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Research Article

In vivo antimalarial and *in vitro* antioxidant activities of hydro-methanol leaf extract of *Fadogia cienkowskii* Schweinf. (Rubiaceae)

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Background: Rapid parasite resistance to antimalarial drugs necessitate continuous search for new drugs. *Fadogia cienkowskii* Schweinf. (Rubiaceae) is one of the several plants utilized in South-East Nigeria for treatment of malaria fever.

Objective: The aim of the study was to evaluate antimalarial and antioxidant potentials of leaf extract of *F. cienkowskii*. The phytochemical profile and toxic heavy metal content were also evaluated.

Methodology: Antimalarial activity of crude leaf extract of *F. cienkowskii* (100 – 400 mg kg^{-1}) was assessed against chloroquine sensitive *Plasmodium berghei* (NK65) using suppressive and curative test models. Chloroquine 10 mg kg^{-1} was used as positive control. Antioxidant activity was evaluated using 1,1-diphenyl-1-picrylhydrazyl and ferric reducing antioxidant power methods. The presence of heavy metals in the raw material was evaluated using Atomic Absorption Spectrophotometric technique and modified Lorke's method was employed to test for oral acute toxicity.

Results: The results showed that the test plant exhibited antimalarial activities in both models in a dose-dependent manner. The comparison analysis showed that at 400 mg kg^{-1}, the suppressive and curative effects were 93.44% and 72.47% respectively. All the animal groups that received test extract lived longer and weight loss was prevented compared to the negative control group. The test extract exhibited antioxidant potential in DPPH scavenging and FRAP assays compare with Vitamin C. All the tested heavy metals were present but within permissible range. Acute toxicity study of extract revealed no immediate adverse effect(s).

Conclusion: These results support the traditional use of *F. cienkowskii* in management of malaria fever.

Key Words: *Fadogia cienkowskii, Plasmodium berghei,* antimalarial, heavy metals, antioxidant

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1. Introduction

Malaria is a life threatening infection caused by blood parasites, *Plasmodium* species. In severe infection, it is associated with high mortality due to associated health challenges such as anemia, cerebral malaria and low birth weight due to malaria in pregnancy. Nigeria has the highest recorded malaria burden in Africa and in the world where it is a risk factor to about 97% of Nigerian population (WHO 2014, Dawaki *at al.,* 2016). The

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populations most affected are the pregnant women and children under the ages of 5 (FMOH, 2009, WHO 2016, Lawal et al*,* 2015). The control of malaria infection in Nigeria like in most part of the globe is currently experiencing challenges because both the parasite and the vector develop resistance easily to available drugs and insecticides (Khozirah et al*,* 2011). There is no vaccine currently in the market for malaria prevention.

Nigeria has good biodiversity and traditional use of medicinal plants in management of malaria fever and other health issues is thus, a common practice (Orimadegun et al*,* 2015). These plants are made up of many bioactive phytocontituents. Quinine and artemisinin are compounds isolated from plants that are currently used in treatment of malaria infection. The search for more possible antimalarial agents or "lead templates" from plants is a continuous one due to the ease at which the parasite develop resistance to any newly available drug.

Increased production of active free radicals and reduction of host cellular antioxidant have been observed in sever malaria infections, thereby possibly indicating the contributory effect of oxidative stress in malaria infection and its systemic complications (Charunwatthana et al*,* 2009, Iribhogbe et al*,* 2013). Medicinal plants and compounds possessing antioxidant potentials have been suggested as being therapeutic beneficial in treatment of malarial infection (Nuchsongsin et al*,* 2007, Percário et al*,* 2012).

Plant raw materials for herbal preparations sourced from the wild are prone to contamination from soil pollutants, and human handling (Obi et al*,* 2006, Igweze et al*,* 2012,). There is an increasing evidence of toxic heavy metal contamination in herbal remedies as reported by Gunturu et al*,* 2011. The cumulative effects of toxic heavy metal contaminants are detrimental to human health (Harries and Cullinan, 1994).

Fadogia cienkowskii Schweinf. (Family: Rubiaceae) is a shrub of less than 1m in height and is locally called 'ufu-ewureje' by Igede tribe of Benue State in Nigeria. The plant has both food and therapeutic values (Burkill 1997). Its reported medicinal value includes, general childhood healthcare, headache, general body debility, inflammation, diarrhea, heamostatic in treatment of bleeding wound, male fertility challenges, analgesic and fever (Burkill 1997, Jiofack et al*,* 2010, Ode et al*,* 2015). The plant has the ability to hyper accumulate unwanted trace elements from it environment and as such could be helpful in phytoremediation of harmful heavy metal (Van der Ent, and Erskine, 2015).

The present study aims at evaluating the efficacy of *F. cienkowskii* in treatment of malarial fever, its value as an antioxidant agent and possible contamination with heavy metals.

2. Methods

2.1 Equipment/Reagents

Giemsa stain and chloroquine phosphate were of Sigma-Aldrich chemical company (USA and Germany respectively), 1, 1-diphenyl-2- picrylhydrazyl (DPPH) (Sigma-Aldrich, USA), Ferric chloride, potassium ferricyanide, trichloroacetic acid, dimethyl sulphoxide, other chemicals and reagents used for this study were of analytical grade.

2.2 Collection and Identification and extraction of plant samples

Leaves of *F. cienkowskii* (FC) were collected at Nsukka, Enugu state in February 2016. It was identified and authenticated by a taxonomist, Mr Ozioko Fred of International Centre for Ethnomedicine and Drug Development (INTERCEDD), Nsukka Enugu state. Voucher number assigned was **INTERCEDD/1607**.

The leaves were cleaned and air dried on laboratory benches. The leaves (400 mg) were extracted by cold maceration using 3600 mL hydro-methanol (90%). The soaked leaf sample was filtered on day 3 and re-soaked for another 3 days. The filtrates were combined and filtered using double-layered white muslin material and subsequently through filter paper. The crude methanol extract was concentrated under vacuum in a rotary evaporator at 40 °C and stored at 4 °C.

2.3 Animals

Healthy Swiss albino mice (18 -22 g) of either sex were used for these experiments. The animals were purchased from College of Medicine Animal Care Center, Department of Physiology, College of Medicine of the University of Lagos and housed in standard cages. They were maintained on a standard pelleted feed and water ad libitum. The animals were allowed to acclimatize seven days preceding the experiment. Dark and light cycles were maintained at 12 h each.

Permission and approval for animal studies were obtained and animals handled in accordance to with the guideline and recommendation of the ethics committee on the use of animals for research of the University of Lagos Ethical Committee, Lagos, Nigeria.

2.4 Malaria parasite

The rodent parasite, chloroquine sensitive *Plasmodium berghei berghei* (NK-65 strain) was sourced from National Institute of Medical Research (NIMR) Yaba Lagos Nigeria. It was then maintained in University of Lagos, Animal House Laboratory by serial passage of the parasite in an uninfected mouse.

2.5 Phytochemical test

The crude leaf extract of FC was subjected to various phytochemical investigation for identification of secondary metabolites using standard procedures (Sofowora 1993).

2.6 Acute toxicity

The method of Lorke's (1983) was employed in determining the median lethal dose (LD_{50}). This involved single oral administration of 3 different doses of FC extract (100–4000 mg kg^{-1}) to groups of six Swiss albino mice each, while the negative untreated group was given the vehicle, 5% DMSO. The animals were observed for manifestation of physical signs of toxicity and distress and death.

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The number of deaths in each group within 24 h was recorded. The observation was initially hourly, daily and continued for up to 7 days.

2.7 Antimalarial assays

2.7.1 Evaluation of suppressive activity of the extract (Four-day test)

Evaluation of the schizontocidal activity of FC extract against early chloroquine sensitive *Plasmodium berghei* infection in mice was done according to method (4-day suppressive test) described by Peters et al, (1967). Twenty five (25) mice were inoculated intraperitoneal (i.p.) with the parasite (1×10^7 parasitized erythrocytes) on the first day and randomly divided into 5 groups of 5 mice each. The mice in groups 1 - 3 were administered with the 100, 200 and 400 mg kg^{-1} of FC crude extract respectively in 0.2 mL volume, 2 h post infection. Groups 4 - 5 were administered with 10 mg kg^{-1} of chloroquine and 0.2 mL of 5% DMSO for standard and negative control respectively.

The groups were treated for four consecutive days (D_0 – D_3). On the fifth day (D_4), thin blood films were made from tail blood of each mouse and stained with Giemsa stain and examined under the microscope. The percentage suppression of parasitaemia was determined.

Body weight of each mice was measured before the animals were parasitized (D_0) and on day 4 (D_4) before collection of the blood for smear. The instrument used was digital weighing balance.

2.7.2 Evaluation of curative activities of extract (Rane's test)

Antimalarial activity of methanol crude extract of FC on established infection was carried out according to the method described by Iyiola et al, (2011). Twenty-five (25) mice were injected intraperitoneally with standard inoculum of 1 x 10^7 *P. berghei* NK- 65 infected erythrocytes on the first day (D_0). Seventy-two hours later (D_3) and confirmation of parasitaemia, the mice were divided into five groups of five mice each. The groups were orally treated with FC leaf extract (100, 200 and 400 mg kg^{-1}), chloroquine (10 mg kg^{-1}) was given to the positive control and 0.2 mL of 5 % DMSO was given to the negative control group. The treatment was carried out once daily for another 4 days (D_4 – D_7), making it a 5 day treatment.

Thin blood smears were prepared from blood collected daily (D_3 - D_7) from the tails of each mice and examined microscopically to monitor parasitaemia level. A final blood smear was prepared on D_8, 24 h after last drug administration, to determine the effect of the last dose of drug administered. The ability to reduce the parasite load in established infection (curative effect /percentage of cure) was calculated.

The mean survival time for each group was determined arithmetically by finding the average survival time (days) of the mice (post inoculation) in each group over a period of 28 days (D_0 - D_{27}).

2.8 Evaluation of Antioxidant Activity

2.8.1 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging assay

The DPPH scavenging capabilities of deleterious effects of free radical was evaluated using slightly modified method of Mensor et al, 2001. Various concentrations of extract (0.05-1.00 mg mL^{-1}) in methanol were prepared. To 1 mL of each prepared concentration, 3 mL of methanol and 1 mL of DPPH solution (1 mM) was added to make up to 5 mL. The resulting solutions were continuously stirred and left to incubate for 30 min in the dark, at room temp. The absorbance of the resulting solutions was recorded at wavelength of 517 nm. The experiment was performed for ascorbic acid as a positive standard and for a blank solution (without the extract) as the negative control. All determinations were carried out in triplicates.

2.8.2 Reducing power assay

Reducing power of FC leaf crude extract was estimated as described in literature by Yen and Chen (1995) with some modifications. Different concentrations of FC extract and the standard were prepared using methanol. One (1) mL of each prepared FC extract was mixed with 2.5 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 2 mL of potassium ferricyanide (1%, w/v) and incubated at 50 °C for 20 min. After addition of 2.5 mL of trichloroacetic acid (10%, w/v) the mixture was centrifuged at 3,000 rpm for 10 min. The upper layer of the supernatant (2 mL) was mixed with 2 mL of deionized water and 0.4 mL of freshly prepared ferric chloride ($FeCl_3$ 0.1%, w/v). The resulting mixture was thoroughly mixed and incubated for 10 min.

Absorbance was measured at 700 nm. Ascorbic acid was used as standard, while blank preparation was used as negative control. A higher absorbance power of test samples compared with that of the blank indicates increased reducing power.

2.9 Heavy metal analysis

The sample of FC leaf extract was analyzed for some heavy metals, Cadmium, Zinc, Copper, Lead, Nickel and Iron content and their compliance with recommended limits of the World Health Organization (WHO) and European Union (EU) were applicable. A 2 g weight of the sample was transferred into the digest tube followed by 10 mL nitric acid. The mixture was evaporated on the Q-block digester for 1.30 h. Deionized water (50 mL) was added and filtered with filter paper. The resultant filtrate was assayed for trace metals using Atomic absorption spectrophotometry (model 210, VGP Bulk Scientific, USA).

2.10 Statistical analysis

Results were reported as mean ± standard error of mean (SEM). Statistical analysis was done using one-way analysis of variance (ANOVA) followed by Tukey's test. Differences were considered significant at $P < 0.05$.

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3. Results

Phytochemical screening of FC leaf crude extract showed that it consists of alkaloids, phenols, flavonoids, terpenes, saponins and glycosides.

Acute toxicity Test

The various doses of leaf crude extract of FC caused no death or any observable physical distress even after animal were treated with a dose of 4000 mg kg^{-1} of the extract.

Effect on suppressive activity of hydro-methanol leaf extract of *F. cienkowskii*

Antimalarial activity of hydro-methanol crude leaf extract of FC against early infection using *P. berghei* showed significant dose-dependent parasitaemia suppression compared to the negative control ($p<0.05$). At 100, 200 and 400 mg kg^{-1} of the extract, 81.82, 87.06 and 93.44% suppression of parasitaemia occurred respectively. The obtained result at 400 mg kg^{-1} is comparably to standard drug, chloroquine which exhibited chemosuppression of 89.36% (**Table 1**). No weight loss was observed for animals at any dose level of the test drug and the positive control, chloroquine. The higher the dose of the text drug, the more the weight gained compared to the weight loss observed in the negative control group.

Effect on Curative activity of hydro-methanol leaf extract of *F. cienkowskii*

The extract showed schizonticidal effect on the parasite that is not dose dependent (**Table 2**). The reduction in the percentage parasitaemia obtained at 400 mg kg^{-1} of the extract is lower than the percentage reduction obtained at 200 mg kg^{-1} of the same drug but significantly higher compared to the negative control ($p<0.05$). Mean survival time on established infection as observed in this study was dose dependent. The animals (mice) lived longer and were more protected as the doses increased (17.28 – 21.41 days). However, the animals were not fully protected at any dose level of the test extract compared with the chloroquine (28 days).

Table 1: Suppressive effect of hydro-methanol leaf extract of *F. cienkowskii* on *P. berghei* in mice

Drug	Dose mg kg^{-1}	Weight D_0	Weight D_3	% Weight change	% Parasitaemia	% Chemosuppression
5% DMSO	0.2 mL	20.17 ± 0.63	18.03 ± 0.13	-10.61	11.28±0.35	0
CQ	10	20.80 ± 0.50	21.10 ± 0.23	1.44	1.20 ± 0.02*	89.36
FC	100	19.82 ± 0.45	20.16 ± 0.45	1.72	2.05 ± 0.06*	81.82
	200	21.40 ± 0.45	21.87 ± 0.45	2.20	1.458 ± 0.11*	87.06
	400	20.51 ± 0.45	21.36 ± 0.45	4.144	0.736 ± 0.10*	93.44

*N = 5; *: Significant at p <0.05; CQ: Chloroquine; FC: F. cienkowskii*

Table 2: Curative effect of hydro-methanol leaf extract of *F. cienkowskii* on *P. berghei* in mice

Drug	Dose mg kg^{-1}	Parasitaemia	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	MST (Days)
5% DMSO	0.2 mL	%Parasitaemia	11.31 ± 0.28	13.49 ±1.04	15.38 ±0.64	18.30 ±0.42	20.47 ±0.34	16.31 ±0.51	7.63 ± 1.1
		% Cure	0	0	0	0	0	0	
CQ	10	%Parasitaemia	11.66 ±0.32	10.70 ±0.84	4.38±0 .68	1.39 ±0.24	0±0	0 ±0	28.00 ± 0.0
		% Cure	-3.09	20.68	71.52	92.40	100	100	
FC	100	%Parasitaemia	11.52 ±0.32	13.00 ±0.42	10.75± 0.34	8.37 ±0.23	6.51 ±0.55	5.20 ±0.52	17.28 ± 1.63
		% Cure	-1.86	3.63	30.10	54.26	68.20	68.12	
	200	%Parasitaemia	11.29 ±0.36	12.16 ±0.26	10.46± 0.23	9.38 ±0.16	7.67 ±0.43	4.60 ±0.41	17.52 ± 1.02
		% Cure	0.18	9.86	31.99	48.74	62.53	87.98	
	400	%Parasitaemia	11.27 ±0.34	11.83 ±0.34	10.30± 0.45	8.28 ±0.16	6.35 ±0.17	4.49 ±0.33	21.41 ± 1.02
		% Cure	0.35	12.31	33.03	54.75	68.98	72.47	

Results are Mean count + SEM; n = 5

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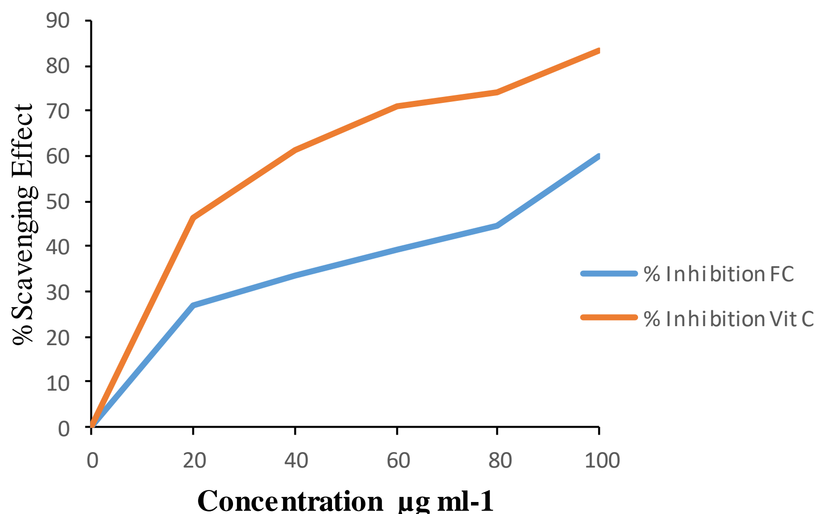

Figure 1: DPPH Radical scavenging activities of FC and Vit. C

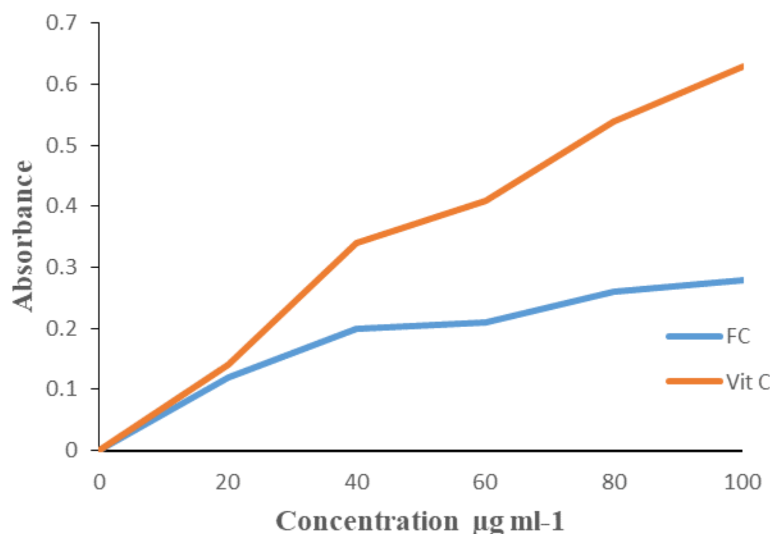

Figure 2: Reducing power of FC leaf hydro-methanol extract in comparison with Vit C. as a reference antioxidant.

DPPH free radical scavenging effect

The ability to scavenge DPPH free radicals was shown to be concentration-dependent for both extract and the standard (ascorbic acid). The IC_{50} (50% or half-maximal inhibitory concentration) values of hydro-methanol leaf extract of FC and ascorbic acid were calculated to be 5.05 µg mL^{-1} and 3.08 µg mL^{-1}, respectively (**Figure 1**). The test extract therefore exhibited antioxidant potential as radical scavengers (Dehpour et al, 2009).

Figure 2 showed the reducing powers of extract and ascorbic acid on Fe^{3+} were concentration dependent. Increasing absorbance at 700 nm is an indication of increasing Fe (III) reduction effect of test samples. The reducing power effect of both extract and standard drug increased with concentration in a strongly linear manner (R^2 = 0.8912 and 0.9809 respectively). However, the reducing power of ascorbic acid was significantly ($P<0.05$) higher than that of FC extract (IC_{50} 956.11 and 396.66 µg/mL respectively).

Heavy metal contaminants

The results obtained showed the presence of all the metals tested but within various documented permissible maximum limits (**Table 3**).

4.0 Discussion

Malaria is an infection associated with poor economic status and drug resistant challenges. These two key factors play crucial role in the constant use of herbal medicine in the treatment of malarial infection (Orimadegun et al, 2015). Herbal products are also believed to come with less side effects and often given/prescribed by people (traditional practitioners) they know, lives among them and highly trusted in the community. Antimalarial potentials of some medicinal plants used in folkloric treatment of malaria have been investigated and reported (Salawu et al, 2010).

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Table 3: Mean heavy metal concentration (mg kg $^{-1}$) dry weight of FC

Heavy Metal	Lead Pd (mg/kg)	Cadmium Cd (mg/kg)	Zinc Zn (mg/kg)	Copper Cu (mg/kg)	Nickel Ni (mg/kg)	Iron Fe (mg/kg)
FC Extract	0.170 ± 0.03	0.001 ± 0.02	0.8108 ± 0.03	0.068 ± 0.02	0.805 ± 0.11	3.661 ± 0.06

The antimalarial activity of hydro-methanol (90%) crude leaf extract of FC was investigated using two different standards *in vivo* models, suppressive and curative models. Data obtained in the study were expressed and reported as the ability to prevent the growth of parasite in the presence of the crude extract (test drug), degree of parasite load clearance, time of onset of activity, mean survival time and changes in body weight.

The crude extract of FC demonstrated a good degree of activity against chloroquine-sensitive strain of *P. berghei* in both models used for the evaluation. The suppressive effect of the crude leaf extract of FC seems to be stronger than its ability to reduce parasite in established malaria infection. A non-dose dependent but significant reduction in parasitaemia was observed on daily bases comparable to the reference drug chloroquine 10 mg kg^{-1} in established infection bioassay. The curative effect or parasite reduction effectively started on the third day post drug administration, indicating that FC has slow or late onset of activity (Table 2). Thus, it cannot be classified as drug of emergency. The mean survival time (MST) of FC extract treated mice on day 28 (D_{27}) showed dose dependent prolonged survival time but the animals were not fully protected as in chloroquine group (100 %). At 400 mg kg^{-1} it was not able to fully protect the infected mice but it was probably able to reduce the quick on-set of recrudescence occurrence. Improved mean survival time support the curative potential of the FC extract. Control of weight loss observed may be due to the ability of the crude extract of FC to control development to full malaria infection and/or reverse some pathological features of malaria infection (Bantie et al*,* 2014). The data obtained from this study are in consistent with previously reported outcome of plants from Rubiaceae family (Odugbemi et al*,* 2007, Lawal et al*,* 2010, Karous et al*,* 2011). A species of the genus Fadogia, *F. agrestis* leaf extract, has been documented to exhibiting antimalarial activity against *P. falciparum in vitro* (Sanon et al*,* 2003).

Oxidative stress in malaria possibly triggers off malaria systemic complications and damage to body organs (Percário et al*,* 2012). Antioxidant therapy may be able to ameliorate such event. The study results suggest that the crude leaf extract of FC contains antioxidants that possibly scavenge free radicles, reducing effect by electron donation, breaking of free radical chain and singlet oxygen quenching. (Owolabi et al*,* 2018).

Secondary metabolites identified from the test extract of hydro-methanol leaf extract of FC include, tannins, alkaloids, flavonoids, and terpenes. Some of these phytochemical constitutes have been reported to have antimalarial parasite clearance potentials or used as adjunct in treatment of malaria infection. Alkaloids are the major classes of compounds possessing antimalarial activity in Rubiaceae family (Koffi et al*,* 2011). Thus the observed antimalarial activity of FC crude leaf extract in this study could be as a result of any of the phytoconstituents or a combination effects of the plant constituents. Malaria parasite are more likely to develop resistance to single active compound or single purified product compared to crude extra that consist of poly phytoconstituents (Lathrop, 2015; Daddy et al*,* 2017). This could explain efficacy and longevity still observed in effective medicinal plants such as *F. cienkowskii* despite long years of use.

The result revealed that the wild collected FC leaf contained all the tested heavy metals in varied concentrations (Igweze et al*,* 2012). Though none of the heavy metals exceeded the permissible limit in studied extract of FC but accumulation of the detectable heavy metals over long term usage may occur and calls for concern. Accumulation of these metals in the human bodies is toxic to the body. They have been associated with many organ damages, including renal and nervous systems, various carcinogenic conditions, miscarriages in pregnant women (Sathiavelu et al*,* 2012). However, the plant is noted to be helpful in phytoremediation of harmful heavy metal (Van der Ent, and Erskine, 2015).

Metals, such as, lead, nickel and iron have negative or toxicological effects on reproduction and other human physiology (Thompson and Bannigan, 2008). Lead is a highly toxic environmental pollutant and its contamination of herbal products is common (Abou-Arab and Abou Donia, 2000; Ziarati, 2012). Exposure to lead poisoning at childhood and prenatally has been documented to interfere with mental development and learning deficit (Johnson 1998, ATSDR 2007). Lead accumulates in male reproductive tissues and interferes with its functionality (Igweze et al*,* 2012).

The study on acute toxicity revealed absence of mortality or untoward change in behavioural status up to the dose of 4000 mg kg^{-1} body weight of extract administered orally, which could be an indication the safety of the extract (Ode et al*,* 2015). All the animals used for 4-day suppressive antimalarial test out lived the days of the experiment. Death occurring before D_5 may be attributed to the effect of the test drugs rather than the parasites (Jutamaad et al*,* 1998).

5.0 Conclusion

Antimalarial potential of *Fadogia cienkowskii* Schweinf. (Rubiaceae) leaf has been demonstrated in this study. The observed high *in vivo* parasite clearance indicates efficacy and possible potential in human malaria and should therefore be pursued further for possible

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development of new antimalarial drugs. Antioxidants potential exhibited by the plant FC may ameliorate the progress of malarial infection and also may aid fast recovery from the infection.

Conflict of Interest declaration

The authors declare no conflict of interest.

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