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ORIGINAL ARTICLE

Prophylactic Effects of ARTAVOL® on *Plasmodium berghei* Infected Mice

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ABSTRACT

Introduction: Despite the efforts of governments and health organisations to eradicate malaria, it is still endemic in sub-Saharan Africa and this could be due to cost of antimalarial drugs, resistance to these drugs and climate change among others. Traditional medicine practitioners and scientists have started developing antimalarial drugs from medicinal plants among which is ARTAVOL®. ARTAVOL® is a herbal product that is used to prevent malaria in some communities in Uganda, however, its prophylactic effects on *Plasmodium berghei* infected mice has not been established yet.

Methods: The infusion of ARTAVOL® powder was prepared, cooled, filtered, concentrated *in vacuo* at 55 ^{0}C and freeze-dried. The freeze-dried extract was reconstituted with distilled water for antimalarial using prophylactic model mice. Thirty-six mice were randomised into 6 groups of 6 mice each. Groups I to III mice were orally administered with the extract at 15 to 60 mg/kg/day while group IV received Pyrimethamine (1.25 mg/kg) while groups V and VI (uninfected) received 0.2 mL distilled water for seven days before the inoculation of *Plasmodium berghei* ANKA parasites on day 7 (D_7). The parasitaemia levels were examined after 72 hours using standard procedure and blood collected through cardiac puncture for haematological study.

Results: The Lethal Dose (LD_{50}) of the crude ARTAVOL extract was greater than 5000 mg/kg. Also, there was calmness and paw licking immediately after dosing which stopped after few minutes. Significant reduction in parasitaemia level was observed in all test doses when compared with negative control. At 30 mg/kg, the extract gave 62.9% suppression, which was not significantly different from that of 60 mg/kg (68.7%) on day 3. On day 5, the extract gave 62.3% and 66.4% Suppressions At 30 And 60 Mg/Kg That Were Not Significantly Different From Each Other. A Dose Dependent Reversal of Hematocrit (HCT) reduction was observed at the 3 dose levels but their parameters did not show any significant difference when compared to the normal group but significant when compared with negative control.

Conclusion: ARTAVOL® extract has shown a dose dependent reducing effect on the level of parasitaemia in *P.berghei* infected mice; it is acutely safe and has ability to increase RBC counts.

Keywords: ARTAVOL®, Parasitaemia, *Plasmodium berghei*, Prophylactic activity

INTRODUCTION

Malaria remains one of the Public Health challenges in Africa[1]. According to WHO malaria report (2018), an estimated 228 million cases of malaria occurred worldwide[2]. The WHO global malaria control programme strategy 2030 is aimed at reducing malaria case incidence by at least 90%, eradicating malaria in at least 35 countries and preventing a resurgence of malaria in countries that are malaria

free[3]. A number of protective interventions are being used in endemic areas to reduce the risk of malaria infection. These include chemoprophylaxis, using conventional medicines recommended by WHO[4, 5]. However, the designed eradication programs have had challenges such as poorly managed Vector Control Programs, emergence of antimalarial drug resistance, poverty and the effect of global warming and climate change among others[6, 7]. Antimalarial drug resistance also poses a challenge to malaria control measures[8-10].

Since 2018, Uganda recorded a surge in the incidence of malaria in some districts due to a number of factors such as climate change, a decline in the use of protective nets and high influx of refugees[11]. A number of strategies are being deployed to control the mosquitoes in Uganda. These include:- use of Long-Lasting Insecticidal Nets (LLINs), Indoor Residual Spraying of Insecticide (IRS), clearing bushes around homesteads and draining of waterlogged ditches around homesteads to prevent mosquito breeding[12].

Traditional medicines have been used to treat malaria for thousands of years and are part of the culture and tradition of African people[5]. It has been reported that up 80% of the human population in developing countries rely on herbal medicinal products as their primary source of healthcare[6]. Many developed and developing countries have also embraced the use of herbal remedies as complementary and alternative medicines[13].

The traditional medical practitioners of Uganda use symptoms like high temperature, shivering, among others in malaria diagnosis[14]. Some people in rural communities in Uganda use herbal plants and plant products to prevent and treat malaria. The most commonly used plants are: - *Aloe* species, *Vernonia amygdalina*, *Azadirachta indica* A Juss, *Moringa oleifera* Lam among others[14-17]. ARTAVOL® is one of the herbal products used in the prevention of malaria in Uganda and was developed by scientists at Natural Chemotherapeutical Research Laboratory and Makerere University[18]. ARTAVOL® is composed of extracts of dried *Artemisia annua*, avocado seed base and lemon grass extract. The artemisinin in the *A. annua* is first removed from the extract by partitioning with petroleum ether and using separating funnel to separate the two-immiscible layers formed. The aqueous layer is further concentrated, and then mixed with avocado seed extract and lemon grass extract for the production of ARTAVOL. The major phytochemical ingredients of ARTAVOL® are coumarins, sterols, triterpenes, flavonoids and lemon grass derivatives[18, 19]. The powder is dissolved in hot water, porridge or hot milk and taken as a beverage to prevent frequent fevers, worm infestation and malaria[18]. Although, ARTAVOL® is being used to prevent malaria, its ability to reduce parasitaemia levels in mice infected with *Plasmodium berghei* has not yet been established. Hence, this research studied the prophylactic effects of ARTAVOL® with a view to providing the missing information on its activity *in vivo* using *Plasmodium berghei*-infected mice.

MATERIALS AND METHODS

ARTAVOL® product was obtained from ARTAVOL LTD (ARTAVAL LTD. P.O. Box. 34 Ntinda, Kampala-Uganda) the manufacturer.

A picture of ARTAVOL® tin.

Malaria parasites

The chloroquine sensitive *Plasmodium berghei* ANKA was obtained from Biodefense and Emerging Infection Research Resources Repository (BEI Resources), United State of America (USA).

Laboratory animals

Adult male and female Swiss albino mice between 18 to 22 grams were obtained from the Animal Laboratory Research, Mbarara University of Science and Technology, Uganda. The mice were fed on grower pellets and had free access to water. The mice were allowed to acclimatise for 2 weeks before the experiments.

Ethical Considerations

Permission to use ARTAVOL® herbal product in the study was obtained from developers of the product. This study also received ethical clearance from Mbarara University Research and Ethics Committee and Uganda National Council for Science and Technology registration number HS465ES. All the animals in the experimental study were treated humanely according the Organization for Economic Co-operation and Development (OECD) guidelines and American Psychological Association[20, 21]

Preparation of Crude ARTAVOL Extracts

The ARTAVOL® extract was prepared using infusion method in which distilled water was boiled at 600c, powered into 173 g powdered ARTAVOL®, and

allowed to stand for 15 minutes. Thereafter, the extract was filtered, concentrated in vacuo at 55 0C and freeze-dried. The aliquot of the stock solution of 100mg/mL concentration was prepared and stored in a fridge 5-8^{0}c. The test doses of 15,30 and 60mg/Kg was separately determined from the stock solution and administered orally to already grouped mice.

Acute toxicity study of the Crude ARTAVOL Extracts

The median Lethal Dose (LD_{50}) of ARTAVOL was determined *in vivo* using the Lorke[22] method. In the first phase, 9 mice were randomly divided into 3 groups of 3 mice each and each group received the extract at 10, 100 and 1000 mg/kg body weight orally (via a feeding cannula). The mice were then observed for signs of adverse effects and mortality for the first 48 hours and then for 12 more days. In the second phase, 4 mice were divided into 4 groups of 1 mouse each and similarly treated but at doses of 1000, 1600, 2900 and 5000 mg/kg orally. The animals were then monitored for any sign of toxicity like stretching, rubbing of nose on the floor and wall of cage, change in body weight and mortality over a period of 24 hours and then 14 days. LD_{50} was calculated using the formula:

$LD_{50} = [A \times B]^{1/2}$ Where A= highest non-lethal dose and B= the lowest lethal dose.

Preparation of malaria parasite Inoculum

Chloroquine (CQ)-sensitive *Plasmodium berghei* ANKA parasites were activated in the mice according to BEI Resources procedures. The vial containing the parasites was defrosted in water bath at 35^{0}C and the lid was wiped with 70% ethanol before opening. About 0.2 mL of inoculum was injected into the donor mouse intraperitoneally and the parasites growth was monitored after 72 hours by preparation of smear from the blood taken from the tail of infected mice. The parasites were maintained by continuous blood passage of the blood collected from the donor mouse into a new group of mice. A standard inoculum of 1×10^7 parasitized erythrocytes was prepared by dilution of blood collected through cardiac puncture from a donor mouse (> 30% parasitaemia) with normal saline and administered intraperitoneally (200 µL) to each test mouse.

Calculation of the inoculum

The percentage parasitaemia of the donor mouse was determined as follows:

Percentage parasitaemia= P.RBC/T.RBC * 100 [23, 24]

Where P.RBC= parasitized red blood cells and T.RBC =Total number of red blood cells counted.

The total number of RBC counted on different fields should be 1000 cells. Using $C_1V_1 = C_2V_2$ and having known the volume of infected blood from the donor, the collected volume from donor mouse was diluted to obtain 2% parasitaemia in infected blood (2% parasitaemia is the recommended concentration of the inoculum). Assuming the percentage of parasitaemia in the donor mouse is 30% and volume of blood collected is 0.5mL; then using the above formula $C_1V_1 = C_2V_2$. C_1 = 30%, V_1= 0.5mL, C_2%, V_2. $C_1V_1 = C_2V_2$= 30% * 0.5/2% * V_2 therefore V_2= 30% * 0.5/2= 7.5mL. Top up 0.5mL to 7mL of PBS to make 7.5mL. The volume needed for all the animals to be inoculated = no of animals x volume needed for each animal. Volume for 30 mice is 30 * 0.2 mL = 6.0 mL.

Prophylactic Test and Determination of Parasitaemia levels.

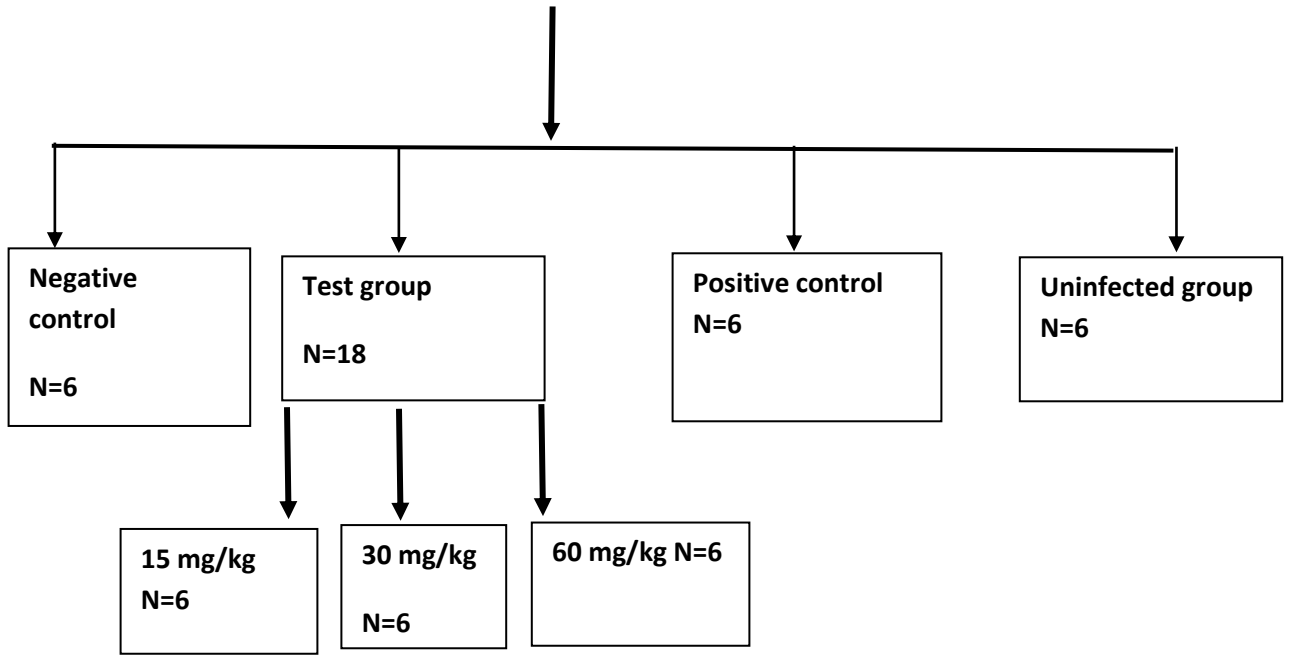
The prophylactic antimalarial potential of ARTAVOL® was carried out according to Peter's method 2002[25]. Thirty six (36) mice were grouped into 6 groups of 6 mice each; Mice in Groups I –III were orally administered with ARTAVOL® by using oral cannula while the animals in Group IV were given Pyrimethamine at 1.25 mg/kg/day (positive control), group V (negative control) and group VI (not infected) received 0.2 ml/mouse/day distilled water for 7 consecutive days ($D_1 - D_7$) respectively. On day 7 (D_7), the mice in group I-V were inoculated with *P. berghei* parasite. On day 3 (72 hours) and day 5 (120 hours) after inoculation, blood samples were collected from the tail vein of the mice and thin blood films on microscope slides were prepared, air-dried and fixed with methanol. The fixed thin smears of blood were stained with 10% Giemsa stain diluted with buffered water (pH = 7.2) for 20 to 30 minutes. The percentage parasitaemia was determined by counting the number of parasitized red blood cells out of 1000 blood cells in 10 randomly selected microscopic fields using oil immersion lens (×100). Percentage parasitaemia level was determined using the formula: Parasitaemia level= $(Np/Nt) \times 100$, where N_p is the number of parasitized red blood cells and N_t is the total number of the red blood cells.

The percentage suppression was determined by:

$$PS = \left\{\frac{X-Y}{X}\right\} \times 100$$[26].

Where 'X'= mean parasitaemia of negative control group, 'Y'= parasitaemia of test group.

FIGURE 1: Schematic diagram of mice grouping for prophylactic study



Determination of Packed Cell Volume, RBC & WBC

On day 6 post inoculation, the mice were sacrificed, blood was collected through cardiac puncture and kept in EDTA tubes to prevent clotting. A hematology analyser (Beckman Coulter AC.T 5Diff – CP by Beckman Coulter Inc, California, USA) was used to measure the PCV, RBC and WBC values.

Statistical analysis

Data was analysed using Graph Pad Prism version 5.0 (GraphPad Software, Inc., San Diego, CA, USA). Results obtained from the study were expressed as mean ± standard error of mean[27]. The variation in a set of data was analysed through One-way Analysis of Variance while the difference among the means was considered at 95% confidence level using the post-hoc method of Tukey's Multiple Comparison Test.

RESULTS

The acute oral toxicity test of aqueous ARTAVOL® extract caused no gross behavioural changes such as loss of appetite, paw licking, body temperature, calmness, locomotion, etc., and there was no mortality within 24 hours as well as in the next 14 days, indicating that the LD_{50} values of the aqueous ARTAVOL® extract were greater than 5000 mg/Kg in mice.

On day 3, the ARTAVOL® extract gave a dose dependent activity by reducing the growth of the parasite at 30 mg/kg to 60 mg/kg from 8.11% to 5.35%, which was significantly different from negative control with 14.4 % (Table 1). In addition, the ARTAVOL® extract gave 62.9% suppression at 30 mg/kg, which was not significantly different from 60 mg/kg with 68.7% suppression on day 3 (Fig.2). On day 5, the extract gave 62.3% suppression at 30 mg/kg, which was not significantly different from 60 mg/kg with 66.4% suppression (Fig 2).

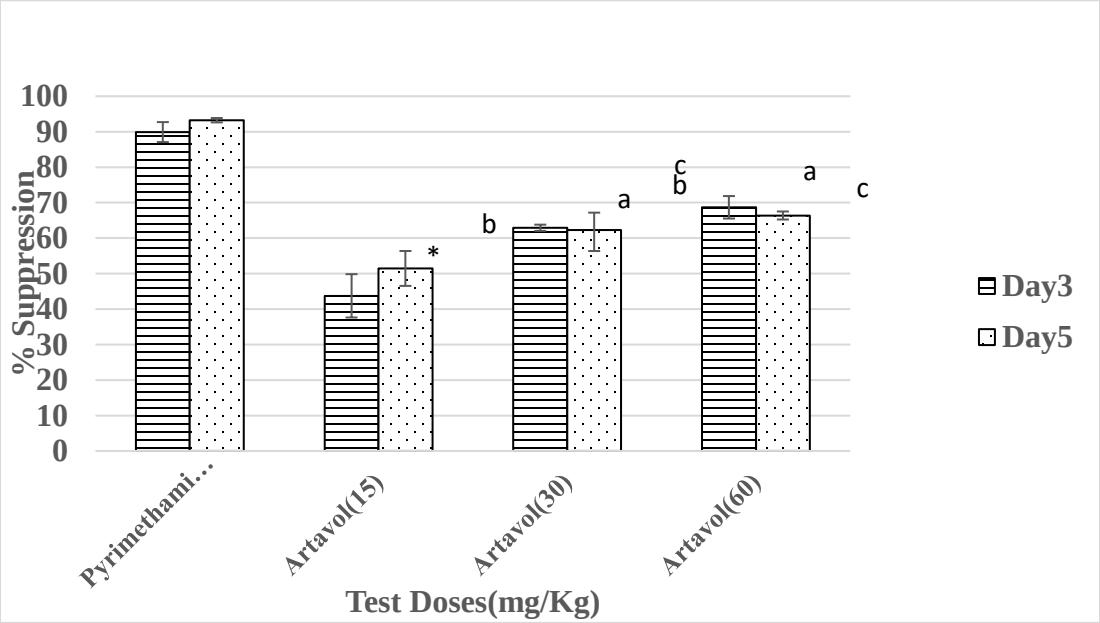
Table 1 shows the parasitaemia level on day 3 and Day 5, Figure 2 shows the percentage parasitaemia suppression in the treated (ARTAVOL® 15-60mg/kg) groups in comparison to positive control group on day 3 and day 5 and Table 2 shows the effect of ARTAVOL® prophylaxis on Monocytes, HCT, and RBC values of P. berghei infected mice.

TABLE 1: The level of Parasitaemia in treated (ARTAVOL® at 15-60mg/kg) and control groups

	Parasitaemia (%)			
	Day 3 after infection		Day 5 after infection	
Test doses (mg/kg)	Parasitaemia	CI	Parasitaemia	CI
Negative Control (0.2mL distilled H_2O)	14.42±0.63	-	23.60 ±2.37	-
Pyrimethamine (1.25)	1.46±0.41***	10.64 -15.28	1.60 ±0.14 ***	16.50 --27.51
ARTAVOL(15)	8.11±0.88***	3.983- 8.624	11.46 ±1.16 ***	6.633 -17.65
ARTAVOL(30)	5.35±0.13**	6.750-11.39	8.91 ±1.40***	8.918- 20.47
ARTAVOL(60)	4.51±0.46**	7.582 -12.22	7.94 ±0.27***	10.16- 21.17

*Values are presented as Mean ± SEM, ***means P<0.0001, ** means P<0.001 when compared to Negative control for parasitaemia*

FIGURE 2: The Percentage parasitaemia suppression in the treated (ARTAVOL® 15-60mg/kg) groups in comparison to positive control group on day 3 and day; Superscripted letters implies P>0.05



All the extract doses significantly reduced parasitaemia when compared to negative, however, the effect was still significantly lower when compared to the positive control drug. The percentage parasitaemia of negative control dramatically increased after Day 3 and Day 5 post treatment with parasitaemia level of 14.42% and 23.60 % respectively (Table 1). The extract showed a relative suppression activity but it was not significant when compared to positive control.

TABLE 1: Effect of ARTAVOL® prophylaxis on Monocytes, HCT, and RBC values of P. berghei infected mice

Treatment Groups	HCT (%)	CI	RBCx10^{12}/L	CI	Monocytes (%)	CI
Normal group	41.80±0.98	-	9.03±0.37	-	12.18±1.62	-
Negative control	19.00±3.73***	9.96-35.63	3.84±0.56***	2.31-8.09	4.37±0.82***	3.62-11.99
Positive control	33.98±2.16[a]	-6.24-21.88	7.68±0.58[a]	-1.82-4.52	5.17±0.75***	2.84-11.19
15mg/Kg	33.15±3.03[a]	-4.19-21.48	6.63±0.90[a]	-0.49-5.29	4.32±0.69***	3.67-12.04
30mg/Kg	39.47±3.77[a]	-12.85-17.52	7.54±0.42[a]	-1.93-4.91	6.77±0.96*	0.46-10.36
60mg/Kg	37.55±0.51[a]	-9.81-18.31	7.43±0.46[a]	-1.56-4.78	8.22±0.86[a]	-0.23-8.14

***P< 0.0001, **P<0.001, *P<0.05, [a]P >0.05 compared to normal group

The 3 test doses of extract in a dose dependent way and positive control were able to reverse the HCT reduction (Table 2) which could have been caused by infection and their parameters did not show any significant difference when compared to the normal group but significant when compared with negative control.

All ARTAVOL® test doses prevented destruction of RBCs (Table 2) due to infection by *P. berghei* and this was not significantly different as compared to normal group but significant when compared to negative control. There was no significant difference in red blood cell count (Table 2) in ARTAVOL® extracts doses when compared positive control but significant when compared to negative control.

The ARTAVOL® extract doses at 15mg/Kg and 30mg/Kg showed a significant rise in the level of monocytes (Table 2) compared to negative control group.

DISCUSSION

The prophylactic anti-plasmodial activity of the aqueous ARTAVOL® extract on *Plasmodium berghei* infected mice produced a dose-dependent activity. The extract elicited suppression activity at the 3 doses investigated. The test doses of ARTAVOL® extract was able to inhibit the growth in the parasitaemia levels and this reduction was significant when compared with the negative group. Despite the overall less suppression effect compared to the positive control group, all test doses of ARTAVOL® extract produced a dose related significant changes in parasitaemia suppression as compared to the negative control group. It has been reported that a compound/product is active when its percentage chemosuppression is more than 30%[28].In this study, the aqueous extract of ARTAVOL® produced chemosuppression in the prophylactic activity with over 62%.

All the test doses of the ARTAVOL extract caused significant increase in monocytes count when compared to negative control group and was not different from the normal group. Widiyantoro[29] reported that secondary metabolites including alkaloids, flavonoids, terpenoids, steroid, phenolic, and saponins stimulate extra release of monocytes in circulation. It was also reported that monocytes are one of the innate immune cells that play a significant role in eliminating parasites through the mechanism of phagocytosis[30-32].

Hematocrit (HCT) values were measured in this study to determine the effectiveness of ARTAVOL® extract in inhibiting hemolysis of the red blood cells (RBCs) and further infection of normal RBCs. The ARTAVOL® extract in this study was able to inhibit further destruction of RBCs in tested mice to level that was significant when compared to the negative control. Many studies have reported that *Plasmodium* infections causes anaemia and this may be due to rapid destruction of infected erythrocytes[33, 34]. The slight increase in HCT values in this study demonstrated a progress in disease regression, which suggests

ARTAVOL® extract could have prevented destruction of RBCs during infection. There was no significant effects of ARTAVOL® extract on RBC counts when compared with the normal group. This implies that ARTAVOL® extract inhibited destruction of red blood cells. This could be probably due to the presence of secondary metabolites including alkaloids, coumarins, flavonoids, sterols, triterpenes, tannins, volatile oils, fatty acids and reducing compounds which were reported to have anti-parasitic effects[18].Flavonoids have also been reported to have antioxidant, anti-inflammation properties and inhibit the parasites protein synthesis[35, 36].

CONCLUSION

In this study, the aqueous ARTAVOL® extract did not show any lethal effect in the tested mice up to 5000mg/kg dose and therefore could be acutely safe. Also, the extract has displayed a dose dependent reducing effect on the level of parasitaemia in the tested mice and could therefore be recommended as an antimalarial prophylaxis. Further investigation on its sub-chronic effect and its immuno-modulatory effects is recommended.

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Competing Interests

None declared.

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