REAGENT BASED CHEMICAL SCREENING AND ANT-*PLASMODIUM BERGHEI* STUDIES OF GREEN ORTHODOX BLACK KENYAN TEA

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Abstract
The Kenyan green ,orthodox, and black tea (*Camellia sinensis*) aqueous extracts were subjected to reagent based chemical screening of bioactive constituents, and their *in vivo* anti-*Plasmodium berghei* ANKA effects on parasitemia, % pcv and serum proteins concentration done using a 105 male swiss mice model to determine their pharmacological significance. The phytochemical results from the colorimetric tests of green, orthodox and black teas showed the presence of alkaloids, flavonoids, saponins, terpenes and tannins as common metabolites. Anthraquinones were not detected in green tea unlike orthodox and black teas. There was significant reduction ($p < 0.05$) in parastemia levels of $p = 2.6$ on day 10 after infection compared to the control. The fall in packed cell volume (pcv) occurred on day 7 after infection of $p = 4.17$. There was a significant difference in pcv levels $p = 4.8$ ($p < 0.05$) on day 11 between the infected mice given tea and the infected control. There was a significant reduction $p=10.4$ ($P < 0.01$) in serum protein reduction on day 11 post infection only in the mice given water. Tea produced significant ($p < 0.01$) elevation of parasite's induced hypoproteinemia ($p = 3.38$) as compared to infected control mice ($p = 10.4$).
The role of these bioactive principles is discussed according to their folkloric use in Kenya and elsewhere in the world. Besides tea is of great importance as far as other clinical applications are concerned. This qualitative analysis can be used for comparative evaluation of bioactive constitutes with other population of *Camellia sinensis* present in different parts of the world, and can be used for selection of superior quality of this herb to be used in pharmaceutical industries.

1.0 Introduction

Tea is the leading cash crop in Kenya and contributes significantly to the country's economy. In 2010, Kenya exported over 441 million kg of tea, earning the country over ksh 97 billion in foreign exchange (Anon, 2011). There are two major commercial types of tea namely; black tea and green teas depending on wheather the leaf is subjected to fermentation (autoxidation) or not. 78% of the tea produced and consumed worldwide is black tea, 20% is green tea and less than 2% Oolong, Orthodox and others (Costa *et al*., 2002., Zuo *et al*.,2002). Each type of tea has several sub-classifications (Benerjee,1992). All of them are prepared from *Camellia sinensis* (L) theaca and its varieties by different manufacturing processes. While green tea is mainly produced from Chinary varieties, the Assam and

Cambodia varieties are largely suitable for processing black teas. Kenyan tea is almost exclusively sold as black (CTC) tea and in bulk (Wachira and Kamunya, (2005). The continued use of tea as a beverage has gained world wide prominence due to the quality of its phytochemicals and other related tea extracts such as polyphenols and catechins and thus its pleasant flavor, stimulating effects and health benefits (Blades, 2000., Benjamin *et al*.,1991). These pharmacological aspects had been perceived to be more in green tea than in black tea and this influences market trends. Tea polyphenols (flavonoids) and their oxidative products are being identified with a number of diverse phamarcotherapeutic effects such as reduction of heart diseases and cancer in humans (Venesa and Williams, 2004). Immunosuppresion and lowering oxidative stress (Katiyer, 2003), antidiabetis including hyperglyceamia (Vinson *et al*., 2001), lowering levels of cholesterol, triglycerides and decreasing fat tissue accumulation (Tokimitsu *et al*., 2004), and the potential improvement in special cognitive learning abilities (Hague *et al*., 2004). These pharmacological roles of tea tend to affect the consumption with tea trade now thriving due to medicinal value associated with catechins. For example in 2003 China exported 800 tons of tea polyphenols, 300 tons of tea pigments (thaflavins and thearubigins), 10 tons of L- theanin and appreciable amount of tea saponins (Wan *et al*., 2004).

In this study green, black and Orthodox teas were extracted in water. The presence of secondary metabolites was determined using colorimetric chemical tests. The anti- *Plasmodium berghei*, studies were done *in vivo* using a 105 mice model treated with tea with the objective of determining wheather tea could down-regulate parastemia and inflammation.

The trend in parastemia, pcv, serum proteins was determined by statistical analysis of data, using ANOVA to give meaningful conclusions.

2.0 Materials and Methods

2.1.1 Processed Tea Samples

Green (non fermented) and orthodox (partially fermented) teas collected from Ngere in Murang'a county, and black tea (completely fermented) from Nyankoba Nyamira county Kenya, in March 2012, were used.

2.1.2 Experimental Animals

Swiss male white albino mice, weighing 20-30g, aged 6-8 weeks were used.

2.1.3 The *Plasmodium berghei* ANKA isolate cryopreserved in Biochemistry department JKUAT were used. The parasites were donated by Kenya Medical research Institute (KEMRI) .

2.1.4 The laboratory reagents used in these experiments were of analytical grade obtained from Sigma, Oxoid, Aldrich, Merck, Biochemical and BDH through local dealer Kobian.

2.2 Reagent-based Chemical Screening

The aqueous extracts of green, orthodox, and black teas were subjected to standard reagent-based phytochemical colorimetric tests for eight major constituents namely; tannins, terpenes, saponins, alkaloids, anthraquinones, cardenolides, cardiac glycosides, flavonoids (Martnez, 2003, Jigna and Sumitra,2007, Herborne,1973., Trease and Evans,1989., Sofowara, 1982.,Trease and Evans,1983).

2.3 *In vivo* Evaluation of Consumption of Aqueous Tea Extract

Preliminary evaluation tests were done to ascertain whether the test animals could voluntarily drink water 10g/l sucrose and various concentrations of green tea extract (GrTE) (0-20g/l). The mice were acclimatized for 2 weeks during which each mouse was treated once using 0.1ml of 1 % ivermectine to exclude any helminthes infections. The animals were then randomly put into 5 groups and each group had 6 mice being housed in cages separately at room temperature in the normal laboratory enviroment, over a period of 10 days. Each group was subjected to either: Water with 10g/l sucrose (Control)
Water supplement with 10g/l sucrose + 5g/l GrTE
Water supplement with 10g/l sucrose + 10g/l GrTE
Water supplement with 10g/l sucrose + 15g/l GrTE
Water supplement with 10g/l sucrose + 20g/l GrTE

The test animals were closely observed thoroughly daily for 10 days once in a day on the consumption of tea water for toxic signs and symptoms. Pcv was determined using the standard micro-haematocrit method. Their overall health and general well being; weight loss, thinning, loss of fare, fare position change, and ceasing to feed, were observed and daily. Significant weight loss (more than 2 fold loss compared to that of water 10g/l sucrose control over the 10 days closing period) and death was considered a key indicator of declining health due to toxicity.

2.4 Determination of *In-Vivo* Efficacy of the Tea Extracts

Green tea, orthodox tea and black tea extracts (10g/ml) were evaluated *in vivo* for *Plasmodium berghei* activity using intra-peritoneal innoculation of each mouse with $1.0x10^4$ parasites (0.2ml). Treatment was administered 24 hours later. A total of 105 mice eight weeks old health adults were used for the experiments. The mice were randomly divided into seven equal groups (n = 15/ group) according to the model adopted and subjected to one of the following treatments; green tea, orthodox tea, black tea at 10g/l, 0.1ml of anti- inflammatory drug (dexamethasone) equivalent to 0.2mg per mouse, water only (infected control), pyremethamine(refrence drug, and water (non-infected) (placebo). Except the placebo group, animals in other groups were infected with *Plasmodium berghei*. All the extracts were freshly prepared in distilled water. Each group of mice was contained in a mice cage. Mice were checked daily during the 18 days of

treatment to estimate the number of parasites in their tail blood in a wet blood film. Subsequent data was obtained by serial sacrificing of three mice per group at each sampling time. The absolute number of parasites per milliliter of blood was taken as log using the rapid matching method for estimating the host's parastemia according to Herbert and Lumsden (1976). At higher level of parasites the parastemia estimation was achieved by matching microscopic fields of wet blood films against charts and when fewer parasites were present by counting the number of parasites in 5, 10 or 20 such microscopic field for the assessment of effect of tea extract, the level of parasitemia (expressed as log of absolute number of parasites per milliliter of blood) in the animal was compared to the control animals. Animals were checked daily for parasites in tail vein for 18 days. Infected and treated animals at the end of the experiment, with no parasites in the blood were considered cured. During the period, pcv, weight and serum protein concentration of mice were also determined and recorded.

2.6 Estimation of Plasmodium *berghei ANKA* Parasites in Blood

The parasites in the blood were estimated according to the rapid matching method of Herbert and lumsden (1976) as described by Atawodi *et al*.(2003); Atawodi (2005). The method employs a matching technique in which microscopic fields were compared with a range of standard logarithmic values. To count the number of parasites in blood, a drop of blood was obtained on a slide by pinching the tip of the pre-sterilized tail with a sterile needle, immediately covered with a cover slip, and the wet mount observed under x40 magnification. The number of malarial parasites per microscopic slide was then compared with the table of logarithmic values. The logarithmic values which matched the microscopic observation were then converted to antilogarithm, from where the absolute number of malarial parasites per ml of blood was obtained.

2.7 Packed Cell volume Determination

Blood samples collected into heparinised capillary tubes with one end of each tube sealed with plasticine were spun at 2000g for five minutes in micro-haematocrit centrifuge. The packed cell volumes (PCVs) were determined with the aid of micro-haematocrit reader and the' values expressed as percentages (Barbara, 1980).

2.8 Determination of Mice Serum Protein Concentration

Serum Protein concentrations were determined using the lowry method (Lowry, 1951). To 10ml, (0.1ml) of sample protein; 9.9ml of distilled water, 3ml of reagent D were added and mixed thoroughly. The mixture was allowed to stand for 15 min at room temperature before adding reagent E, and allowing the mixture to stand for 30 min at room temperature. Absorbance was measured at 670nm using a spectrophotometer. Bovine serum albumin (BSA) was used as the standard protein.

2.8 Statistical Analysis

Data obtained was subjected to ANOVA and analyzed using statisview SPSS.

3.0 Results

3.1 Reagent-Based Phytochemical Screening of Crude Aqueous Extracts of Tea

Reagent-based phytochemical screening of the crude aqueous extracts of the three types of tea revealed the presence of flavonoids, tannins, saponins, terpenes, cardiac glycosides, and alkaloides as secondary metabolites. Cardenolides were present in green and black teas but absent in orthodox tea. Anthraquinones were not detected in green tea unlike orthodox and black teas (Table 1).

Table 1: Phytochemical constituents of crude extract of tea samples

	Green tea	Orthodox tea	Black tea
Flavonoids	+	+	+
Terpenes	+	+	+
Cardia glycosides	+	+	+
Cardenolides	+	-	+
Anthraquinones	-	+	+
Alkaloids	+	+	+
saponins	+	+	+
Tannins	+	+	+

\+ Presence of secondary metabolite - Absence of secondary metabolite

3.2.0 *In-Vivo* Studies

3.2.1 Tea Dosage Determination

The determination of the appropriate tea dosage to be adopted in the subsequent experiment was done using green tea extract on swiss male healthy mice and the results indicated a significant difference ($p<0.05$) on daily water intake but no significant difference on pcv for all the treatments . Of the five different dosages used, 20mg/ml was the most consumed and tolerated concentration (Fig 1a&b). The dosage had no toxicity and ensured the highest amount of tea intake thus activity,as there were no significant weight loss (more than two fold loss compared to that of water 10g/l sucrosecontrol over closing period nor death. Their overall health was normal.

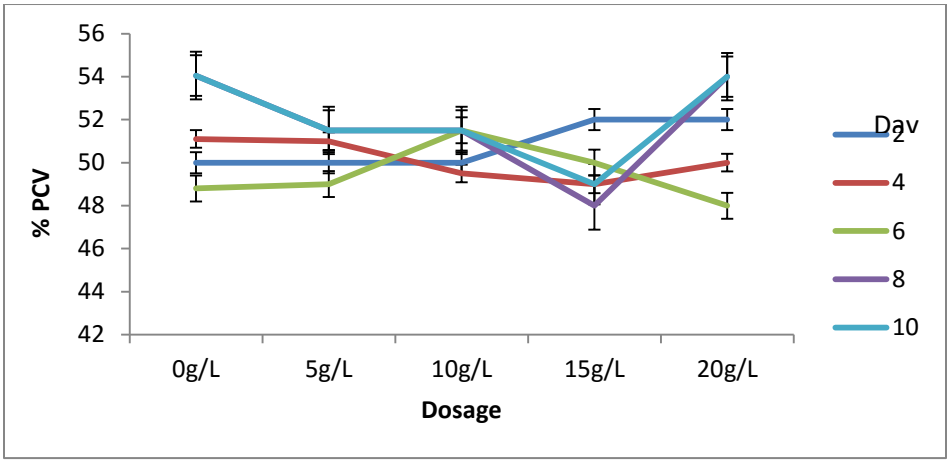

Fig.1a: Effect of oral administration of green tea extract on pcv for a period of 10 days on male swiss mice

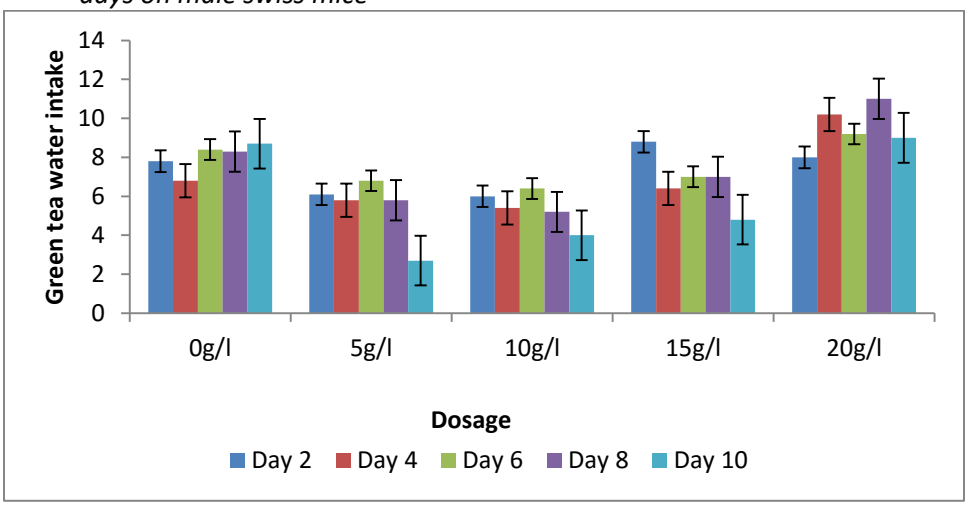

Fig .1b: Effect of oral administration of green tea extract on water intake on male swiss mice

3.2.2 Effect of Tea on Parastemia

To study the effect of tea on *Plasmodium berghei* in mice, the data on mean parastemia level $\log_{10}$ was recorded for 18 days.

All the *Plasmodium berghei* infected mice portrayed similar clinical symptoms for the pre patent period of five days. The observation of mice on day five after infection was in line with the parasites` known incubation period of 5-10 days. Results on parastemia levels in infected control and experimental mice portrayed an exponential increase at similar rates. Day 8 after infection was the peak of parastemia and the parasites were at similar densities.

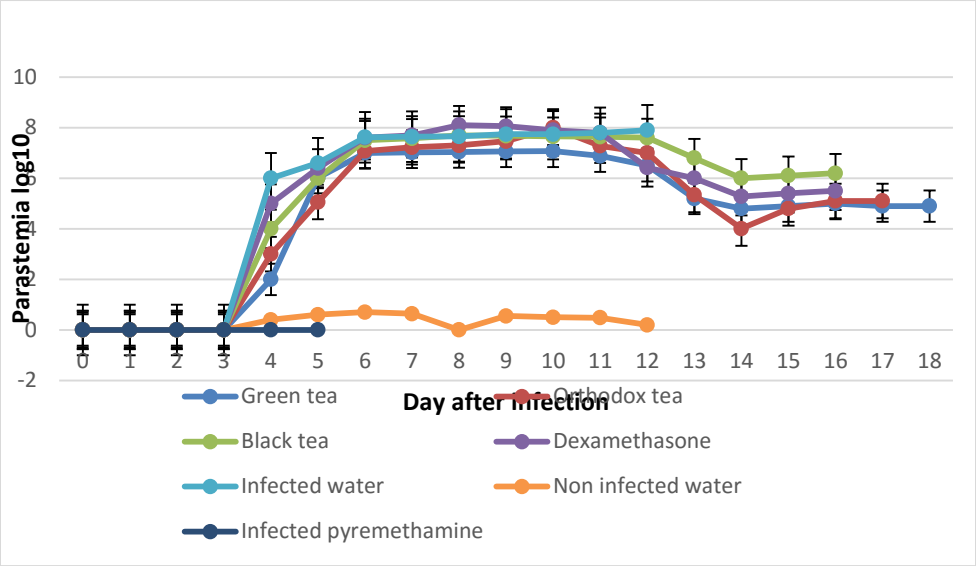

Fig. 2 Time course of Plasmodium berghei for different treatments plotted as log_{10} of parasites per milliliter of blood

Statistical analysis of parastemic levels declined after parastemic peak on day 2 post infection was significantly different (P<0.05) between the various treatment groups (Table 2).

Table 2: Values (means ± SEM) of log_{10} parastemia in mice infected with Plasmodium berghei.

Treatment	Day 11	Day 14
Infected and green tea	7.10 ± 0.442^{L}	5.60 ± 0.055^{L}
Infected and orthodox tea	7.39 ± 0.458^{L}	5.45 ± 0.032^{L}
Infected and dexamethasore	7.85 ± 0.044^{M}	5.90 ± 0.076^{M}
Infected and black tea	7.63 ± 0.172^{M}	6.80 ± 0.014^{M}
Infected and water only	7.70 ± 0.423^{M}	ND
Non- infected and water only	No parasites	No parasites
Infected and pyrimethemine	5.76 0+.588	No parasites
	C.V 5.3 P< 0.05	C.V 1 P< 0.01

Treatments marked with the same letter are not significantly different at P<0.05. ND-not done since all mice had died on day 11 after infection.

On day 2 post infection mice given tea had a significant reduction in parastemia levels compared to the ones infected and given water. However the parasitic reduction levels observed between the tea treatments indicates that there were

no significant difference (P>0.05). There was also significant parasitic level reduction by 14 DPI with green tea having the highest reduction in parastemia and significant difference (P<0.01) than other treatments including dexamethasone but not pyremethamine (Table 2).

3.2.3 Packed Cell Volume

The trend of packed cell volume was followed along with the parasitemia, and thus a companying the events on parasitemia above the fall in packed cell volume had occurred on by 7 day post infection (Fig 3). To study the progressive reduction of pcv and effect of various treatments over time, the mean change in pcv was carried out daily for 18 days and analyzed on day11 and 17 (table 3). The results shows that there was significant PCV difference (P<0.05), between the mice treated using different teas. However, there was no significant difference observed between the tea treatment on day 11 and 17 (Table 3).

Table 3: Mean change (means ± SEM) in % PCV of the treated animals and the control group from day 0 to day 11 and 17 post infection.

Treatment	Day 11	Day 17
Infected and green tea	12.0 ±1.33^{D}	22.0 ± 2.24^{D}
Infected and orthodox tea	13.5± 1.60^{D}	18.0± 2.60^{D}
Infected and dexamethasone	14.5± 1.70^{D}	24.02± 2.80^{D}
Infected and black tea	15.0± 1.68^{D}	12.0 ± 2.62^{D}
Infected and water only	22 ±2.00^{E}	ND
Non- infected and water only	No change in PCV	No change in PCV
Infected and pyrimethemine	2 ±1.22^{D}	23.02 ± 4.42^{D}
	C.V 3.4, P<0.037	C.V 37.8

ND; Not done since all mice in this group had died on 11th day after infection.

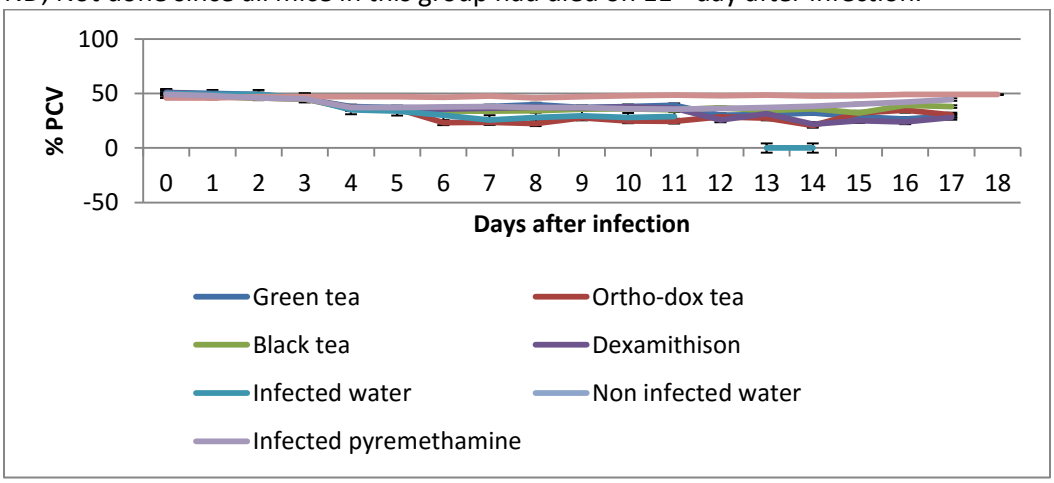

Fig 3: Changes in PCV (means) during the period of study

3.2.4 Mice Serum Protein Concentrations

The mice serum protein concentrations were carried out daily from day 0 to 17. However the statistical analysis of data was done on day 11 and day 17 post infection to determine the effect of various tea treatments over time (Table 4).

Table 4: Serum protein concentrations g/dl (means ± SEM) of the treated mice, the infected and non-treated mice on day 11 and 17

Treatment	Day 11	Day 17
Infected and green tea	2.472 ±0.023^{Q}	2.602 ± 0.14^{Q}
Infected and orthodox tea	2.422 ±0.042^{Q}	2.300 ±0.18^{Q}
Infected and dexamethasone	2.488 ±0.094^{Q}	2.568 ±0.09^{Q}
Infected and black tea	2.023 ±0.462^{Q}	2.00 ± 0.14^{R}
Infected and water only	1.4.4 ± 0.201^{R}	NAD
Non- infected and water only	2.846 ±0.245	2.824 ± 0.26
Infected any pyrimethemine	2.522 ±0.348	2.742 ± 0.338
	C.V 13.7, P<0.01	C.V 29 P<0.01

ND- not done since all the mice in this group had died.

Treatments marked with the same letters in the whole table are not significantly different at P<0.05. Statistical analysis on reduction of mice serum protein concentrations (Fig 4) indicates that there was a significant amelioration (P<0.01) of parasite induced hypoproteinemia due to the treatment with various teas (Table 4), the placebo (non infected group of mice) had serum protein concentrations within the normal range for the whole experiment period. There was a significant reduction in serum protein concentration on day 11 post infection only in the infected control (P<0.01). Data analysis on day 17 post infection shows that mice given black and orthodox tea had significantly (P<0.01) lower seum protein concentrations, than mice treated with green tea, pyremethamine and dexamethasone (Table 4).

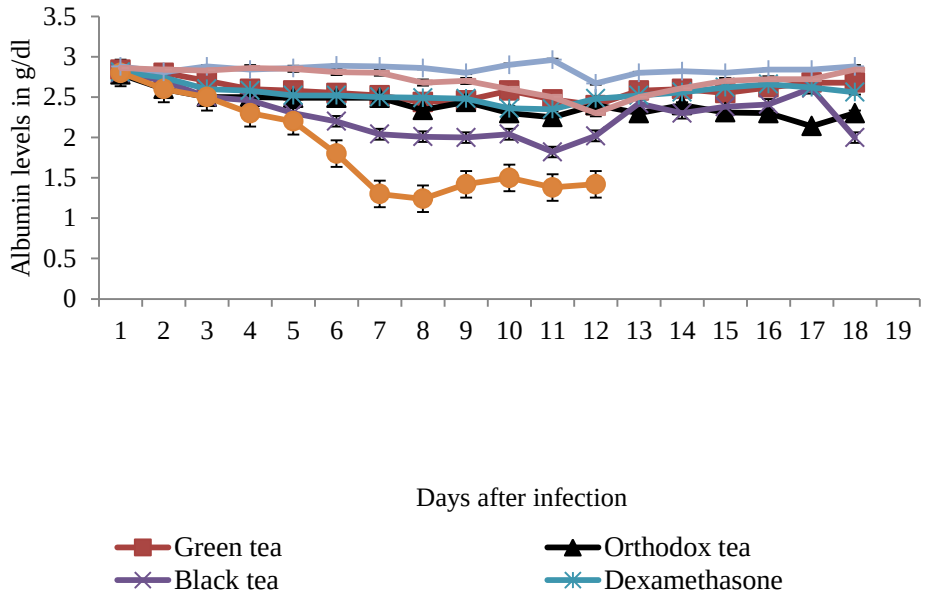

Fig 4: Changes in serum protein (means) concentrations during the period of study

4.0 Discussion

The results on pcv values during dosage determination were within the reference values range of 42-53% for males and 30-48 for females. Thus the green tea extracts had no effect on pcv values neither did the 20mg/ml dosage potray ill symptoms suggesting that it was nontoxic.

The results on *in vivo* studies showed that tea had the ability to lower parasitemia.The anti-plasmodial effect of green and orthodox teas was more than that of black tea. This is probably because they contain catechnins (flavan-3-ols) notably epicatechin, epigallocatechin-3-gallate that are highly hydroxylated compared to black tea that have oxidized polyphenols in the form of thearubigins and theflavins. The environment is known to potentially influence the monopoly and expression of compounds in plants (Folkers *et al*.,2008.,Tsukanya *et al*.,2007). Etimotic factors and therefore agronomic factors affect both the flavonol content of the green tea shoot and can therefore be speculated that high amounts of phytochemicals could reduce *Plasmodium berghei* in the host. However due to the presence of other secondary metabolities, there could be synergistic effects in terms of therapeutic effects of tea. The antiparastic effects shown by green tea against *Plasmodium berghei* ANKA are in line with the previous antimicrobial works in the species of *Streptococcus mutans* linking antimicrobial activities with presence of secondary metabolites (Ebana *et* al.,1991). There is therefore need for studies towards fractionation and isolating the specific phytochemicals by use of ethyl acetone or methyl acetone (Ajayeioba and Sama,2006), understanding the

mode of action of these active therapeutic compounds and identifying of the drug targets together with the development of parasite specific drug formulations.
Pcv determination accompanying the above parastemia events indicated that there was reduction in total pcv early in the infection which might have been due to the binding of the *Plasmodium berghei* antigens with specific receptors on the ethrocytes giving rise to complexes which elicit antibodies with subsequent lysis of the fetal erthrocytes. The experimental infected mice given tea extracts, showed significantly high level of pcv compared to the infected control. This can be attributed on enhanced resistance to erythrocyte haemolysis conferred by tea.

This demonstrates that tea containing polyphenols posses *in vivo* ability to prevent red blood cells from haemolysis which can be accredited to flavonoids. Further to that, the red blood cells structurally have membranes with high content of polyunsaturated lipids and a rich oxygen supply making then susceptible to lipid peroxidation. The reactive oxygen radicals generated during infection of *Plasmodium berghei* can attack erthrocytes membrane, induce its oxidation and trigger haemolysis. We can also speculate that the antioxidant activity of tea could have elicited an increase in plasma antioxidant activity leading to a reduction on the vulnerability of red red blood cell membrane destruction. From the study it is clear that the taking of tea could decrease the effect of free radical individual oxidative damange of the red blood cells. The levels of certain plasma constituents (proteins) were expected to decrease due to damage to the organ or tissues responsible for their synthesis. However the oral administration of tea extracts had a significant ($p<0.01$) prevention of serum concentration reduction in *Plasmodium berghei* infected mice, thereby indicating a decreased effect on inflammation induced by plasmodium parasite. The effect can be due to the presence of flavonoids. The flavonoids and evidence for their role in the prevention of many digenerative diseases is emerging (Amie *et al*., 2003).

The ability of tea to prevent decline in serum protein concentration and subsequent ant-inflammatory effects can be attributed to various properties.The compound that posses these have the ability to exert strong antioxidant effects based in part on their structural characteristics especially the 3,4-dilydroxylation of the 3-ring in the catechol moiety. These structure configurations of flavonoids represent the molecule basis for the radical scavaging and reduction of oxygen species which have been implicated in the pathogens of inflammatory diseases (Hansey *et al*., 2001).

The parasite causes a severe inflammatory response, extensive tissue and organ damage. During inflammation, pro-inflammatory cytokines are activated leading to release of acute phase proteins, which are recognized markers of inflammation (Hebert and Lumsden ,1976). A sustained inflammatory response in critical illness may also tend to prolong inhibition of negative acute phase proteins such as

albumin. The reduction in albumin therefore could be used as aprognostic marker of inflammation (Dey, 1972).

The green tea contains flava -3 -ol which includes epigallo catechins gallate (EGCG), epicatechin (EC), with EGCG being the major constituent and also the component with the highest antioxidant property (Picard, 1996). Catechins undergo major enzymatic transformation to form theaflavins and thearubins which are the characteristic constituents of black tea but which have less antioxidant capacity (Murray *et al.,* 1974). During inflammation among the events which comes into play is that toxic oxidates including oxygen radicals, superoxide, nitrites are generated. The phenolic hydroxyl subsitution present in the tea catechins mainly in EGCG act as a potent radical scavenger increasing the capacity of endogenous antioxidant defences and thereby modulating the cellular redox state (Tasdemir *et al.,* 2006). Therefore the study gives a clear, indication that tea phytochemicals elevated the serum protein concentrations and this is promising for tea as beverage, being on auxillary ant-inflammatory agent in chronic inflammatory diseases. Inflammation and several other pathological conditions leading to disease often result from the effect of free radicals, the most important one being oxygen radical, superoxide, nitrites and hydroxyls.
The efficient radical scavenging property of tea extract is due to the presence of polyphenols and is important property in the management of digenerative diseases (Murray *et al*., 1974). Among the health beneficial effects of tea, the radical scavenging and anti-oxidant properties of tea polyphonols are the most attributes. The evidence supporting an anti-oxidant function of tea polyphenols is derived from assay of their anti-oxidant activity *in vitr*o (Tas demir *et al*., 2006, Murray *et al.,* 1974). Although evidence that tea polyphenols are acting as direct or indirectly as antioxidant *in vivo* is limited, this study shows clearly the indirect action prospect of tea as an anti-inflammatory agent since some serum proteins concentration *in vivo* are regarded as indirect or direct makers of inflammation.This animal model offer unique opportunity to study the contribution of the oxidant properties of the polyphenols and other secondary metabolites. Individual tea polyphenols need to be evaluated to gauge their usefulness in rational design of synthetic drug anologues with higher *in vitro* and *in vivo* activities having more favourable chemical properties.

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