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Antimalarial Effect of Combined Extracts of the Leaf of *Ficus exasperata* and Stem Bark of *Anthocleista vogelii* on Mice Experimentally Infected with *Plasmodium berghei* Berghei (Nk 65)

[1]Okpok Eta Okon, [2]Lebari B. Gboeloh and [3]Samuel Effiong Udoh
[1]Department of Zoology and Environmental Biology, University of Calabar, Cross River State, Nigeria
[2]Department of Biology, Ignatius Ajuru University of Education, Port Harcourt, Rivers State, Nigeria
[3]Cross River University of Technology, Calabar, Cross River State, Nigeria

Corresponding Author: Okpok Eta Okon, Department of Zoology and Environmental Biology, University of Calabar, Cross River State, Nigeria

ABSTRACT

Malaria has known to be resistant to current synthetic drugs, hence there is urgent need to develop new antimalarial drugs to control the disease. This study was designed to investigate the antiplasmodial effect of the combined ethanolic extracts of the leaf of *Ficus exasperata* and stem bark of *Anthocleista vogelii* at different doses in albino mice infected with *Plasmodium berghei* berghei (NK 65). Thirty six mice were divided into six groups of six mice each. Five groups (C_1, C_2, C_3, D and G) were infected with *Plasmodium berghei* berghei parasitized red blood cells. Groups D, H and G served as the controls. Six days post infection, mice in group C_1, C_2 and C_3 were treated orally with 100, 200, 400 mg kg^{-1} b.wt. of *F. exasperata*, respectively for six executive days. Group D was treated with 5 mg kg^{-1} b.wt. of chloroquine while Group G was given distilled water. Group H was neither infected nor treated thus acting as the normal control. The combined extracts exhibited significant ($p<0.05$) dose-dependent chemosuppression of *P. berghei*. The extract exhibited average chemosuppressive effects of 68.8, 79.5 and 91.7% at dose levels of 100,200 and 400 mg kg^{-1} b.wt., respectively. There was also increase in size of the liver and spleen of the infected mice observed in the study compared with the uninfected mice (control). Preliminary qualitative phytochemical screening of the plant extract revealed the presence of tannins, saponins, cardiac glycosides, flavonoids alkaloids and steroid in *F. exasperata*. Saponins, cardiac glycosides, flavonoids, terpenes, alkaloids and steroid were found in *A. vogelii*. The acute toxicity (LD_{50}) of the combined extract was estimated to be 3162 mg kg^{-1} b.wt. The above result showed that the leaf of *F. exasperata* and stem bark of *A. vogelii* possesses antiplasmodial property.

Key words: Antiplasmodial, combined extracts, *Ficus exasperata*, *Anthoclesta vogelii*, chemosuppression, *Plasmodium berghei*

INTRODUCTION

Malaria is one of the most dangerous parasitic diseases in underdeveloped countries especially in Sub-Saharan Africa where it remains a major cause of infant mortality (WHO, 2008). The World Health Organization estimated the mortality rate of malaria in Africa at 781,000 people per year (WHO, 2010) and despite various declarations by African governments in the 1990s and complementary effort promised in the main content of the Roll back Malaria Declaration in Abuja in 2000, malaria remains a major health challenge (Alaba and Alaba, 2003).

In Nigeria, human malaria is mostly caused by *P. falciparum* and *P. malariae* and transmitted by the female anopheles mosquito. The burden of malaria is well documented and has been shown to be a big contributor to the economic burden of disease in communities where it is endemic and is responsible for annual economic loss of 132 billion Naira (WHO, 2009; Onwujekwe *et al*., 2000). There is strong correlation between malaria and poverty and it has been established that malaria impedes economic growth and keeps households in poverty (Teklehaimanot and Mejia, 2008). It is estimated that 300,000 deaths occur each year, 60% of outpatient visits and 30% hospitalizations are all attributed to malaria (FMOH, 2009). Again, at least 50% of the population has at least one episode of malaria annually resulting in high productivity losses while children that are aged less than 5 years have 2-4 attacks annually (FMOH, 2009). The disease is particularly virulent among pregnant women and children under 5 years of age, due to their low levels of immunity. The World Health Organization estimated malaria mortality rate for children under five in Nigeria at 729 per 100,000 (WHO, 2000).

The high mortality rate is largely attributed to resistance of the parasite to commonly used anti-malarial drugs (Slatter *et al*., 1983; Alli *et al*., 2011). In Africa, chloroquine resistant Plasmodium falciparum was first found in 1978 in non-immune travellers from Kenya and Tanzania (Campbell *et al*., 1979; Fogh *et al*., 1979). This was followed two to three years later by reports from Madagascar (Aronson *et al*., 1981). Resistance spread from the African coastal areas inland and by 1983, resistance had been observed in Sudan, Uganda, Zambia and Malawi (Onori, 1984; Ekue *et al*., 1983; Fogh *et al*., 1984; Slatter *et al*., 1983). The emergence of the ineffectiveness of chloroquine in combating malaria had led to additional studies, which had produced a new and effective anti-malaria drug, Artemisin (WHO, 2005; Odugbemi *et al*., 2007). Despite the success recorded with the Artemisin Combination Therapy (ACT), most malaria endemic communities still rely on traditional herbal medicines which are often readily available and affordable (Etkin, 2003).

In view of the problems associated with anti-malaria drugs, poverty and continuous dependence on herbal medicine by about 80% of Africans (Agbedahunsi, 2000), researchers have now focused on the investigation of medicinal plants with a view to scientifically establish the viability or otherwise of these plants in the treatment of malaria. According to UNESCO (1998), the usefulness of these medicinal plants may hold the key to another new and effective anti-malaria drug in the future. In consonance with the enormous dependence on herbal medicine/ traditional health practice and in recognition of the vital role of herbal medicine in health care delivery, the World Health Organization (WHO), in 1978, approved the use of these natural products in the Alma Mata Declaration of Health for All by the Year 2000 A.D.

In Africa, plant extracts are employed in the treatment of various ailments due to their antibacterial, antifungal and antiparasitic properties (Ryley and Peters, 1970; Pamplona-Rogers, 2004). It is known that more than 400,000 species of tropical flowering plants have medicinal properties and this has made traditional medicine cheaper than modern medicines (Ladipo and Doherty, 2011). These plants are generally referred to as medicinal plants. Medicinal plant refers to any plant which, in one part or more of its organs, contains substances that can be used for therapeutic purpose or which are precursor for the synthesis of useful drugs. The medicinal value of the plant lies in some chemical substances that produce a definite physiological action on the human or animal body (Edeoga *et al*., 2005). Some plant decoctions are of great value in treatment of malaria, typhoid fever, diarrhoea or gastrointestinal disorder, urinary tract infections and abscesses (Meyer *et al*., 1996).

The objective of our research was to investigate the antiplasmodial effect of the combined ethanolic extracts of the leaf of *Ficus exasperata* and stem bark of *Anthocleista vogelii* at different doses in albino mice infected with *Plasmodium berghei* berghei.

MATERIALS AND METHODS

Collection of plant materials: The plant materials (leaves and stem bark of *F. exasperata* and *A. vogelii* respectively) were obtained from Awi forest, Akamkpa Local Government Area of Cross River State, Nigeria. The plants were properly identified using appropriate identification keys. Voucher specimens of the two plants were deposited in the herbarium of the Department of Botany, Faculty of Science, University of Calabar, Calabar.

Acute toxicity studies (LD_{50}): The median lethal dose (LD_{50}) of the combined extract of *A. vogelii* and *F. exasperata* that will kill 50% of the animals in a population was determined orally using the method described by Alaribe *et al.* (2011). The mice were divided into five groups of four mice each weighing between 18-20 g. The mice were subjected to 24 h fasting (with only water) before administration of the combined extracts. The extract was dissolved in 20% Tween-80 and administered in doses of 500, 1,000, 2,000, 3,000 and 4,000 mg kg^{-1} b.wt. orally. The sixth group served as the control and received only 20% Tween-80. The mice were then observed for toxicity and fatalities over 72 h.

The LD_{50} was calculated using the equation of Lorke (1983):

$$LD_{50} \sqrt{ab}$$

Where:
a = Least tolerable dose
b = Maximum tolerable dose

Preparation of powdered leaf and ethanolic leaf extract of *Ficus exasperata*: The fresh leaves and stem bark were washed with clean water and air dried at room temperature for five days under shade. The dried leaves were pulverized to powder form using an electric blender while the dried stem bark were ground into powder in a mortar with pestle before being pulverized into powder form using an electric blender. About 500 g of each plant was macerated in 80% ethanol in separate plastic bottles with intermittent shaking to enhance the extraction process and left overnight. The supernatants from each mixture were carefully drained into well labelled cleaned stainless basins and evaporated to dryness in a water bath at 45°C overnight. Equal volume of the two extracts was constituted and mixed together to form the combined extract.

Acquisition of *Plasmodium berghei*, mice and chloroquine: Mice already parasitized with *Plasmodium berghei* berghei (NK 65) were bought from National Institute for Medical Research (NIMR), Lagos and maintained alive. The mice for the study were obtained from the animal house of the Faculty of Basic Medical Sciences, University of Uyo, Uyo, Nigeria. The mice were housed in standard cages in the laboratory and stabilized for 7 days during which they were fed on Standard livestock feed (Vital Feed Growers) obtained from Brand Cereals and Oil Mills Limited, Bukuru, Jos, Nigeria and clean drinking water. Standard chloroquine obtained from Sigma-Aldrich

Company POB 1450B, Louis MD 63178, United States of America (USA) was used in this study. The mice were handled in accordance with the guidelines for the care and use of laboratory animal by the Institute for Animal Research (NRC, 2003).

Experimental design and administration of extracts: At the commencement of the experiment, 36 albino mice weighing between 13-23 g were divided into 6 groups (C_1, C_2, C_3, D, G, H) of 6 mice each. These were labelled Groups C_1,C_2,C_3 and control Groups D,G and H. Groups C_1, C_2 and C_3 were treated for 6 executive days with 100, 200 and 400 mg dose of the combined extracts of *F. exasperata* and *A. vogelii* kg^{-1} b.wt. orally, respectively. Control group D was infected with the parasite and treated with 5 mg chloroquine kg^{-1} b.wt. orally while control group H was neither infected with the parasite nor treated with the extract. The mice parasitized with *Plasmodium berghei* berghei (Nk 65) were sacrificed after six days, having been observed to have shown clinical symptoms of malaria. The mice were anaesthetized in a glass jar containing cotton wool soaked in chloroform. Blood was collected from the sacrificed mice by cardiac puncture using sterile syringes and needles. The blood was diluted in normal saline in the ratio of 1:10 (1 mL of blood in 10 mL of normal saline). The parasitized erythrocyte in volume of 0.3 mL was used to infect each of the experimental mice intraperitoneally six days before treatment.

Determination of baseline parasitaemia: Six days after inoculation of parasite, blood was collected from the tail of each mouse in the various groups before administration of extracts. This was used to make thin and thick blood smear, to determine the baseline parasitaemia.

Percentage of parasitaemia was determined by counting the number of parasitized erythrocytes out of 200 erythrocytes in random fields of the microscope. Percentage parasitaemia and average percentage parasitaemia were calculated according to the following formula adopted by Kundu *et al.* (2010):

$$PP = \frac{\text{Total No. of PRBC}}{\text{Total No. of RBC}} \times 100$$

Where:
PP = Percentage parasitaemia
PRBC = Parasitized red blood cells
RBC = Red blood cells

Average percentage parasitaemia:

$$APP = \frac{APPC\text{-}APPT}{APPC} \times 100$$

Where:
APP = Average percentage parasitaemia
APPC = Average percentage parasitaemia in the control
APPT = Average percentage parasitaemia in the test group

Determination of percentage average suppression: The percentage chemo suppression was determined using the method of Godwin *et al.* (2012). It was calculated by subtracting the average percentage parasitaemia in the test group from average percentage parasitaemia in control group G (infected untreated group). The value obtained was expressed as a percentage of the average percentage parasitaemia in the control group G:

$$AS = \frac{APC\text{-}APT}{APC} \times 100$$

AS = Average Suppression
APC = Average Parasitaemia in control
APT = Average Parasitaemia in test group

Collection of samples for analysis: The extracts were administered for 6 days. Thereafter (first day post-administration), the mice were sacrificed and blood collected from each mouse in all the groups by cardiac puncture. Fresh blood from the sacrificed mice was used to make thin and thick blood films for parasite count and determination of parasitaemia.

HISTOLOGICAL STUDIES

The liver of Plasmodium infected mice treated with 400 mg kg^{-1} b.wt. of the combined extract (Group C_3) was removed and compared with the liver of mice in Group H (mice that were neither infected nor treated with the combined extracts) after staining with haematoxylin and eosin. They were carefully examined under the microscope using X400 objectives and a photomicrograph was taken to show the histological structure of the liver.

RESULTS

Phytochemical composition of *F. exasperata* and *A. vogelii*: Preliminary qualitative phytochemical analysis of the ethanolic extract of the leaves of *F. exasperata* revealed the presence of tannins, flavonoid, saponins, cardiac glycosides, steroid and alkaloid. Also, stem bark of *A. vogelii* were found to contain saponins, cardiac glycosides, flavonoids, terpenes, alkaloids and steroid (Table 1).

Table 1: Phytochemical composition of the ethanolic extracts of the leaf of *F. exasperata* and stem bark of *A. vogelii*

Phytochemical medicinal plants components	*F. exasperata* (Leaves)	*A. vogelii* (Stem bark)
Tannins	+	-
Phlobatannins	-	-
Saponins	++	+
Anthraquinones	-	-
Cardiac glycosides	++	++
Flavonoids	++	+
Deoxy-sugar	-	-
Terpenes	-	++
Alkaloids	+++	-
Steroid	+	+

+++: Highly present, ++: Moderately present, +: Presence in trace, -: Absent

Table 2: Acute toxicity (LD_{50}) of the combined extract after 72 h

Group	No. of mice	Dosage (mg kg^{-1} b.wt.)	Mortality (%)
1	4	1000	0
2	4	2000	0
3	4	3000	75
4	4	5000	100
5	4	6000	100

LD_{50} of the combined extract (x) = $\sqrt{ab}$, Where a: Maximum dose with 0% mortality = 2000, b: Minimum dose with 100% mortality = 5000, $x = \sqrt{2000 \times 5000}, \sqrt{10000000} = 3162$ mg kg^{-1} b.wt.

Table 3: Baseline parasitaemia and Chemosuppression

Group	Extract dosage (mg kg^{-1} b.wt.)	Average parasitaemia before administration of extracts (%)	Average suppression after administration of extracts (%)
C1	Combined extract	100±6.25	68.8±1.62
C2	Combined extract	200±5.25	79.5±1.10
C3	Combined extract	400±4.0	91.7±1.62
D	Chloroquine	5±9.1	100± 2.90
G	-	6.2±0.4	-
H	-	-	-

Values are Mean±SEM of n = 4 values significantly different ($p<0.05$) from control (Group G)

Acute toxicity of the combined extract on the animals showed behavioural signs of toxicity at doses above 2000 kg kg^{-1} b.wt. Signs such as weakness/reduced physical activity, salivation, dizziness and death were observed (Table 2).

Determination of parasitaemia/chemosuppressive activities in the mice: After inoculation of the *Plasmodium berghei* berghei into mice in the various experimental groups, blood was collected after six days for determination of parasitaemia. The results obtained from the baseline parasitaemia from the various experimental groups before administration of extracts ranged between 4.0-9.1% (Table 3). The results indicated that there was very high parasitaemia in all the groups experimentally infected with *Plasmodium berghei* before administration of extracts. The average percentage chemosuppression at the dose levels of 100, 200 and 400 mg kg^{-1} b.wt. were 68.8, 79.5 and 91.7%, respectively (Table 3). One of the infected mice from group G was also sacrificed to obtain the liver and spleen for comparison with same organs from the unparasitized control group H. The two organs of the mouse from group G (parasitized) were physically seen to be dark in colour and about twice the size of the organs of the mice in group H which also had a pink or red colour (Fig. 1).

The standard drug, chloroquine (5 mg kg^{-1} day^{-1}) produced 100% chemosuppression in the control group D. An increase in the size of the liver and spleen of infected mice when compared to the normal control was observed in this study (Fig. 1). The photomicrograph of a normal mouse (Group H) showed normal lobular architecture of the liver having polygonal hepatocytes with granular cytoplasm and centrally placed round nucleus, few spaced hepatic sinusoids between cords (Fig. 2). The photomicrograph of Group C_3 showed areas of cellular proliferation of marked central vein, portal triad, containing red blood cells, vascular congestion and hyperplastic hepatocytes and inflammation of the cells of the liver (Fig. 3).

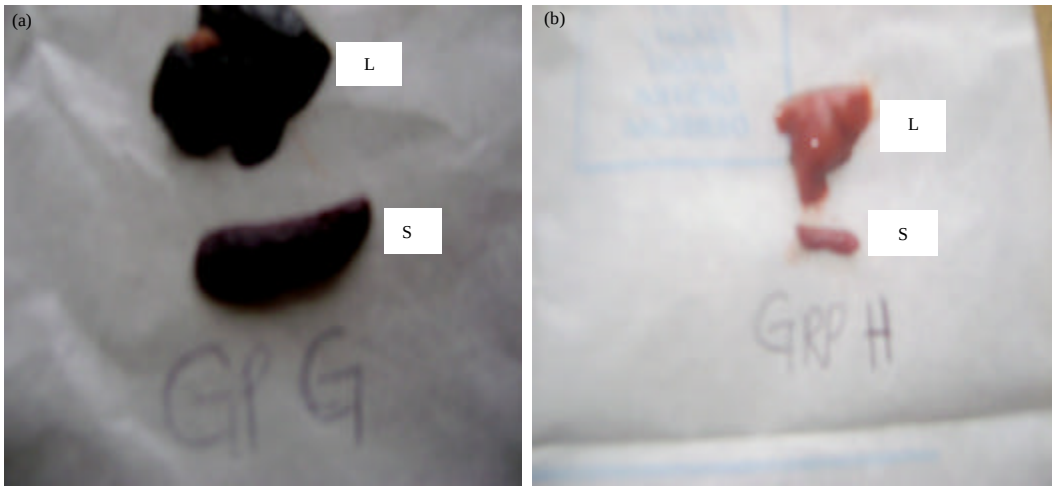

Fig. 1(a-b): Liver (L) and spleen (S) from Liver (l) and spleen (L) from parasitized mouse in (a) Group G and unparasitized mouse in (b) Group H

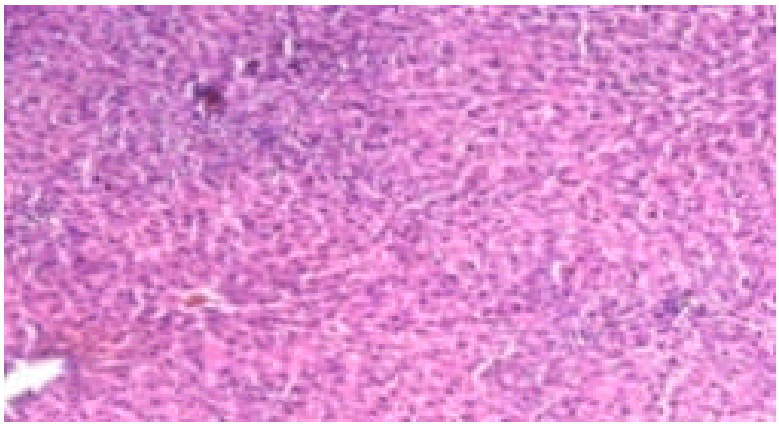

Fig. 2: Cross section of liver of the control group (Group H) showing normal lobular architecture of the liver having polygonal hepatocytes with granular cytoplasm and centrally placed round nucleus, few spaced hepatic sinusoids between cords (Haematoxylin and Eosin stain, ×400)

DISCUSSION

The combined extracts of F. exasperata and *A. vogelii* were well tolerated by the mice up to the dose level of 2000 mg kg^{-1} b.wt. within 24-72 h. However, physical signs of toxicity were noticed in mice administered with 3000 mg kg^{-1} b.wt. of the combined extracts 24-72 h after administration. Using the method of Lorke (1983), the LD_{50} of the combined extracts was estimated to be 3,162 mg kg^{-1} b.wt., far above the highest administered dose level of 400 mg kg^{-1} b.wt. This indicated that the combined extracts administered in this study was relatively safe and was not toxic to the liver of the mice.

From our studies, preliminary phytochemical screening of the ethanolic extracts of the leaf of *F. exasperata* and stem bark of *A. vogelii* showed the presence of saponins, cardiac glycosides,

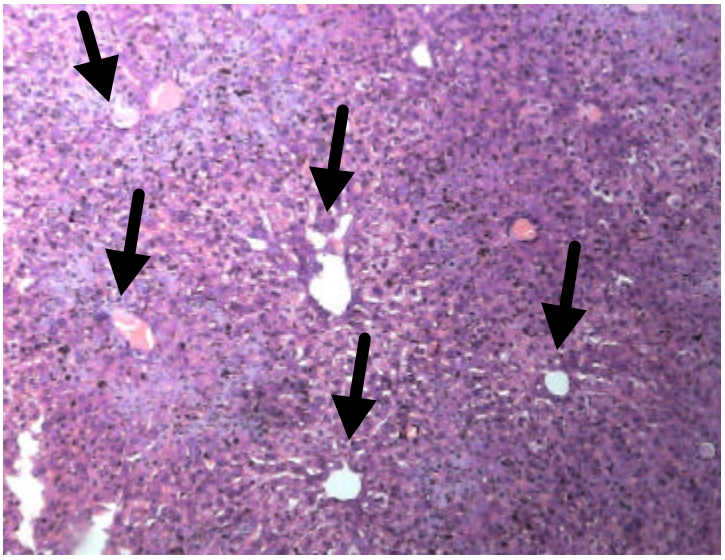

Fig. 3: Histologic photomicrographs of liver tissue of mice treated with 400 mg kg^{-1} b.wt. of combined extract of *F. exasperata* and *A. vogelii* (x400) showing area of cellular proliferation of marked central vein, portal triad, containing red blood cells, billiary epithelium, bile duct and hyperplastic hepatocytes radiating from the sinusoidal layer with inflammation and granulated eosinophilic cells, vascular congestion, vacuolization, pigments against background of inflammation (arrowed)

flavonoids and steroids. The components found exclusively in *F. exasperata* that were not in *A. vogelii* were tannins and alkaloids while terpenes were found only in *A. vogelii*. Similar results were recorded by Irene and Iheanacho (2007), Adebayo *et al.* (2009), Omotayo and Borokini (2012) and Oseni and Owusu (2012) in *F. exasperata*. Ayinde and Owolabi (2009) also recorded the presence of tannins, saponins, cardiac glycosides and flavonoids in the leaf extract of another species, *F. capensis*. Omotayo and Borokini (2012) reported the presence of similar phytochemicals including alkaloids in *F. thonningii*. The phytochemicals recorded in *A. vogelii* were previously reported by Tene *et al.* (2008), Jegede *et al.* (2011) and Alaribe *et al.* (2011). Bassey *et al.* (2009) also reported the presence of these phytochemical compounds in another species, *A. djalonenesis*. Moreso, Odeghe *et al.* (2012) also recorded the presence of similar phytochemicals in *A. grandiflora*.

These constituents have been found in other natural products which exhibited antimalarial activity (Ayoola *et al.*, 2008). Saganuwan *et al.* (2011) reported that plants which contain many phytochemicals with biological activities like alkaloids and flavonoids could serve as sources of antimalarial drugs. Studies have recorded that some of these phytochemical compounds are antioxidants. This property of the plants has been implicated in creation of an intracellular environment that is unfavourable to plasmodial growth (Kirby *et al.*, 1989; Levander and Ager, 1993; Alli *et al.*, 2011). This suggested that the antiplasmodial properties of the combined extract could be based on the antioxidant and antiparasitic effects of these phytochemicals (Ayoola *et al.*, 2008). This observation is validated by Etkin (2003) who reported that artimisinin (a modern antimalarial drug) depends on its oxidant action for its potency against Plasmodium species. This does not however rule out plants that lack oxidant property from having antiplasmodial activity

since they may be active through other biochemical mechanisms (Alli *et al*., 2011). Therefore, the antiplasmodial activity of the combined extract could be attributed to the presence of these phytochemical compounds (Philipson and Wright, 1991; Bassey *et al*., 2009).

The percentage average parasitaemia obtained by the method of Kundu *et al*. (2010) showed high level of infection in all the groups after five days of inoculation of *P. berghei*. The average parasitaemia range was 5.25±1.10-9.1±2.90. This result is consistent with previous reports by Fidock *et al*. (2004) and Ene *et al*. (2008) who observed high percentage of parasitaemia in *P. berghei* infected mice after five days and death of infected mice after seven days of inoculation. Hence, high level of parasitaemia is an important feature of Plasmodium infection which could result in severe anaemia and death of infected animals.

Infected mice treated with 200 and 400 mg kg^{-1} b.wt. experienced a significant reduction ($p<0.05$) in parasitaemia level (79.5 and 91.7%, respectively). The results obtained using the combined extract is consistent with the traditional use of parts of the plants as ethnotherapeutic agent against malaria in West and Eastern Nigeria as reported by Igoli *et al*. (2005) and Adebayo and Krettli (2011). The combined therapy was very effective and was also dose-dependent. The higher potency attained by the combined therapy at the maximum doses administered (200 and 400 mg kg^{-1} b.wt.) may be due to the presence of certain phytochemical compounds which were present in the two extracts (Kingsley *et al*., 2012). For instance, saponins, flavonoids and steroids were present in both extracts and their combined effects might have caused the 79.5 and 91.7% reduction in parasitaemia level at dose levels of 200 and 400 mg kg^{-1} b.wt., respectively. Flavonoid is known to elevate red blood cell oxidation and inhibit the parasite's protein synthesis (Chandel and Bagai, 2010).

This activity nullifies the oxidative damage induced by the malaria parasite (Ayoola *et al*., 2008). The strong antiplasmodial effect of the combined extract of *F. exasperata* and *A. vogelii* may also be due the presence of components with similar mode of actions thus not leading to additive or synergistic effects (Kingsley *et al*., 2012).

In our studies, a maximum of 91.7% chemosuppression of *P. berghei* in mice were reported after administration of the combined extracts of *F. exasperata* and *A. vogelii* at dose level of 400 mg kg^{-1} b.wt. Similar results of 35.6% chemosuppression of *P. berghei* in mice was recorded by Kingsley *et al*. (2012) using combined extracts of *Carica papaya, Azadirachta indica* and *Magnifera indica* at a dose level of 50 mg kg^{-1} b.wt. Paula *et al*. (2012) also reported 87% chemosuppression of *P. berghei* in mice using combined exracts of *Cymbopagan citrates* and *Veronia amygdalina* at a dose level of 400 mg kg^{-1} b.wt. The mechanism of action of these extracts may be similar to that of chloroquine, a standard antimalarial drug which induced the destruction of the asexual forms of the Plasmodium parasite (Bennet and Brown, 2005).

In our studies, chloroquine, used as a positive control, was observed to significantly ($p<0.05$) decrease the parasitaemia in the infected mice at higher rate (100%) than the extracts. According to Kamei *et al*. (2000), when a standard antimalarial drug is used in the management of P. berghei in mice, it suppressed parasitaemia . The 100% chemosuppressive effect of chloroquine recorded in the present study showed that it is still one of the drugs of choice against malaria parasite (Fidock *et al*., 2004). The result agreed with previous report on the antimalarial effect of chloroquine by Oyewole *et al*. (2008) and Odeghe *et al*. (2012). An increase in the size of the liver and spleen of infected mice observed in this study when compared to the normal control is also a feature of severe Plasmodium infection (Ibrahim *et al*., 2011). Moreover, changes in the histological structures of the liver tissues of the mice in the present study as shown in Fig. 1, 2 and 3 are also

characteristics of tissues previously exposed to parasitic infection (*Plasmodium berghei* berghei), although the 400 mg kg^{-1} b.wt. of the combined extract gave 91.7% chemosuppression of the infection.

However, the potency of the combined extracts of *F. exasperata* and *A. vogelii* at different dose levels were not in doubt as up to 91.7% chemosuppresion of *P. berghei* berghei were observed. These extracts could be utilized for the trial of some newer antimalarial drugs in future in view of the constant development of resistance of malaria to currently used drugs.

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