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Anti-Plasmodial And Ifn-y Immunosuppressive Activities of an Aqueous Crude Extract from Aerial Parts of Tephrosia Purpurea

Lydia I. Wanyaga
Senior Lecturer, Department of Health and Applied Science,
Nairobi Technical Training Institute, Kenya
Michael M. Gicheru
Professor. Department of Zoological Sciences, Kenyatta University, Kenya
Faith A. Okalebo
Professor, Department of Pharmacology and Pharmacognosy,
The University of Nairobi, Kenya.
Lois M. Mutisya
Senior Lecturer, Department of Health and Applied Science,
Thika Technical Training Institute, Kenya
Boniface G. Mukabane
Senior Lecturer, Department of Mechanical and Chemical Engineering,
Nairobi Technical Training Institute, Kenya

Abstract:
Plasmodium infections present a high risk that lead to high mortality and mobidity among human populations globally.Cellular and humoral mmune responses during inate and adaptive stages of malaria have been recorded. The most effective malaria medications are plant-derived but are costly and remain a challenge in the developing world, which is curretly focused on ethnomedicines for malaria management. Many medications contain immunomodulatory components which exhibit diverse action such as downregulation or upregulation of the immune responses. An earlier study confirmed that Tephrosia purpurea, a herb growing in arid and semi-arid regions of the world contains terpurinflavones, which were anti-plasmodial in vitro. In this study, effects of an orally administered aqueous crude extract from T. purpurea aerial parts and Chloroquine on peripheral IFN-γ and parasitemia in P. berghei infected BALB/C mice were compared. Groups of mice peritoneally inoculated with 1×10^7 parasitized RBC were treated orally daily for four days with aqueous crude extract (200 mg / kg mouse body weight) or with Chloroquine (5 mg / kg mouse body weight)or normal saline. Blood samples for parasitemia and mouse IFN-γ assays were collected through the tail tip and cardiac punctures respectively. There was a significant (P< 0.05) curative activity of the extract against the established infection of P. berghei in the mice. Activities of the crude extract and chloroquine against the infection were not significantly (P< 0.05) different. The extract also significantly (P< 0.05) limited the potentially dangerous inflammatory response of IFN-γ in the infected mice. Comparatively, chloroquine significantly (P< 0.05) lowered concentration of the cytokine in infected mice more than the extract. This study has thus revealed that T. purpurea aerial parts possess potent in vivo antiplasmodial characteristics and also contains components that are immunosuppressive.

Keywords: *Tephrosia purpurea, BALB/C mice,IFN-γ, plasmodium berghei*

1. Introduction

Malaria is a serious disease that affects human and several other vertebrate. The human malaria is common in the tropics and is caused by any of the five species of Plasmodium parasites namely: *Plasmodium falciparum*, Plasmodium malariae, Plasmodium vivax, Plasmodium ovale and Plasmodium knowlensi) (Biamonte et al., 2013; Omotosho and Adebiyi, 2014;). It causes morbidity and even death to foetus, putting the pregnant women at high risk. Fever, chills, muscle aches, headache, nausea, vomiting, and diarrhea are among the symptoms of malaria infections (Surve et al., 2017).

Plasmodium berghei are rodent malaria parasites. Their life cycle and physiology typify malaria infections in man and other primates (Carter and Diggs, 1977). This has made them useful in research study, for instance, using BALB/C mice to test immunological effects of human anti-malarial drugs and vaccine (Goodman et al., 2013; Siciliano and Alano, 2015;Somsak et al., 2016). Nevertheless, blood collection presents a challenge because mice contain a small blood volume (Rainy et al., 2016) and lack easily accessible blood vessels. Approximately, only 300 µL (<20% of the mouse total blood volume) can be drawn

within 24 hrs, making blood collection for some experimental procedures such as the mouse IFN-γ ELISA terminal (Rainy et al., 2016) due to the large volume required for the analysis (Golde et al., 2005).

For quite a long time, there has been consistent failure of antimalarial drugs especially in Africa, and this has made ethnobotany important in exploration of vegetation for anti-malarial agents (Otieno and Analo, 2012; Shikov et al., 2014; Weathers et al., 2014). Besides, need for reserve malaria drugs in this era of drug resistance by Plasmodium parasite has been cited (Miller et al., 2013). In vitro activities of many plant products against Plasmodium parasites have been demonstrated, but in vivo immunomodulatory effects have remained unknown. Flavonoids are a part of biomolecules occurring in the aerial parts (stems, leaves, flowers, seedpods, seeds and twigs) of *Tephrosia purpurea*, a plant wildly distributed in Kenya and other regions of the world. These biomolecules have in a past study demonstrated antiplasmodial activity in vitro(Juma et al., 2011). The current study investigated in vivo immunomodulatory effects of an orally administered aqueous crude extract of T. purpurea aerial parts on peripheral parasitemia and levels of IFN-γ in P. berghei infected BALB/C mice.

2. Materials and Methods

2.1. Ethical Approval

This study was approved by the Scientific Steering Committee (SSC) of Kenyatta University and the Animal Care and Use Committee (ACUC) of the University of Nairobi (UON). The committee guidelines were strictly observed throughout the research period.

2.2. Animals and parasites

Eighty four (84) ten weeks old (18 g to 20 g) female BALB/C mice (Kruisbeek, 2001)) were purchased from Kericho, KEMRI animal breeding unit in Kenya. The mice were housed in sterile cages and allowed toacclimatize for 7 days in the University of Nairobi School of Pharmacy animal breeding unit, prior to their randomization into the various experimental groups (Fawcett, 2012). Groups of experimental mice were housed in plastic cages with saw dust as beddings andmaintained at a temperature ranging from 22 to 24°C and a 12 h light / dark cycle(Mazzaccara et al., 2008; Fawcett, 2012). The animals were allowed free access to pellet food and fresh water. Proper hygiene was observed daily through constant cleaning and removal of faces and spilled feed from cages, tables and floor. Cryopreserved chloroquine (CQ)-sensitive rodent malaria parasite, *Plasmodium berghei* ANKA (Alli et al., 2011; Basir et al., 2012) was sourced from the Institute of Primate Research, Kenya. The parasites were kept alive by continuous intraperitoneal re-infestation in mice every three days (Basir et al., 2012). The re-infected mice were used for the study.

2.3. Plant Collection and Identification

Aerial parts of *Tephrosia purpurea* plant wereharvestedfrom Kilifi County, Kenya. The plant was identified and authenticated by Mr. Patrick C. Mutiso of the University Herbarium, Botany Department, the University of Nairobi, Kenya. A voucher specimen (Mutiso-520-August 2007) was deposited at the herbarium (Juma et al., 2011).

2.4. Preparation of Aqueous Crude Drug

The aerial parts of the plant were chopped using a sharp knife and air dried under a shade, then crushed into a coarse powder using sterile pestle and mortar under strict aseptic conditions. A decoction was made by boiling 500g of the powder in 2 liters of distilled water for about 30 minutes under sterile conditions. The resultant concoction was filtered by passage through a sterile stainless-steel sieve with a millipore membrane. Obtained filtrate was deep frozen at −70°C inside the American Thermo Scientific™ X series ultra-low freezer. The frozen filtrate was then lyophilized (freeze dried) using the American Lyophilizer, 2004, under low pressure, to collect a crude aqueous extract (Sacchetti et al., 2007). About 50g of the freeze-dried porous crude drug was collected and stored inside a sterile airtight bottle within a desiccator at room temperature until used later in preparing a water suspension for treatment of the infected mice.

2.5. Curative Test of the Plant Extract on Established Infection

Thirty-six (36) mice were randomly divided into three groups of twelve (n=12) each, then housed in wire mesh 67 by 75 square inch stainless steel cages labeled A1, A2 and A3. The animals were allowed supply of food and water ad libitum with daily change of beddings. From each cage, three uninfected animals were removed for the first (baseline) blood sample. Curative potential of the extract against established P. berghei infection in the mice was carried out as described by Ryley & Peters, 1970 (Okokon et al., 2006 ; Alli et al., 2011; Kehinde et al., 2014). The mice previously infected with P. berghei (parasite donor) were euthanized and EDTA blood collected by cardiac puncture into a disposable 1 ml syringe and a 26 × 6-gauge needle. The blood was dispensed into heparinized vacutainer tube containing 0.5% trisodium citrate and then diluted with normal physiological saline in such a way so that 1ml blood contained 5×10^7 infected RBCs (Arrey et al., 2014). A standard inoculum of 1×10^7(Kehinde et al., 2014; Chandel et al., 2015) of these parasitized erythrocytes in volumes of 0.2 ml (Arrey et al., 2014; Okokon et al., 2017) was used to infect each experimental mouse intraperitoneally (Okafor et al., 2013; Arrey et al., 2014), on day 0. The mice were then left to rest for 72 hours (Okafor et al., 2013; Chandel et al., 2015; Christian et al., 2017) for disease to develop, after which they were treated to evaluate efficacy of the crude drug. Each mouse was restrained gently

and firmly by gathering the loosed skin of the neck region backwards and slightly upwards to position the esophagus as straight as possible. The oral gavage tube was then carefully inserted between the tongue and the roof of the mouth until a space was observed for delivering the drug into the stomach (Okafor et al., 2013; Chandel et al., 2015) using 1000 microliter (µL) syringe (Turner et al., 2011). Each mouse in various groups received treatments as follows: A1 (crude extract, 200 mg / kg mouse body weight (Alli LA et al., 2011; Christian et al., 2017) in water suspension; A2 (chloroquine, 5 mg / kg mouse body weight (Kehinde et al., 2014; Chandel et al., 2015; Christian et al., 2017); A3 (normal saline, 10 ml / kg mouse body weight (Alli LA et al., 2011; Okokon et al., 2017). The treatments were given once daily for 4 days (Alli et al., 2011; Chandel et al., 2015; Christian et al., 2017).

Animal Groupings	Treatments
A1	T. purpurea extract (200 mg extract / kg body weight / day)
A2	Chloroquine diphosphate salt (5 mg / kg body weight / day)
A3	Normal saline (10 ml / kg body weight / day)

Table 1: Summary of Animal Groupings Showing Administration of Treatments

2.6. Collection and Preparation of Blood Samples

Blood samples were taken across the groups of mice four times in the course of experiment. The first sample (baseline sample) was collected at day 0 (before infection), second sample at day 3 (before treatment), third sample at day 8 (24 hrs. post treatment) and the fourth sample at day 13 (6 days post treatment). At every sampling time, blood was collected separately from three mice per cage at the tail (Okafor et al., 2013) and heart (Hoff, 2000). For tail samples, about 1 cm of the tail tip of each mouse was chopped off using a pair of sharp sterile scissors, and a drop of blood from each individual mouse collected directly onto the edge of a frosted microscope slide for parasitemia and WBC differential counts (Adetutu et al., 2016). A little pressure was then immediately applied at the freshly cut end of the tail for a few seconds to stop the bleeding. Blood smears were made using a spreader slide held at an angle of 45^0 over the drop of blood and then slowly pushed forward to disperse the blood over the slide's length. This helped in getting a monolayer region, where the cells were spaced far enough apart to be counted and differentiated. The blood films were air dried then fixed by dipping them in absolute methanol for 30 seconds. The fixed slide smears were then arranged on a staining rack and flooded with10% May-Grunewald-Giemsa stain at pH 7.2 for 20 minutes (Adetutu et al., 2016). Excess stain was rinsed off carefully but thoroughly with neutral distilled water from a water dispenser and left to dry on a rack in an upright position. After tail bleeding, the three mice were immediately sacrificed by cardiac puncture to provide blood for mouse IFN-γ ELISA. In this procedure, the animals were individually subjected to inhalation anesthesia with methoxyflurane (Baxter Caribe, Inc., Guayama, PR), (Doeing et al., 2003). The mouse was then placed dorsally on a clean dissection board and the left chest wall felt between the thumb and forefinger to locate the beating heart. A 25-gauge needle (Becton Dickinson and Co., Franklin Lakes, NJ) attached to a 1 ml syringe pre-coated with heparin lock flush solution (100 USP units/ml, Abbot Laboratories, Chicago, IL) was lined up laterally above the chest wall in order to estimate how far the needle would be inserted into the chest to reach the heart. The needle was then carefully inserted and slowly introduced into the chest cavity to about 5 mm to 10 mm until the heart was punctured and blood began to rush into the syringe. The plunger of the needle was then gently aspirated so as to collect as much blood from each cardiac cycle as possible taking care not to collapse the heart. When blood stopped flowing, the needle was pulled out gently. Approximately 0.7 ml to 1.0 ml of blood was collected per mouse and dispensed into separate labeled 2 ml blood tubes designated for serum isolation. The blood samples were left on test tube holder to clot at room temperature for 50 minutes, after which the formed serum was aliquoted using separate sterile micropipette into well labeled sterile 0.5 ml clear microtiter Eppendorf tubes and stored at −80 °C (Tuck et al., 2010).

2.7. Parasite Enumeration in the Blood Samples

The percent of parasites on each slide smear was determined by enumerating the number of infected RBC in relation to the number of uninfected RBC under a standard light microscope using the ×100 oil immersion objective (Adetutu et al., 2016). This was accomplished by noting the number of parasitized RBC seen in 4 monolayer microscope cell fields which are estimated to contain a total of 1000 RBC, considering that one monolayer microscope field of view is estimated to have on average 250 RBC. The exercise was conducted by moving the slide to new fields until the parasites were counted in all the four fields. Parasitemia was expressed as percentage (%) of the erythrocytes infected with malarial parasites and was calculated by dividing the number of infected RBC by the total number of RBC counted in the four fields (1000) multiplied by 100 as indicated in the following formula:

{(No. of infected Red Blood Cells × 100) ÷ 1000 Red Blood Cells} = Percentage Parasitemia (Adetutu et al., 2016).

2.8. Quantification of Circulating Interferon Gamma in the Blood Samples

Quantification of circulating IFN-γ was done using mouse IFN-γ ELISA kit (Mabtech, Nacka Strand, Sweden) (E. Taylor et al., 2017). Wells were coated with 100 µl of monoclonal antibody, (1 µg / ml) to capture IFN-γ from serum samples and recombinant mouse IFN-γ standard. 100µl of monoclonal IFN-γ detecting antibody, (0.5 µg / ml) was added per well and the plate incubated for 1 hour at room temperature. Streptavidin-horse radish peroxidase (HRP) was added to the wells at a

dilution of 1:1000 and the plates incubated again for 1 hour at room temperature before the addition of 100 µl / well of Tetramethlebenzidine (TMB) microwell peroxidase substrate. Optical densities were read at 630 nm in a micro-plate reader (Dynatech Laboratories). The limit of detection of the assay was 2 Pg. / ml. Cytokine levels were expressed as amount produced under experimental conditions after subtracting the cytokines released by control cultures containing media alone.

2.9. Statistical Data Analysis and Presentation

Numerical data obtained in this study were organized and managed with EXCEL spreadsheet for windows and presented as Mean±SD (standard deviation) of the means using line graphs or tables. The data were analyzed using the Minitab 17 data analysis tool. Two sample t-tests were carried out to determine the statistical significance in the mean parasitemia and IFN-γ between the respective groups of experimental mice before and after treatments. Paired t-tests were done to determine statistical significance in the means of data within the groups of mice. For all the data obtained, results were considered statistically significant at 95% confidence level and $P< 0.05$.

3. Results

		T. purpurea extract-treated mice										
Sample days		**Day 0**			**Day 3**			**Day 8**			**Day 13**	
Individual mice		**$A1_1$**	**$A1_2$**	**$A1_3$**	**$A1_4$**	**$A1_5$**	**$A1_6$**	**$A1_7$**	**$A1_8$**	**$A1_9$**	**$A1_{10}$**	**$A1_{11}$**	**$A1_{12}$**
	Para	0	0	0	1.2	2.1	1.5	0.6	1.0	0.9	0.5	0.7	0.5
	IFN-γ	29.11	28.782	30.102	1025.47	1019.8	1030.4	901.26	897.2	895.3	807	799.31	801.5
		Chloroquine-treated mice										
Sample days		**Day 0**			**Day 3**			**Day 8**			**Day 13**	
Individual mice		**$A2_1$**	**$A2_2$**	**$A2_3$**	**$A2_4$**	**$A2_5$**	**$A2_6$**	**$A2_7$**	**$A2_8$**	**$A2_9$**	**$A2_{10}$**	**$A2_{11}$**	**$A2_{12}$**
	Para	0	0	0	2.0	2.2	1.0	0.7	0.6	0.7	0.4	0.9	0.3
	IFN-γ	28.644	31.224	30.512	998.31	1010.05	995.79	682.68	675.11	678.25	500.01	510.22	505.7
		Saline-control mice										
Sample days		**Day 0**			**Day 3**			**Day 8**			**Day 13**	
Individual mice		**$A3_1$**	**$A3_2$**	**$A3_3$**	**$A3_4$**	**$A3_5$**	**$A3_6$**	**$A3_7$**	**$A3_8$**	**$A3_9$**	**$A3_{10}$**	**$A3_{11}$**	**$A3_{12}$**
	Para	0	0	0	2.5	1.0	2.6	1.4	2	2.3	1.9	2.3	3.0
	IFN-γ	30.122	27.644	31.051	1007.89	1012.17	1020.91	985.47	981.29	990.85	1817.57	1824.5	1821.303

Table 2: Parasitemia (%) and Interferon Gamma Levels (Pg. / Ml) in Individual Mice

The table above presents data for parasitemia (%) and IFN-γ (Pg. / ml) in infected mice; A1- test mice; A2- positive control mice; A3- saline control mice; subscripts ($_1$ to $_{12}$)- Individual mice; Para-Parasitemia; IFN-γ-interferon gamma.

		T. Purpurea Extract-Treated Mice			
		Day 0 to Day 3	**Day 3 to Day 8**	**Day 3 to Day 13**	**Day 8 to Day 13**
	Para	0.000±0.000 to * 1.6±0.458	1.600±0.458 to * 0.833±0.208	1.600±0.458 to * 0.600±0.361	0.833±0.208 to 0.600±0.361
	IFN-γ	29.33±0.69 to * 1025.22±5.30	1025.22±5.30 to * 897.92±3.04	1025.22±5.30 to * 802.60±3.96	52.58±10.74 to 36.75±0.57
		Chloroquine-treated mice			
		Day 0 to Day 3	**Day 3 to Day 8**	**Day 3 to Day 13**	**Day 8 to Day 13**
	Para	0.000±0.000 to * 1.733±0.252	1.733±0.252 to * 0.667±0.58	1.733±0.643 to * 0.533±0.252	0.667±0.058 to 0.533±0.321
	IFN-γ	30.13±1.33 to * 1001.38±7.61	1001.38±7.61 to * 678.68±3.8	1001.38±7.61 to * 505.31±5.12	82.5±45.9 to 36.7±7.0
		Saline-control mice			
		Day 0 to Day 3	**Day 3 to Day 8**	**Day 3 to Day 13**	**Day 8 to Day 13**
	Para	0.000±0.000 to * 2.033±0.208	2.033±0.896 to 1.900±0.458	2.033±0.896 to 2.400±0.557	1.900±0.458 to * 2.400±0.577
	IFN-γ	29.61±1.76 to * 1013.66±6.64	276.5±129.7 to 125.5±13.2	1013.66±6.64 to * 1821.12±3.47	985.87±4.79 to * 1821.12±3.47

Table 3: Curative Anti-Plasmodial and IFN-Γ Immunosuppressive Activity of Aqueous T. Purpurea Extract and Chloroquine in Infected Mice

In the table above, data are expressed as Mean ±SDM (Standard deviation of mean); N = 3; 95 % CI; * indicate P < 0.05 (significant paired t-results); Day 0-before infection; Day 3-post infection; Day 8-twenty-four hours post treatment; Day 13-six days post treatment; Para-Parasitemia; IFN-γ-interferon gamma.

3.1. Trends of Parasitemia in Peripheral Blood of Experimental Mice

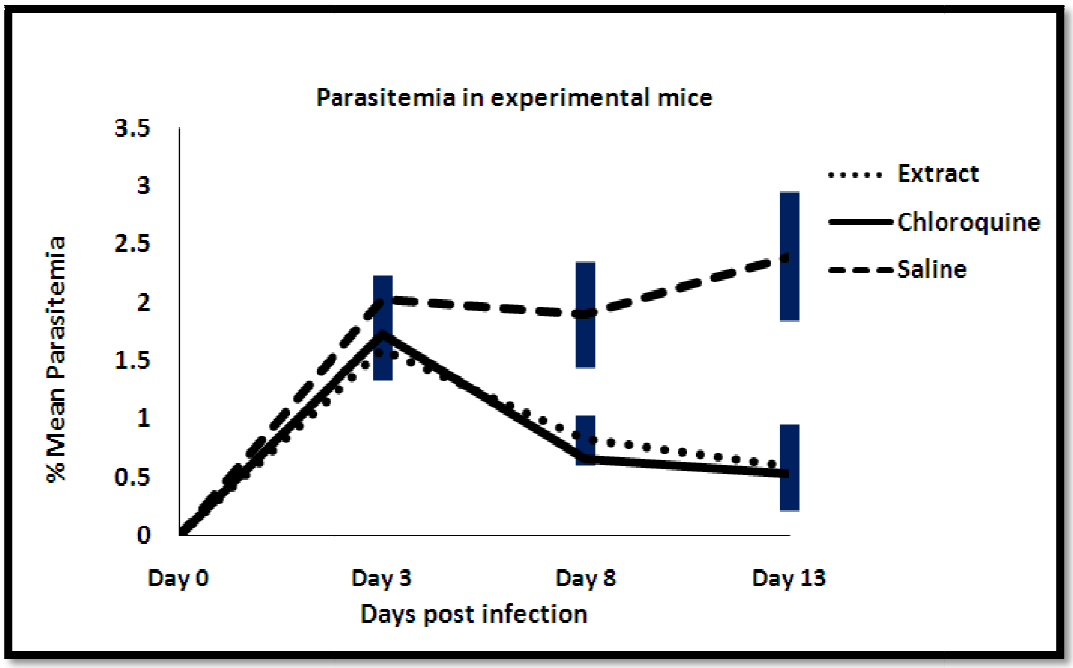

Figure 1: Comparative Parasitemia (% Mean±SD, N=3) In Groups of Experimental Mice

Groups of mice were treated with the T. purpurea crude extract or chloroquine or normal saline respectively. Days 0, 3, 8 and 13 indicate the state of mice before infection, after infection, 24 hours post treatment and 6 days post treatment respctively. Treatment of mice on day 3 with aqueous T. purpurea crude drug and chloroquine respectively, significantly (P< 0.05) reduced their peripheral parasitemia, while parasitemia of the saline control mice prominently increased significantly (P< 0.05). The crude drug produced antiplasmodial effect that was comparable to that of chloroquine.

Paired samples t-tests on data collected from peripheral parasitemia of the experimental mice (table 1) indicated significantly (P< 0.05) higher levels following infection with P. berghei (day 3) than before (day 0)(table 2). Thus, parasitemia in the test mice on day 3 (1.6±0.265 % mean) and on day 0 (0.000 ± 0.000 % mean); a statistical increase of 1.6 (95 % CI, 0.943 to 2.257), t (2) = 10.47, P< 0.009; parasitemia in the positive control mice on day 3 (1.733±0.252 % mean) and on day 0 (0.000±0.000 % mean); a statistical increase of 1.733 (95 % CI, 1.108 to 2.358), t (2) = 11.93, P< 0.007; parasitemia of the saline control mice on day 3 (2.033±0.208 % mean) and on day 0 (0.000 ± 0.000 % mean); a statistical increase of 2.033 (95 % CI, 1.516, 2.55) parasitemia, t (2) = 16.92, P< 0.003.

In table 2 and figure 1, mice had significantly (P< 0.05) lower parasitemia twenty four hours post treatment with the crude extract (day 8) (0.833±0.208 % mean) than before the treatment (day 3) (1.6±0.265 % mean); a statistical decrease of -0.767 (95 % CI, -1.484, -0.050), t (2) = -5.28, P< 0.044. The mice had significantly lower parasitemia six days post treatment (day 13) (0.6±0.265 % mean) than before the treatment; a statistical decrease of 1.0 (95 % CI, -1.2484 to -0.7516), t (2) = -17.32, P< 0.003.

The positive control mice had significantly lower parasitemia twenty four hours post treatment with chloroquine (day 8) (0.667±0.58 % mean) than before the treatment (day 3) (1.733±0.252 % mean); a statistical decrease of -1.067 (95 % CI, -1.826 to -0.308), t (2) = -6.05, P< 0.026. The mice had significantly lower parasitemia six days post treatment (day 13) (0.533±0.252 % mean) than before the treatment; a statistical decrease of -1.2 (95 % CI, -1.448 to -0.952), t (2) = -20.78, P< 0.002. 2.033±0.896

Although the negative control mice did not indicate significant (P< 0.05) change in parasitemia between the day of established infection, day 3 (2.033±0.896) and 24 hours post saline administraion, day 8 (1.900±0.458 % mean), the mice parasitemia increased significantly (P< 0.05) from day 8 to day 13 (2.400±0.577 % mean); a statistical increase of 0.500 (95 % CI, 0.003 to 0.997), t (2) = 4.33, P< 0.049.

3.2. Trends of Interferon Gamma Levels in Peripheral Blood of Experimental Mice

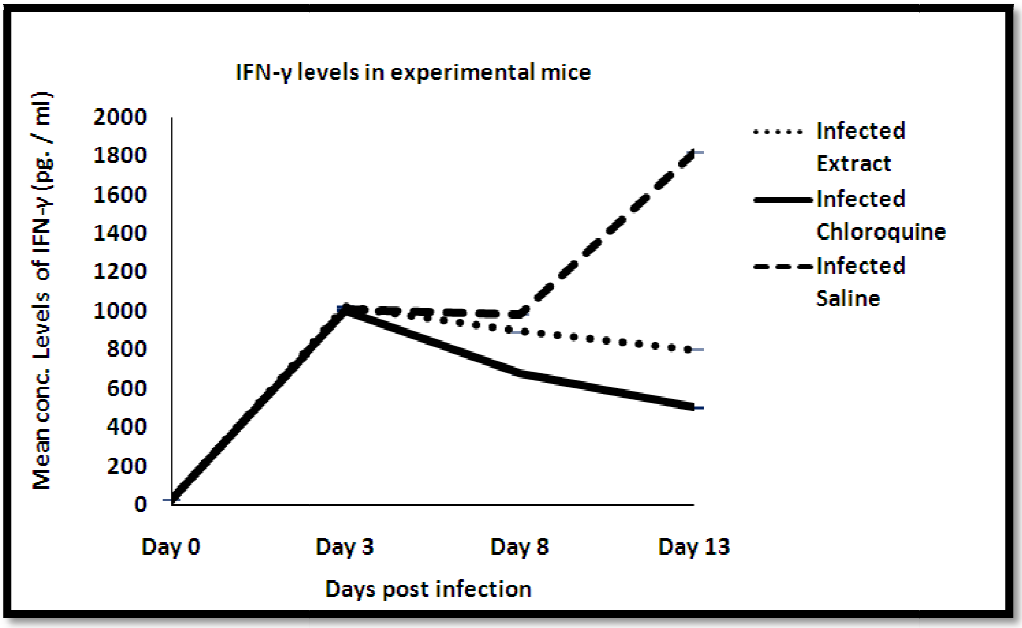

Figure 2: Comparative Levels of Interferon Gamma (Pg. / Ml Mean±SD, N=3) in Groups of Experimental Mice

Groups of mice were treated with T. purpurea crude extract or chloroquine or normal saline respectively. Days 0, 3, 8 and 13 indicate the state of mice before infection, after infection, 24 hours post treatment and 6 days post treatment respectively. Infection with P. berghei elevated levels of peripheral IFN-γ in the groups of mice. Subsequently, treatment of the animals on day 3 with aqueous crude drug and chloroquine respectively, significantly (P< 0.05) downregulated concentration of the IFN-γ throughout the experimental period, while levels of the cytokine in the saline-control mice significantly (P< 0.05) increased.

Paired-samples t-test on data from the infected mice (table 1), indicated significan (P< 0.05)increase in peripheral IFN-γ levels from day 0 (before infection) to day 3 (post infection) (table 2). Thus, IFN-γ levels in the test mice on day 0 (29.33 ± 0.7 Pg. /ml mean) and day 3 (1025.22 ± 5.3 Pg. /ml mean); an increase of 995.89 (95% CI, 984.32, 1007.46) Pg. /ml, t (2) = 370.34, P < 0.000; IFN-γ levels in the positive control mice on day 0 (30.1 ± 1.3 Pg. /ml mean) and day 3 (1001.38 ± 7.61 Pg. /ml mean); an increase of 971.26 (95% CI, 954.08 to 988.43) Pg. /ml, t (2) = 243.36, P < 0.000; the saline control animals on day 0 (29.61 ± 1.8 Pg. /ml mean) and day 3 (1013.66 ± 6.64 Pg. /ml mean); an increase of 984.05 (95% CI, 969.00 to 999.10) Pg. /ml, t (2) = 281.28, P < 0.000

After treating the mice with the crude drug, levels of cytokine declined significantly within 24 hours from 1025.22±5.3 Pg. /ml mean, on day 3, to 897.92±3.04 Pg. /ml mean by day 8; a mean decrease of -127.30 (95% CI, 144.20, 110.41) Pg. / ml, t (2) = 32.43, P < 0.001, and then further decline to 802.60± 3.96 Pg. /ml mean, by the 6th day post treatment, day 13; a mean decrease of -222.62 (95% CI, 236.36 to 208.88) Pg. /ml, t (2) = -69.71, P < 0.000.

Treatment of the mice with chloroquine on day 3, significantly (P< 0.05) reduced peripheral cytokine levels within 24 hours from 1001.38±7.61 Pg. /ml mean to 678.68±3.80 Pg. /ml mean on day 8; a decrease of -322.70 (95% CI, 349.14 to 296.27) Pg. /ml mean IFN-γ, t (2) = 52.53, P < 0.000. This was followed with further significant (P< 0.05) decline to 505.31± 5.12 Pg. /ml mean on day 13, a decrease of -496.07 (95% CI, 509.08, 483.06) Pg. /ml, t (2) = 164.04, P < 0.000.

Following administration of the normal saline, infected non-treated mice had a significant (P< 0.05) increase in the cytokine levels from day 3 (1013.66±6.64 Pg. /ml mean) to day 6 (1821.12±3.47 Pg. /ml mean); an increase of 807.47 (95% CI, 791.90 to 823.04) Pg. /ml, t (2) = 223.11, P< 0.000.

4. Discussion

Medicinal plants are benefitial because their crude material contain bioactive components (Juma et al., 2011; Jain et al., 2013; Weathers et al., 2014). Unfortunately, while in the crude material, these compounds are not fully active against parasites in vivo, which has been attributed to failure of the active compounds'uptake to physiologically active levels (Savjani et al., 2012; Kalepu and Nekkanti, 2015). Medicinal plant biomolecules such as saponins, flavonoids, tannins, cardiac glycosides and steroids have been recorded to be anti-plasmodial (Kruisbeek, 2001; Ferreira et al., 2010; Sasidharan et al., 2011; Weathers et al., 2014; Taylor et al., 2017). Earlier investigations on an aqueous crude extract of T. purpurea aerial parts revealed the presence of the phytochemical flavonoids (Patil et al., 2011; Juma et al., 2011; Sharma et al., 2013b; Atilaw et al., 2017). In the current study treatment of P. berghei infected mice with the aqueous crudedrugsignificantly reduced the mice

peripheral parasitemia (figure 1). It is therefore possible to suggest that these compounds are partly responsible for the in vivo anti-plasmodial effect of the crude drug.

The extent to which the crude drug reduced peripheral parasitemia was not significantly different from chloroquine (figure 1). Therefore, although the plant extract did not eradicate parasites in the mice completely, its impact in the peripheral parasitemia indicates its ability for direct action on the parasites. However, the lower impact of the crude drug on P. berghei compared to the CQ may be attributed to low dose of the drug and possibly its short duration of action accompanied with rapid metabolism (Savjani et al., 2012; Kalepu and Nekkanti, 2015), which could have made it impossible to totally clear the parasites. The similarity in the manner at which the extract and CQ acted on malaria parasites as was observed in this study (figure 1), suggests the probable use of this plant in the management of malaria. Nevertheless, the crude drug reduced the parasite density in the mice to a less extent in comparison with the standard drug. Report from earlier studies have suggested the importance of monitoring the mice for about four weeks to confirm the efficacy of any anti-malarial drug (Chandel et al., 2015). Although there is an obvious indication that the T. purpurea crude drug has activity against P. berghei parasites, this study took less than one month which makes it necessary to do the study for a longer period of time.

Different views suggest that crude drugs have better plasmodistatic than plasmodicidal effects due to the fact that a greater range of unpurified bioactive molecules need first to be converted into various forms, which may take long allowing parasites time to proliferate. Many oral medications exhibit poor bioavailability caused by poor solubility and low permeability into active physiological sites (Savjani et al., 2012; Kalepu and Nekkanti, 2015). Observations made in this study suggest that the active molecules in the T. purpurea extract might not have been present in enough concentrations to cause fast and complete eradication of the parasites, as has been earlier elucidated(Savjani et al., 2012; Kalepu and Nekkanti, 2015). It would therefore be important to test the anti-plasmodial activity of the extract's purified active compound (Iwu, Duncan and Okunji, 1999). Plant biomolecules can cause anti-plasmodial activities through increasing red blood cell oxidation and by preventing the parasite's protein synthesis (Rasoanaivo et al., 2011; Shears et al., 2015), thereby preventing the oxidative damage induced by the parasites to the red blood cells. The exact mechanism by which the T. purpurea crude drug exhibits its antiplasmodial property may not be clearly elucidated from the current study.

Malaria research using peripheral blood has provided information on human immune responses against Plasmodium infection. Peripheral blood has remained a readily accessible avenue for harvesting white blood cells. In spite of this fact, earlier reports indicate that peripheral white blood cells may not reflect fully the immune response to malaria since the activated cells during infections often appear in secondary lymphoid organs (Mougneau et al., 2011; Azcárate et al., 2014). This truth has indicated need for more understanding of quantifiable immune response proteins in the circulation to help identify the exact clinical states in humans and animal malaria models. Interferon gamma is a pro-inflammatory cytokine known to play a central role in the cellular immune response, by stimulating several immune-regulatory pathways and cellular responses (Gostner et al., 2012). The IFN-γ produced by CD4 T helper (CD4 Th) cells during blood stage malaria infection has an inflammatory role that cannot be ignored(Awandare et al., 2006; Basir et al., 2012; Wykes et al., 2014; Boyle et al., 2017). The CD4 Th1 cells produce interleukin (IL)-2 (Mougneau et al., 2011; Julius et al., 2013) and tumor necrosis factor (TNF), which serve a pivotal role in cell-mediated immunity (CMI). These cells stimulate macrophages and other cells to produce mediators by releasing cytokines that stimulate inflammation (Wykes et al., 2014). Cells of the Th1 subclass cause production of opsonizing antibodies and promote phagocytosis in human and rodent malaria (Mougneau et al., 2011; Bijker et al., 2015). Interferon gamma is to date the predominant cytokine produced during early phases of acute malaria infection (Mougneau et al., 2011; Gostner et al., 2012; Wykes etal., 2014), but it declines later in infection, as was demonstrated in P. chabaudiinfected mice where exhaustion of CD4+ T cells prevented lasting immunity against malaria after one month(Wykes et al., 2014). The current study indicated a decrease in concentration of peripheral IFN-γ ingroups of P. berghei infected mice following treatment with T. purpurea crude drug and chloroquine respectively (figure 2), with subsequentparasitemia decline (figure 1). This was contrary to the infected negative control mice which registered increasing levels of the cytokine with disease progression (figure 2).

Cells of the CD4 Th2 subclass produce IL-4, IL-5, IL-6 and IL-10 cytokines(Horne-Debets et al., 2013; Boyle et al., 2017). Th1IFN-γ, and Th2IL-4 and IL-10 cytokines are antagonistic (Boyle et al., 2017). Th1 cells are involved in primary parasitemia (Horne-Debets et al., 2013; Wykes et al., 2014) whereas Th2 cells are required to finally eradicate Plasmodium parasites during the infection. In an earlier study using mice, the peripheral IFN-γ was increased within 24 hours after infection as innate protective measure (Awandare et al., 2006). Interferon gamma influences proliferation, differentiation and responses of B and T cells. Several in vitro studies have confirmed that IFN-γ may be produced rapidly by the innate immune system in response to Plasmodium infected RBC (Awandare et al., 2006; Mougneau et al., 2011; Julius et al., 2013).Similarly, this study recorded increased levels of the cytokine in the mice following infection with P. berghei(figure 2). Comparatively, non-infected mice did not concurrently indicate any significant increase in concentration of the peripheral IFN-γ (figure 2).

Interferon gamma is vital in modulating the developing immune response, the very reason it is produced by cells of the innate immunity (Souza et al., 1997; Julius et al., 2013). Research records indicate increased levels of peripheral IFN-γ within 24 hours post infection of P. yoelli in mice (King and Lamb, 2015). Natural killer cells or gamma delta (γδ) T cells are argued to be the source of IFN-γ in human peripheral blood mononuclear cells (PBMC) after introducing them to P. falciparum iRBC in vitro, and also in several other murine malaria models(King and Lamb, 2015). It has been recorded that chloroquine plays an immunomodulatory role by suppressing production of IFN-γ and suppressing PBMC production (Gostner et al., 2012;

Golenser, 2014). In the current study, treatment of mice with the crude drug suppressed peripheral IFN-γ, indicating its immunomodulatory characteristics. An earlier study in which a fraction of T. purpurea flavonoids suppressed cellular phagocytosis and production of circulating antibodies (Juma et al., 2011; Patil et al., 2011)makes it possible to suggest that theflavonoidsin thetest drugwere responsible for the immunosuppressive activityonperipheral IFN-γ ofthe experimental mice.

5. Limitations of the Study

The procedures used in this study were far too costly. Reagents, mice and the manpower employed were very expensive and almost unaffordable. Additionally, the manual microscopic differential cell counts were tiresome, tedious and time consuming.

6. Conclusions

In vivo anti-plasmodial effects of the aqueous crude extract of T. purpurea aerial parts at a concentration of 200 mg / kg mouse body weight / day for three days produced a significant reduction in peripheral parasitemia in the treated P. bergheiinfected mice,concurrently with a significant decline in peripheral IFN-γ concentration. Thiswas unlike the negative control animals which showed increased levels of the cytokine alongside increasing parasitemia. The extract therefore indicated curative potential against established infection of P. berghei in the mice, and also contains immunomodulatory components with ability to suppress IFN-γ. These are confirmed by the drug's ability to significantly reduce mice parasitemia and to limit the potentially dangerous host immune inflammatory response of IFN-γ. The extract however was found to downregulate IFN-γ to a lesser extent than the standard drug. Specifically, the extract of T. purpurea aerial parts possesses potent in vivo anti-plasmodial andIFN-γ immunomodulatory components and may therefore serve as potential source of antimalarial agents.

7. Recommendations

- Investigation of anti-plasmodial effect of the aqueousT. purpurea crude extract atdifferent concentrations in the peripheral blood of P. berghei infected and non-infected BALB/C mice.
- Investigation of the exact mechanism through which the crude drug exerts its anti-plasmodial activity in the mice.
- Elucidation of the exact biomolecules that are involved in downregulating IFN-γ cytokinein the infected mice.

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