# IMMUNOMODULATORY EFFECTS OF ARTAVOL® IN MALARIA PROPHYLAXIS USING A MOUSE MODEL

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MAY, 2023

## DECLARATION

I Martin Amanya, declare that the work presented in this thesis is my original work that has never been submitted to any University or Institution for an award. Acknowledgment for the use of other people's work has been clearly stated.

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## DEDICATION

I dedicate this Thesis to my parents Mr. Charles Bagatuzayo and Mrs. Constance Bagatuzayo (Late), Mr. Joab Mwebaze (Late) and Mr. Celestine Kambanda

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| ATCC | American Type Culture Collection |
| CD | Cluster of Differentiation |
| CQ | Chloroquine |
| DAMPS | Danger Associated Molecular Patterns |
| DP | Dihydroartemisinin-Piperaquine |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| FACS | Fluorescence-Activated Cell Sorting |
| FCAP | Flow Cytometric Analysis Program |
| HLA | Human Leukocyte Antigen |
| IFN-$\gamma$ | Interferon gamma |
| IgG | Immunoglobulin G |
| IgM | Immunoglobulin M |
| IL | Interleukin |
| $LD_{50}$ | Lethal Dose 50 |
| MHC | Major Histocompatibility Complex |
| MOH | Ministry of Health |
| PAMPS | Pathogen Associated Molecular Patterns |
| RBC | Red Blood Cells |
| SP | Sulfadoxine-Pyrimethamine |
| TMP-SMX | Trimethoprim-Sulfamethoxazole |
| TNF-$\alpha$ | Tumor necrosis Factor- alpha |
| WHO | World Health Organization |

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## DEFINITION OF OPERATIONAL TERMS

ARTAVOL®..... A herbal remedy formulated using extracts de-arteminized *Artemisia annua*, Lemon grass and avocado seeds powder used as malaria in prophylaxis of malaria in Uganda

Chemoprophylaxis.... Use of medicines to prevent diseases

Immunomodulation.....Modification of the immune response or the functioning of the immune system by the action of an immunomodulator

Immunomodulator .....A substance that either enhances or suppress the ability of the body to fight infection and disease

Prophylaxis..... ..... Use of drugs/chemical agents to prevent diseases

## ABSTRACT

**Background:** ARTAVOL® is a herbal product for malaria prophylaxis in Uganda, derived from de-artemisinized extracts of *Artemisia annua* combined with lemon grass (*Cymbopogon citratus*) extract and avocado seed powder as a base. Although, this product is being used widely in Uganda, its acute toxicity, innate and adaptive immune responses in malaria prophylaxis have not been assessed. This study assessed the acute toxicity and hematological effects of ARTAVOL® in malaria prophylaxis, the innate and adaptive immune response activity of the aqueous extract of ARTAVOL® and the flavonoid extract of *A. annua* activity.

**Methods:** The acute toxicity study was assessed using Lorke's method while hematological effects of aqueous ARTAVOL® extract against *Plasmodium berghei* was assessed using a prophylactic test model of Peters and Wright. Ninety-six (96) mice were used for all the study objectives (36 for objective one, 30 for objective two and 30 mice for objective three). The mice were randomized into groups each with 6 mice. Groups I - III were orally administered with the extract at 15 - 60 mg/kg/day while group IV received Pyrimethamine (1.25 mg/kg) as positive control while groups V (infected and negative control) and VI (uninfected group) received 0.2 mL distilled water for seven days before the inoculation of *P. berghei* ANKA parasites on day 7 (D7). The parasitemia levels were examined on day 3 and day 5 using standard procedure, at the end of 7days, all the mice were sacrificed, blood collected through cardiac puncture for hematological study and the spleen collected from each mouse for innate and adaptive immune responses study. The frequency and functionality of innate and adaptive cell immune responses of ARTAVOL® and flavonoids extracts were determined using Cytoflex- LX flow Cytometer by measuring the percentage of expression surface markers (CD4+ T cells, CD8+ T cells, CD 11b,

and CD335) and intracellular markers (IFN-γ, IL-2, IL-4, IL-10, IL-17A and TNF-α). Data was analyzed using FlowJow and GraphPad prism software.

**Results:** Acute toxicity test of ARTAVOL® extract did not show any adverse effect or mortality up to 5000 mg/kg. ARTAVOL® extract showed a dose-dependent suppression of parasite levels at 15, 30 and 60 mg/kg with 43.7, 62.9 and 68.7% on days 3 and 51.4, 62.3 and 66.4 % on day 5, respectively. Compared to the uninfected group (12.18%), mice that were prophylactically treated with ARTAVOL® extract at 60 mg/kg had no significant result in the frequency of expression of monocytes. The ARTAVOL® at 15 to 60 mg/kg increased expression of IFN-γ by natural killer cells from 1.5 to 3.5% and increased expression of TNF-α by monocytes from 2.8 to 11.7%. There was a dose-dependent increase in frequency of expression of CD4 + T cells of ARTAVOL extract at 15, 30 and 60 mg/kg with 31.0, 44.0 and 56.8% activity while there was a dose-independent activity reported in the frequency of expression of CD8+Tcells with 34.6, 42.4 and 25.2%, respectively, though the increase was not significant compared to the negative control.

**Conclusion:** ARTAVOL® did not cause any adverse effect and mortality in all the tested doses in animals and is acutely safe. There was a dose-dependent suppression of the parasites in the activity of ARTAVOL® with an increase in monocytes and natural killer cells count. However, there is need for future studies on effect of ARTAVOL on humoral mediated immune responses malaria prophylaxis, its pharmacokinetic and pharmacodynamic profiles prior to its clinical trial in human to ascertain the innate, adaptive and humoral mediated immune responses.

# CHAPTER ONE

# INTRODUCTION

## 1.1 Background

Malaria remains one of the leading causes of mortality in Africa and the most affected are children under five and pregnant women (WHO, 2021). The World Health Organization (WHO) reported an estimated 14 million malaria clinical cases and about 47000 deaths in the year 2020 (WHO, 2021). According to WHO 2019 world malaria report, Uganda had the 3rd highest global burden of malaria cases (5%), 8th highest level deaths (3%) and highest proportion of malaria cases in East and Southern Africa (World Health Organization, 2020. In Uganda, the Ministry of Health estimates that malaria is responsible for 30–50% of outpatient visits and up to 15–20% hospital admissions(Ogwang et al., 2018). Current prophylaxis against malaria involves the use of conventional medicines such as doxycycline, dihydro-artemisinin-piperaquine (DP), Mefloquine, and trimethoprim-sulfamethoxazole (DeVos and Dunn, 2021). Chemoprophylaxis has been effective in preventing morbidity and mortality from malaria among high risk groups such as pregnant women, infants and children (Tizifa et al., 2018). Tourists travelling to endemic and high risk areas are also advised to take chemoprophylactic agents (Schlagenhauf et al., 2019, Shellvarajah et al., 2017). Studies examining the efficacy of the antimalarial prophylactic agents have found them all to be equally effective in the prevention of malaria in short-term travelers, but Atovaquone-proguanil and doxycycline appear to have the fewest side effects. Mefloquine targets blood schizonts and is effective against chloroquine-resistant *P. falciparum*, though Mefloquine resistance has emerged in parts of Southeast Asia (DeVos and Dunn, 2022). Doxycycline targets blood schizonts and is effective against chloroquine-resistant P. falciparum, however, results in a lack of protection if a traveler misses even a single dose. When taken as

indicated, doxycycline has been shown to have an efficacy rate of 92 to 96% for P. falciparum and 98% for P. vivax (Gaillard, Madamet and Pradines, 2015). There is considerable resistance of parasites to Chloroquine and hydroxychloroquine, and they should only merit consideration for travelers to areas with chloroquine-sensitive *P. falciparum* (the Caribbean, Central America west of the Panama Canal, and some parts of the Middle East. (Lei et al., 2020; DeVos and Dunn, 2022). Primaquine targets liver hypnozoites and can be used as prophylaxis in areas with only *P. vivax*. Primaquine is more common as presumptive antirelapse therapy (PART), where it is taken daily upon returning from a P. vivax or P. ovale endemic area in the final 14 days of post-travel treatment (Ebringer et al., 2011). WHO has also recommended the use of recombinant protein-based malaria vaccine (S/AS01) among children in sub-Saharan Africa and other regions with moderate to high Plasmodium falciparum transmission (Trottier and Elliott, 2021).

Currently, there are no chemoprophylactic agents that can provide 100% protection against malaria and using these agents for long period might facilitate the emergence and spread of resistant drug strains (Rasmussen et al., 2022). With the problems of increasing levels of drug resistance among conventional antimalarials, herbal medicines could be an alternative source of treatment of malaria parasites. The use of herbal medicines has also been embraced in many developed and developing countries (Ekor, 2014) and medicinal plants have been used in the prophylaxis and treatments of malaria in sub-Saharan Africa for many years (Chinsembu, 2015). In Uganda, a number of medicinal plants and plant products used to prevent and treat malaria has been reported. Tibuti (2008) studied the medicinal plants used in malaria in selected villages in Budiope county Uganda. In his work, 27 medicinal plants were reportedly used as antimalarial remedies (Tibuti, 2008). Tugume *et al.* (2016) conducted a survey on 190 species of plants used

for treatment of various ailments in Eastern, Central and Southwestern Uganda, 20 species were commonly used for malaria (Tugume et al., 2016). Anywar et al. (2016) conducted an ethno-botanical survey in Cegere sub-county Uganda. In his report, 12 plants were used for malaria treatment and 8 plants for malaria prevention. ARTAVOL® is one of the herbal plant products that is used to prevent malaria in Uganda. It is composed of de-arteminized *Artemisia annua* (16.7%), *Cymbopogon citratus* (lemon grass) extract (8.3%) which are the active medicinal plants and *Persea americana* (avocado) seed (75.0%) base as the excipient and its major phytochemicals include alkaloids, coumarins, flavonoids, sterols and triterpenes (Ogwang et al., 2011). It was de-arteminized to avoid parasite resistance to artemisinin and dihydroartemisinin one of the phytochemicals in *Artemisia annua*. ARTAVOL® is taking as prophylactic agent through infusion preparation in hot water, porridge or hot milk and taken as a beverage. ARTAVOL® can be taken once daily for two consecutive weeks and there after once a week for people in endemic areas (Ogwang et al., 2011). Despite its general use, there was no information on its immunomodulatory activity as a prophylactic agent which was reported in this study. The manufacturer also claimed that flavonoids could be the main active ingredient in ARTAVOL® responsible for activity after de-arteminizing the *A. annua*, hence the reason for investigating the immunomodulatory effect of flavonoids in this study.

## 1.2 Statement of the Problem

In 2020, Uganda had the highest proportion of malaria morbidities (23.2%) in East and Southern Africa (WHO, 2021). Both conventional and alternative herbal medicines have been used to prevent malaria and their efficacy is reported to be influenced by the immune system activation. ARTAVOL® has been in use as a prophylactic agent for malaria since 2008 and is widely distributed in Ugandan drug shops and pharmacies. However, its effects on innate and adaptive immune responses has neither been established nor documented.

Hence, this study focused on evaluating prophylactic effects on P. berghei ANKA-infected mice and assessing immunomodulatory effects of ARTAVOL® in malaria prophylaxis with a view to addressing the missing information.

## 1.3 Significance of the study

ARTAVOL® is currently being used by some people in Uganda to prevent and reduce the severity of malaria infection. Understanding the immunological events that occur after prophylactic treatment with ARTAVOL® will expand our knowledge on specific immune responses to *Plasmodium* antigens and thus facilitate exploitation of these responses to expedite progress in the development of this herbal product.

Assessing the immunomodulatory effects of ARTAVOL® in malaria prophylaxis will also offer an additional understanding on its possible mechanism of action, enable its further development and potential isolation of active compounds. The key findings of this study will contribute new knowledge and understanding of this products mode of action.

The findings of this research will also benefit the people who are already using this product to prevent malaria. Additionally, the findings of this research will contribute the existing literature hence increase on the volume of published scientific information available for this product.

Lastly, the findings of this research will help the future researchers to explore other capabilities of this product and others in malaria prophylaxis.

### 1.4.Hypothesis

**Null hypothesis**:

ARTAVOL® and flavonoid extracts do not increase the frequency and function of CD4+T cells and CD8+ T cells repertoire in *Plasmodium berghei* infected mice.

**Alternative hypothesis:**

ARTAVOL® and flavonoid extracts increases the frequency and function of CD4+T cells and CD8+ T cells repertoire in *Plasmodium berghei* infected mice.

## 1.5 Research questions

1. At what dose could ARTAVOL® cause mortality in the animals?
2. What are the hematological effects of ARTAVOL® in *Plasmodium berghei* prophylaxis in mice?
3. What are the innate immune responses of ARTAVOL® and flavonoid extracts in *Plasmodium berghei* prophylaxis in mice?
4. What are the adaptive immune responses of ARTAVOL® and flavonoid extracts in *Plasmodium berghei* prophylaxis in mice?

### 1.6 OBJECTIVES

#### 1.6.1 Main objective:

The main objective of this study is to evaluate the immunomodulatory activity of the aqueous ARTAVOL® extract in malaria prophylaxis.

### 1.6.2 Specific objectives:

The specific objectives of the study are to:

a. Evaluate the acute toxicity and hematological effects of ARTAVOL® in *Plasmodium berghei* prophylaxis.
b. Assess innate immune responses in mice infected with *Plasmodium berghei* after prophylactic treatment with ARTAVOL® and flavonoid extracts
c. Determine the adaptive immune response in mice infected with *Plasmodium berghei* after prophylactic treatment with ARTAVOL® and flavonoid extracts.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Etiology and pathogenesis of malaria

Malaria is a protozoan parasite of the genus Plasmodium transmitted by female anopheles' mosquitoes (Cox and vectors, 2010). More than 100 species of Plasmodium infect numerous species such as birds, reptiles and various mammals but only four species of parasites infect humans (*P. falciparum, P. malariae, P.ovale* and *P.vivax*) under natural conditions (Tuteja, 2007). The zoonotic *P.knowlesi* affects both humans and monkeys (Antinori et al., 2013). The rodent parasites include: *P. berghei, P.chabaudi, P. yoeli* and *P.vinckei* but they do not infect humans (Gowda and Wu, 2018).

Malaria is initiated when sporozoites are injected into a vertebrate host from the saliva of infected female anopheles 'mosquito (Pimenta et al., 1994). The sporozoites are transported via the blood to the liver where they invade hepatocytes which are the sites for schizogony (Douglas et al., 2015). The intrahepatic sporozoites develop into schizonts. The fully differentiated schizonts rupture and release thousands of merozoites that invade erythrocytes to initiate the erythrocytic phase of infection. Some merozoites infect other red blood cells and some develop into asexual forms (gametocytes) which are taken by anopheles' mosquitoes to start another cycle. The entire process takes 5-7days for parasites infecting humans and 2days for parasites infecting rodents. The first symptoms of malaria are associated with rupture of erythrocytes when erythrocytic stage schizonts mature (Lindner et al., 2012).

Clinical symptoms such as fever, chills and sweat may develop a few days after an infected mosquito bite. The disease can in some cases result into life threatening complications such as severe anemia and cerebral malaria.

## 2.2 Immune responses to Plasmodium

### 2.2.1 Innate immune responses to malaria

In trying to fight against the parasites, the host releases a number of pro and anti-inflammatory cytokines, chemokines, growth factors and effector molecules as part of the innate immune response. The innate immune responses limit the initial phase of parasite replication and controls the first wave of parasitemia and allows the host to develop specific adaptive immune responses (Gowda and Wu, 2018, Stevenson and Riley, 2004). Innate immune response detects malaria parasites both at liver and blood stages (Beutler, 2004). It responds by inducing pro-inflammatory cytokines and chemokines (Gowda and Wu, 2018). Cytokines prime phagocytes (monocytes and natural killer cells to clear uptake parasites and also help to recruit effector cells to the target sites (Dobbs et al., 2020). The host responds by sensing certain evolutionary conserved molecules of the pathogens called pathogen-associated molecular patterns (PAMPs) through receptors called pathogen recognition receptors (PRRs) (Beutler and Hoffmann, 2004, Vijay, 2018). Prominent transmembrane pathogen recognition is Toll like receptors, C-type lectin receptors, Scavenger receptors such as CD36, CD 204 (Kawai, Kawasaki and Kawai, 2014, Wu and Chen, 2014). These receptors are present on outer surface membrane, luminal surface of endosomal membrane, outer membrane of mitochondria and in the cytosol. The host also detects certain endogenous factors called danger associated molecular patterns (DAMPs) released during infection (Rubartelli and Lotze, 2007, Vénéreau et al., 2015). After recognition of PAMPs and DAMPs by PRRs the innate immune response is activated.

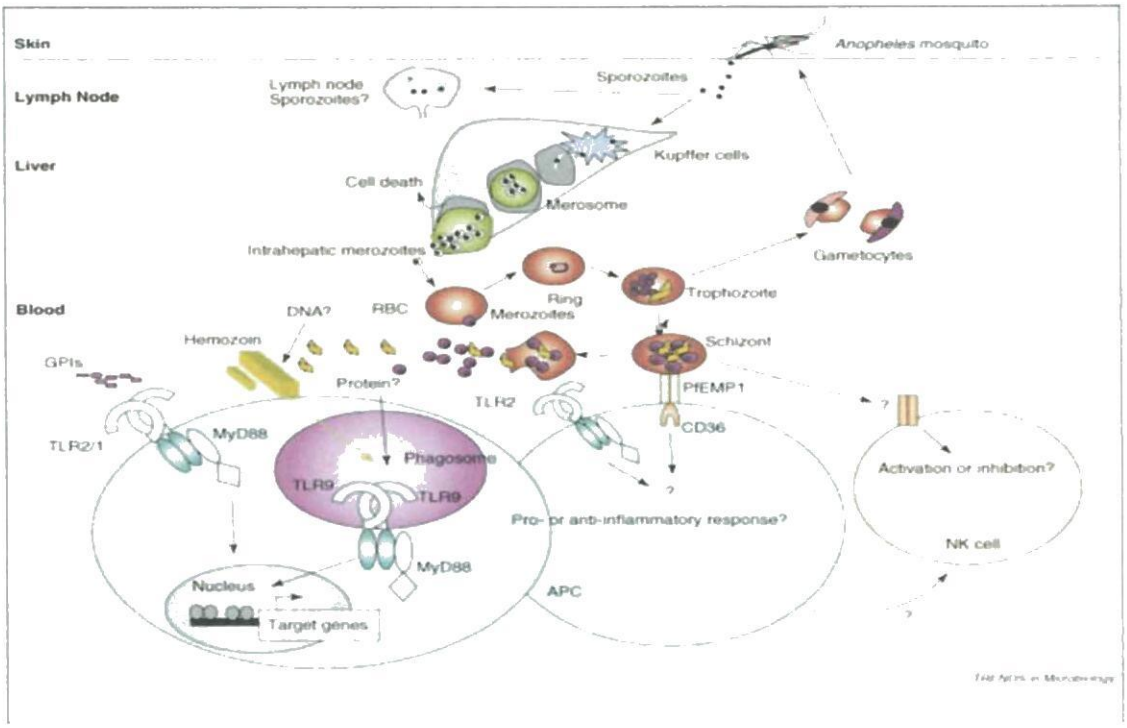



**Figure 1:** Life cycle of *Plasmodium falciparum* in a vertebrate host and its recognition by the innate immune system. Adopted from the Trends in microbiology.Vol.15 No.6 (Coban et al., 2007).

### 2.2.2 Adaptive Immune Responses to Malaria

The adaptive immune responses involve both T and B lymphocytes and these come into play at different parasite attack. The link between innate and adaptive immune responses are dendritic cells (DCs) (Gomes et al., 2016). Also chemokines and chemotactic cytokines play important roles in bridging the innate and adaptive immune systems (Boström et al., 2012). The dendritic cells have a function of antigen presentation and stimulates the T cells which are major mediators of adaptive immune response (Gowda and Wu, 2018). CD4+ T cells play a critical role in priming phagocytic cells to capture and kill malaria parasites as well as helping B cells to produce functional anti-parasitic immunoglobulins (Imai et al., 2015, Kumar et al., 2020). During malaria infection CD8+ T cells are activated and the activated CD8+ T cells primes the

release of interferon gamma (IFN-γ) that enhances the phagocytosis of parasitized cells (Imai et al., 2015).

### 2.2.3 Role of cytokines during malaria infection

During malaria infection, pro-inflammatory cytokines produced include: - Interleukin-4 (IL-4), interleukin-8 (IL-8), interleukin-10 (IL-10), interleukin-12 (IL-12) and interferon (IFN)-γ, tumor necrosis factor (TNF)-α (Jason et al., 2001). In acute malarial infection interferon (IFN)-γ and tumor necrosis factor (TNF)-α are released into circulation (Gun et al., 2014). Interleukin-10 (IL-10), interferon (IFN)-γ and tumor necrosis factor (TNF)-α help to minimize parasitemia and preserve erythropoiesis (Angulo et al., 2002). TNF-α is critical for parasite killing and preventing parasite replication. TNF-α also mediates its effects by inducing production of macrophage migration inhibitory factor (MIF) and release of nitric oxide which also has a direct parasite killing effect (Perkins et al., 2011).

### 2.2.4 Humoral Responses to Malaria

During malaria infection marked changes in IgG and IgM levels occur. Antibodies are directed against the sexual stages of plasmodium, block the invasion of sporozoites into hepatocytes and merozoites into erythrocytes (Fabra-García et al., 2022). Antibody mediated protection is primarily mediated by cytosolic immunoglobulins G (IgG) which activates the cytotoxic and phagocytic functions of neutrophils and monocytes (Bournazos et al., 2020). Malaria infection also involves elevated production of IgE antibodies (Perlmann and Troye-Blomberg, 2000). Antibodies produced in humoral response mediates protection against the parasites in blood stages. Antibodies also limit the growth of parasites in blood by blocking erythrocyte invasion (Persson et al., 2013), opsonizing parasitized erythrocytes for phagocytic clearance (Hill et al., 2013), monocyte-mediated antibody dependent cellular killing (Bouharoun-Tayoun et al., 1995)

and complement mediated lysis (Boyle et al., 2015) as well as interfering with adherence of erythrocytes to vascular endothelium. Plasmodium species have evolved biomolecular strategies to escape immune responses and survive within the host (Gomes et al., 2016). The strategies include the clonal antigenic variation (Scherf et al., 2008) and interfering with induction of B cell memory. This pauses a great challenge for developing more effective drugs to overcome the various immune evasion strategies and parasite antigenicity.

### 2.3 Current Prevention and Treatment of malaria

The global strategy for malaria prevention and control according to WHO (2021) is reduce the morbidity, mortality and the long term vision of malaria eradication (WHO, 2021). Globally, malaria prevention has mainly relied on the vector control using insecticide treated mosquito nets (ITNs) and indoor residual spraying (WHO, 2020).

The WHO recommends that patients in endemic areas be treated within 24 hours after the first symptoms appear. The treatment of patients with malaria depends on the country's national guidelines which considers the species of the infecting parasites, clinical status of the patient, co-morbidities, pregnancy, drug allergies and other drugs taken by the patient, and drug resistance (Fischer, 2022, WHO, 2021). In Uganda, the ministry of health (MOH) has provided free insecticide treated mosquito nets to children under 5 years, pregnant women and other vulnerable groups since 2006 and in 2020 the government of Uganda through Ministry of Health distributed 27.5 million long lasting insecticidal nets (LLINs) in 133 districts across the country (MOH, 2020). The Ministry of Health has also been issuing clinical guidelines on the management of uncomplicated and complicated malaria among the vulnerable groups in Uganda (MOH, 2016).

Chemoprophylaxis is effective in preventing mortality and morbidity from malaria especially among high-risk groups such as pregnant women, infants and children (Tizifa et al., 2018).

Tourists travelling to endemic areas are advised to take prophylactic antimalarial drugs (Mabayoje and Longley, 2021). However, no chemoprophylaxis regime provides 100% protection and it is difficult to sustain for prolonged periods because it may interfere with the development of naturally acquired immunity and will facilitate the emergence and spread of drug resistant strains (Agudelo Higuita et al., 2021). The treatment of malaria with conventional medicines depends on the type of the parasite causing the infection and severity of the disease. The drugs recommended for treatment of uncomplicated malaria include: - Artemisinin-based combination treatments (e.g., artemether-lumefantrine, artesunate-amodiaquine), chloroquine, doxycycline, mefloquine and quinine (WHO, 2022) although Chloroquine and Mefloquine are no longer effective in some or many parts in the world. The WHO also recommends parenteral artesunate for severe *P. falciparum* malaria in both children and adults, however, if artesunate is not available, artemether and quinine are acceptable alternatives for severe malaria.

**2.4 Epidemiology of Malaria in Uganda**

All species of human malaria parasites do exist in Uganda but *Plasmodium falciparum* is responsible for the majority of the cases diagnosed (Yeka et al., 2012). A total of 158,188 malaria cases were reported in January 2021. The highest positivity rates were reported in Busia (83%), Pader (82%), Kaliro (73%), Namayingo (73%), Kayunga (70%), Luuka (70%) and Amolatar (68%) (MOH, 2021).

**Map of Uganda showing malaria endemicity**

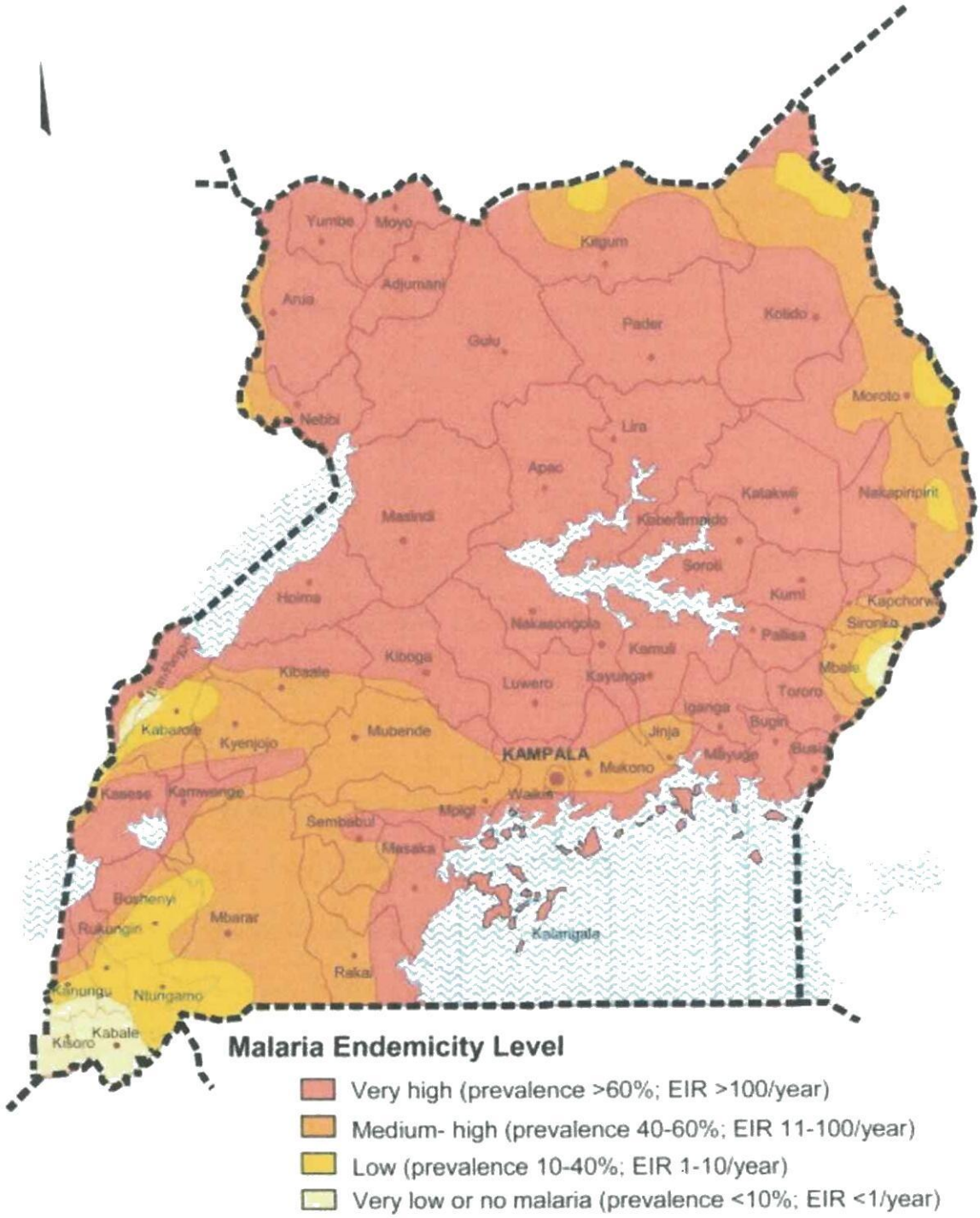



**Fig. 2. Malaria endemicity in Uganda (Uganda Bureau of Statistics,2014)**

## 2.5 Economic burden of Malaria

The economic burden of malaria affects households, health systems and economic development and growth (Alonso et al., 2019). The costs of prevention, treatment and loss of productivity as a result of malaria morbidity and mortality represents a significant portion of the annual income of poor agricultural households (Alonso et al., 2019, Pan and Singhal, 2019,). According to the WHO malaria report 2020, there were 241 million reported cases of malaria with estimated deaths at 627,000. Sub-Saharan Africa had a high share of the global malaria burden with 95% of all the reported cases of malaria and 96% deaths (WHO, 2021). Children under 5 years of age accounted for about 80% of all malaria deaths in the region. Endemic malaria distresses economies of low developing countries, at a country level there is increased government expenditure on control and treatment and reduced economic development. Malaria causes a negative impact on the economic growth and attempts should be made to reduce burden by developing new efficacious drugs.

## 2.6 Antimalarial drug resistance

The emergence and spread of resistance to antimalarials is a major health problem in sub-Saharan Africa, a region with highest morbidity and mortality (Enato and Okhamafe, 2015).

The emergence of resistance in *Plasmodium* depends on multiple factors, including (i) the mutation rate of the parasite, (ii) the fitness costs associated with the resistance mutations, (iii) the overall parasite load, (iv) the strength of drug selection, and (v) the treatment compliance (Petersen, Eastman and Lanzer, 2011). Antimalarial resistance has been observed for all human plasmodium parasites (P. falciparum, P. vivax, P. malarie and P. ovale). Quinine resistance is associated with polymorphisms in several transporters (Cui et al., 2015). Resistance of Malaria Parasites to Antifolates is caused by point mutations in dihydrofolate reductase and

dihydropteroate synthase, the two key enzymes in the folate biosynthetic pathway that are targeted by the anti-folates (Gregson and Plowe, 2005).

Resistance to chloroquine (CQ) and Sulfadoxine-Pyrimethamine (SP) led the World Health Organization (WHO) to recommend changes in national drug policies (Flegg et al., 2013).

WHO also recommends that national malaria programmes adopt antimalarial medicines with a parasitological cure rate of more than 95% (WHO, 2015). The global technical strategy for malaria 2016-2030 was adopted by World Health assembly in 2015. It provides a comprehensive framework to guide countries in their efforts towards elimination of malaria.ie. reducing global malaria incidence and mortality by at least 90% by 2030 (WHO, 2015). The strategy also centers on access to malaria testing and treatment, enhanced surveillance and continued research and innovation among others. Due to the increasing problem of drug resistance towards *Plasmodium* species, there is urgent need to screen and develop new lead compounds to treat this disease. ARTAVOL® was de-arteminized to avoid parasite resistance to artemisinin one of the phytochemicals in *Artemisia annua*.

### 2.7 Antimalarial herbal plants as alternative drugs

Medicinal plants are resources of traditional medicines and many of the modern medicines have been produced directly or indirectly from plants (Hosseinzadeh et al., 2015). It has been reported by WHO that herbal medicines serve the health needs of about 80% of the world's population especially for millions of people living in rural areas of developing countries (Ozioma and Chinwe, 2019, WHO, 2015). Similarly, developed countries have adopted the use of alternative and complementary medicines including herbal medicines in their health care systems.

## 2.8 Medicinal plants used for prevention and treatment of malaria in Uganda.

Throughout history, plants have been a source of medicines and nutrient supplement for both humans and animals. It has been reported that four out of five people in Uganda seek care from traditional herbalists (Schultz et al., 2020). In a review study conducted by Clement et al. (2020), fifteen medicinal plants were found to be commonly used to control malaria in Uganda Clement et al. (2020). The plants are either used as bark, roots, leaves or whole plants. Extractions from these plants are prepared from single species or in combinations, the remedies are prepared as decoctions or infusions and administered in variable doses over varied period of time. Several others studies on the use of medicinal plants for prevention and treatment of malaria in Uganda have also been carried out and these include; Katuura et al. (2007) in Mbarara and Rwampara; Tabuti (2008) in Budiope county; Phillip et al. (2017) in Butebo county in Palisa; Anywar et al. (2015) in Cegere county and Tugume et al. (2016) in central and western regions (Anywar et al., 2015, Katuura et al., 2007, Philip et al., 2017, Tabuti, 2008, Tugume et al., 2016). Some of the plants investigated for their antimalarial/anti-plasmodial activity have been found to lack efficacy and safety aspects (Okello et al., 2019). Approximately 182 plant species used for treatment of malaria in Uganda have been documented. Among these, 112 plant species, including *Artemisia annua,* were reported to have been investigated for anti-malarial activities, with 96% showing positive results.

## 2.9 Use of ARTAVOL® in prevention of malaria

According to the National Drug Authority guidelines on clinical research on herbal medicinal products 2020, a herbal medicinal product is a finished, labelled herbal medicine product that contains active ingredients aerial or underground parts of plants or other plant materials or a combination of these (NDA 2020). There are few registered herbal medicinal products used in

prophylaxis of malaria. ARTAVOL® is a herbal product composed of extracts of de-arteminized *Artemisia annua*, lemon grass and avocado seed base (Ogwang et al., 2011). Its main phytochemical constituents include alkaloids, coumarins, flavonoids, sterols and triterpenes, tannins, volatile oils and fatty acids.

### 2.10. Safety of herbal medicines

Traditional or folk medicine comprises practices, approaches, knowledge and beliefs not based on scientific evidence that are applied to treat, diagnose and prevent illness within a society (Moreira et al., 2014). There is no scientific rationale to assume that plants, their parts and/or derived products, including those of long-standing popular use, are intrinsically safe and/or beneficial; or that, compared to conventional medicines, they would require fewer and simpler pre-clinical and/or clinical studies (Moreira et al., 2014; Saad et al., 2017). Uganda National Drug Authority certifies all medicines, regardless of their origin and development to meet the safety and efficacy standards before they are allowed in Ugandan drug shops and pharmacies. However, herbal products continue to be widely sold and consumed as herbal and dietary supplements, a category of consumer products more loosely regulated by the Uganda National Drug Authority.

# CHAPTER THREE

# METHODOLOGY

## 3.1 Study design

The study was a laboratory based experimental design.

## 3.2 Study area

The study was carried out at the Pharmacy analytical laboratory and Animal house facility of Mbarara University of Science and Technology and Department of Immunology laboratory, Collage of Health Sciences, Makerere University.

This study was carried out on ARTAVOL® a malarial herbal product that is being used in malaria prophylaxis in Uganda. The product with the same batch number to ensure consistency was purchased from the manufacturers in Kampala and was prophylactically given to Swiss albino mice and there after infected with *Plasmodium berghei* ANKA parasites.

## 3.3 Evaluation of the hematological effects of ARTAVOL® in malaria prophylaxis

### 3.3. 1 Study population

Adult male and female Swiss albino mice between 18 to 22 grams (8-10 weeks) totaling 96 used for this study 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 acclimatize for 2 weeks before the experiments.

### 3.3.2 Sample size determination

The number of animals in each study group was calculated using the formula: **n= Log β/Log p** (National Research Council, 2010) where **β** is the chosen power (usually 0.10 or 0.05) and **p** represents the proportion of the animals in the group that are not infected. Assuming that 40% of the animals are infected and there is a 95% chance of detecting that infection, the number, n, of animals was calculated as follows:

**Number of animals (n)= $\frac{log0.05}{log0.6}$ =5.8. ≈6 animals**

Six (6) animals were involved in each study objective. The mice were randomly selected for the inoculation without bias based on sexes before they were grouped into six mice per group (3males and 3 females). Test groups I-III, the positive control group IV, Group V for negative control and Group VI for uninfected group.

### 3.3.3 Inclusion criteria

Healthy, adult mice with the weight between 18-22g (8-10weeks) were used in the study.

### 3.3.4. Exclusion criteria

Mice with weight below 18g or above 22g were not be included in the study.

### 3.3.5 Sampling method

Randomization was done for allocation of experimental and control groups, cages and treatments (Kilkenny et al., 2009). A simple random sampling method was used in which 36 small papers containing equal numbers of 1-5 was put in a tin. After shaking the tin, each paper was picked and if it had number 3, the first animal was assigned to group 3 and the 2$^{nd}$ animal was assigned to group 2 if the picked paper had number 2. The exercise continued until all the papers were picked and all the mice were randomly assigned to the respective groups. In order to determine which, cage a group belong to, the cages were labelled 1-5. The cages were also randomly selected.

### 3.3.6 Preparation of Crude ARTAVOL Extracts

About 173 g of ARTAVOL® with batch no: 0028 was purchased from the manufacturer; Artavol® Ltd, Kampala, Uganda. ARTAVOL® extract was prepared using infusion method in which 60 mL of distilled water was boiled at 80 °C, poured into 173 g powdered ARTAVOL®, and allowed to stand for 15 minutes. Thereafter, the extract was filtered, concentrated *in vacuo* at 55 °C and freeze-dried. The aliquot of the stock solution of 5 mg/mL concentration was prepared and stored in a refrigerator below 4°C. The test doses of 15, 30 and 60 mg/kg was separately

determined from the stock solution to have a final volume of 60, 120 and 240 µL and administered orally to already grouped mice.

### 3.3.7 Acute toxicity study of the Crude ARTAVOL Extracts

The median lethal dose ($LD_{50}$) of ARTAVOL®, was determined in vivo using Lorke's method (Lorke, 1983). In the first phase, nine mice were randomly divided into three groups of three mice each and each group received the extract at 10, 100 and 1000 mg/kg body weight orally. 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 following the same Lorke's method, three mice were divided into 3groups of 1 mouse each. The animals were given higher doses (1600, 2900 and 5000 mg/kg) of the test extract. 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:

$$\mathbf{LD_{50} = [D_0 \times D_{100}]^{1/2}}$$

Where $D_0$ = highest that caused no mortality and $D_{100}$ = the lowest dose that produced mortality (Lorke, 1983).

### 3.3.8 Preparation of malaria parasite Inoculum

Chloroquine (CQ)-sensitive *Plasmodium berghei* ANKA parasites were activated in the mice according to Biodefense and Emerging Infections Research Resources Repository (BEI Resources) user reference. About 200 µL of the 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 mouse. A standard inoculum of $1\times10^7$ parasitized erythrocytes was prepared by dilution of blood collected through

cardiac puncture from a donor mouse (> 30% parasitemia) with normal saline and administered intraperitoneally (200 µL) to each test mouse.

### 3.3.9 Calculation of the inoculum

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

**Percentage parasitemia=** $(\boldsymbol{P.RBC/T.RBC}) * \mathbf{100}$ (Adetutu et al., 2020, SUNITA and SATISH, 2020). 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 not be below 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% parasitemia in infected blood (2% parasitemia is the recommended concentration of the inoculum). Assuming the percentage of parasitemia in the donor mouse is 30% and volume of blood collected is 0.5mL; then using the above formula;

$C_1V_1 = C_2V_2$. $C_1 = 30\%$, $V_1 = 0.5$mL, $C_2 = 2\%$, $V_2 = ?$

Therefore, $V_2 = 30\% * 0.5/2 = 7.5$mL

To prepare 2% inoculum the blood collected from donor mice of 0.5 mL should be topped up with 7 mL of PBS to make 7.5mL.

Hence, the volume needed for all the animals to be inoculated = number of animals' x volume needed for each animal. Volume for 30 mice is 30 * 0.2 mL = 6.0 mL.

### 3.3.10 Prophylactic Test and Determination of parasitemia levels.

The prophylactic antimalarial potential of ARTAVOL® was carried out according to Peter's method 2002 (Peters et al., 2002). Thirty-six (36) mice were grouped into 6 groups of 6 mice each; Mice in Groups I –III were orally administered with 15, 30 and 60mg/kg of 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 (D1 – D7) respectively. On day 7 (D7), 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 parasitemia 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 parasitemia level was determined using the formula:

**% Parasitemia = [Number of parasitized red blood cells/ Total number of red blood cells] x 100** (Adetutu et al., 2016).

The percentage suppression was determined by: **% suppression = [% parasitemia in Negative control - % parasitemia in study groups] / [% parasitemia in Negative control] x 100** (Misganaw et al., 2019).

**3.3.11 Determination of hematological parameters.**

On day 6 post inoculation, the mice were sacrificed, blood was collected through cardiac puncture and kept in EDTA tubes to prevent clotting. The hematological parameters including Packed cell volume (PCV), Red blood cell (RBC) and white blood count (WBC) values were determined using automated hematological analyzer (Beckman Coulter AC. T 5Diff – CP by Beckman Coulter Inc, California, USA).

### 3.4 Assessment of the immune responses

#### 3.4.1 Determination of the effect of ARTAVOL® aqueous extract on innate and adaptive immune responses in mice.

Thirty (30) mice were grouped into 5 groups of 6 mice each; Mice in Groups I –III were orally administered with ARTAVOL® (15, 30 and 60mg/kg respectively) by using oral cannula while the animals in Group IV were given Pyrimethamine at 1.25 mg/kg/day (positive control) and group V (negative control) received 200 µL/mouse/day distilled water for seven consecutive days (D1 – D7) respectively. On day 7, all the mice were inoculated with P. berghei parasites. On day 7 post infection, mice were euthanized and the spleen collected from each mouse under sterile conditions. The spleens were placed in Roswell Park Memorial Institute (RPMI1640) media (Thermo Fisher Scientific, Waltham, MA USA), crushed through a cell strainer. The cell suspension was washed twice using 1X Phosphate Buffer Solution (PBS) (washing buffer) and centrifuged at 1500 rpm for 10 min at 25 °C. RBCs in the pellet were lysed using 2.5 mL of lysing buffer (Gibco, USA) for 2 minutes at room temperature. The lysing buffer was washed off and splenocytes counted using a hemocytometer machine. The splenocytes were cryopreserved in liquid nitrogen until use.

#### 3.4.2 Splenocytes Thawing and Staining

Cryopreserved splenocytes were retrieved from liquid nitrogen at -196$^0$C and immediately transferred to a preset 37$^0$C water bath. Upon thawing, cells were washed in complete RPMI 1640 medium and kept overnight at 37°C in 5% carbon dioxide (CO2). Thereafter, the cell yield viability using trypan blue (Thermo Fisher Scientific, Waltham, MA USA,) solution was determined. Cells were stained using 0.4% Trypan blue solution at a 1:1 dilution ratio. Samples with at least 70% viability were used for stimulation assays.

### 3.4.3 Cell Surface Staining

About 4.0 x$10^5$ cells/mL were surface stained and incubated for 30 minutes with the following antibodies.

**Table 1: Mouse CD markers used to assess Innate and Adaptive immune responses**

| CD | Phenotype/Function | Fluorophore Marker | Manufacturer |
|---|---|---|---|
| CD3ε | T cell lineage marker | Brilliant Violet 510™ anti-mouse CD3ε (Cat No. 100353) | Biolegend |
| CD4 | CD4+ lineage | PE-Cy5 anti-mouse CD4 Antibody (100410) | Biolegend |
| CD8 b2 | CD8+ T lineage | APC/Fire™ 750 anti-mouse CD8b.2 (Cat. No. 140420) | Biolegend |
| CD 11b | Lineage marker for monocytes, macrophages | FITC anti-mouse/human CD 11Bb (Cat. No. 101206) | Biolegend |
| CD25 | Marker for regulatory CD4+ T cells | PE/Dazzle™ 594 anti-mouse CD25 Antibody (102047) | Biolegend |
| CD69 | Early marker of lymphocyte activation | PE/Cyanine7 anti-mouse CD69 (Cat. No. 104512) | Biolegend |
| CD279 | Promotes apoptosis (programmed cell death) of antigen-specific T-cells | Brilliant violet 421 anti-mouse CD279 (PD-1) antibody (Cat.No 109121) | Biolegend |
| CD335(NKp46) | NK cell activation marker | APC anti-mouse CD335 (NKp46) Cat. No. 137608 | Biolegend |

1uL of each of the surface markers were added to the samples and incubated for 30 min at room temperature in the dark. The cells were washed twice by adding cell staining buffer and centrifuged at 1800 rpm for 5 min. The cells were acquired on a 19 color CytofleX LX flow Cytometer (Beckman coulter, New Jersey, USA). At least 400,000 events were recorded for analysis. Gating was standardized and set using fluorescence minus one control (FMOs).

### 3.4.4 Intracellular staining of cytokines

Following surface staining, the cells (splenocytes) were fixed by adding 0.5 mL of fixation buffer to each sample tube, incubated in the dark for 20 minutes at room temperature and then centrifuged at 1800rpm for 5 minutes and the supernatant discarded. The fixed cells were permeabilized by re-suspending in intracellular staining perm wash buffer (diluted to 1X in deionized water) and centrifuged at 1800 rpm for 5 minutes. A cocktail of intracellular cytokine antibodies was prepared by adding 1ul of each of the following intracellular markers in the tube.

**Table 2: Mouse Cytokine markers used to assess Innate and Adaptive immune responses**

| Cytokine | Cytokine source | Function | Fluorophore Marker | Manufacturer |
|---|---|---|---|---|
| IL-2 | T cells | Proliferation; enhancement of cytotoxicity, IFN-γ secretion, and antibody production | Brilliant Violet 785™ anti-mouse IL-2 (Cat. No. 503843) | Biolegend |
| IL-4 | T cells, mast cells | Proliferation; differentiation of Th2; promotes IgG and IgE production; inhibits cell-mediated immunity and Th17 development | PerCP/Cyanine5.5 anti-mouse IL-4 504124 | Biolegend |
| IL-10 | Differentiated T helper cells, Tregs, B cells, dendritic cells, others | Immune suppression; decreases antigen presentation and MHC class II expression of dendritic cells; down- regulates pathogenic Th1, Th2, and Th17 response | PE anti-mouse IL-10 (Cat No. 505008) | Biolegend |
| IL-17A | Th17 cells and others | Pro-inflammatory; promotes mobilization of neutrophils and cytokine production by epithelial cells; promotes angiogenesis | Alexa Fluor® 700 anti-mouse IL-17A 506914 | Biolegend |
| IFN-γ | Th1 cells, NK cells, CD8 T cells | Promotes activation of APCs and cell-mediated immunity; increased MHC class II expression | Brilliant Violet 605™ anti-mouse IFN-γ 505839 | Biolegend |
| TNFα | Macrophages, monocytes, T cells, others | Promotes activation and cytotoxicity | Brilliant Violet 650™ anti-mouse TNF-α | Biolegend |

The antibody cocktail was added to each of the sample tubes and incubated for 20 minutes in the dark at room temperature. Cells were washed 2 times with 2 mL of Intracellular Staining Perm Wash Buffer and centrifuged at 1800 rpm for 5 minutes. The cells were then re-suspended in 200 µL Cell Staining Buffer. The samples were acquired on the Cytoflex LX flow cytometer. Compensations and fluorescent minus ones (FMOs) controls for each of the antibody fluorochromes were ran together with the optimization gains for every channel using unstained cells. Compensation calculations were done and applied to the samples. At least one hundred thousand events were acquired and recorded per sample. Data was exported for analysis using flowJo software.

### 3.5 Determination of the effect of flavonoids on innate immune and adaptive responses in mice

#### 3.5.1 Extraction of flavonoids

Ten kilograms (10 kg) of *Artemisia annua* powder was used to extract flavonoids using maceration method with 70% ethanol (manufactured by Sugar Corporation of Uganda) for 72 hours. Thereafter, it was filtered using muslin cloth and further filtered with Whitman filter paper. The filtrate was concentrated *in vacuo* at $40^0$c and freeze dried. The freeze-dried extract was weighed and reconstituted with little distilled water to form a slurry as a support. The slurry was poured into a separating flask. About 500 mL of 99.5% Hexane (LOBA Chemie, CatLog number 141-78-6) was used to partition the extract, shaken carefully and allowed to stand and two immiscible layers created. The aqueous phase was decanted in order to remove hexane layer before the aqueous phase returned back to the separating flask and this process was repeated 5 times until the nonpolar compound having affinity for hexane was exhaustively removed and monitored with TLC profiling. The aqueous phase was poured into separating flask before

500mL of Ethyl acetate (LOBA Chemie, CatLog 110-54-3) was added to partition the extract and the process repeated until the flavonoids were removed. The flavonoids which was extracted with ethyl acetate was retained for the study after confirming its presence using Ferric chloride.

### 3.5.2 Acute toxicity study of the Flavonoid extracts

The median lethal dose ($LD_{50}$) of flavonoids, was determined in vivo using Lorke's method (Lorke, 1983). In the first phase, nine mice were randomly divided into three groups of three mice each and each group received the extract at 10, 100 and 1000 mg/kg body weight orally. 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, four 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: $\mathbf{LD50 = [D_0 \times D_{100}]^{1/2}}$ Where $\mathbf{D_0}$ = highest that caused no mortality and $\mathbf{D_{100}}$ = the lowest dose that produced mortality (Lorke, 1983).

### 3.5.3 Determination of the effect flavonoid extract on innate and adaptive immune responses in mice.

Thirty (30) mice were grouped into 5 groups of 6 mice each; Mice in Groups I –III were orally administered with flavonoids (15, 30 and 60mg/kg respectively) by using oral cannula while the animals in Group IV were given Pyrimethamine at 1.25 mg/kg/day (positive control) and group V (negative control) received 200 µL/mouse/day distilled water for seven consecutive days (D1 – D7) respectively. On day 7, all the mice were inoculated with P. berghei parasites. On day 7 post infection, mice were euthanized and the spleen collected from each mouse under sterile conditions. The spleens were placed in Roswell Park Memorial Institute (RPMI1640) (Thermo

Fisher Scientific, Waltham, MA USA) media, crushed through a cell strainer. The cell suspension was washed twice using 1X Phosphate Buffer Solution (PBS) (washing buffer) and centrifuged at 1500 rpm for 10 min at 25 °C. RBCs in the pellet were lysed using 2.5 mL of lysing buffer (Gibco, USA) for 2 minutes at room temperature. The lysing buffer was washed off and splenocytes counted using a hemocytometer machine. Cell viability and yield was determined before the splenocytes were cryopreserved in liquid nitrogen until use.

### 3.5.4 Splenocytes Thawing and Staining.

Cryopreserved splenocytes were retrieved from liquid nitrogen at -196$^0$C and immediately transferred to a preset 370C water bath. Upon thawing, cells were washed in complete RPMI 1640 medium and kept overnight at 37°C in 5% carbon dioxide (CO2). Thereafter, the cell yield viability using trypan blue (Thermo Fisher Scientific, Waltham, MA, USA,) solution was determined. Cells were stained using 0.4% Trypan blue solution at a 1:1 dilution ratio. **Samples with at least 70% viability were used for stimulation assays.**

### 3.5.5 Cell Surface Staining

About 4.0 x105 cells/mL were surface stained and incubated for 30 minutes with the following antibodies; CD3ε Brilliant Violet 510™ (Cat No. 100353), CD4 PE-Cy5(Cat No 100409), CD8b.2 APC/Fire™ 750(Cat. No. 140419), CD 11Bb FITC (Cat. No. 101206), CD25 PE/Dazzle™ 594 (Cat No 102047), CD69 PE/Cyanine7 (Cat. No. 104512), CD279 (PD-1), Brilliant violet 421(Cat. No 109121), CD335 (NKp46) APC (Cat. No. 137608) in which 1uL each of surface markers were added to the samples and incubated for 30 min at room temperature in the dark. The cells were washed twice by adding cell staining buffer and centrifuged at 1800 rpm for 5 min. The cells were acquired on a 19 color CytofleX LX flow Cytometer (Beckman

coulter, New Jersey, USA). At least 400,000 events were recorded for analysis. Gating was standardized and set using fluorescence minus one control (FMOs).

### 3.5 6 Intracellular staining of cytokines

Following surface staining, the cells (splenocytes) were fixed by adding 0.5 mL of fixation buffer (4% paraformaldehyde, Biolegend) to each sample tube, incubated in the dark for 30 minutes at room temperature and then centrifuged at 1800rpm for 5 minutes and the supernatant discarded. The fixed cells were permeabilized by re-suspending in intracellular staining perm wash buffer (Biolegend) (diluted to 1X in deionized water) and centrifuged at 1800 rpm for 5 minutes. A cocktail of intracellular cytokine antibodies was prepared by adding 1ul of each i.e., IFN-γ BV605 (Cat. No 505839), TNF-αBV650 (Cat. No 506333), IL-2 BV785 (Cat. No. 503843), IL-4 PerCP Cy5.5 (Cat. No 504123), IL-10PE (Cat No. 505008) and IL-17A Alexa F700 (Cat. No 506914) in the tube. The antibody cocktail was added to each of the sample tubes and incubated for 45 minutes in the dark at room temperature. Cells were washed 2 times with 2 mL of Intracellular Staining Perm Wash Buffer (Biolegend) and centrifuged at 1800 rpm for 5 minutes. The cells were then re-suspended in 200 µL Cell Staining Buffer. The samples were acquired on the Cytoflex LX flow cytometer. Compensations and fluorescent minus ones (FMOs) controls for each of the antibody fluorochromes were ran together with the optimization gains for every channel using unstained cells. Compensation calculations were done and applied to the samples. At least one hundred thousand events were acquired and recorded per sample. Data was exported for analysis using flowJo software.

### 3.6 Ethical Considerations

Permission to use ARTAVOL® herbal product in the study was obtained from developers of the product. All the animals in the experimental studies were treated humanely according to the

Organization for Economic Co-operation and Development (OECD) guidelines and American Psychological Association (Bedi and Krishan, 2020, Loue, 2020). This study also received ethical clearance from Mbarara University Research and Ethics Committee approval number 07/05-19 and Uganda National Council for Science and Technology registration number HS465ES.

### 3.7 Data analysis

#### 3.7.1. Data analysis for haematological parameters

Haematological study data was analysed using Graph Pad Prism version 9.1.0 (GraphPad Software, Inc., San Diego, CA, USA). Results obtained from the haematological study were expressed as mean ± standard error of mean (Chandel et al., 2015). 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.

#### 3.7.2. Data analysis for flow cytometry

##### 3.7.2.1 Gating Strategy

A gating strategy was developed to analyze the *P. berghei* activation marker expression on CD4+ and CD8+ T-cells. Representative flow cytometry dot plots to identify CD3, CD4, CD8T cells, CD25, CD69 and CD279(PD-1) cell subsets from *Plasmodium berghei* infected mice prophylactically treated with aqueous extract of ARTAVOL® in the figure 4(A-I) below.

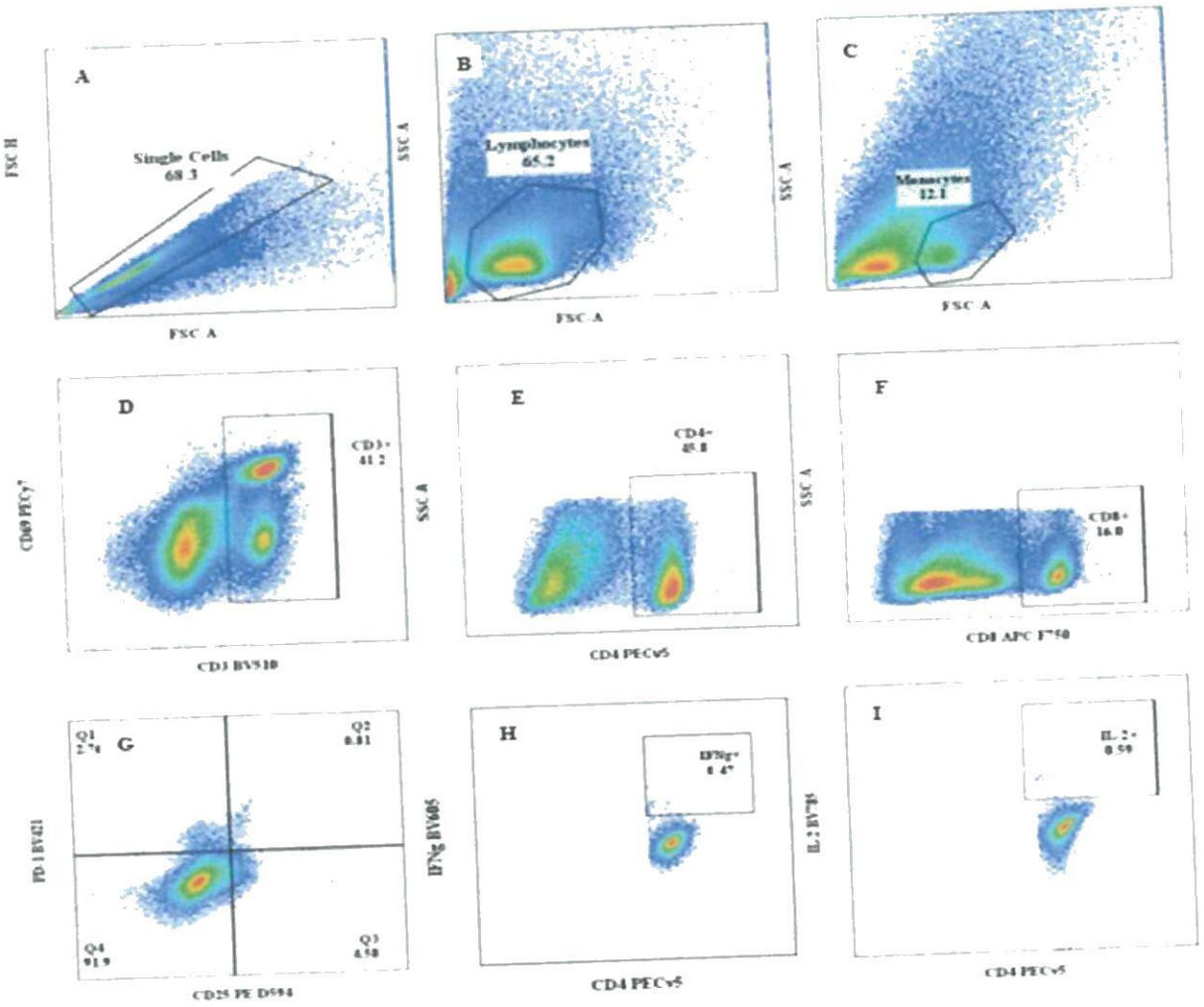



**Figure 3:** Innate and adaptive T cell subsets panel gating strategy on mice splenocytes samples.

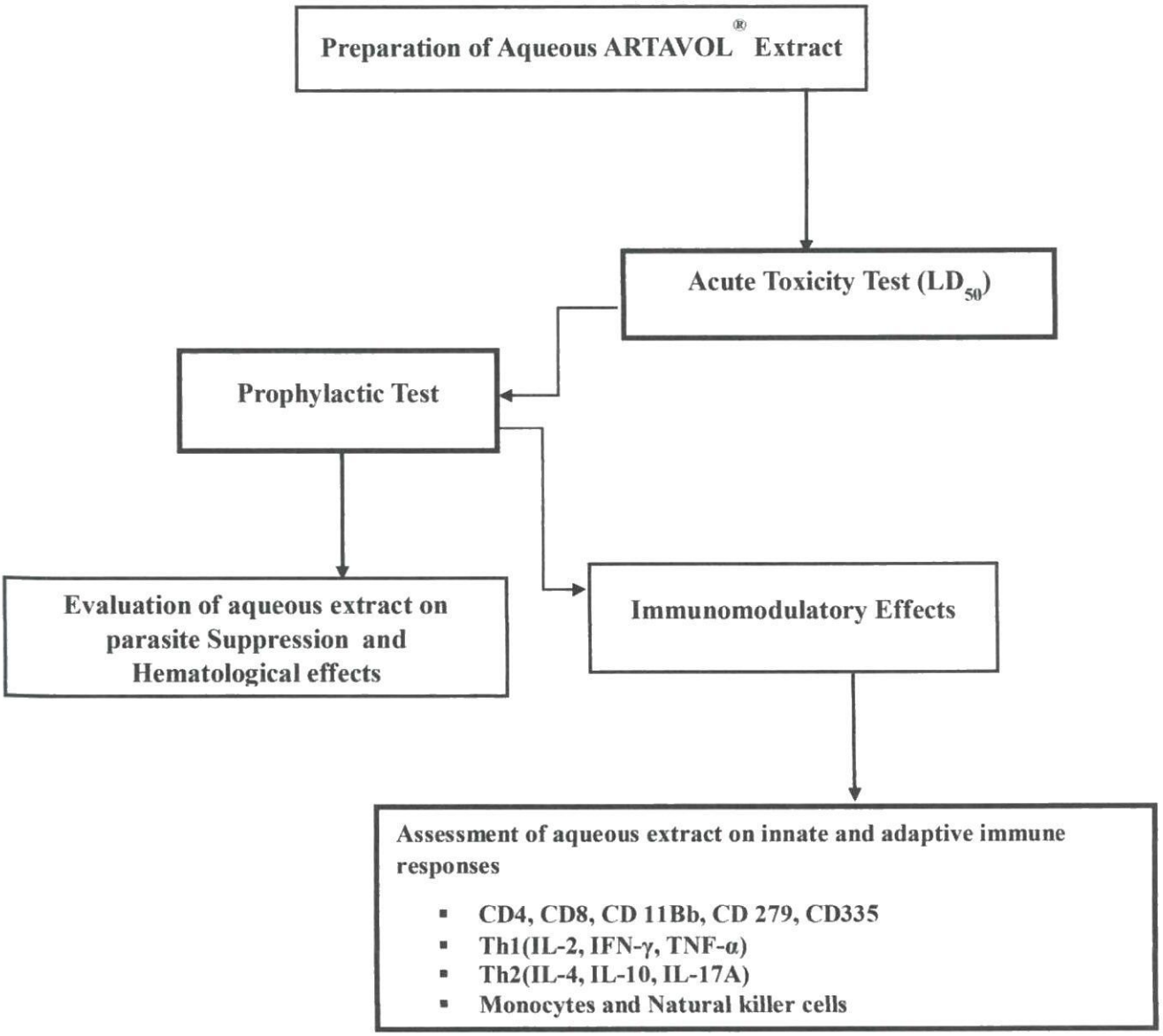
*To identify Plasmodium berghei specific CD 11Bb(monocytes), CD335(Natural killer cells), CD CD4+ and CD8+ T cells and their functional responses, we first gated single cells on a forward scatter -height (FSC-H) versus forward scatter-area (FSCA) channels (Fig 3A), followed by Monocytes (Fig 3B) and Lymphocytes (Fig 3C), then from lymphocyte population, we gated out CD3 positive population cells (Fig 3D), fromCD3 positive cells, CD4+T cells were gated on a side scatter-A(SSC-A) versus CD4PECy5 (Fig 3E) and CD8+Tcells (Fig 4F) respectively. Using the individual CD4+ and CD8+ T cell subpopulations, we identified markers of activation using CD25 and CD279(PD-1) (Fig 3G) and functional markers IFN-γ (Fig 4H), IL-2 (Fig 3I) among others.*

The percentage of positive cells for each marker was determined on the basis of the numbers of viable lymphocytes. The frequencies of T cells expressing activation and memory markers were measured as a percentage of the CD4+ and CD8+ T cell parent cell populations. Bivariate dot plots or probability contour plots were generated upon data analysis to display the frequencies of and patterns by which individual cells co-express certain levels. of cell surface antigen and intracellular cytokines

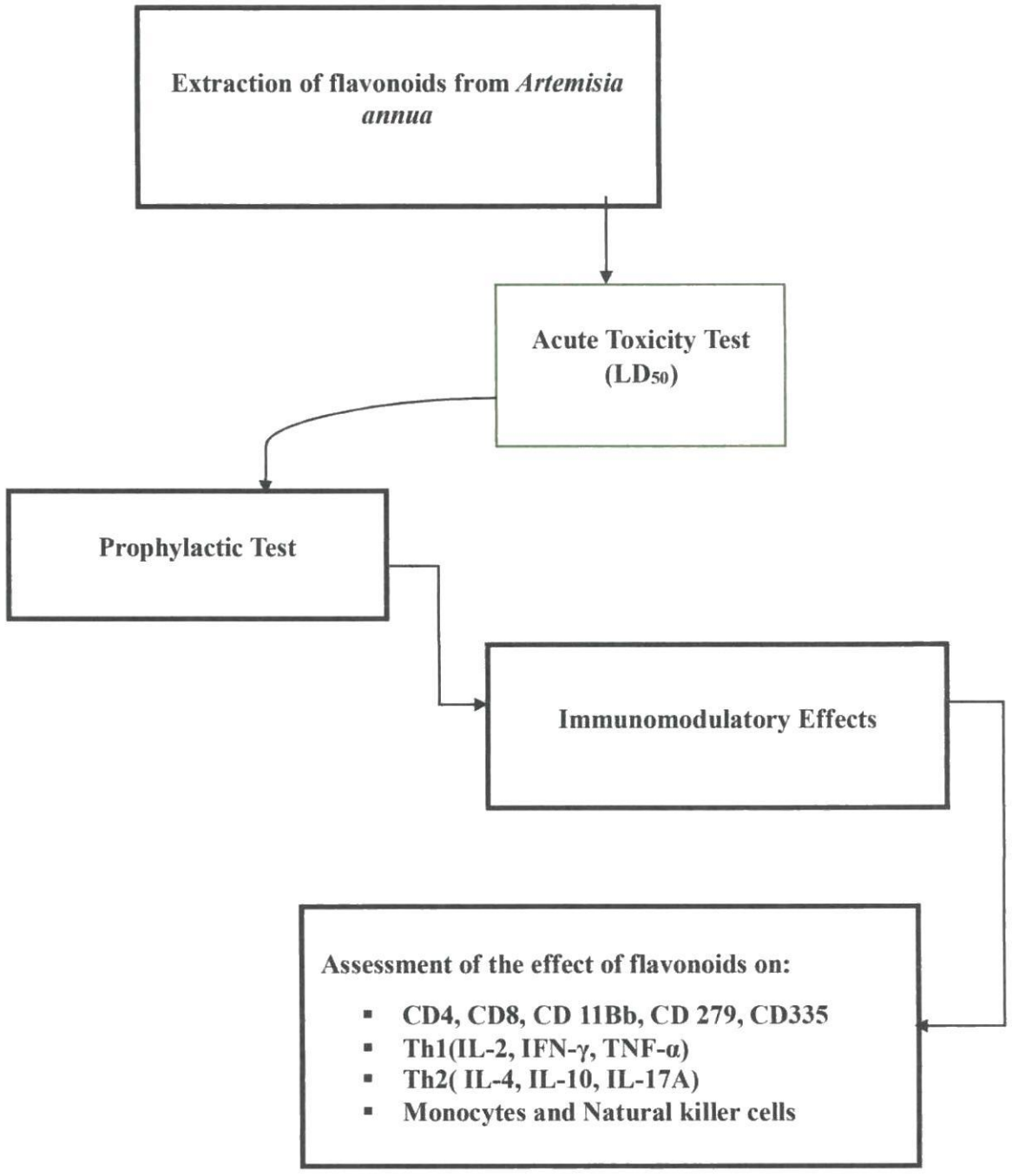
Flow cytometry data were analysed using FlowJo version 10.7.1 software (Becton Dickinson, New Jersey, USA). Results obtained were expressed as mean ± standard error of mean. Differences in the frequencies of the T cell subsets were evaluated using a One-way Analysis of Variance while the difference among the means was considered at 95% confidence interval using the post-hoc method of Dunnett's Multiple Comparison Test.

### 3.8 Study flowcharts

#### 3.8.1 Study flow chart for Objectives 1 and 2



### 3.8.2 Study flow chart for Objective 3



# CHAPTER FOUR
# RESULTS

## 4.1 Acute toxicity tests of ARTAVOL® and Flavonoids

The acute oral toxicity test of aqueous ARTAVOL® extract caused no stretching, rubbing of nose on the flow and wall of cage, change in body weight and mortality within 24 h 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.

The acute oral toxicity test of flavonoid extract caused no gross behavioral changes such as loss of appetite, paw licking, body temperature, calmness, locomotion and there was no mortality within 24 h as well as in the next 14 days, indicating that the LD50 values of the flavonoid extract were greater than 5000 mg/kg in mice.

## 4.2 Effect of ARTAVOL® on hematological values

**Table 3: Prophylactic effects of ARTAVOL® on *P. berghei* ANKA-infected mice at 15 – 60 mg/kg on days 3 and 5**

| | Day 3 | | | | Day 5 | | | |
|---|---|---|---|---|---|---|---|---|
| | % Parasitaemia | 95%CI | % suppression | CI | % Parasitaemia | CI | % suppression | CI |
| NC | 14.42 ± 0.62 | 12.80-16.03 | 0.0 | - | 23.60 ± 2.37 | 17.49-29.17 | 0.0 | - |
| PC | 1.46 ± 0.40*** | 0.41-2.50 | 89.89± 2.82** | 82.64-97.13 | 1.60 ± 0.14*** | 1.23-1.96 | 93.24 ± 0.61** | 91.68-94.80 |
| 15 mg/kg | 8.11 ± 0.88*** | 5.84-10.38 | 43.73 ± 6.11** | 28.03-59.03 | 11.46 ± 1.16** | 8.45-14.44 | 51.44 ± 4.92** | 38.80-54.09 |
| 30 mg/kg | 5.34 ± 0.13*** | 5.02-5.67 | 62.92± 0.88** | 60.64-65.19 | 8.91 ± 1.39** | 5.04-12.78 | 62.27 ± 5.91* | 45.87-78.66 |
| 60 mg/kg | 4.51 ± 0.45*** | 3.38-5.69 | 68.69 ± 3.17** | 60.53-76.85 | 7.93± 0.26** | 7.25-8.62 | 66.38 ± 1.13* | 63.46-69.29 |

Data are expressed as mean ± SEM; n=6; PC= positive control. * means P=0.001, ** means P<0.001 *** Means P<0.0001,

All the ARTAVOL® extract doses significantly suppressed parasite growth when compared to positive control.

**Table 4: Effect of ARTAVOL® prophylaxis on Monocytes, HCT, and RBC values**

| Treatment Groups(mg/kg) | HCT (%) | 95%CI | RBCx$10^{12}$/L | 95%CI | Monocytes (%) | 95%CI |
|---|---|---|---|---|---|---|
| **Group 6** (Uninfected group) | 41.80±0.98 | 38.68-44.92 | 9.03±0.37 | 7.86-10.20 | 12.18±1.62 | 7.03-17.3 |
| **Group 5** NC(0.2mL $H_2O$) | 19.00±3.73*** | 9.42-28.58 | 3.84±0.56*** | 2.39-5.28 | 4.37±0.82*** | 2.25-6.48 |
| **Group 4** PC( 1.25mg/kg) | 33.98±2.16[a] | 27.09-40.86 | 7.68±0.58[a] | 5.84-9.52 | 5.17±0.75*** | 3.25-7.09 |
| **Group1** (15mg/kg) | 33.15±3.03[a] | 25.37-40.93 | 6.63±0.90[a] | 4.32-8.94 | 4.32±0.69*** | 2.53-6.1( |
| **Group2** (30mg/kg) | 39.47±3.77[a] | 23.24-55.69 | 7.54±0.42[a] | 5.75-9.34 | 6.77±0.96* | 2.67-10.8 |
| **Group 3** (60mg/kg) | 37.55±0.51[a] | 35.94-39.16 | 7.43±0.46[a] | 5.97-8.88 | 8.22±0.86[a] | 6.02-10.4 |

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

The three test doses of extract in a dose-independent way and positive control were able to reverse the HCT reduction which could have been caused by infection and their parameters did not show any significant difference when compared to the uninfected group but significant when compared with negative control. All test doses prevented destruction of RBCs due to infection by *P. berghei* and this was not significantly different as compared to uninfected group but significant when compared to negative control. There was no significant difference in red blood cell count in ARTAVOL® extracts doses when compared positive control but significant when compared to negative control. The ARTAVOL® extract doses at 30 and 60 mg/kg showed a significant rise in the level of monocytes compared to negative control group.

### 4.3 Effect of ARTAVOL® extract on innate immune response

#### 4.3.1 Effect of ARTAVOL® extract of CD11b (monocytes) response

We performed a phenotypic and functional analysis CD11b (monocytes) of mice splenocytes infected with *Plasmodium berghei* with both surface (CD11b)) and intracellular markers (TNF-α). We hypothesized that ARTAVOL® prophylaxis alters the frequency (Clonal expansion) and function of monocyte (CD11b) repertoire in *Plasmodium berghei* infected mice. Results revealed a variation in the expression of the frequency and function.

The frequency of expression of monocytes was significantly higher in 30 and 60mg/kg of the extract compared to the negative control) (Fig.4a) below.

Similarly, the frequency of expression of TNF-α by CD 11(monocytes) was significantly higher in the extract doses of 30 and 60mg/kg compared to the negative control, however, the 15mg/kg dose of the extract did not (Fig.4b).

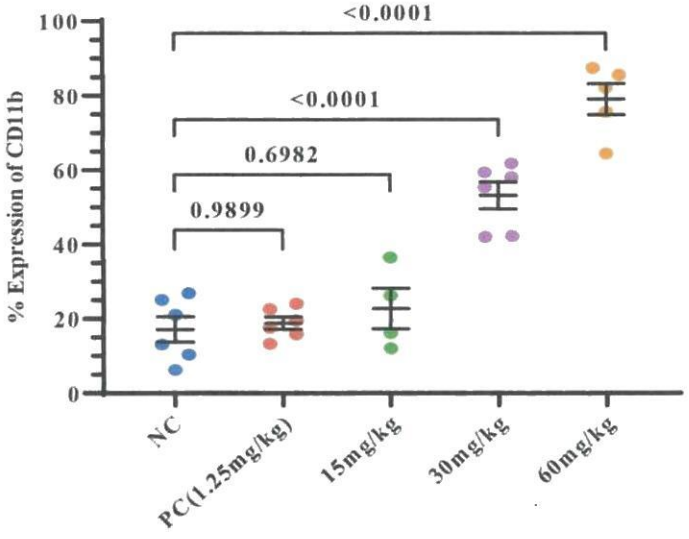



**Fig 4a:** Effects of ARTAVOL® extract on the frequency of CD11b (Monocytes) in *Plasmodium berghei* infected mice.

*Differences in the frequency of CD11b(monocytes) were evaluated using a One-way Analysis of Variance while the difference among the means was considered at 95% confidence level using the post-hoc method of Dunnett's Multiple Comparison Test. On the scatter plots, the horizontal axis represents concentrations of the ARTAVOL® extracts, negative and control groups. PC refers to Pyrimethamine (a positive control drug). P values < 0.05 were considered significant.*

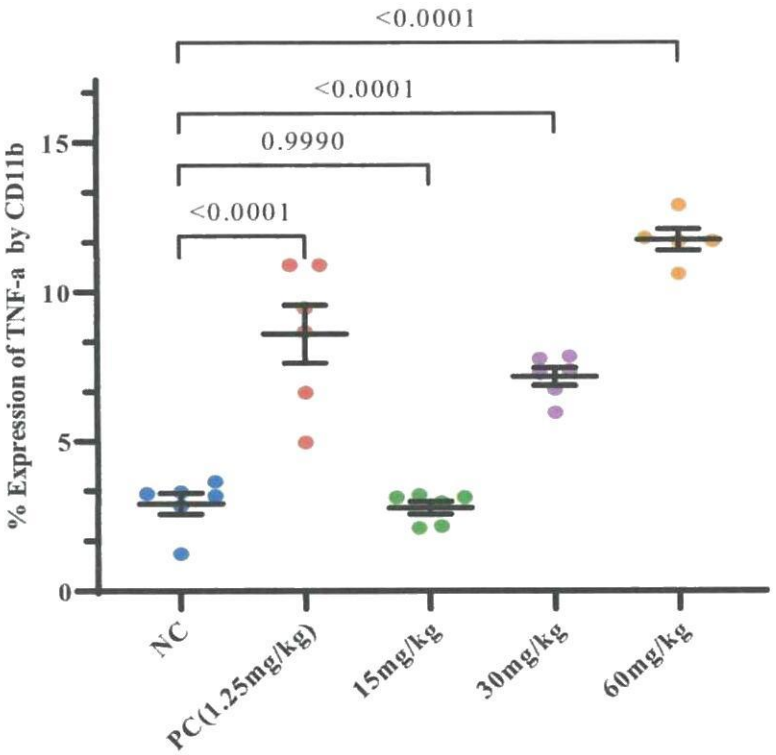



**Fig 4b:** Effects of ARTAVOL® extract on the frequency TNF-α by CD11b in *Plasmodium berghei* infected mice.

*Differences in the frequency of expression of TNF-α by CD11b (monocytes) were evaluated using a One-way Analysis of Variance while the difference among the means was considered at 95% confidence level using the post-hoc method of Dunnett's Multiple Comparison Test. On the scatter plots, the horizontal axis represents concentrations of the ARTAVOL® extracts and*

*control groups. PC refers to Pyrimethamine (a positive control drug). P values < 0.05 were considered significant.*

### 4.4.2 Effect of ARTAVOL® extract of CD 335 (Natural killer cells) response

We also performed a phenotypic and functional analysis of CD335 (natural killer cells) of mice splenocytes infected with *Plasmodium berghei* with both surface (CD335) and intracellular markers (IFN-y and TNF- α). We hypothesized that ARTAVOL® prophylaxis alters the frequency (Clonal expansion) and function of monocyte (CD335) repertoire in *Plasmodium berghei* infected mice.

Results revealed a variation in the expression of the frequency and function. We observed a significant increase in frequency of expression of CD335 (Natural killer cells) by all the extracts 15, 30 and 60mg/kg compared to the negative control (Fig.5a) below.

We also noted a significant increase in the frequency of expression of Interferon gamma (IFN-γ) by CD335 (Natural killer cells) by all extract doses compared to the negative control (Fig.5b) below.

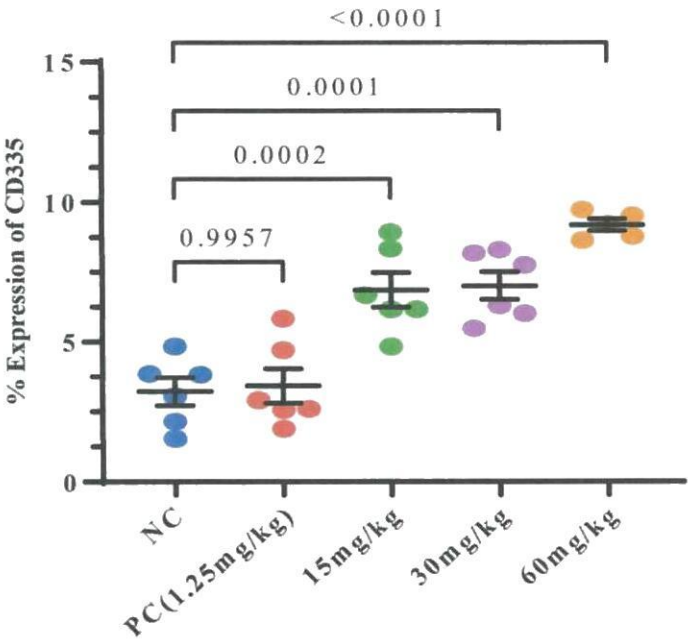



**Fig 5a:** Effects of ARTAVOL® extract on the frequency of CD335 (NK cells) in *Plasmodium berghei* infected in mice.

*Differences in the frequency of CD335 (NK cells) were evaluated using a One-way Analysis of Variance while the difference among the means was considered at 95% confidence level using the post-hoc method of Dunnett's Multiple Comparison Test. On the scatter plots, the horizontal axis represents concentrations of the ARTAVOL® extracts and control groups. PC refers to a positive control drug (Pyrimethamine). P values < 0.05 were considered significant.*

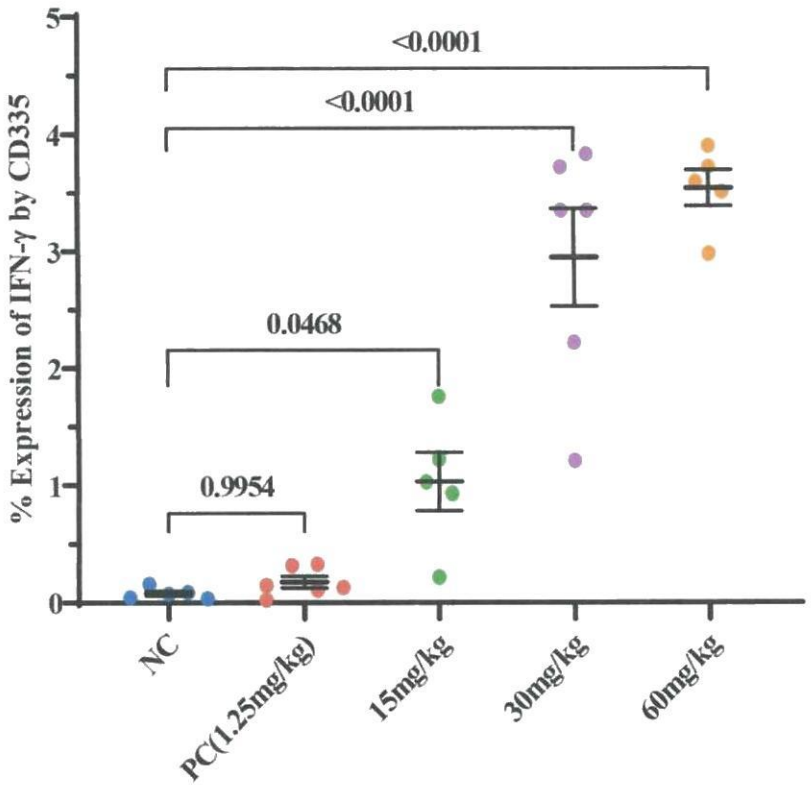



**Fig 5b:** Effects of ARTAVOL® extract on the frequency of IFN-γ by CD335 in *Plasmodium berghei* infected in mice.

*Differences in the frequency of expression of IFN-γ by CD335 (NK cells) were evaluated using a One-way Analysis of Variance while the difference among the means was considered at 95% confidence level using the post-hoc method of Dunnett's Multiple Comparison Test. On the scatter plots, the horizontal axis represents concentrations of the ARTAVOL® extracts and control groups. PC refers to a positive control drug (Pyrimethamine). P values < 0.05 were considered significant.*

### 4.5 Effect of ARTAVOL® extract on adaptive immune responses

#### 4.5.1 Effect of ARTAVOl® extract of CD4+ T cells

A phenotypic and functional analysis of CD4+ T cells in adaptive immune response of the mice splenocytes infected with *Plasmodium berghei* with both surface (CD4, CD 279) and intracellular markers (IL-10, IL-17A and TNF- α) was done. We hypothesized that ARTAVOL® prophylaxis alters the frequency and function of CD4+ T cells repertoire in *Plasmodium berghei* infected mice. Results revealed a variation in the expression of the frequency and function.

In figure 6a, we noted a gradual dose increase in frequency of expression CD4 + T cells by all extract doses, although not statistically significant when comapred with the negative and positive control (Fig. 6a) below.

We also noted a significant increase in the frequency of expression of CD279(PD-1) by CD4 + T cells in the extract dose of 15mg/kg, the other doses of extract(30 and60mg/kg) showed a decline in the expression of CD279(PD-1) although not statistically significant( Fig 6b) below.

In figure 6c, we noted a significant increase in the frequency of expression of IL-10 by CD4+T cells by all the extract doses compared to the negative control.

There was no significant increase in the frequency of expression of IL-17A by CD4+ T cells for all the extract test doses when compared with the negative control(Fig.6d) below.

In the figure 6e, shows a significant increase in the expression of TNF-α by CD4+ T cells for the extract doses of 15 and 30mg/kg respecticely,however, a decline in expression was noted as the concentration of extract increased.

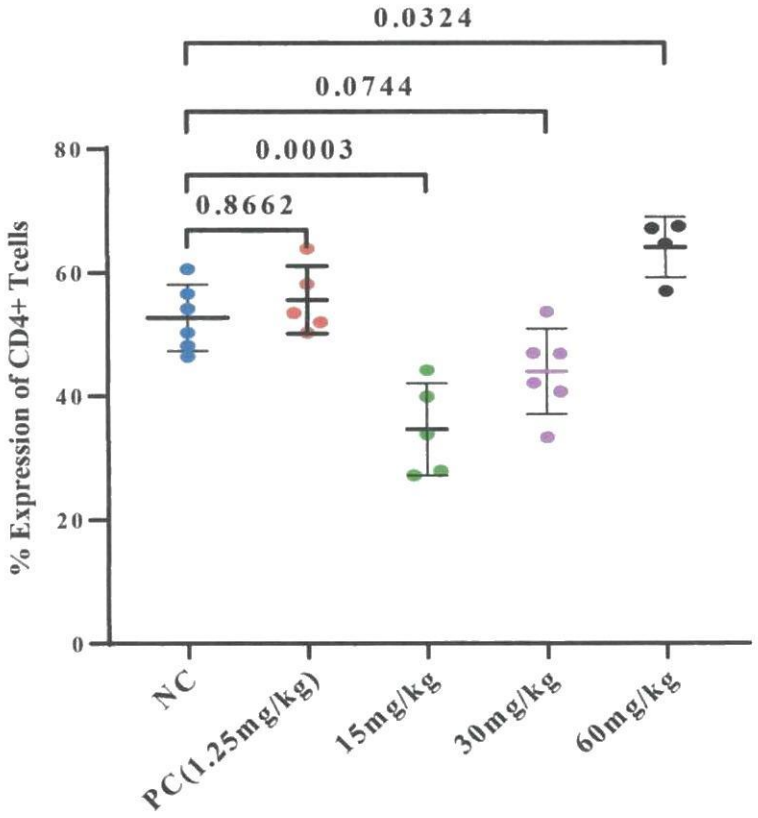



**Fig 6a:** Effects of ARTAVOL® extract on the frequency of CD4+T cells in *Plasmodium berghei* infected in mice.

*Differences in the frequencies of CD4+T cells were evaluated using a One-way Analysis of Variance while the difference among the means was considered at 95% confidence level using the post-hoc method of Dunnett's Multiple Comparison Test. On the scatter plots, the horizontal axis represents concentrations of the ARTAVOL® extracts and positive control. PC refers to a positive control(Pyrimethamine. P-values < 0.05 were considered significant.*

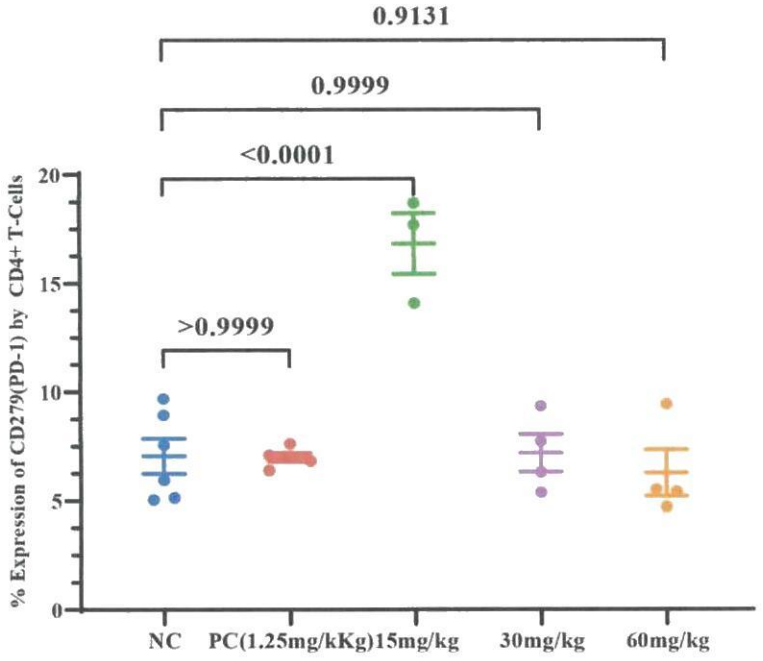



**Fig 6b:** Effects of ARTAVOL® extract on the frequency of CD279(PD-1) by CD4+ T cells in *Plasmodium berghei* infected in mice.

*Differences in the frequencies of expression of CD279(PD-1) by CD4+T cells were evaluated using a One-way Analysis of Variance while the difference among the means was considered at 95% confidence level using the post-hoc method of Dunnett's Multiple Comparison Test. On the scatter plots, the horizontal axis represents concentrations of the ARTAVOL® extracts and positive control. PC refers to a positive control(Pyrimethamine. P-values < 0.05 were considered significant.*

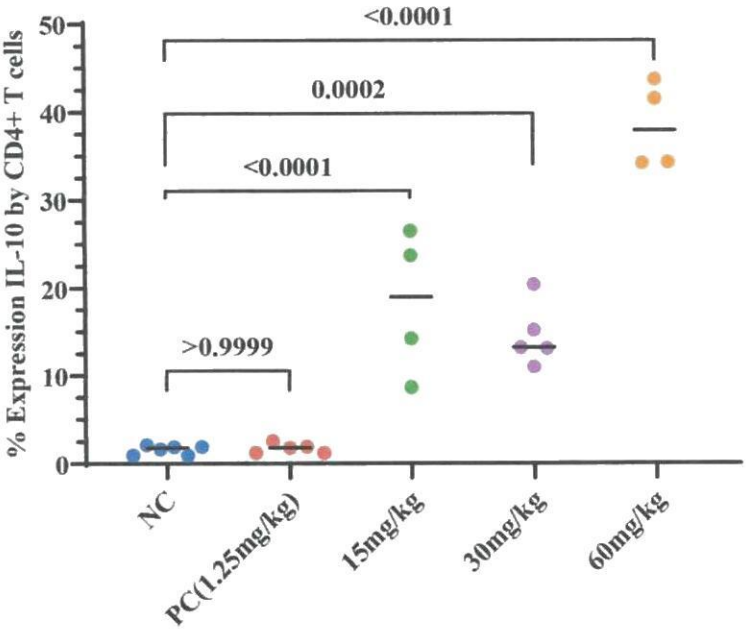



**Fig 6b:** Effects of ARTAVOL® extract on the frequency of IL-10 by CD4+T cells in *P. berghei* infected in mice.

*Differences in the frequencies of expression of IL-10 by CD4+T cells were evaluated using a One-way Analysis of Variance while the difference among the means was considered at 95% confidence level using the post-hoc method of Dunnett's Multiple Comparison Test. On the scatter plots, the horizontal axis represents concentrations of the ARTAVOL® extracts and positive control. PC refers to a positive control (Pyrimethamine). P-values < 0.05 were considered significant.*

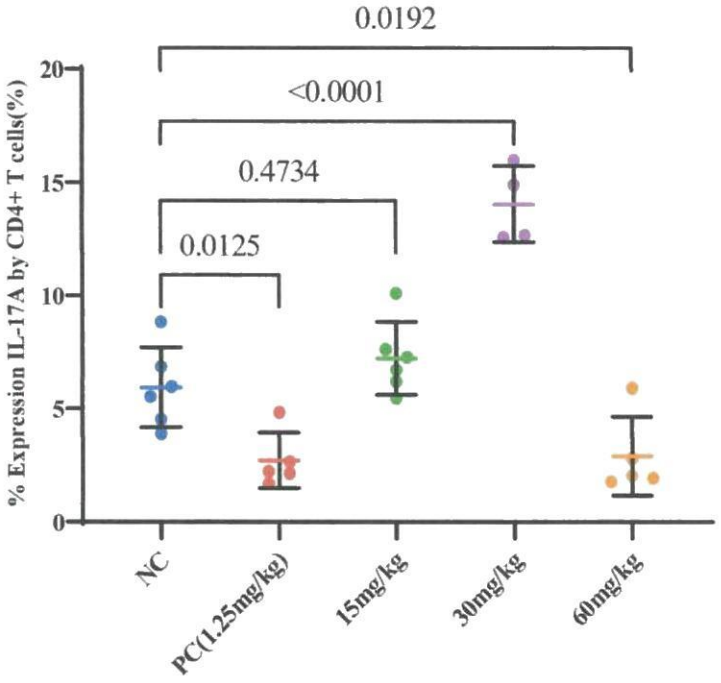



**Fig 6d:** Effects of ARTAVOL® extract on the frequency of IL-17A by CD4+ T cells in Plasmodium berghei infected in mice.

*Differences in the frequencies of expression of IL-17A by CD4+T cells were evaluated using a One-way Analysis of Variance while the difference among the means was considered at 95% confidence level using the post-hoc method of Dunnett's Multiple Comparison Test. On the scatter plots, the horizontal axis represents concentrations of the ARTAVOL® extracts and positive control. PC refers to a positive control (Pyrimethamine). P-values < 0.05 were considered significant.*

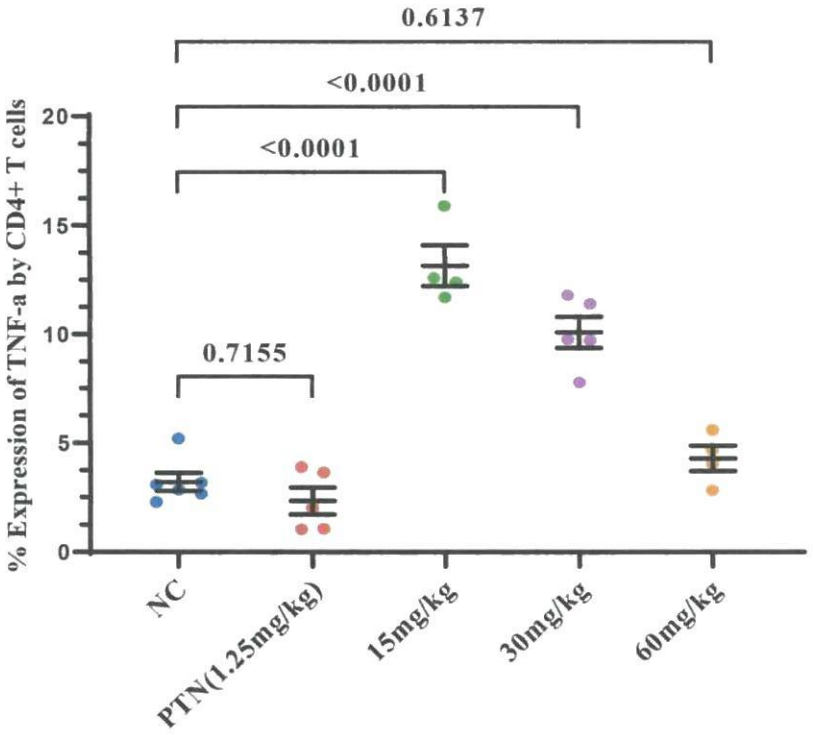



**Fig 6e:** Effects of ARTAVOL® extract on the frequency of TNF-α by CD 4 + T cells in *Plasmodium berghei* infected in mice.

*Differences in the frequencies of expression of TNF- α by CD4+T cells were evaluated using a One-way Analysis of Variance while the difference among the means was considered at 95% confidence level using the post-hoc method of Dunnett's Multiple Comparison Test. On the scatter plots, the horizontal axis represents concentrations of the ARTAVOL® extracts and positive control. PC refers to a positive control (Pyrimethamine). P-values < 0.05 were considered significant.*

### 4.5.2 Effect of ARTAVOL® extract of CD8 T cells

We performed a phenotypic and functional analysis of CD8+T cells of the splenocytes of mice prophylactically treated with ARTAVOL® and then infected with *Plasmodium berghei* with both surface (CD8, CD279(PD-1)) and intracellular markers (IFN-γ and TNF- α). We hypothesized that ARTAVOL® prophylaxis alters the frequency) and function of CD8+ T cells repertoire in *Plasmodium berghei* infected mice.

Figure 7a shows non-significant increase in the frequency of expression of CD8+T cells in all extract doses.

In figure 7b, there was a decline in the frequency of CD8 T cells expressing PD-1 although statistically significant in extract doses of 15 and 60mg/kg.

The frequency of CD8 T cells expressing IFN-γ for showed a significant increase in the extract doses 15 and 30mg/kg when compared with the negative control (Fig.7c). There was a significant increase in the frequency of CD8+T cells expressing TNF-α in the extract dose of 30mg/kg (Fig.7d).

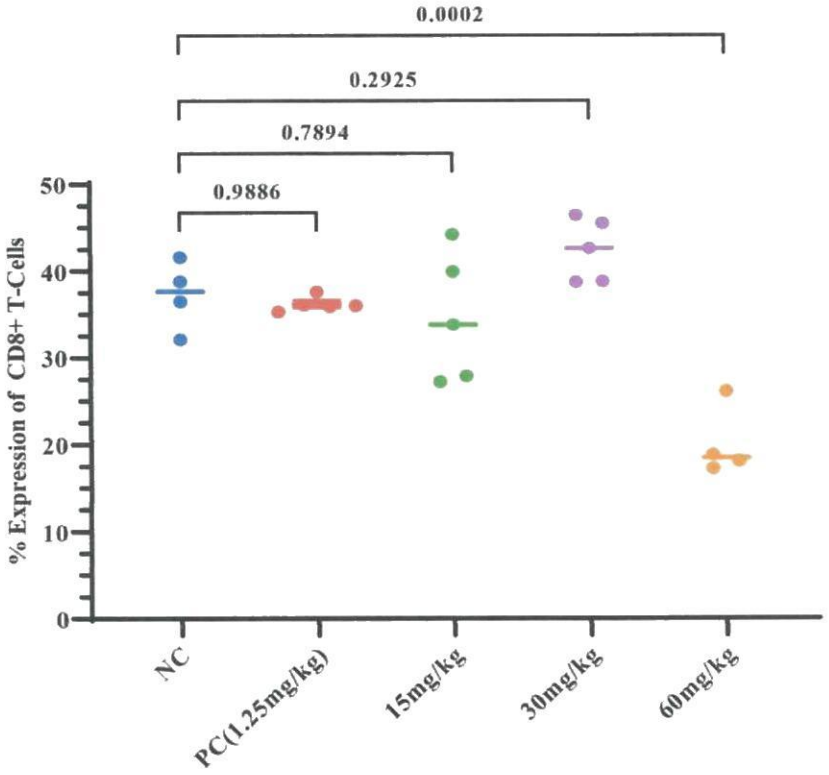



**Fig 7a**: Effects of ARTAVOL® extract on the frequency of CD8+ T cells in *Plasmodium berghei* infected in mice.

*Differences in the frequency of expression of CD8+T cells were evaluated using a One-way Analysis of Variance while the difference among the means was considered at 95% confidence level using the post-hoc method of Dunnett's Multiple Comparison Test. On the scatter plots, the horizontal axis represents concentrations of the ARTAVOL® extracts and positive control. PC refers to a positive control (Pyrimethamine. P-values < 0.05 were considered significant.*

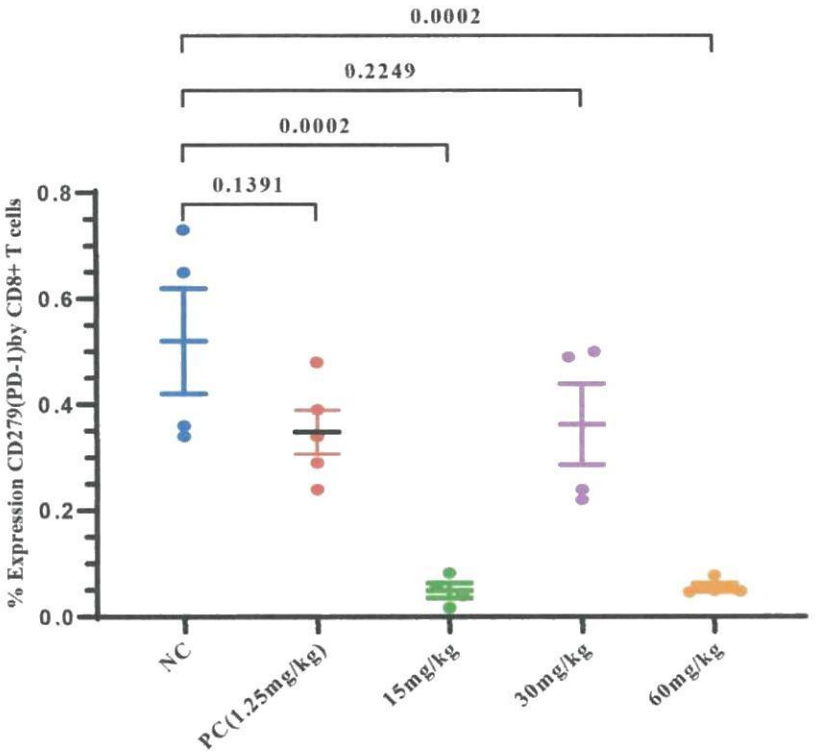



**Fig 7b:** Effects of the ARTAVOL® extract on the frequency of CD279(PD-1) by CD8+ T cells in *Plasmodium berghei* infected in mice

*Differences in the frequency of expression of CD279(PD-1) by CD8+ T cells were evaluated using a One-way Analysis of Variance while the difference among the means was considered at 95% confidence level using the post-hoc method of Dunnett's Multiple Comparison Test. On the scatter plots, the horizontal axis represents concentrations of the ARTAVOL® extracts and positive control. PC refers to a positive control (Pyrimethamine. P-values < 0.05 were considered significant*

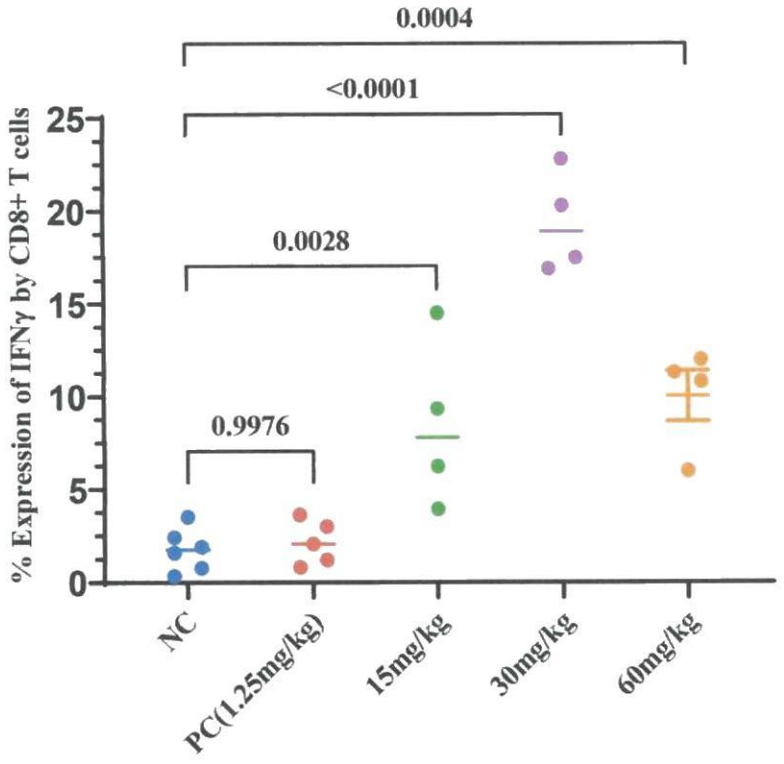



**Fig 7c:** Effects of the ARTAVOL® extract on the frequency of IFN-γ by CD8+ T cells in *Plasmodium berghei* infected in mice

*Differences in the frequency of expression of IFN-γ by CD8+T cells were evaluated using a One-way Analysis of Variance while the difference among the means was considered at 95% confidence level using the post-hoc method of Dunnett's Multiple Comparison Test. On the scatter plots, the horizontal axis represents concentrations of the ARTAVOL® extracts and positive control. PC refers to a positive control (Pyrimethamine). P-values < 0.05 were considered significant*

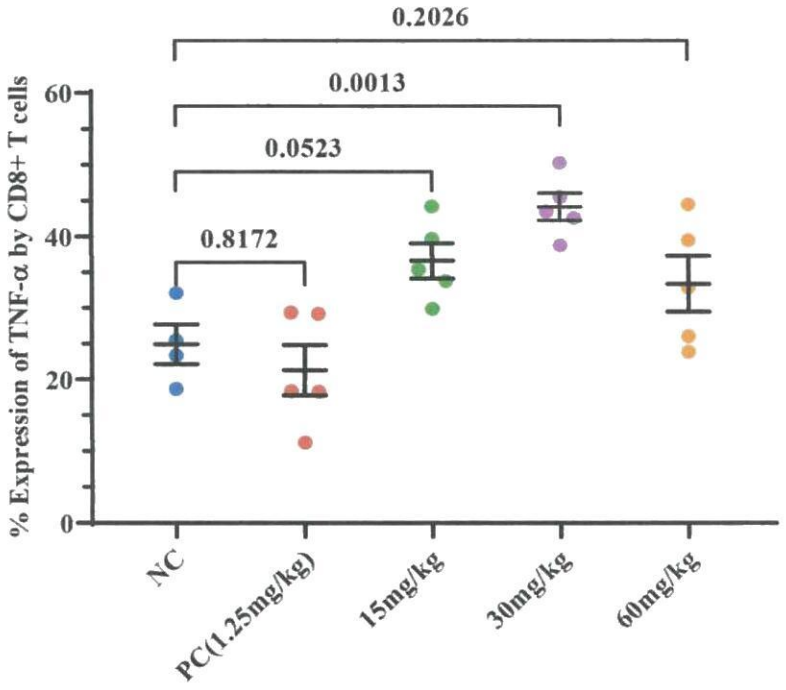



**Fig 7d**: Effects of the ARTAVOL® extract on the frequency of TNF-α by CD8+ T cells in *Plasmodium berghei* infected in mice

*Differences in the frequency of expression of TNF-α by CD8+T cells were evaluated using a One-way Analysis of Variance while the difference among the means was considered at 95% confidence level using the post-hoc method of Dunnett's Multiple Comparison Test. On the scatter plots, the horizontal axis represents concentrations of the ARTAVOL® extracts and positive control. PC refers to a positive control (Pyrimethamine). P-values < 0.05 were considered significant.*

### 4.6 Effect of Flavonoid extract on innate immune responses

We hypothesized that flavonoid extracted from ARTAVOL® increases the frequency and function of innate and adaptive immune cells during *Plasmodium berghei* infection in mice. Results revealed a variation in the expression of the frequency and function.

#### 4.6.1 Effect of Flavonoid extract on CD 11b (Monocytes)

The flavonoid extract did not increase the frequency of expression of CD11b (monocytes) significantly in all the test doses when compared with the positive and negative control (Fig 8a). There was a significant increase in the frequency of expression of TNF-α by CD11b (Monocytes) only in the extract test dose of 15mg/kg (Fig 8b) when compared with the negative control.

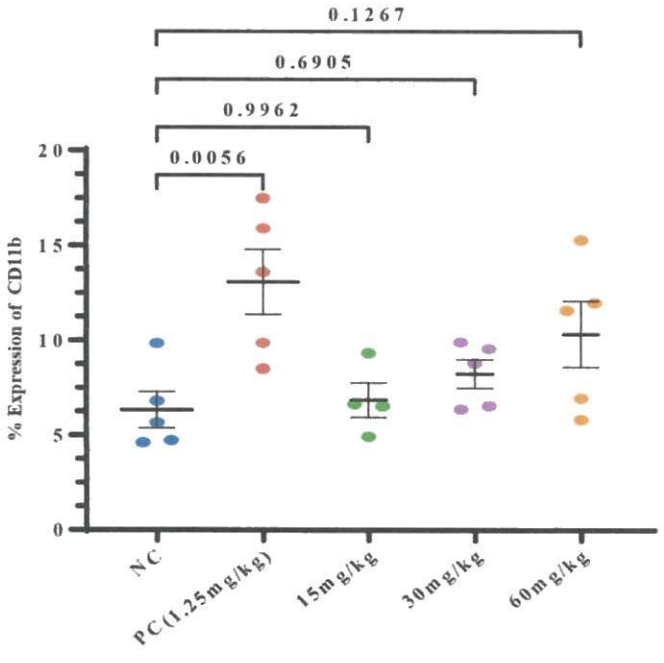



**Fig 8a:** Effects of the flavonoid extract on the frequency of CD 11b in *Plasmodium berghei* infected in mice

*Differences in the frequency of expression of CD 11b(Monocytes) were evaluated using a One-way Analysis of Variance while the difference among the means was considered at 95% confidence level using the post-hoc method of Dunnett's Multiple Comparison Test. On the scatter plots, the horizontal axis represents concentrations of flavonoid extracts, negative and positive control. PC refers to a positive control (Pyrimethamine). P-values < 0.05 were considered significant.*

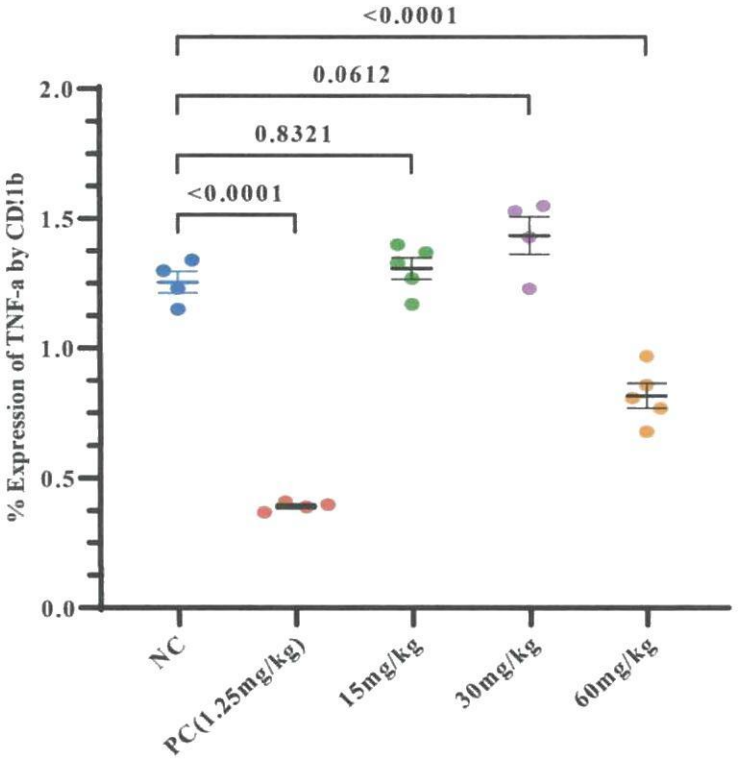



**Fig 8b:** Effects of the flavonoid extract on the frequency of TNF-α by CD11b in *Plasmodium berghei* infected in mice

*Differences in the frequency of expression of TNF-α by CD 11b(Monocytes) were evaluated using a One-way Analysis of Variance while the difference among the means was considered at 95% confidence level using the post-hoc method of Dunnett's Multiple Comparison Test. On the scatter plots, the horizontal axis represents concentrations of flavonoid extracts, negative and*

*positive control. PC refers to a positive control (Pyrimethamine). P-values < 0.05 were considered significant.*

### 4.6.2 Effect of Flavonoid extract on CD 335 (Natural Killer cells)

All the extract test doses caused a significant increase in the frequency of CD335 (NK) when compared with to the negative control (p=0.0053, 0.0013, and 0.0019 respectively) (Fig.9a). The extract did not cause a significant increase in the frequency of CD 335 expressing IFN-γ compared to the negative control (Fig.9b).

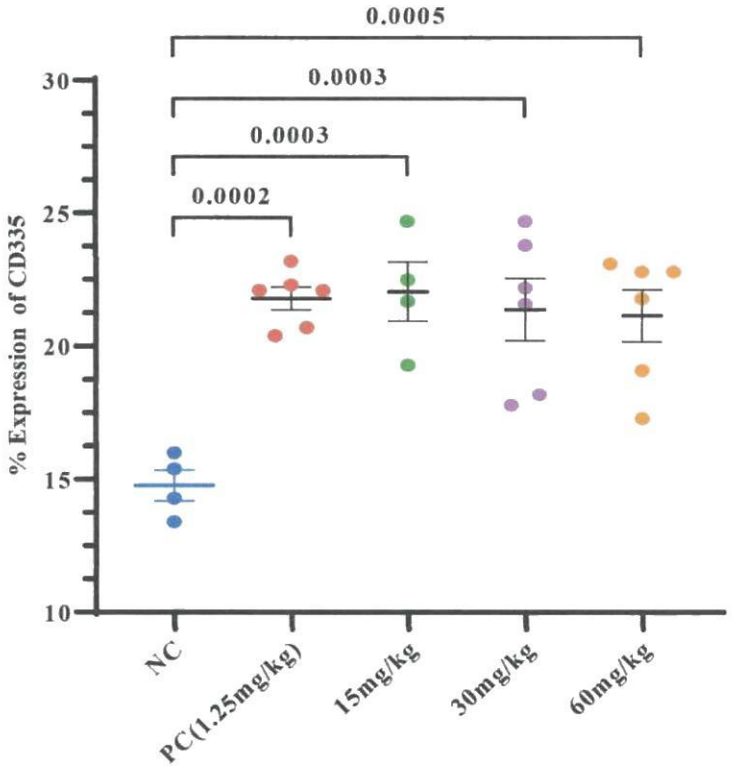



**Fig 9a:** Effects of the flavonoid extract on the frequency of expression of CD335 (NK cells) in *Plasmodium berghei* infected in mice

*Differences in the frequency of expression of CD335(Natural killer cells) were evaluated using a One-way Analysis of Variance while the difference among the means was considered at 95% confidence level using the post-hoc method of Dunnett's Multiple Comparison Test. On the*

*scatter plots, the horizontal axis represents concentrations of the Flavonoid extracts and positive control. PC refers to a positive control (Pyrimethamine). P-values < 0.05 were considered significant.*

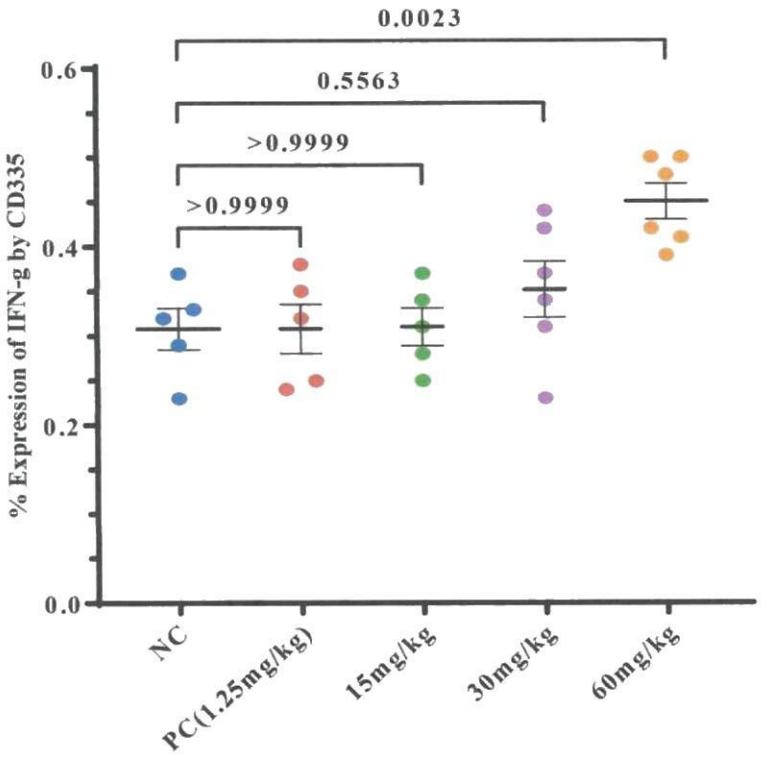



**Fig 9b:** Effects of the flavonoid extract on the frequency of IFN-γ by CD335 in *Plasmodium berghei* infected in mice

*Differences in the frequency of expression of IFN-γ by CD335(Natural killer cells) were evaluated using a One-way Analysis of Variance while the difference among the means was considered at 95% confidence level using the post-hoc method of Dunnett's Multiple Comparison Test. On the scatter plots, the horizontal axis represents concentrations of the Flavonoid extracts and positive control. PC refers to a positive control (Pyrimethamine). P-values < 0.05 were considered significant.*

### 4.7 Effect of Flavonoid extract on adaptive immune responses

The frequency of expression and functional analysis of the adaptive immune responses in mice prophylactically treated with flavonoid extract and then infected with *Plasmodium berghei was* determined. We hypothesized that flavonoids prophylaxis alters the frequency and function of CD8+ T cells repertoire in *Plasmodium berghei* infected mice.

#### 4.7.1 Effect of Flavonoid extract on CD4 T cells

The flavonoid extract increased the frequency of expression of CD4+ T cells, however, the increase was not statistically significant compared with negative control (Fig.10a).

In the figures10b, 10c, 10d and 10e, we noted no significant increase in the frequency of expression of CD279 (PD-1), IL-10, IL-17A and NF-α by CD4+ T cells when compared with the negative control.

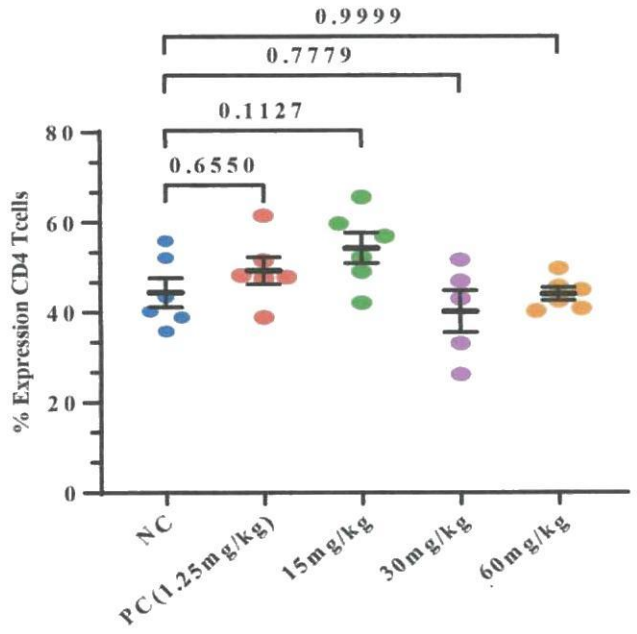



**Fig 10a:** Effects of the flavonoid extract on the frequency of CD4+ T cells in *Plasmodium berghei* infected in mice

*Differences in the frequency of expression of CD4+ T cells were evaluated using a One-way Analysis of Variance while the difference among the means was considered at 95% confidence level using the post-hoc method of Dunnett's Multiple Comparison Test. On the scatter plots, the horizontal axis represents concentrations of the Flavonoid extracts and positive control. PC refers to a positive control (Pyrimethamine). P-values < 0.05 were considered significant.*

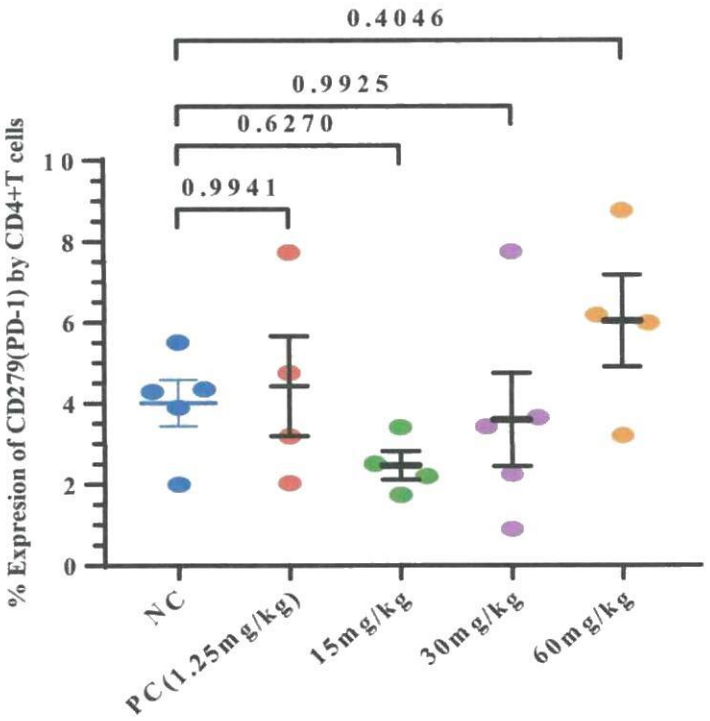



**Fig 10b:** Effects of the flavonoid extract on the frequency of CD279 (PD-1) by CD4+ T cells in *Plasmodium berghei* infected in mice

*Differences in the frequency of expression of CD279(PD-1) by CD4+ T cells were evaluated using a One-way Analysis of Variance while the difference among the means was considered at 95% confidence level using the post-hoc method of Dunnett's Multiple Comparison Test. On the scatter plots, the horizontal axis represents concentrations of the Flavonoid extracts and positive*

*control. PC refers to a positive control (Pyrimethamine). P-values < 0.05 were considered significant.*

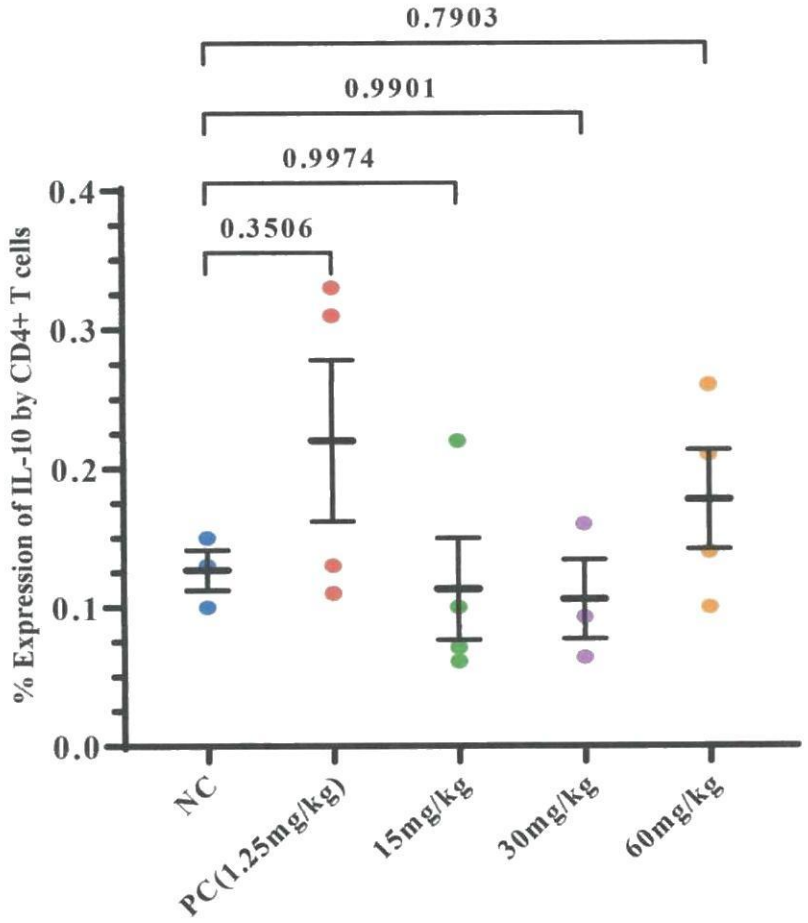



**Fig 10c:** Effects of the flavonoid extract on the frequency of IL-10 by CD4+ T cells in *Plasmodium berghei* infected in mice

*Differences in the frequency of expression of IL-10 by CD4+ T cells were evaluated using a One-way Analysis of Variance while the difference among the means was considered at 95% confidence level using the post-hoc method of Dunnett's Multiple Comparison Test. On the scatter plots, the horizontal axis represents concentrations of the Flavonoid extracts and positive control. PC refers to a positive control (Pyrimethamine). P-values < 0.05 were considered significant.*

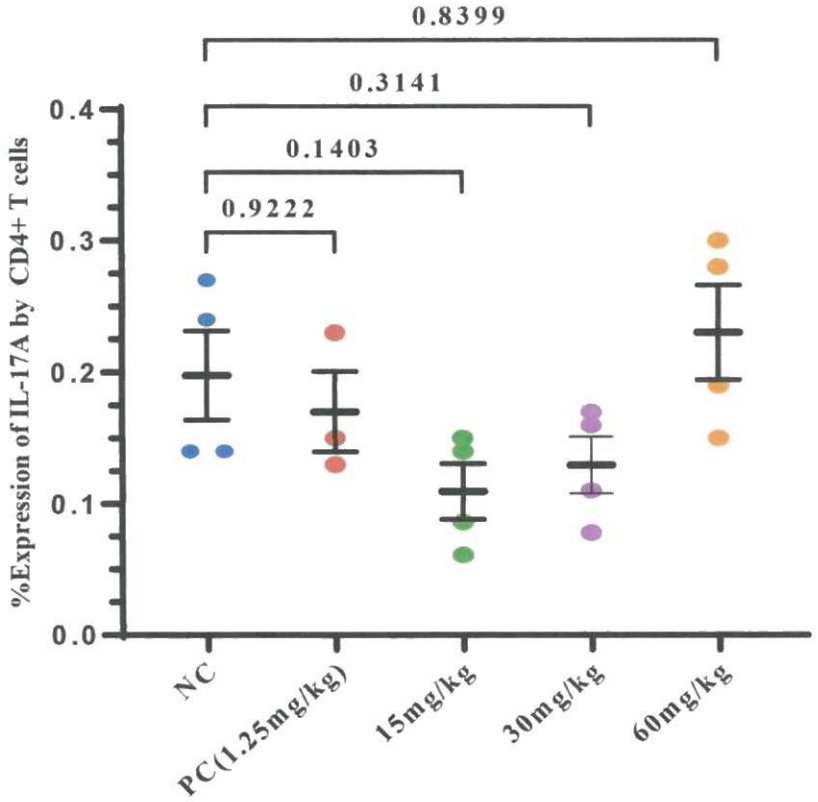



**Fig.10d:** Effects of the flavonoid extract on the frequency of IL-17A by CD4+ T cells in *Plasmodium berghei* infected in mice

*Differences in the frequency of expression of IL-17A by CD4+ T cells were evaluated using a One-way Analysis of Variance while the difference among the means was considered at 95% confidence level using the post-hoc method of Dunnett's Multiple Comparison Test. On the scatter plots, the horizontal axis represents concentrations of the Flavonoid extracts and positive control. PC refers to a positive control (Pyrimethamine). P-values < 0.05 were considered significant.*

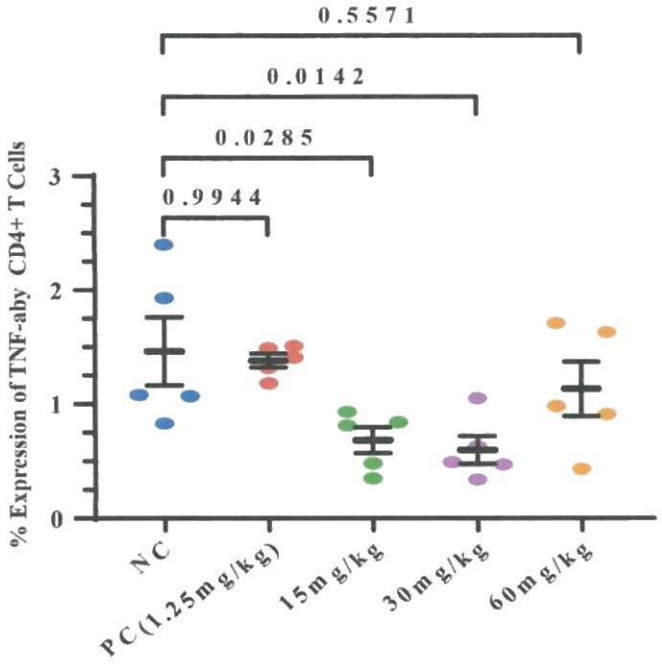



**Fig 10e:** Effects of the flavonoid extract on the frequency of TNF-α by CD4+ T cells in *Plasmodium berghei* infected in mice

*Differences in the frequency of expression of TNF-α by CD4+ T cells were evaluated using a One-way Analysis of Variance while the difference among the means was considered at 95% confidence level using the post-hoc method of Dunnett's Multiple Comparison Test. On the scatter plots, the horizontal axis represents concentrations of the Flavonoid extracts and positive control. PC refers to a positive control (Pyrimethamine). P-values < 0.05 were considered significant.*

### 4.7.2 Effect of Flavonoid extract on CD8+T cells

We hypothesized that flavonoids prophylaxis alters the frequency and function of CD8+ T cells repertoire in *Plasmodium berghei* infected mice.

Figure 11a shows the flavonoid extract increased frequency of expression of CD8+ T cells although not statistically significant when compared with the negative control.

Likewise, there was no significant increase in the frequency of expression of CD279 (PD-1), IFN-y and TNF-α by CD8+ T cells when compared with the negative control (Figs.11b, 11c and 11d).

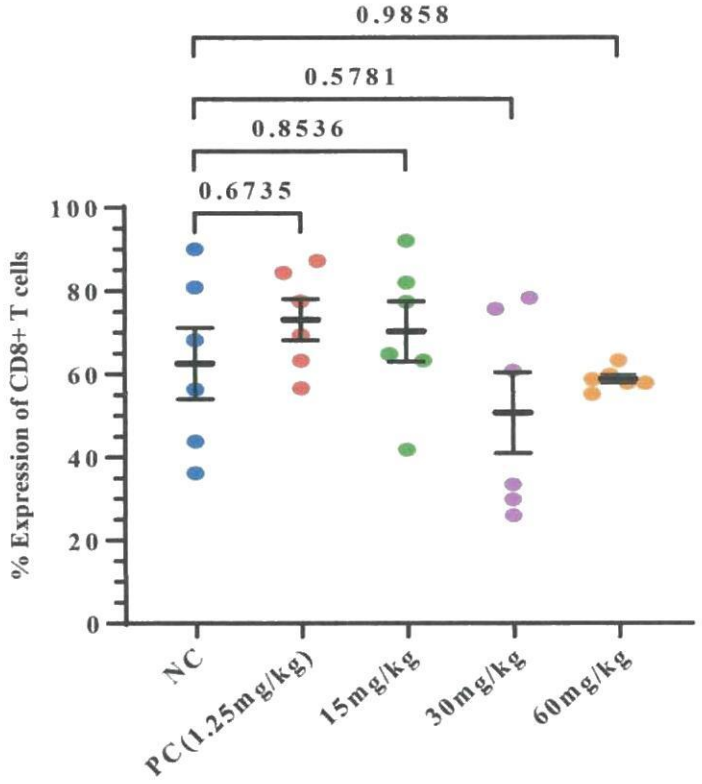



**Fig 11a:** Effects of the flavonoid extract on the frequency of CD8+ T cells in *Plasmodium berghei* infected in mice

*Differences in the frequency of expression of CD8+ T cells were evaluated using a One-way Analysis of Variance while the difference among the means was considered at 95% confidence level using the post-hoc method of Dunnett's Multiple Comparison Test. On the scatter plots, the horizontal axis represents concentrations of the Flavonoid extracts and positive control. PC refers to a positive control (Pyrimethamine). P-values < 0.05 were considered significant.*

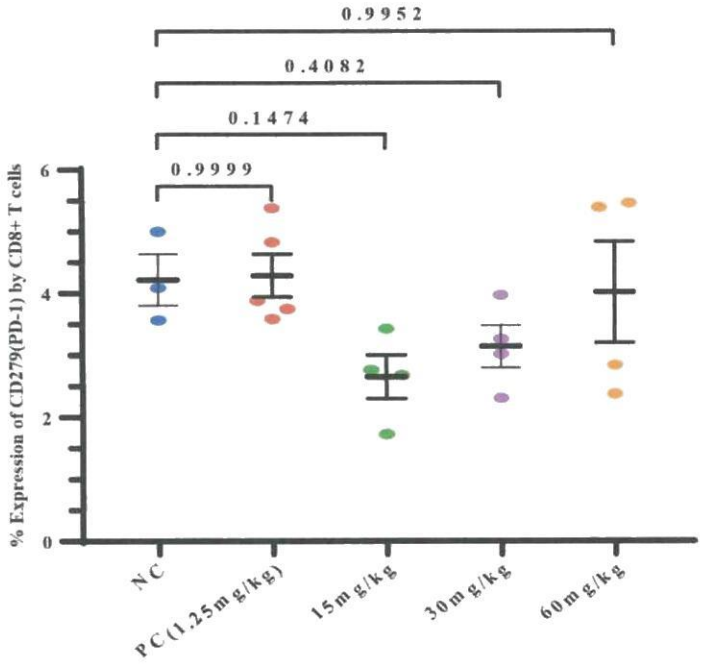



**Fig 11b**: Effects of the flavonoid extract on the frequency of CD279 (PD-1) by CD8+ T cells in *Plasmodium berghei* infected in mice

*Differences in the frequency of expression of CD279(PD-1) by CD8+ T cells were evaluated using a One-way Analysis of Variance while the difference among the means was considered at 95% confidence level using the post-hoc method of Dunnett's Multiple Comparison Test. On the scatter plots, the horizontal axis represents concentrations of the Flavonoid extracts and positive*

*control. PC refers to a positive control (Pyrimethamine). P-values < 0.05 were considered significant.*

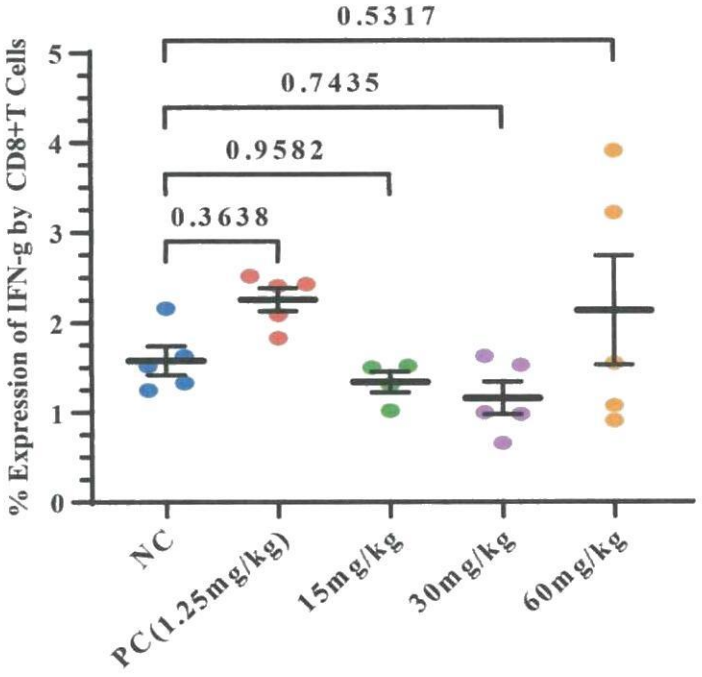



**Fig 11c:** Effects of the flavonoid extract on the frequency of IFN-y by CD8+T cells in *Plasmodium berghei* infected in mice

*Differences in the frequency of expression of IFN-y by CD8+ T cells were evaluated using a One-way Analysis of Variance while the difference among the means was considered at 95% confidence level using the post-hoc method of Dunnett's Multiple Comparison Test. On the scatter plots, the horizontal axis represents concentrations of the Flavonoid extracts and positive control. PC refers to a positive control (Pyrimethamine). P-values < 0.05 were considered significant.*

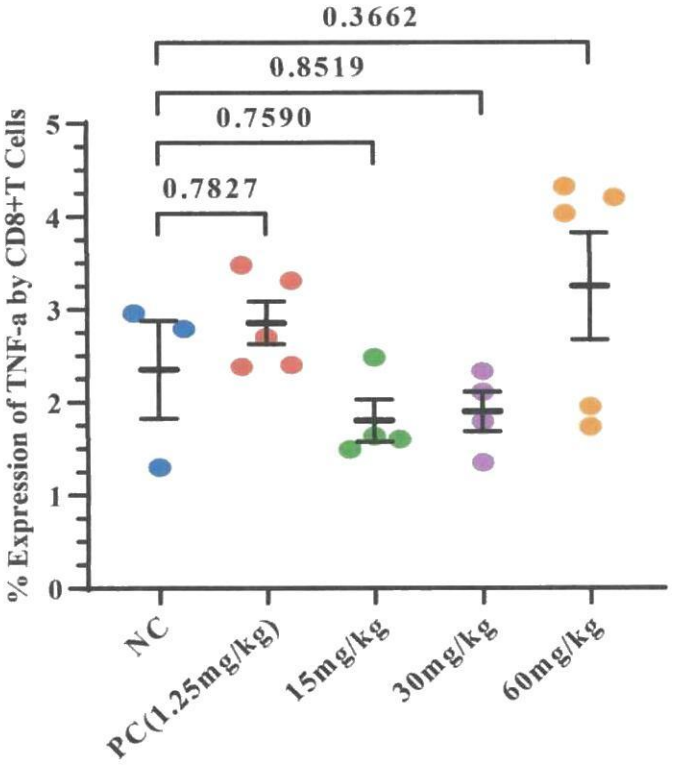



**Fig 11d:** Effects of the flavonoid extract on the frequency of TNF-α by CD8+ T cells in *Plasmodium berghei* infected in mice

*Differences in the frequency of expression of TNF-α by CD8+ T cells were evaluated using a One-way Analysis of Variance while the difference among the means was considered at 95% confidence level using the post-hoc method of Dunnett's Multiple Comparison Test. On the scatter plots, the horizontal axis represents concentrations of the Flavonoid extracts and positive control. PC refers to a positive control (Pyrimethamine). P-values < 0.05 were considered significant.*

# CHAPTER FIVE

# DISCUSSION

## 5.1 Hematological effects of ARTAVOL® in *Plasmodium berghei* prophylaxis

The prophylactic anti-plasmodial activity of the aqueous ARTAVOL® extract on *Plasmodium berghei* ANKA-infected mice produced a dose dependant suppression on day3 and day5 suggesting the extract has ability to suppress parasite growth. The extract elicited suppression activity at the three doses (15 – 60 mg/kg) investigated. The test doses of ARTAVOL® extract was able to inhibit the increase in the parasitemia levels and this effect was significant when compared with the negative group (Table 3). Despite the overall suppression effect compared to the positive control group, all test doses of ARTAVOL® extract produced a parasitemia suppression as compared to the negative control group. This activity could be attributed to the presence of alkaloids, flavonoids, terpenoids and tannins in ARTAVOL® (Ogwang *et al.*,2011). Petros and Melaku (2012) stated in their study that a compound/product is active when its percentage suppression is more than 30%. In the present study, the aqueous extract of ARTAVOL® produced suppression in the prophylactic activity with above 40% suggesting the ARTAVOL® extract to be a promising prophylactic agent.

The extract dose 60 mg/kg caused comparative increase in the monocyte count to the uninfected group (Table 4). A similar observation was made by Ketema *et al.* (2015) who studied the effect of Khat (*Catha edulis*) on *Plasmodium berghei* ANKA infection in mice (Ketema et al., 2015). An increase in monocyte count by extract could be probably be associated with alkaloids, flavonoids, terpenoids and tannins in ARTAVOL®. It was reported that flavonoids are capable of increasing the monocyte count among the phytochemicals reported by Setyawan et al (2021), Kiani et al (2022) and Kayani *et al* (2021) which was the reason of further investigating the effects of flavonoids in this study.

In the present study, the effect of ARTAVOL® extract in inhibiting hemolysis of the red blood cells (RBCs) and further infection of normal RBCs were assessed. There was no significant effect of ARTAVOL® extract on RBC counts when compared with the uninfected group but significant compared to the negative control which was an indication of positive response (Table 4). This is contrary to the findings of Audomkasok *et al.* (2014) who carried a similar study and reported that the crude extracts of Green tea, Safflower and Mulberry exerted a dose dependent anti-hemolytic effects against *P. berghei* ANKA infection in mice (Audomkasok et al., 2014).

A non-significant slight increase in HCT values in this study (Table 4) was observed which suggests ARTAVOL® extract could prevent destruction of RBCs during infection. 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 (Ogwang et al., 2011).

**5.2 Effect of ARTAVOL® extract on innate and adaptive immune responses.**

The influence of ARTAVOL® on innate and adaptive immune responses in mice prophylactically was studied in *Plasmodium berghei* ANKA infected mice. This was aimed at assessing whether the ARTAVOL® extract could increase the frequency and function of immune cells.

In the innate immune response, the extract at 30 and 60 mg/kg showed varied significant increase in the frequency of frequency of CD 11b (Monocytes) compared to negative control which could have resulted into parasite clearance Fig 4a). In a previous study, it was found out that alkaloids, flavonoids, terpenoids, steroids, phenolic and saponins extracted from eggplant peel effectively increased the number of monocytes in mice infected with *P. berghei* (Widiyantoro, 2019*)*. The present study also significantly increased the frequency of the frequency of TNF-α by CD 11Bb

at higher doses (Fig 4b). A similar observation was made by Afolayan *et al.* (2020) who reported the extract from *Moringa lucida* stimulated both pro-inflammatory and anti-inflammatory cytokines with percentage immunomodulatory activity of 45% for TNF-α among others (Afolayan *et al.*, 2020). Dobbs et al. (2020) also reported that monocytes are innate immune cells that protect the host by reducing parasite burden through phagocytosis, cytokine production and antigen presentation (Dobbs et al., 2020).

Furthermore, in this study, all tested doses showed significantly increased in the frequency of the frequency of CD335 (natural killer cells) (Fig5a) and IFN-γ by natural killer cells compared to the negative control (Fig 5b). Flavonoids have been reported to increase the release of intracellular cytokines (Grudzien and Rapak, 2018). IFN-γ controls Plasmodium infection in both the liver and blood stages of the parasite life cycle (Thayer et al, 2015).

In the adaptive response, a significant increase in the frequency of CD4+T cells expressing IL-10 was observed in all the test doses (15–60 mg/kg) of the extract(Fig6c). Interleukin 10 (IL-10) is regarded as necessary for suppressing severe pathology during plasmodium infection (Niikura et al., 2011). Kumar et al. (2019) also reported that IL-10 directly dampens pro-inflammatory cytokine and chemokine production and increases the expression of immune checkpoint molecules.

Though there was a dose-independent effect on the frequency of the frequency of CD4+ T cells at 15 to 60 mg/kg in the frequency of CD4+ T cells Fig 6a) for instance at 30 and 60 mg/kg, there was no significant increase in the frequency of CD4+ T cells compared to the negative control. This could possibly be as a result of an increase in antagonistic component resulting to the inhibition of the active one inducing proliferation. Kurup *et al.* (2019) reported that extracts may induce T-cell exhaustion which can down-regulate T-cell function (Kurup et al., 2019).

In addition, the increase in the frequency of frequency of CD8+ T cells (Fig 7a) by all the test doses was not significant suggesting that the extract did not cause enough frequency of CD8 T cells. This could be due to reduction in the oral bioavailability of flavonoids. It was reported that flavonoids have a low bioavailability of 16% in rats (Kaşıkçı and Bağdatlıoğlu, 2022).

Although the frequency of CD8+ T cells was not significant, there was a significant increase in the frequency of IFN-γ(Fig 7c)and TNF-α(Fig7d) by CD8+ T cells in all extract tests. This means that the few CD8+ T cells generated by the extract were able to induce the release of IFN-γ and TNF-α.

The ARTAVOL® extract test doses at 15 and 30 mg/kg showed a significant increase in the frequency of CD 279(PD-1) Fig 7b). This means the extract activates PD-1 at lowest and medium doses promote immune extortion thus decreasing further inflammation. Liu *et al.* (2020) reported that CD279(PD-1) acts as an important immune check point by inhibiting CD8+ T cell activation and antibodies against CD279(PD-1) enhances T cell mediated cytotoxic function. Konkel et al., 2010 also reported that CD279 (PD-1) provides inhibitory signals that dampen T-cell function and restrains their clonal expansion (proliferation) to an immunogenic stimulus.

**5.3 Effect of Flavonoid extract on innate and adaptive immune responses**

All the extract test doses at 15, 30 and 60mg/kg showed a significant gradual frequency of CD335 (natural killer cells) (Fig 9b) but the extract was not able to induce the release of IFN-y (Fig. 9b) by natural killer cells. The findings of this study agrees with Han et al, (2015) who reported that total flavonoids extracted from seeds of *Astragali complanati* had significant effect on natural killer cell frequency and function (Han et al., 2015).

However, there was increase in the frequency of CD 11 (Monocytes) (Fig 8a) at all doses though not significant compared to the negative control. Likewise, the extract test doses at 15 to 60

mg/kg induced expression of intracellular of cytokines (TNF-α) by CD 11(Fig. 8b) which was significant at the lowest dose (15 mg/kg) compared to the negative control. Monocytes have been reported to exhibit protection against malaria and reduce parasite burden through cytokine production and phagocytosis (Chua et al., 2013, Ortega-Pajares and Rogerson, 2018, Dobbs et al., 2020).

In this study, the flavonoids extract did not increase the frequency (clonal expansion) and expression of intracellular of cytokines (IL-10, IL-17A and IFN-y and TNF-α) of CD4+ T cells (Fig.10a-10b and Fig.11a-11b). The non-frequency and function exhibited by the flavonoid extract on CD4+ T cells could be due to short period of study.

The results of this study also showed that the flavonoids extract did not significantly increase the frequency(clonal expansion) and expression of intracellular of cytokines (IFN-y and TNF-α) of CD8+ T cells(Fig 11c and 11d) This could be attributed to the low oral stability and solubility of extracted flavonoids as reported by Li *et al.* (2019) who informed that water solubility and gastric stability are major limiting factors for flavonoids to pass through the biological membranes and to be absorbed systematically following oral administration.

**5.4 Limitations of the study.**

In this study, the ARTAVOL® and flavonoids extract were given prophylactically to mice for 7 days. There could have been a possible extension of the period of study but the *P. berghei* ANKA used in the study was highly virulent and the negative control mice could not survive beyond 7 days.

Humoral mediated immune responses of ARTAVOL® in malaria prophylaxis was not done because of limited finances.

# CHAPTER SIX:
# CONCLUSION AND RECOMMENDATIONS

## CONCLUSION

ARTAVOL® did not cause any adverse effects and mortality in the animals hence both extracts are acutely safe. ARTAVOL® showed a dose-independent suppression of the parasites. This study showed that ARTAVOL® increased monocytes and Natural killer cells count.

ARTAVOL® extract at higher dose stimulated the frequency of monocytes and natural killer cells and subsequent release of intracellular cytokines like Interferon gamma (IFN-γ) and Tumor necrosis factor- alpha (TNF-α) hence, this product can modulate the innate immune responses.

ARTAVOL® showed a dose-independent increase in the frequency of CD4+ T and CD8+ T cells although not statistically significant.

The flavonoid extract did not show increase the frequency of CD4+ and CD8+Tcells.

## RECOMMENDATIONS

Future studies should explore the humoral mediated immune responses of ARTAVOL® in malaria prophylaxis.

There is need to carryout Pharmacokinetic and Pharmacodynamic analysis of ARTAVOL®

There is need to carry out the study for at least 14 to 28 days to fully assess the adaptive immune responses.

A clinical trial in humans should be carried out to ascertain the innate, adaptive and humoral mediated immune responses.

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## Appendices

### Appendix i: Faculty Research Committee approval

MBARARA UNIVERSITY OF SCIENCE AND TECHNOLOGY
FACULTY OF MEDICINE RESEARCH COMMITTEE

Ref: DMS 6

May 2, 2016

Dr Martin Amanya
Reg no: 2016/PHD.028/PS
Department of Pharmacology
MUST

Dear Dr Amanya

Submitted Proposal "**Immunomodulatory Effects of Artavol® in Malaria Prophylaxis Using a Mouse Model**"

Reference is made to the above protocol you re-submitted to the Faculty Research Committee (FRC) for review.

The Committee reviewed and approved your protocol.

You are now advised to proceed to produce enough copies of the **Protocol Form** and **Research Proposal** for eventual submission to the Institutional Research Ethics Committee. The Secretary to that Committee will advise you on the number of copies you are to submit.

Please remember to attach a letter from the Head of Department or your supervisor when submitting.

Since rely,

Associate Professor Paul Mele
Chairperson, Faculty Research Committee

Copy: Professor Abia Akabai and Dr Bernard Natukunda, Supervisors

## Appendix ii: MUST research Ethics committee



MBARARA UNIVERSITY OF SCIENCE AND TECHNOLOGY

P.O. Box 1410, Mbarara Uganda. Tel: +256 4854 33795. Fax: +256 4854 20932

RESEARCH ETHICS COMMITTEE

E-mail: [redacted]

**Our Ref.** MUREC 1/7

**Date:** 11 June 2019

Mr Martin Amanya
Postgraduate Student

**Re:** **"Immunomodulatory effects of ARTAVOL [redacted] malaria prophylaxis using a mouse model" 07/05-19**




**Type:** [✓] Initial Application
[ ] Protocol Amendment
[ ] Letter of Amendment (LOA)
[ ] Continuing Review
[ ] Material Transfer Agreement
[ ] Other, specify _____

Reference is made to the above protocol which was submitted to the Research Ethics Committee for reconsideration and *approval*.

The committee at its 109th sitting held on Tuesday 28th and Wednesday 29 May 2019, unanimously *agreed to approve* your proposal.

I am pleased to inform you that your study has been approved for a period of one year from **June 11, 2019** up to ***June 10, 2020.***

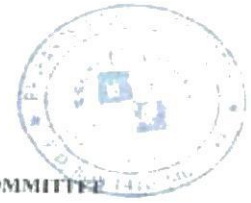
As Principal Investigator of the research, you are responsible for fulfilling the following requirements of approval

1. All co-investigator must be kept informed of the status of the research
2. Changes, amendments, and addenda to the protocol or the consent form must be submitted to the REC for review and approval **prior** to the activation of the changes. The REC application number assigned to the research should be cited in any correspondence.
3. Reports of unanticipated problems involving risks to participants or other must be submitted to the REC. New information that becomes available which could change the risk: benefit ratio must be submitted promptly for REC review.
4. Only approved consent forms are used in enrolment of participants. All consent forms signed by subjects and/or witness should be retained on file. The REC may conduct audits of all study records, and consent documentation may be part of such audits.
5. Regulations require review of an approved study not less than once per 12-month period. **Therefore, a continuing review application must be submitted to REC eight weeks prior to the above expiration date of June 10 2020 in order to continue the study beyond the approved period.** Failure to submit a continuing review application in timely fashion may result in suspension or termination of the study, at which point new participants may not be enrolled and currently enrolled participants must be taken off the study.
6. You are required to register the research protocol with the Uganda National Council for Science and Technology (UNCST) for final clearance to undertake the study in Uganda

The following is the list of documents approved in the application.

| Document | Language | Version |
|---|---|---|
| Proposal | English | Version 1 |
| Protocol forms | English. | Version 1 |

I wish you all the best



**Dr. Francis Bajunirwe**
**CHAIR,**
**MUST RESEARCH ETHICS COMMITTEE**

## Appendix iii: UNCST Approval



**Uganda National Council for Science and Technology**

*(Established by Act of Parliament of the Republic of Uganda)*

**Our Ref: HS465ES** **17th October 2019**

Mr. Martin Amanya
Principal Investigator
Mbarara University of Science and Technology
**Mbarara**

Dear Mr. Amanya

I am pleased to inform you that on **08/10/2019** the Uganda National Council for Science and Technology (UNCST) approved your study titled, **Immunomodulatory Effects of Artavol in Malaria Prophylaxis Using a Mouse Model.** Approval is valid for the period of **08/10/2019** to **08/10/2021**.

Your study reference number is **HS465ES**. Please, cite this number in all your future correspondences with UNCST in respect of the above study.

Please, note that as Principal Investigator, you are responsible for:

1. Keeping all co-investigators informed about the status of the study.
2. Submitting any changes, amendments, and addenda to the study protocol or the consent form, where applicable, to the designated local Research Ethics Committee (REC) or Lead Agency, where applicable, for re-review and approval prior to the activation of the changes.
3. Notifying UNCST about the REC or lead agency approved changes, where applicable, within five working days.
4. For clinical trials, reporting all serious adverse events promptly to the designated local REC for review with copies to the National Drug Authority.
5. Promptly reporting any unanticipated problems involving risks to study subjects/participants to the UNCST.
6. Providing any new information which could change the risk/benefit ratio of the study to the UNCST for final registration and clearance.
7. Submitting annual progress reports electronically to UNCST. Failure to do so may result in termination of the research project.

Please, note that this approval includes all study related tools submitted as part of the application.

Yours sincerely,

Hellen Opolot
For: Executive Secretary
**UGANDA NATIONAL COUNCIL FOR SCIENCE AND TECHNOLOGY**


***LOCATION/CORRESPONDENCE***

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*KAMPALA, UGANDA*

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**Appendix iv: Publication 1**




ORIGINAL ARTICLE

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

[redacted]


## 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 one of 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 °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 1). 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 Haematocrit (HCT) reduction was observed at the 3 dose levels but their parameters did not show any significant differences 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 $1\times10^7$ 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.*

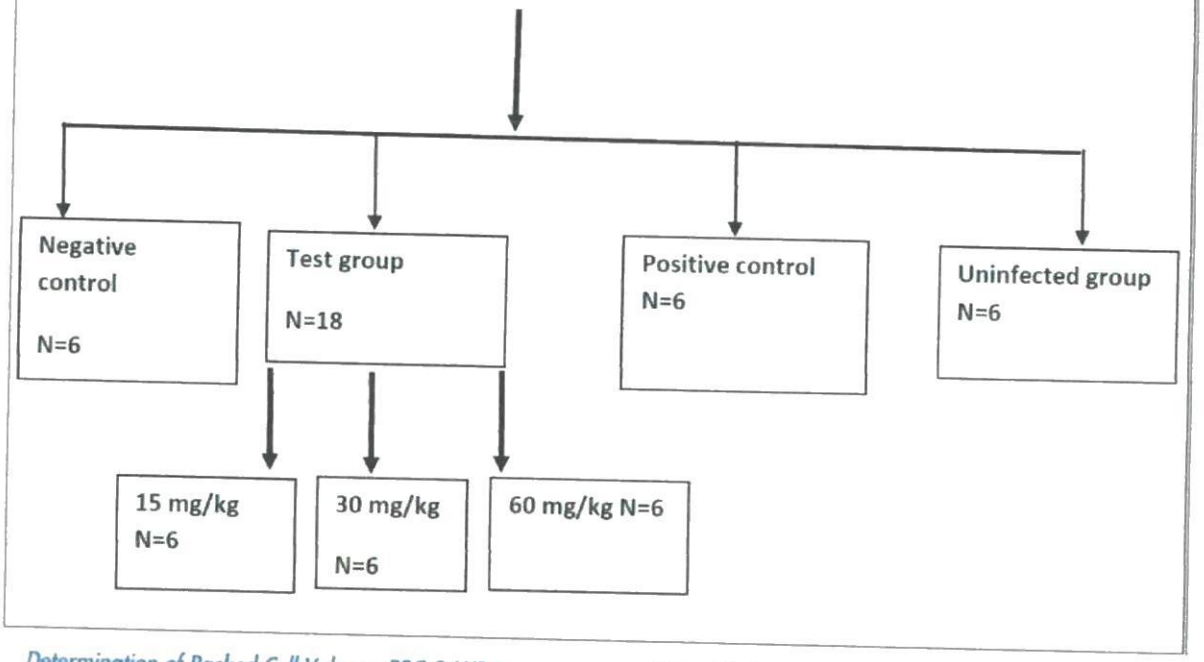
The prophylactic antimalarial potential of ARTAVOL® was carried out according to Peter's method 2002[25]. Thirty six (36) mice were grouped into 6 groups of 6 mice each; Mice in Groups I –III were orally administered with ARTAVOL® by using oral cannula while the animals in Group IV were given Pyrimethamine 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).

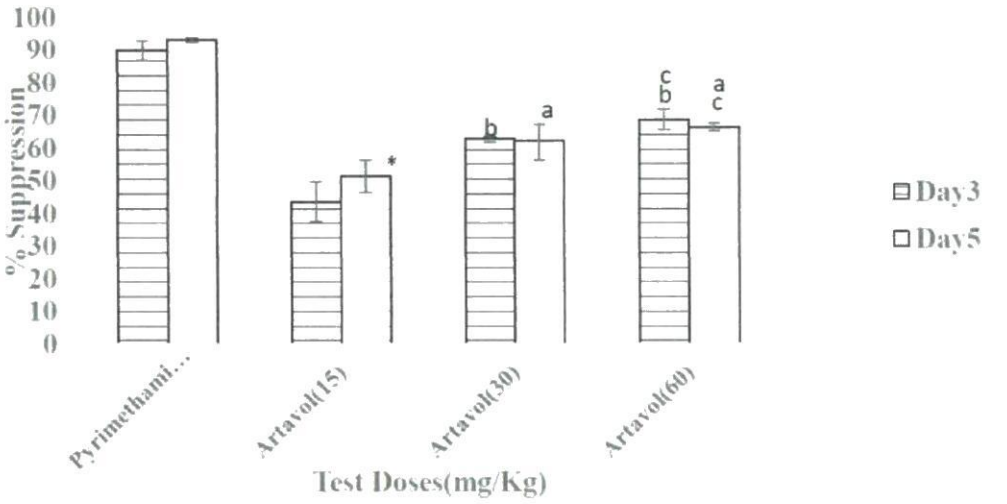
Table 1 shows the parasitaemia level on day 3 and Day 5. Figure 2 shows the percentage parasitaemia suppression in the treated (ARTAVOL® 15-60mg/kg) groups in comparison to positive control group on day 3 and day 5 and Table 2 shows the effect of ARTAVOL® prophylaxis on Monocytes, HCT, and RBC values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

| Treatment Groups | HCT (%) | 95%CI | RBCx$10^{12}$/L | 95%CI | Monocytes (%) | 95%CI |
|---|---|---|---|---|---|---|
| **Normal group** | 41.80±0.98 | 38.68-44.92 | 9.03±0.37 | 7.86-10.20 | 12.18±1.62 | 7.03-17.33 |
| **Negative control** | 19.00±3.73*** | 9.42-28.58 | 3.84±0.56*** | 2.39-5.28 | 4.37±0.82*** | 2.25-6.48 |
| **Positive control** | 33.98±2.16[a] | 27.09-40.86 | 7.68±0.58[a] | 5.84-9.52 | 5.17±0.75*** | 3.25-7.09 |
| **15mg/Kg** | 33.15±3.03[a] | 25.37-40.93 | 6.63±0.90[a] | 4.32-8.94 | 4.32±0.69*** | 2.53-6.10 |
| **30mg/Kg** | 39.47±3.77[a] | 23.24-55.69 | 7.54±0.42[a] | 5.75-9.34 | 6.77±0.96* | 2.67-10.87 |
| **60mg/Kg** | 37.55±0.51[a] | 35.94-39.16 | 7.43±0.46[a] | 5.97-8.88 | 8.22±0.86[a] | 6.02-10.42 |

***$P$< 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%[24].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[25] 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[26, 27].

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[28, 29]. The slight increase in HCT values in this study demonstrated a progress in disease regression, which suggests

TAVOL® extract could have prevented destruction RBCs during infection. There was no significant cts of ARTAVOL® extract on RBC counts when pared with the normal group. This implies that TAVOL® extract inhibited destruction of red blood s. This could be probably due to the presence of ondary metabolites including alkaloids, coumarins, onoids, sterols, triterpenes, tannins, volatile oils, y acids and reducing compounds which were orted to have anti-parasitic effects[15].Flavonoids e also been reported to have antioxidant, anti-ammation properties and inhibit the parasites tein synthesis[35, 36].

## ONCLUSION

this study, the aqueous ARTAVOL® extract did not w any lethal effect in the tested mice up to 0mg/kg dose and therefore could be acutely safe. o, the extract has displayed a dose dependent lucing effect on the level of parasitaemia in the ted mice and could therefore be recommended as antimalarial prophylaxis. Further investigation on sub-chronic effect and its immuno-modulatory ects is recommended.

***knowledgement:*** We would like to thank BEI Resources USA provision of *Plasmodium berghei* ANKA parasites. Pharmbiotrax Mr. David Nkwangu for Laboratory extraction.

### *mpeting Interests*

re declared.

### *nding*

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**Appendix iv: Publication 2**

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ACADEMIC JOURNALS

African Journal of Pharmacy and Pharmacology

*Full Length Research Paper*

# Assessment of the immunomodulatory activity of Artavol® aqueous extract in *Plasmodium berghei* infected mice

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**ARTAVOL® is an herbal product derived from de-artemisinised extracts of Artemisia annua combined with lemon grass extract and avocado seed powder as a base locally used in Uganda for malaria prevention. This study assessed the immunomodulatory activity of the aqueous extract of Artavol® in *Plasmodium berghei* infected mice. The immunomodulatory activity of the aqueous extract of Artavol® was evaluated on Chloroquine-sensitive *P. berghei* ANKA-infected mice using 7 days test at 15, 30, and 60 bmg/kg with Pyrimethamine (1.25 mg/kg) as positive control; and distilled water as negative controls. Seven days post – infection, mice were euthanized and the spleen collected from each mouse under sterile conditions. Phenotypic and functional characterization was T-cell surface and intracellular markers was done by CytofleX LX flow Cytometer, and the data obtained were analyzed using FlowJo version 10.7.1 software. All the extracts (15-60 mg/kg) doses showed a significant increase in the frequency of monocytes ($p=0.0078$, $p=0.0084$, and $p<0.0001$, respectively) compared to the negative control. The extract at 30 and 60 mg/kg showed a gradual significant increase ($p<0.0001$ and $p< 0.0001$, respectively) in the frequency of natural killer cells (NK cells) compared to the negative control. The aqueous extract of ARTAVOL® possesses variable immunomodulatory effects and showed ability to increase the frequency of monocytes and natural killer cells. IFN-γ and TNF-α levels showed a significant increase suggesting the extract induced their responses.**

**Key words:** Artavol®, Immunomodulatory effect, *Plasmodium berghei*.

## INTRODUCTION

The world health organization promotes the use of chemoprophylactic drugs to treat and prevent malaria in endemic areas (World Health Organization, 2015). Chemoprophylaxis has been effective in preventing

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morbidity and mortality from malaria among high-risk groups such as pregnant women, infants and children (Tizifa et al., 2018) hence, tourists travelling to endemic and high-risk areas are advised to take prophylactic antimalarial agents (Schlagenhauf et al., 2019; Shellvarajah et al., 2017). World health organization has also recommended the use of recombinant protein-based malaria vaccine (RTS, S/AS01) among children in sub-Sharan Africa and other regions with moderate to high *Plasmodium falciparum* transmission (Trottier and Elliott, 2021). Currently, all available chemoprophylactic anti-malarial agents do not provide 100% protection and using these agents for long period might interfere with the development of naturally acquired immunity and facilitate the emergence and spread drug resistant strains (Greenwood, 2010). With the problems of increasing levels of drug resistance among conventional antimalarials, herbal medicines could be an important and sustainable source of treatment of malaria parasites (Willcox and Bodeker, 2004).

Medicinal plants andl products have been used for prophylaxis and treatment of malaria in sub-Saharan Africa for years (Chinsembu, 2015). Use of herbal remedies has also been embraced in many developed and developing countries as complementary and alternative medicines (Ekor, 2014).

In Uganda, a number of medicinal plants used to treat malaria have been reported (Ajayi et al., 2020; Tugume et al., 2016). ARTAVOL® is one of the prophylactic antimalarial herbal products used locally in Uganda (Mesa et al., 2017; Ogwang et al., 2011). It is composed of Avocado seed extracts, Lemon grass, and *Artemisia annua* without artemisinin.

The ARTAVOL® powder can be mixed in hot water, porridge or hot milk and taken as a beverage to prevent malaria (Ogwang et al., 2011). Although ARTAVOL® has been proven to exhibit antimalarial activity, there is need to assess its immunomodulatory effects. This study, therefore, assessed the immunomodulatory responses of the aqueous extract of ARTAVOL® in Swiss albino mice infected with *P. berghei* ANKA parasites.

## MATERIALS AND METHODS

### Study design

The study employed a controlled *in vivo* experimental design in which the effects of ARTAVOL® aqueous extract on the cell Mediated immune response in *P. berghei* infected mice was evaluated.

### Malaria parasite strain

Chloroquine sensitive *P. berghei* ANKA MRA 311 was obtained from BEI resources, NIAID, NIH contributed by Thomas F. McCutchan. The vial containing 0.5 mL *P. berghei* ANKA strain parasite in dried ice was activated by thawing frozen cryovial in water bath at 35°C for 2 min. This was followed by wiping the outside of the vial with 70% ethanol before opening and 50 µL injected intraperitoneally into each mouse. The growth of the parasites was monitored by tail vein blood sampling and Giemsa-stained thin blood smear microscopically daily, starting on day -3 post infections.

### Laboratory animals, care and ethical approval

Adult male and female Swiss albino mice between 20-30 g were obtained from the Animal Facility Research, Mbarara University of Science and Technology, Uganda. The mice were fed on grower pellets purchased from Kuyoyo Feeds, Uganda and had free access to water. The mice were acclimatized for two weeks before the experiments. The study was carried out between September 2021 to December 2021. All the animals in the experimental study were treated humanely according to the Organization for Economic Co-operation and Development (OECD) guidelines and American Psychological Association (Bedi and Krishan, 2020, Loue, 2020). ARTAVOL® product (Batch number: 005) was obtained from ARTAVOL Ltd (ARTAVAL Ltd. P.O. Box.34 Ntinda, Kampala-Uganda the manufacturer 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 with approval number 07/05-19 and the Ugandan National Council for Science and Technology registration number HS465ES.

### Preparation of crude ARTAVOL extracts

ARTAVOL® extract was prepared using infusion method in which 60 mL of distilled water was boiled at 80°C, poured into 173 g powdered ARTAVOL®, and allowed to stand for 15 min. Thereafter, the extract was filtered, concentrated *in vacuo* at 55°C and freeze-dried. The aliquot of the stock solution of 5 mg/mL concentration was prepared and stored in a fridge below 4°C. The test doses of 15, 30, and 60 mg/kg was separately determined from the stock solution to have a final volume of 60, 120 and 240 µL 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's method (Lorke, 1983). In the first phase, nine mice were randomly divided into three groups of three mice each and each group received the extract at 10, 100 and 1000 mg/kg body weight orally. The mice were then observed for signs of adverse effects and mortality for the first 48 h and then for 12 more days. In the second phase following the same Lorke's method, three mice were divided into 43 groups of 1 mouse each. The animals were given higher doses (1600, 2900, and 5000 mg/kg) of the test extract. 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:

$$LD_{50} = [D_o \times D_{100}]^{1/2}$$

Where $D_o$ = highest that caused no mortality and $D_{100}$ = the lowest dose that produced mortality (Lorke, 1983).

### Preparation of malaria parasite Inoculum

Chloroquine (CQ)-sensitive *P. berghei* ANKA parasites were activated in the mice according to BEI Resources user reference.. About 200 µL of the inoculum was injected into the donor mouse intraperitoneally and the parasites growth was monitored after 72 h

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 $1\times10^7$ parasitized erythrocytes was prepared by dilution of blood collected through cardiac puncture from a donor mouse (> 30% parasitemia) with normal saline and administered intraperitoneally (200 µL) to each test mouse.

### Determination of immune response

Thirty (30) mice were grouped into 5 groups of 6 mice each: Mice in Groups I – III were orally administered with ARTAVOL® (15, 30 and 60 mg/kg, respectively) by using oral cannula while the animals in Group IV were given Pyrimethamine at 1.25 mg/kg/day (positive control) and group V (negative control) received 200 µL/mouse/day distilled water for seven consecutive days ($D_1$ – $D_7$) respectively. On day 7, all the mice were inoculated with *P. berghei* parasites. On day 7 post infection, mice were euthanized and the spleen collected from each mouse under sterile conditions. The spleens were placed in RPMI 1640 (Thermo Fisher Scientific, Waltham, MA USA) media, crushed through a cell strainer. The cell suspension was washed twice using 1X PBS (washing buffer) and centrifuged at 1500 rpm for 10 min at 25°C. RBCs in the pellet were lysed using 2.5 mL of lysing buffer (Gibco, USA) for 2 min at room temperature. The lysing buffer was washed off and splenocytes counted using a hemocytometer machine. The splenocytes were cryopreserved in liquid nitrogen until use.

### Splenocytes thawing and staining

Cryopreserved splenocytes were retrieved from liquid nitrogen at -196°C and immediately transferred to a preset 37°C water bath. Upon thawing, cells were washed in complete RPMI 1640 medium and kept overnight at 37°C in 5% carbon dioxide ($CO_2$). Thereafter, the cell yield viability using trypan blue solution was determined. Cells were stained using 0.4% Trypan blue solution at a 1:1 dilution ratio. Samples with at least 70% viability were used for stimulation assays.

### Cell surface staining

About 4.0 $\times10^5$ cells/mL were surface stained and incubated for 30 min with the following antibodies; CD3ε Brilliant Violet 510™ (Cat No. 100353), CD4 PE-Cy5(Cat No 100409), CD8b.2 APC/Fire™ 750(Cat. No. 140419), CD11b FITC (Cat. No. 101206), CD25 PE/Dazzle™ 594 (Cat No 102047), CD69 PE/Cyanine7 (Cat. No. 104512), CD279 (PD-1), Brilliant violet 421 (Cat. No 109121), and CD335 (NKp46) APC (Cat. No. 137608) in which 1 uL each of surface markers were added to the samples and incubated for 30 min at room temperature in the dark. The cells were washed twice by adding cell staining buffer and centrifuged at 1800 rpm for 5 min. The cells were acquired on a 19 color CytofleX LX flow Cytometer (Beckman coulter, New Jersey, USA). At least 100,000 events were recorded for analysis. Gating was standardized and set using fluorescence minus one control (FMOs).

### Intracellular staining of cytokines

Following surface staining, the cells (splenocytes) were fixed by adding 0.5 mL of fixation buffer to each sample tube, incubated in the dark for 20 min at room temperature and then centrifuged at 1800rpm for 5 min and the supernatant discarded. The fixed cells were permeabilized by re-suspending in intracellular staining perm wash buffer (diluted to 1X in deionized water) and centrifuged at 1800 rpm for 5 min. A cocktail of intracellular cytokine antibodies was prepared by adding 1ul of each that is, IFN-γ BV605 (Cat. No 505839), TNF-αBV650 (Cat. No 506333), IL-2 BV785 (Cat. No. 503843), IL-4 PerCP Cy5.5 (Cat. No 504123), IL-10PE (Cat No. 505008), and IL-17A Alexa F700 (Cat. No 506914) in the tube. The antibody cocktail was added to each of the sample tubes and incubated for 20 min in the dark at room temperature. Cells were washed 2 times with 2 mL of Intracellular Staining Perm Wash Buffer and centrifuged at 1800 rpm for 5 min. The cells were then resuspended in 200 µL Cell Staining Buffer. The samples were acquired on the Cytoflex LX flow cytometer.

Compensations and fluorescent minus ones (FMOs) controls for each of the antibody fluorochromes were ran together with the optimization gains for every channel using unstained cells. Compensation calculations were done and applied to the samples. At least one hundred thousand events were acquired and recorded per sample. Data was exported for analysis using flowJo software.

### Statistical analysis

Flow cytometry data were analyzed using FlowJo version 10.7.1 software (Becton Dickinson, New Jersey, USA). Statistical data were analyzed using Graph Pad Prism version 8.0.3 (Prism, 2018). Lymphocytes were carefully gated on a forward scatter and side scatter plot. The percentage of positive cells for each marker was determined on the basis of the numbers of viable lymphocytes. The frequencies of T cells expressing activation and memory markers were measured as a percentage of the CD4+ and CD8+ T cell parent cell populations. Bivariate dot plots or probability contour plots were generated upon data analysis to display the frequencies of and patterns by which individual cells co-express certain levels of cell surface antigen and intracellular cytokines. Results obtained were expressed as mean ± standard error of mean. Differences in the frequencies of the T cell subsets were evaluated using a One-way Analysis of Variance while the difference among the means was considered at 95% confidence interval using the post-hoc method of Dunnett's Multiple Comparison Test.

## RESULTS

The acute oral toxicity test of aqueous ARTAVOL® extract caused no gross behavioral changes such as loss of appetite, paw licking, body temperature, calmness, locomotion, etc., and there was no mortality within 24 h 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.

### Gating strategy

A gating strategy was developed to analyze *P. berghei* activation marker expression on CD4+ and CD8 T-cells (Figure 1).

### Effect of ARTAVOL® extract on CD4 T-cell response in *P. berghei*-Infected mice

There was no increase in the frequency of CD4+ T-cells in all the test samples compared to both the positive and

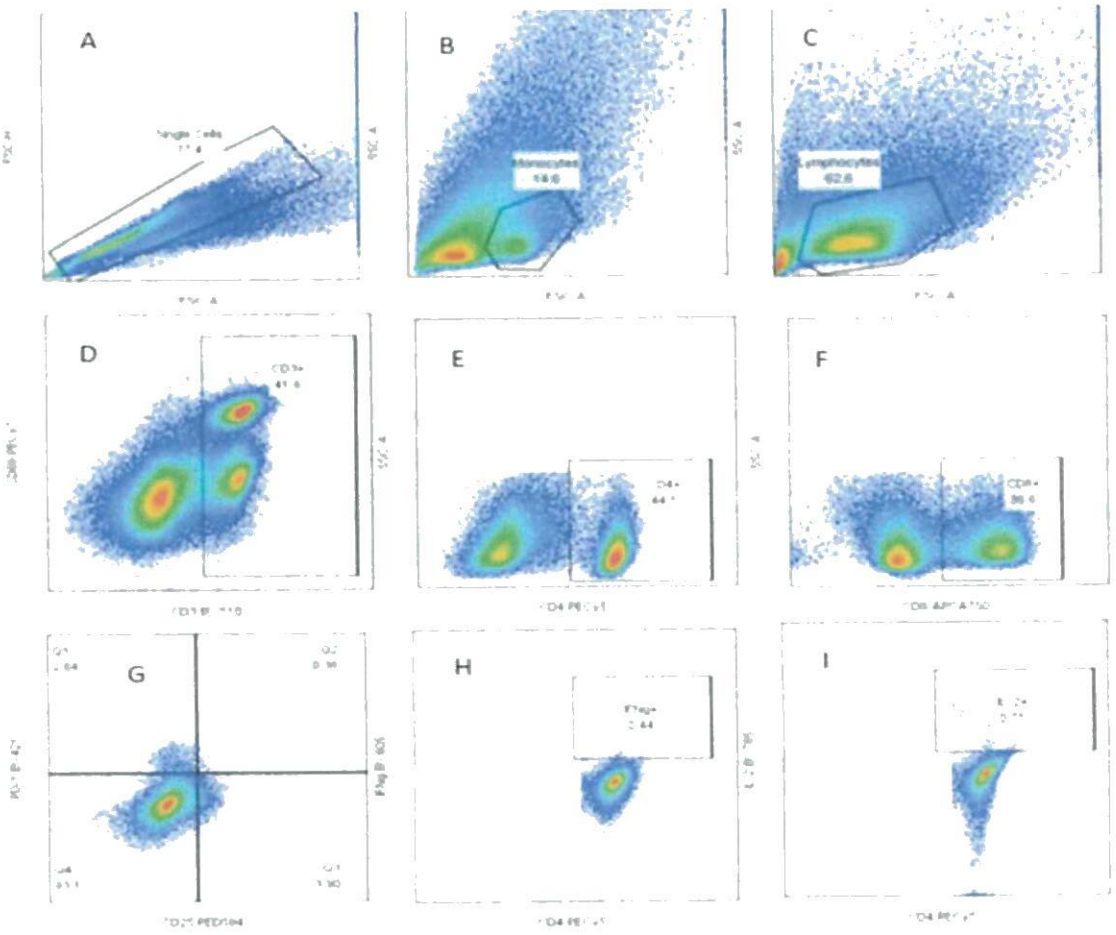


**Figure 1.** Representative flow cytometry dot plots to identify the specific CD3+, CD4+, CD8+ T cells, CD25, CD69 and CD279(PD-1) cell subsets from P. berghei infected mice prophylactically treated with ARTAVOL®. \
Single cells were gated on a FSC-H versus FSC-A channels (A). Monocytes (B) and Lymphocytes (C) were gated using a SSC-A and FSC-A. From the lymphocyte population, we gated out CD3 positive population (D). From the CD3 positive cells, CD4+T cells were gated on a SSC-A versus CD4 PECy5 (E) and CD8+T cells (F) respectively. Using the individual CD4+ CD8+ subpopulations, we identified markers of activation using CD25 and PD-1 (G) and cytokine production i.e , IFN-γ (H), IL-2 (I) among others.
Source: Author 2022

negative controls but the frequency of CD4+ T-cells increases as the test dose increases (Figure 2a). Interestingly, the percentage of CD4 T-cells expressing I L-10, was significantly higher in all the test groups compared to the negative control (Figure 2c).

### Effect of ARTAVOL® extract on CD8 T-cell response in *P. berghei*-Infected mice

There was no significant increase in the frequency of CD8+ T-cells in the test compared to negative control (Figure 3a). However, the frequency of CD8 T-cells expressing IFN-γ for the test group 15mg and 30mg/kg was significantly higher compared to negative control (Figure 3b). The frequency of CD8+ T-cells expressing TNF-α for the test group 30 mg/kg was significantly higher ($p = 0006$) compared to negative control. There was increase in the frequency of CD8 T-cells expressing TNF-α in the other test doses (15 mg and 60 mg/kg) and positive control but it was not significant when compared with the negative control (Figure 3c). The frequency of PD1-expressing CD8+ T cells was higher in the test dose of 30 mg/kg, but lower in the test doses of 15 and 60

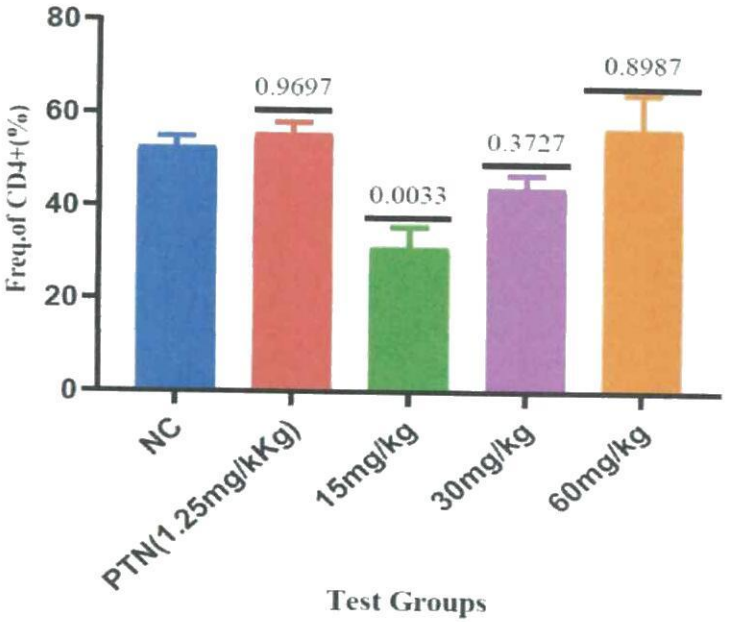


**Figure 2a.** Frequency of CD4+ T Cells among NC, PC(Pyrimethamine), and test groups.
Source: Author 2022

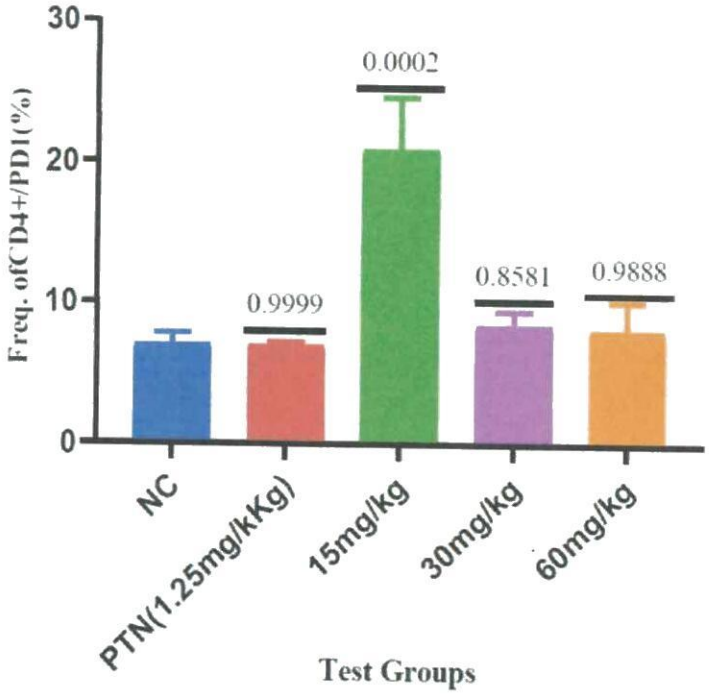


**Figure 2b.** Frequency of CD4 T cells among expressing CD279 (PD1) NC, PC, and test groups.
Source: Author 2022

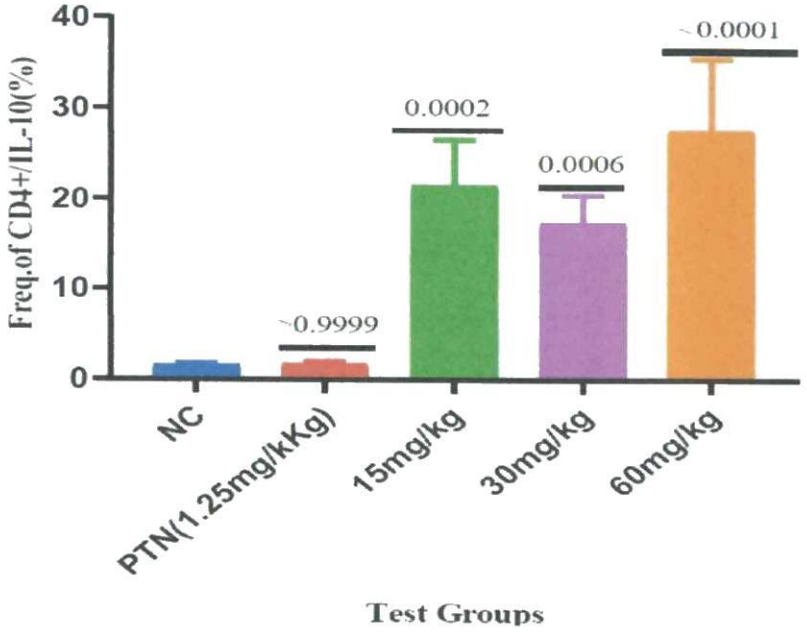


**Figure 2c.** Frequency of CD4T cell expressing IL-10 among NC, PC, and test groups.
Source: Author 2022

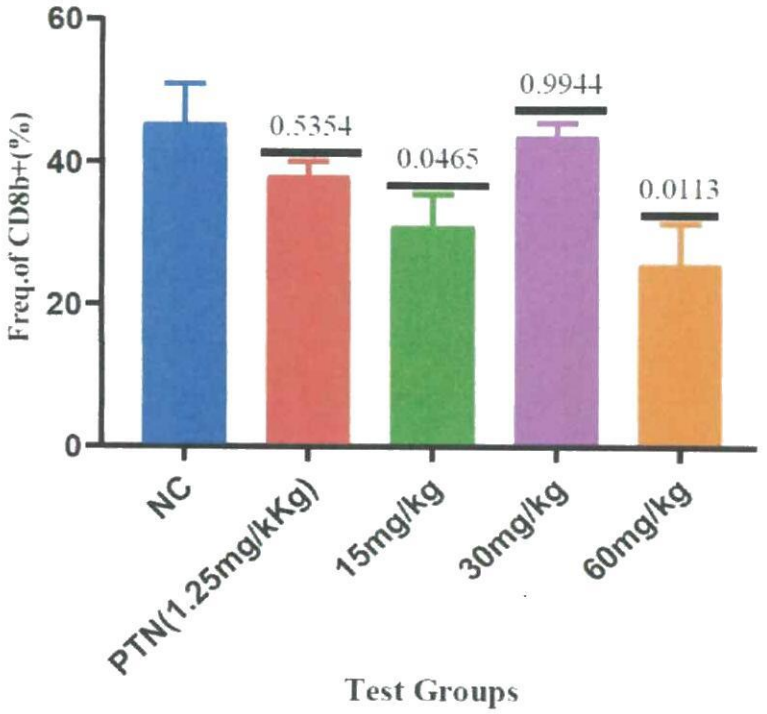


**Figure 3a.** Frequency (%) of CD8.2+ T cells among NC, PC (Pyrimethamine) and test groups.
Source: Author 2022

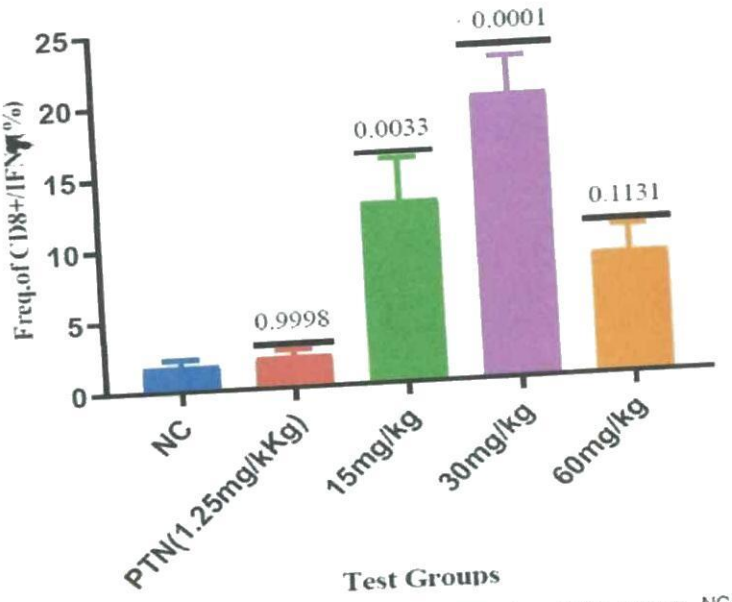


**Figure 3b.** Frequency (%) of CD8b.2+ expressing IFN-γ among NC, PC(Pyrimethamine), and test groups.
Source: Author 2022

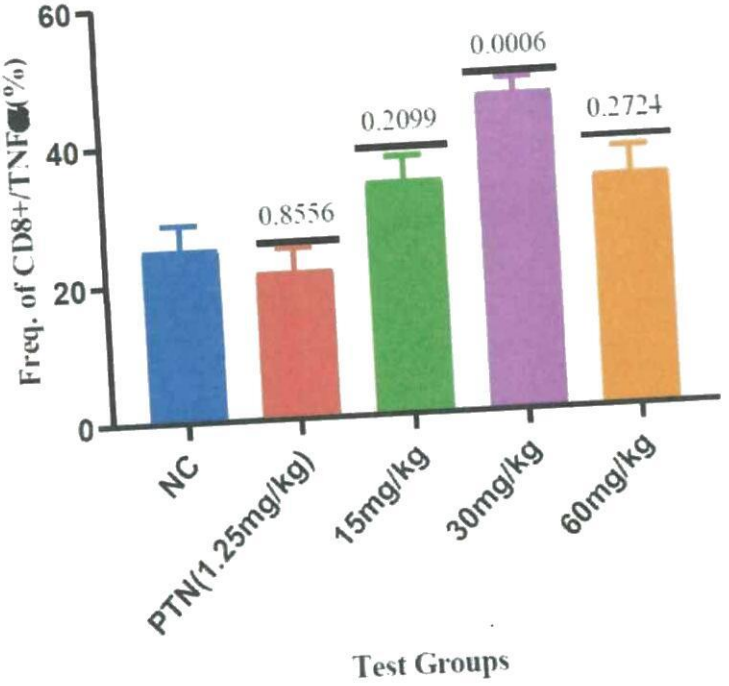


**Figure 3c.** Frequency (%) of CD8b.2+ expressing TNF-α among NC, PC (Pyrimethamine), and test groups.
Source: Author 2022

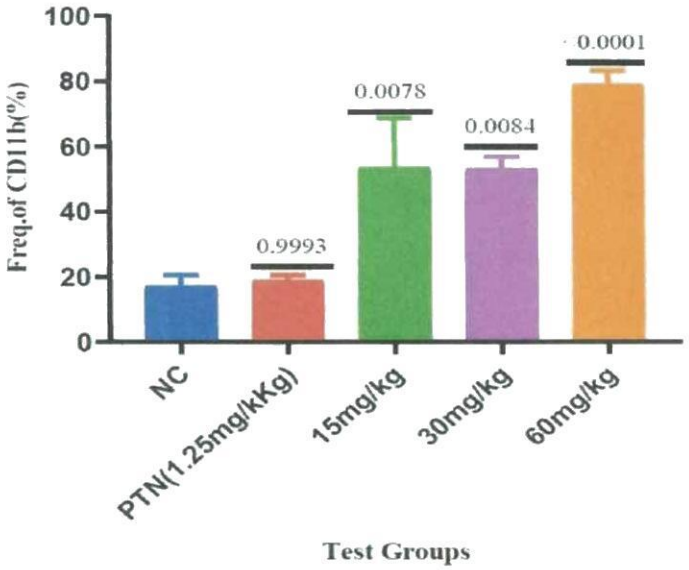


**Figure 4a.** Frequency (%) of monocytes (CD11b+ cells) among NC. PC (Pyrimethamine) and test groups.
Source: Author 2022

mg/kg when compared to the negative control (Figure 3d).

### Effect of ARTAVOL® extract on CD11 (monocytes) response in *P. berghei*-Infected mice

It was found that the frequency of CD11 cells (monocytes) was significantly higher in all the test groups when compared with the negative control. (Figure 4a). Similarly, there was a significant increase of monocytes expressing TNF-α in the test groups of 30mg and 60mg/kg and in the positive control when compared with the negative control. In test dose of 15 mg/kg there was a decrease in the frequency of CD 11 expressing TNF-α and it was not significant when compared to the negative control (Figure 4b).

### Effect of ARTAVOL® extract on CD335 (Natural Killer Cells) response in *P. berghei*-Infected mice

It was observed that a significant increase in the frequency of CD335 in the test groups (15, 30, and 60 mg/kg) when comapred with the negative conrol (Figure 5a). Similarly, there was a significant increase in frequency of CD335 expressing IFN-γ in the all the test groups (15, 30, and 60 mg/kg) when compared with the negative control (Figure 5b).

## DISCUSSION

Herbal medicines have been used over the years to treat malaria and have been reported to regulate the immune system in malaria (Afolayan et al., 2020; Kamau, 2022). ARTAVOL® is an herbal product used to prevent malaria in Uganda. Investigations *in vivo* reported the antimalarial effects of the product (Ogwang et al., 2011b). However, there was no available data on its immunomodulatory activity during *P. berghei* infection which has been reported in this study.

In this study, a dose- dependent was observed from 15 to 60 mg/kg in the frequency of CD4+ T cells (Figure 2a). For instance, at 30 and 60 mg/kg, an insignificant frequency increase was exhibited compared to the negative control which could be from the increase in antagonizing component(s) as the concentration increased. Kurup et al. (2019) reported that drugs may induce T cell exhaustion which can down- regulate T cell function (Kurup et al., 2019). The cell surface expression of the exhaustion marker PD-1(CD279)) was assessed (Figure 2b).

The herbal test doses (30 and 60 mg/kg) did not show significant increase in the frequency of CD4+ T cells expressing PD-1(CD 279). This suggests that the herbal extract in the test doses of 30 and 60 mg/kg did not induce CD4+ T cell proliferation. Konkel et al. (2010) reported that PD-1 provide inhibitory signals that dampen T cell function and restrains their clonal expansion

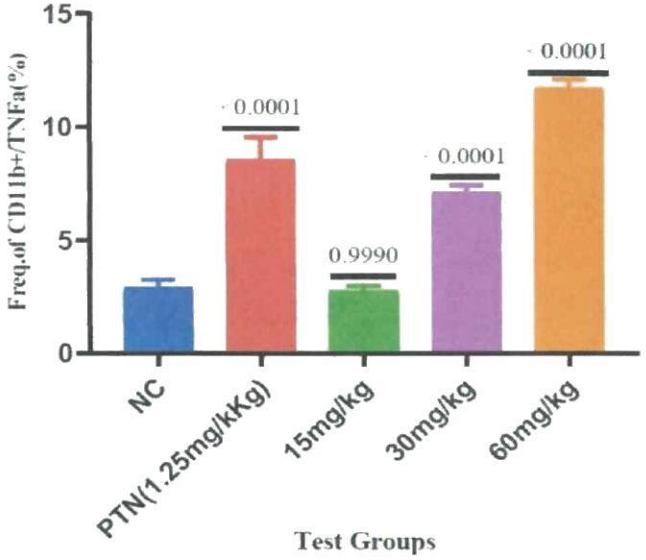


**Figure 4b.** Frequency of CD11b+Tcells expressing TNF-α among NC, PC, and test groups.
Source: Author 2022

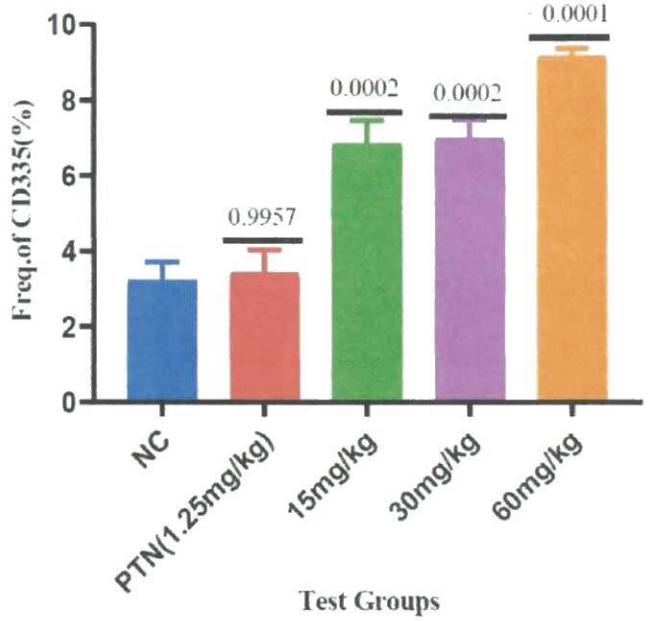


**Figure 5a.** Frequency of CD335+Tcells among NC, PC, and test groups.
Source: Author 2022

(proliferation) to an immunogenic stimulus (Konkel et al., 2010). There were observable significant activities in the

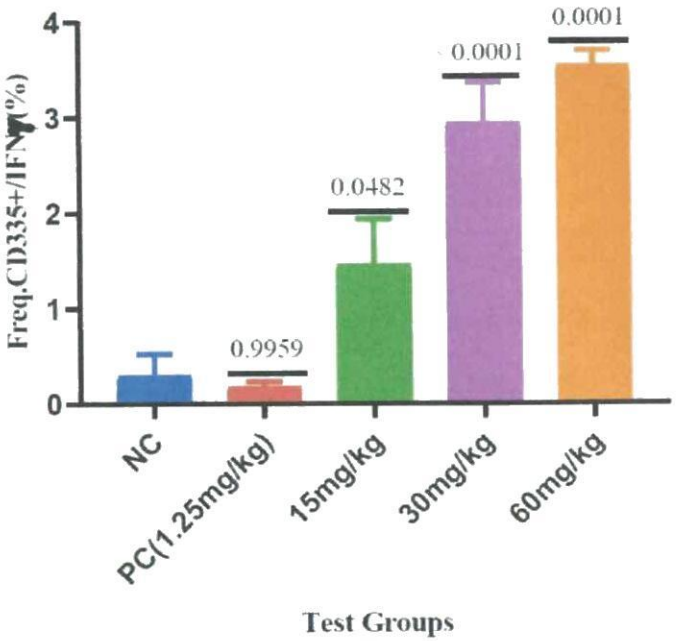


**Figure 5b**. Frequency of CD335+Tcells expressing IFN-γ among, PC. and test groups.
Source: Author 2022

frequency of CD4+ T cells expressing IL-10 (Figure 2c) in all the test doses (15-60 mg/kg) of the extract. A review study by Rajiv Kumar et al., (2019) reported that IL-10 regulates infection mediated inflammation during malaria.

This study observed no significant increase in the frequency of CD8+ T cells (Figure 3a) in all the test doses compared to both positive and negative controls suggesting that the extract did not cause enough proliferation of CD8+T cells. This study's findings indicated there was a significant increase of CD8+T cells expressing IFN-γ in all extract tests (Figure 3b) compared to the negative control. Similarly, the CD8+ T cells expressing TNF-α increased significantly in the test doses of 30 and 60 mg/kg (Figure 3c). ARTAVOL® contains phytochemicals like coumarins, flavonoids, triterpenes, and sterols among others. Flavonoids and triterpenes have been reported to induce the release of flavonoids, NF-α, and to modify intracellular signaling pathways in immune cells (Afolayan et al., 2020; Brindha, 2016; Mavondo et al., 2019).

In this present study, all the test doses of extract showed a significant increase in the frequency of CD 11(Monocytes) compared to negative control (Figure 4a). Likewise, the extract test doses (30 and 60 mg/kg) induce a significant release of TNF-α by CD 11 compared to the negative control (Figure 4B). Monocytes have been reported to exhibit protection against malaria and reduce parasite burden through cytokine production and phagocytosis (Chua et al., 2013; Dobbs et al., 2020; Ortega-Pajares and Rogerson, 2018).

Furthermore, the extract test doses at 15, 30 and 60 mg/kg showed a significant gradual increase in the frequency of CD 335 (Natural killer cells) compared to the negative control (Figure 5a). Also, in this study there was observable significant increase in production of IFN-γ by CD 335 (Natural killer cells) by all the extract doses compared to the negative control (Figure 5B). Various phytochemicals (e.g., allagic acid, flavonoids, curcumin, and quercetin) have been reported work by either directly affecting the pathogens or indirectly stimulating the innate and adaptive defense mechanisms of the host (Grudzien and Rapak, 2018; Sodagari et al., 2018).

## Conclusion

The aqueous extract of ARTAVOL® possesses variable immunomodulatory activity and showed ability to increase the frequency of monocytes and natural killer cells. IFN-γ and TNF-α levels showed a significance increase suggesting the extract induced their responses. However, there is need to investigate the effect of the

extract antibody mediated immune response.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

## ACKNOWLEDGMENTS

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