baboons (*Papio anubis*). Cryopreserved parasites were retrieved from liquid nitrogen using the Behring-Werk method [14]. Briefly, an ampoule containing parasitized erythrocytes was collected from liquid nitrogen and thawed at 37°C after which the parasites were aseptically transferred into a labelled 50ml sterile centrifuge tube and kept in an overnight culture before use.

Experimental animals

Six health male adult baboons (*P. anubis*) weighing between 19-20kg were used to assess for antiplasmodial activity and safety of the extract. The animals were obtained from the Animal Resources Facility at the Institute of Primate Research (IPR), Kenya. The animals were kept in standard single cages at room temperature and supplied with food and drinking water *ad libitum*. Visual observations for mortality, behavioral pattern, changes in physical appearance, pain and signs of illness were conducted daily and recorded for each individual animal over a period of 21 days.

Antiplasmodial activity and safety of *W. ugandensis*

The antiplasmodial activity and safety of the extracts from *W. ugandensis* was investigated on *P. knowlesi* infected olive baboons. Approximately 1.0×10^6 test parasites were intravenously inoculated into the experimental animals. Once an infection was confirmed (Day 5 Post-inoculation) through daily smears from peripheral blood, a phytomedicine capsule from a methanolic extract of *W. ugandensis* was administered to test animals. A single oral dose of 5000 mg/kg/body weight of the extract was administered to three groups of experimental animals as: infected and treated; infected and not treated; and, not infected but treated. Bleeding for biochemical and was done at three time points over a period of 21 days. The antiplasmodial activity of the extracts was determined by daily parasitaemia profiles over the same

period. In order to determine the safety of the phytomedicine, physical changes, hematological profiles and biochemical alterations were analyzed. Further, histopathological alterations of tissue organs on necropsy were also determined once the animals were euthanized.

Microscopy and parasites suppression

Percentage parasitemia suppression was established by counting the number of parasitized erythrocytes out of 1000 erythrocytes on random fields under the microscope. Infected cells were then expressed as percentage suppression relative to parasitemia level of the control group. The difference between the mean value of the negative control group (taken as 100%) and those of the experimental groups was calculated and expressed as percent reduction (activity) according to Tona *et al.* [15] as follows:

$$\% \text{ Suppression} = \left(\frac{\% \text{ Parasitaemia in control} - \% \text{ Parasitaemia in test}}{\% \text{ Parasitaemia in control}} \right) \times 100\%$$

Statistical analyses

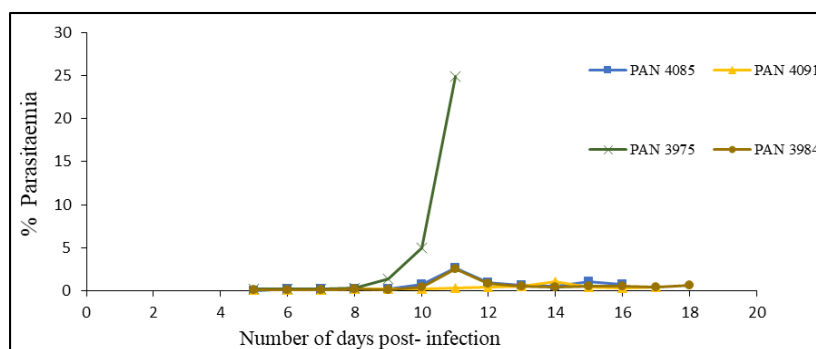
Data were managed and analyzed using GraphPad Prism Version 5.00 and reported as means \pm SEM. When parasitaemia profiles from preclinical studies were subjected to statistic test, significant variation was observed in means between treated and untreated animals. For calculated mean values of 4.60 (PAN 3975), 0.54 (PAN 3984), 0.67 (PAN 4085) and 0.34 (PAN 4091), the variances *P*-value $<$ 0.0001; were significantly different (*P* $<$ 0.05). The Tukeys' Multiple Comparison Test revealed significant difference between PAN 3975 and PAN 4091 (*q* = 4.19) and PAN 3975 and PAN 3984 (*q* = 3.99).

Results

Parasitaemia profiles of *P. knowlesi* infection in olive baboons treated with stem bark extracts from *W. ugandensis*, hematological alterations, biochemistry and gross pathology on necropsy were the parameters used to establish the antiplasmodial activity and safety of the extract on mammalian tissue. The results are presented in Figure 1 and Tables 1 and 2 respectively.

Parasitaemia profiles extract-treated animals

The percentage parasitaemia of PAN 3975 (untreated control) rose exponentially to 25%, killing the animal on the 11th day post inoculation. The parasitaemia levels in treated animals however remained significantly low, reaching a peak of 2% in PAN 3984 and PAN 4085 treated with root and stem bark extract respectively. Lowest parasitaemia were recorded in PAN 4091, whose highest value was recorded as 1.1% on the 14th day PI. All experimental animals were euthanized on the 21st day post infection (Figure 1).



PAN 4085 and PAN 4091 - Infected and treated with extract from stem bark; PAN 3984 - Infected and treated with extract from root bark; PAN 3975 - infected, untreated control; PAN 3763* (not shown) - not infected, treated with stem bark extract.

Fig 1: Activity of *W. ugandensis* on *P. knowlesi*- infected olive baboons

Haematological profiles of extract-treated baboons

All *P. knowlesi*-infected animals presented with varying degrees of anaemia and other haematological alterations. PAN 3982 and PAN 4085 were severely anaemic (Hb < 5 g/dL) with PAN 4091 and 3984 being mildly anaemic (Hb < 11g/dL). PAN 3763 (non-infected but treated) remained non-anaemic, presenting with a mean Hb value of 12.3g/dL. Significant lymphocytes increase resulted following infection and treatment in all experimental animals while the of granulocytes in all treated animals significantly declined (Tables 1).

Table 1: Hematological profiles for extracts-treated *Papio anubis*

PAN	3763			4091			4085			3975			3984			STD Range
	A	B	C	A	B	C	A	B	C	A	B	A	B	C		
WBC	6	13	6	8	8	7	11	7	14	10	7	8	10	15	4-10	
GRA	57	3	5	63	39	6	50	5	12	57	5	64	2	5	40-70	
LYM	31	93	90	26	50	88	40	88	80	34	89	26	94	88	20-45	
HGB	14	12	11	15	16	7	15	12	4	12	11	17	15	6	12-18	
RBC	5	8	5	5	6	3	6	5	1	4	5	6	6	2	4-6	
MCV	79	83	82	87	88	98	84	87	99	93	82	83	87	99	76-98	
MCHC	32	30	29	29	32	26	32	29	25	34	29	32	32	26	31-46	
MCH	25	25	24	26	28	26	27	26	30	31	23	27	27	27	25-35	
HCV	42	39	39	40	49	26	47	40	14	35	38	51	46	22	36-54	
PLT x10 ²	1.9	1.5	1.2	2.8	3.1	1.7	1.9	2.8	1.4	3.0	1.3	2.4	1.7	1.9	1.5-4.5	

PAN 4085 and PAN 4091- Infected and treated with extract from *W. ugandensis* stem bark; PAN 3984- Infected and treated with extract from *W. ugandensis* root bark; PAN 3975 - infected, untreated control; PAN 3763- not infected, treated with extract from *W. ugandensis* stem bark. LYM - lymphocyte count, WBC - white blood cell count, GRA - granulocyte count, HGB - haemoglobin level, MCH- average weight of hemoglobin per red cell; MCV - mean corpuscular volume MCHC-average concentration of hemoglobin per erythrocyte. A, B and C are time points. Before treatment (A); 1 week after treatment (B) and two weeks after treatment (C).

Biochemical profiles of extract-treated animals

All creatinine values and values of liver enzymes; Gamma-glutamyl transpeptidase and Alanine Aminotransferase, as well as serum urea in experimental animals were within normal as indicated in Table 2.

Table 2: Biochemical profiles for extracts-treated *Papio Anubis*

Factor	Range	PAN 3975		PAN 3963		PAN 3984		PAN 4085		PAN 4091	
		A	B	A	B	A	B	A	B	A	B
Urea	2.5-7.8 mmol/L	6	-	6	6	5	7	9	4	7	4
CRT	53-124 mmol/L	81	-	100	109	108	92	46	79	122	79
ALT	7-56 U/L	72	-	43	12	34	63	46	61	18	55
GGT	9-45 U/L	22	-	21	22	18	18	19	26	32	29

CRT-Creatinine; GGT- Gamma-glutamyl transpeptidase; ALT - Alanine Aminotransferase, PAN- *P. anubis*. Urea. A and B are time points before and after treatment.

Discussion

Antiplasmodial activity of *W. ugandensis*

PAN 3975 (untreated control) died by 11th day post infection from high parasitaemia of 24% while parasitaemia levels in treated animals remained significantly low (under 2%) until the 21st day post infection when they were euthanized. This clearly indicates a remarkably high level of antiplasmodial activity in extract-treated animals. Apart from maintaining low parasitaemia levels, the extract also increased survivorship of treated animals for over 10 days. This

observation agrees with our earlier observation [16, 17] in which extracts from the same plant cleared parasites and increased survivorship in *P. berghei*-infected mice and, yielded low *in vitro* IC₅₀ values against *P. knowlesi*.

Gross pathology of extract-treated baboons

Examination of tissues from treated animals showed mild enlargement of spleen, haem metabolism and follicular proliferation. The liver tissues were of normal size though appeared darkened owing to raised haem metabolism. The heart and kidneys were normal, and no obvious lesions were seen in lungs. PAN 3763, the negative control, presented with normal organs: i.e no signs of cardiac atrophy, no signs of nephritis, no indications of haem metabolism, normal liver, pancreas and spleen sizes, non-engorged gall bladder and clear lungs. Overall, no abnormalities were detected (NAD), implying that the extract had no adverse effect on the mammalian tissue. In fact, necropsy examinations of PAN 3975 (infected and not treated) revealed severe hepatosplenomegaly, engorged gall bladder and bile imbibition, proliferation of white follicles, haem metabolism and jaundice. No pathological changes were however observed in the lungs, clearly demonstrating that the animal died due to anaemia, resulting from heavy parasitaemia.

Haematological profiles of extract-treated baboons

All experimental animals that were infected with *P. knowlesi* presented with varying degrees of anaemia and other haematological alterations. Severe anaemia, defined as Hb < 5 g/dL [18] was observed in PAN 3982 and PAN 4085, while PAN 4091 and 3984 were mildly anaemic, Hb < 11g/dL, (18). PAN 3763 (non-infected but treated) remained non-anaemic, presenting with a mean Hb value of 12.3g/dL. Clearly, the observed anaemia and low haematocrit values as well as corresponding low erythrocytes count resulted from the rapture of parasitized RBCs. The sharp rise in parasitaemia observed in PAN 3975 (positive control) as opposed to treated animals strongly displayed the effect of the extract in limiting parasitaemia and increasing animal survivorship. PAN 3975 died early as it suffered hypovolemic shock due to massive destruction of RBCs resulting from exponential parasitaemia levels observed. In terms of differential counts, granulocytes and lymphocyte presented the most important leukocytic changes observed in this study. The levels of granulocytes in all infected animals were significantly low, which is in line with the report of Francis *et al.*, [19]. This observation however, contrasted previous studies that recorded increase in neutrophils attributable to activated neutrophil production and suppressed peripheral removal [20, 21]. It is also not clear why the amount of granulocytes in non-infected but treated animal declined. Moreover, significant lymphocytosis resulted following infection and treatment in all experimental animals, just as was the case in a study by Ourives *et al.*, [22] but in sharp contrast to decrease in peripheral blood lymphocytes usually observed in patients with acute malaria due to lymph nodes sequestration and apoptosis [23, 24]. Interestingly, an increase in lymphocytes was also observed in non-infected treated animal, mechanistically implying that the extract was highly immunostimulatory in its antiplasmodial activity.

Biochemical profiles of extract-treated animals

Creatinine is critically important in assessing renal function [25]. The normal range for creatinine in the blood may be 74.3-107 mmol/dL. In this study, it was observed that all creatinine values fell within normal range for all experimental animals

(Table 2). The reduction in creatinine levels in PAN 4085 points at sudden decrease in activity, arising from massive destruction of RBCs by *P. knowlesi* (H) parasites. On the other hand, serum concentration of urea reflects the balance between production by the liver and elimination by the kidneys. It is therefore one of all important tools of assessing the renal function status [25]. From table 2, all values were within normal range of 2.5 - 7.8 mmol/L, confirming normal liver and kidney function. Further, the normal functioning of the liver in treated animals is indicated by liver enzymes; Gamma-glutamyl transpeptidase (GGT) and Alanine Aminotransferase (ALT). Gamma-glutamyl transpeptidase is concentrated in the liver, but it's also present in the gallbladder, spleen, pancreas, and kidneys whereas ALT is produced in liver. A low level of ALT in the blood is expected and is normal in liver between 7 to 56 IU/L [26, 27]. Mild elevations are generally considered to be 2-3 times higher than the normal range. Very high levels suggest drug-induced hepatitis [26, 27]. In this study, all experimental animals, including PAN 3763 had ALT values within normal range, an indication of normal liver function, confirming safety of extracts.

Conclusion

Treated animals presented with low parasitemia and longer survival times, clearly indicating that the treatment was effective in limiting parasite growth and development of anaemia. Most hematological and biochemical parameters were observed to be within normal ranges, with a few exceptions, though not significantly varied. At the concentration used, the phytomedicine from *W. ugandensis* exhibited significantly high antiplasmodial effects justifying its use in herbal medicine. It also did not display any toxicities on the mammalian tissue implying that its safe for use as herbal medicine. Limitations in this study worthy of mention was the small sample size, single dose and route of administration control which narrowed the scope of comparison. Further assessment could be carried out to determine the optimal concentration of the phytomedicine that would completely kill the *Plasmodium* parasites. Such experiments could be designed based on dose escalation experiments and other treatment regimens as well as different routes of drug administration.

Authors' contributions

PSW: Designed the study, carried out the experimental work and drafted the manuscript. HSO participated in the design and co-supervised the work. WW co-supervised the work. LHK co-supervised the work and provided the overall co-ordination of the study. All authors read and approved the final manuscript.

Conflict of interests

Authors declare no conflict of interests.

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Approval for Animal use

The olive baboons were obtained from the Animal Resources Facility at IPR, Kenya. Ethical approval for animal use was

granted by the Institutional Review Committee (IRC).

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