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β-HEMATIN INHIBITION ACTIVITY OF THE METHANOL LEAF EXTRACT AND RESIDUAL AQUEOUS FRACTION OF *FICUS ASPERIFOLIA*

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ABSTRACT

Chemotherapy is still regarded as the major intervention in fighting the malaria scourge despite the recent introduction of malaria vaccine. Screening of medicinal plants provides opportunities for the discovery of potential drug molecules. *Ficus asperifolia* (Miq), family Moraceae has been traditionally employed in the treatment of many diseases including pain, fever and malaria. The aim of this study was to examine the antimalarial activity of both the methanol leaf extract (MEFA) and residual aqueous fraction of *Ficus asperifolia* (RAF) through the determination of their heme polymerization inhibition ability. The powdered leaf was extracted using 70% methanol and further fractionated using solvents of varying polarity to obtain the residual fraction. Standard protocols were used to conduct preliminary phytochemical screening and oral acute toxicity studies of the extracts. The colorimetric assay method of β-hematin inhibition synthesis was applied using a 96-well plate and determination of UV absorbance using micro plate reader at 405 nm. Percentage inhibition was determine and IC_{50} calculated using Microsoft excel by plotting percentage inhibition vs log concentration. Both MEFA and RAF exhibited significant antimalarial activity with highest percentage of 95.51 and 92.27 and IC_{50} of 4.373 and 4.768 respectively compared to the standard drug chloroquine with 97.27% inhibition and IC_{50} of 5.334 respectively. This activity suggests that the plant may be useful against malarial parasite and justify the claim of its effectiveness in folkloric medicine.

Keywords: Antimalarial, β-hematin, Chloroquine, *Ficus asperifolia*, Heme-polymerization

INTRODUCTION

Malaria constitutes a global health challenge and remains a great cause of morbidity and mortality. It is an endemic infectious disease that is widespread in tropical and sub-tropical areas of the world (NIAID, 2007). This infectious disease is caused by microorganisms of the *Plasmodium* specie. In clinical practice, *Plasmodium falciparum* has been implicated in the most severe form of the disease and is responsible for more deaths in humans. Most fatalities are as a result of *Plasmodium falciparum* infections, whereas *Plasmodium vivax, Plasmodium ovale*, and *Plasmodium malariae* generally cause a milder form of malaria. The species *Plasmodium knowlesi* rarely causes disease in humans (WHO, 2020). This life threatening parasitic disease is most commonly spread by an infected female Anopheles mosquito. The mosquito bite introduces the parasites from the mosquito's saliva into a person's blood which then travels to the liver where they mature and reproduce. Malaria is typically diagnosed by the microscopic examination of blood using blood films, or with antigen-based rapid diagnostic tests. Methods that use the polymerase chain reaction to detect the parasite's DNA have been developed, but are not widely used in areas where malaria is common due to their cost and complexity (Nadjm and Behrens, 2012).

Sub-Saharan Africa carries a disproportionately high share of the global malaria burden. In 2021, the region was home to 95% of malaria cases and 96% of malaria deaths (WHO, 2022). Like other developing countries of the world, malaria prevalence has been observed in Nigeria with about 31.3% of global death burden (WHO, 2022). A research conducted in Kano north western Nigeria revealed 60.06% Prevalence of malarial infection (Dawaki *et al*. 2016). The disease has a huge economic impact in Africa and is one of the major factors that slow down the economy due to loss of income and lives associated with disease (Maigemu and Hassan, 2015). According to Chima *et al.* (2003), the economic costs of malaria can be classified as direct and indirect. The direct costs expenditure resulting from prevention and treatment of the disease while indirect impact is the costs of productive labor and time lost due to malaria morbidity and mortality (Niringiye and Douglason, 2010). Hence, over the past decade, there has been renewed interest in research and innovations in diagnostic methods, drugs, vaccines, and the development of control measures with the aim of eradicating the disease. As a result, between 2000 and 2013, the incidence rates of malaria fell by 30 % globally, and by 34 % in Africa (Dawaki *et al*. 2016).

Medicinal plants have history of wide usage since time immemorial and form the basis of ethno-medical practices worldwide (Cragg and Newman, 2013; Mondal *et al*. 2019). They are regarded as vital sources of medicine, and in Africa, they form an important component of the health care delivery system (Cragg and Newman, 2013). This is because they possess several compounds with diverse pharmacological activities and are considered safer when compared to conventional therapies (Seo *et al*. 2018; De Olivera *et al*. 2019). The use of herbal products in the management of feverish conditions, pain and inflammation has been extensive and recently, there is a resurgence of interest in medicinal plants that could be used against such ailments (Solati *et al.* 2017; Oguntibeju, 2018). The plant *Ficus asperifolia* has been ethnomedicinally employed in the treatment of diseases including malaria (Burkill, 1997; Ojo *et al*. 2016; Bello *et al.* 2017).

MATERIALS AND METHODS

Preparation of plant material

The fresh leaves of *Ficus asperifolia* Miq were collected from Toro district, Toro Local Government Area of Bauchi State, Nigeria. The plants were identified and authenticated at the

herbarium unit of the Department of Biological Sciences, Bayero University, Kano. A voucher specimen number BUKHAN 0106 was collected for future reference.

Drugs and Chemicals

The drugs and chemicals used for the studies include: Ethyl acetate (MERCK Eurolab); N-Butanol (KESHI, USA); Chloroform (Sigma Aldrich, St. Louis Mo, USA); Hydrochloric acid, Sulphuric acid (May and Baker, UK), Hemine chloride, Sodium acetate, Ferric chloride anhydrous (Avishkar, India), ammonia (Loba chemie, India), Agappe diagnostic kit (Switzerland), Distilled Water, HEPES, DMSO, Chloroquine (Fluka, Germany) etc.

Equipment

Thermostat Oven (DHG-9101, USA), Water Bath (HH-4 ENGLAND Lab science), Electric Weighing Balance (FA2104A, Gulfed Medical and Scientific England), Digital (DB-1A, PEC MEDICAL USA), Animals weighing balance (SF-400), Animal cages, Pestle and mortar, Syringes (1 ml, 2 ml, 5 ml and 10 ml), What man's Filter Paper No. 1, Crucibles, Separating funnel, Conical flask, Beakers and Retort stand. Electronic balance, Microscope, Microscope slides, syringes, Mortar and Pestle, Animal cages, Spatula, Whatman No. 1 filter paper (1mm mesh size), 96 well Micro plates, Eppendorf micro pipettes, Desiccator, Candle jar, Centrifuge (England), Thermostat oven (DHG-9101-ISA), Micro-hematocrit reader (Hawksley-15006, England), Biobase auto hematological analyser (BK 6300) etc.

Extraction

Fresh leaves of *Ficus asperifolia* were collected from the plant, rinsed with clean water and shade-dried. The plant materials were then pulverized into a fine powder using a porcelain mortar and pestle and sieved. Powdered plant material weighing 2 kg was macerated with 7 L of 70% v/v methanol at room temperature for 7 days with occasional agitation of the mixture. At the end of the extraction, the crude methanol leaf extract of *Ficus asperifolia* (MEFA) was filtered using What man filter paper (1mm mesh size) and then concentrated in a water bath maintained at 45 ^{o}C until greenish-black residues were obtained and stored in a desiccator.

Fractionation of crude extract

The methanol crude extract of *Ficus asperifolia* leaf (MEFA) was subjected to liquid-liquid partitioning to separate the extract into different fractions. The extract was reconstituted with 300 ml of distilled water. The reconstituted extract was placed in a separating funnel and 300 ml of chloroform was added sequentially as a 1:1 (v/v) solution and shaken (Abdullahi *et al*. 2023). The sample was left to stand for 30 minutes in the separating funnel until a fine separation line appeared indicating the supernatant from the sediment before desorption. The process was repeated at least thrice until the residual aqueous (RAF) fraction was obtained and concentrated 'in a water bath at 45 ^{o}C. The concentrate was then kept in a desiccator for further use.

Phytochemical Screening

The chemical composition of methanol leaf extract and residual aqueous fraction of *Ficus asperifolia* was determined using a phytochemical screening (Abdullahi *et al*., (2023). The extracts were screened for the presence or absence of secondary metabolites including alkaloids, flavonoids, saponins, cardiac glycosides, tannins, triterpenes and anthraquinones.

Safety Studies on the extract and fraction

Acute toxicity study in mice (LD_{50})

LD_{50} determination was conducted using Organization for Economic Co-operation and Development guide lines 420 (OECD, 2001) in mice. In this method, two groups each of three animals were fasted prior to dosing (food but not water was withheld for 3 hours for mice). The fasted body weight was determined for each animal and the dose was then calculated according to the body weight. Food was then further withheld for 1-2 hours in mice after the extract had been administered. The MEFA and RAF was administered in a single oral dose using an oral cannula. A start dose of 2000 mg/kg was used for each animal in the first phase. Animals dosed in the first phase were observed for 48 hours after which there was no death and the test proceeded to the second phase. The same procedure was used but at a dose of 5000 mg/kg. Animals were observed individually at least once during the first 30 minutes after dosing and periodically during the first 24 hours with careful observation during first 4hours and then daily for 14 days.

β-hematin inhibition assay

Inhibition of β-hematin synthesis was conducted according to the method described by Baelmans *et al*. (2000). A 50 µL solution of hemine chloride in DMSO (Dimethylsulfoxide) (5.2 ug/mL) was distributed in 96-well micro-plates. Different concentrations of the extract was dissolved in DMSO and added in triplicates in the test wells (50 µl) final concentrations was between 2 and 32 ug/mL. Controls contained 5.2 ug/ml DMSO only (negative) and chloroquine (2 to 32 ug/mL) (positive). β-hematin synthesis was initiated by the addition of 100 µL of 0.2 M sodium acetate buffer at pH 4.4. Plates were incubated at 37 ^{O}C for 48 h. The incubated plates were then centrifuged at 4000 rpm for 15 min. After discarding the supernatant, the pellet was washed three times with DMSO (200 uL) and finally dissolved in 200 ul, 0.2N NaOH solution. The solubilized aggregates were further diluted at 1:2 with NaOH solution (0.1 N) and absorbance recorded at 405 nm (Microplate Reader, BIORAD-550). The results were expressed as percentage inhibition of β-hematin synthesis compared to negative control. The effective concentrations of sample required to inhibit the β-hematin synthesis by 50 % (IC_{50} value) was then determined.

Statistical Analysis

The experimental procedures in the β-hematin inhibition assay were performed in triplicates and data was presented as mean ± standard mean of error. Data were analysed using one way analysis of variance (ANOVA). The IC_{50} values were determined using Microsoft excel and calculated by regression from the plot of the percentage inhibition and the log concentration of both the extract and standard drug chloroquine.

RESULTS AND DISCUSSION

Preliminary phytochemical constituents

The results of the preliminary phytochemical screening of MEFA and RAF revealed the presence of cardiac glycosides, tannins, flavonoids, alkaloids, anthraquinone, saponins, steroids and triterpenes. The crude extract and the RAF showed similarity in the content of secondary metabolites found in them (Table 1).

Acute toxicity study

The estimated oral median lethal dose of the methanol leaf extract of *Ficus asperifolia* and the RAF were found to be

similar and are greater than 5,000 mg/kg in both rats and mice (Table 2).

Effect of MEFA and RAF on the production of β-Hematin

The MEFA and RAF showed significant ($p<0.05$) reduction in mean absorbance values as a result of inhibition of β-hematin formation when compared to the negative control as was equally observed with the standard drugs chloroquine. Furthermore, the MEFA and RAF in a concentration dependent manner (2, 4, 8, 16 and 32 ug/mL) exhibited the reduction with varying percentage of β-hematin inhibition. The MEFA had 37.16, 49.38, 53.37, 78.55 and 95.51% while the RAF revealed 34.66, 48.88, 51.62, 75.81 and 92.27 as percentage of β-hematin inhibition respectively (Table 3, 4 and Figure 4). However, the standard drug chloroquine also in a concentration dependent manner (2, 4, 8, 16 and 32 ug/mL) had the following percentage inhibition 28.43, 45.14, 52.37, 75.81 and 97.27 respectively (Table 5). The IC_{50} values for MEFA, RAF and chloroquine were found to be 4.373, 4.768 and 5.334 ug/mL respectively (Figure 1; 2 and 3).

Table 1: Phytochemical Constituents of Methanol Leaf and Residual Aqueous Fraction of *Ficus asperifolia*

Chemical constituents	MEFA	RAF
Alkaloids	+	+
Anthraquinone	-	-
Steroids	+	+
Terpenoids	+	+
Cardiac glycosides	+	+
Saponins	+	+
Tannins	+	+
Flavonoids	+	+

+ =present, **-** = absent, MEFA = *Ficus asperifolia* methanol leaf extract, RAF = Residual aqueous fraction

Table 2: Median Lethal Dose (LD_{50}) of MEFA and RAF

Extract	Animal Specie	Route	Value (mg/kg)
MEFA	Mice	Oral	>5000
RAF	Mice	Oral	>5000

MEFA = *Ficus asperifolia* Methanol leaf extract*,* RAF = Residual aqueous fraction

Table 3: Effect of the Methanol Leaf Extract of *Ficus asperifolia* on β-Hematin Inhibition

Treatment	Conc (ug/ml)	Average Absorbance	% Inhibition	IC_{50} (ug/mL)
Control	-	0.401±0.00	-	
MEFA	2	0.252±0.37	37.16	
MEFA	4	0.203±0.63*	49.38	
MEFA	8	0.187±0.41*	53.37	**4.373**
MEFA	16	0.086±0.09*	78.55	
MEFA	32	0.018±0.04*	95.51	

Data presented as Mean ± SEM, * significantly different from negative control analysed using one-way ANOVA at p<0.05 followed by Dunnett's post hoc test: NControl = negative control, MEFA = *Ficus asperifolia* methanol leaf extract.

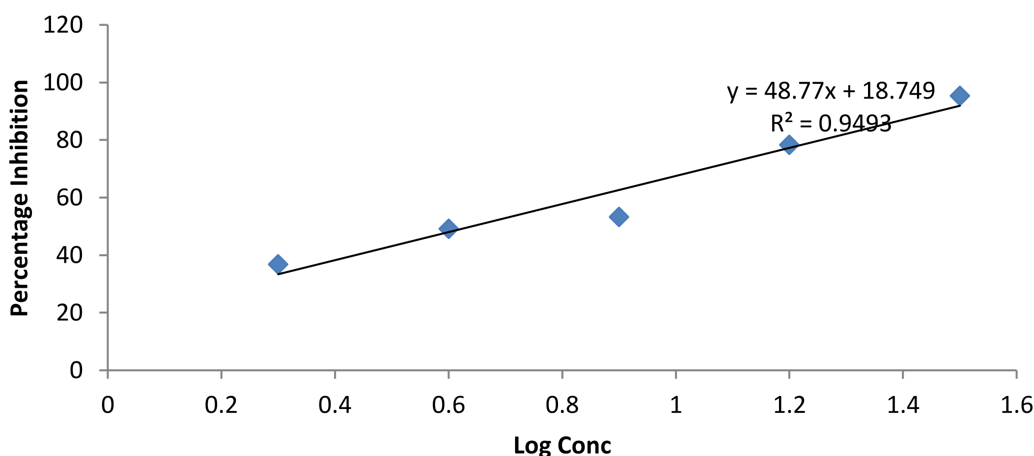

Figure 1: Determination of IC_{50} on the Effect of the Crude Methanol Leaf Extract of *Ficus asperifolia* on β-hematin Inhibition

Table 4: Effect of the Residual Aqueous Fraction of Methanol Leaf Extract of *Ficus asperifolia* on β-Hematin Inhibition

Treatment	Conc (ug/ml)	Average Absorbance	% Inhibition	IC_{50} (ug/mL)
Control	-	0.401±0.00	-	
RAF	2	0.262±0.02	34.66	
RAF	4	0.205±0.13*	48.88	
RAF	8	0.194±0.06*	51.62	**4.768**
RAF	16	0.097±0.11*	75.81	
RAF	32	0.031±0.01*	92.27	

Data presented as Mean ± SEM, * significantly different from negative control analysed using one-way ANOVA at p<0.05 followed by Dunnett's post hoc test: NControl = negative control, RAF = residual aqueous fraction

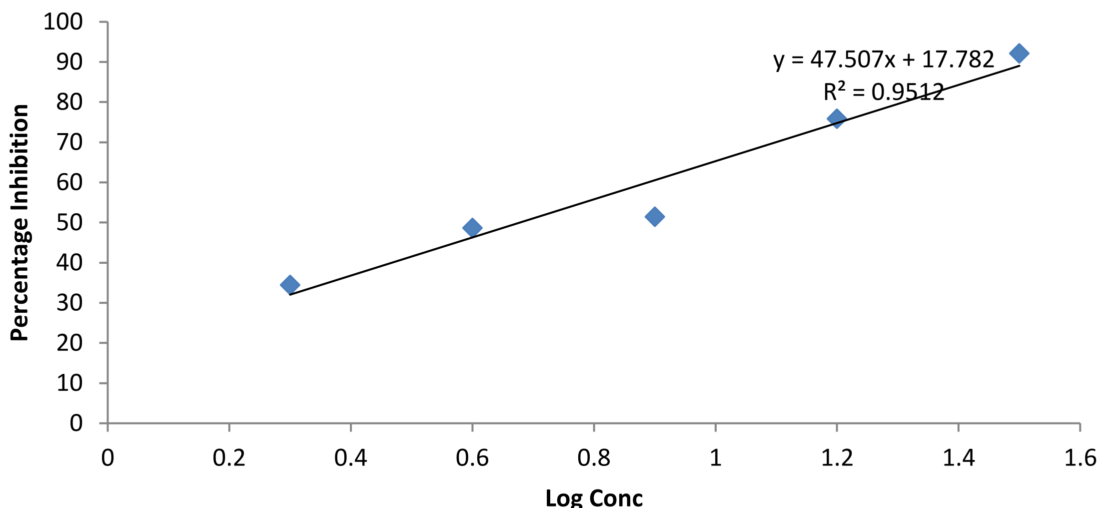

Figure 2: Determination of IC_{50} on the Effect of the Residual Aqueous Fraction of Methanol Leaf Extract of *Ficus asperifolia* on β-hematin Inhibition

Table 5: Effect of the Standard drug Chloroquine on β-Hematin Inhibition

Treatment	Conc (ug/ml)	Average Absorbance	% Inhibition	IC_{50} (ug/mL)
Control	-	0.401±0.00	-	
CQ	2	0.287±0.19	28.43	
CQ	4	0.220±0.31*	45.14	
CQ	8	0.191±0.33*	52.37	**5.334**
CQ	16	0.097±0.14*	75.81	
CQ	32	0.011±0.01*	97.27	

Data presented as Mean ± SEM, * significantly different from negative analysed using one-way ANOVA at p<0.05 followed by Dunnett's post hoc test: NControl = negative control, CQ = chloroquine

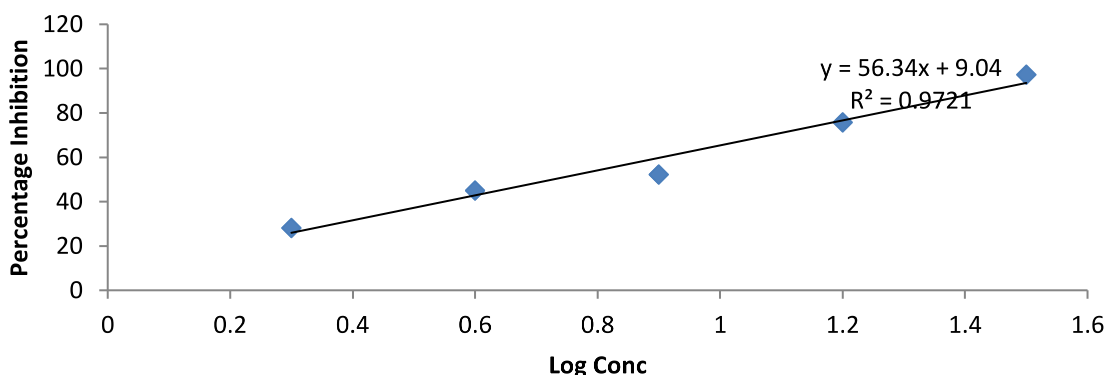

Figure 3: Determination of IC_{50} on the Effect of the Standard Drug Chloroquine on β-hematin Inhibition

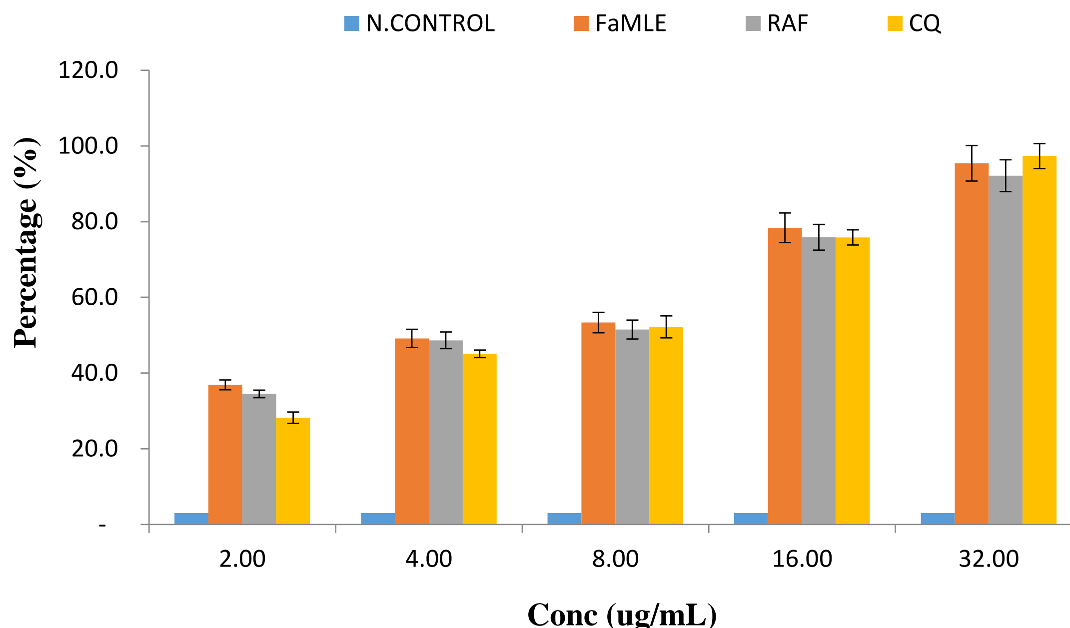

Figure 4: Comparison of the effect of crude extract and the residual aqueous fraction of methanol leaf extract of *Ficus asperifolia* on β-hematin inhibition. Control = Negative, MEFA= Methanol leaf extract of *Ficus asperifolia*, RAF = Residual aqueous fraction, CQ = Chloroquine

Discussion

Ficus asperifolia has been known as one of the useful plants of West Africa (Burkill, 1997). The plant has been ethnomedicinally used to treat various conditions including pain, fever and malaria. The present study attempted to investigate the *in vitro* antimalarial activity of the methanol leaf extract and residual aqueous fraction of *Ficus asperifolia* using the β-hematin inhibition assay.

Preliminary phytochemical screening revealed the presence of flavonoids, alkaloids, anthraquinones, cardiac glycosides, tannins, saponins, steroids and triterpenes which agrees with reported work of Omoniwa *et al*., (2013) and Abdullahi *et al*., (2020). Secondary metabolites such alkaloids, flavonoids, tannins, saponins, terpenoids, cardiac glycosides and steroids are responsible for plants' biological activities (Edewor-Kuponiyi, 2013; Rungsung *et al*., 2015). Phytochemicals constitute an integral part of medicinal plants. These phytochemicals are also referred to as the plant's secondary metabolites that are responsible for their numerous bioactivities. Numerous plants have a wide variety of phytochemicals as their bioactive principles and are reported to possess antiplasmodial activity (Alshawsh *et al*., 2007; Matur *et al.,* 2009). Although some studies have tracked antiplasmodial activity of plants to their alkaloids, flavonoids and terpenoids contents (Akuodor *et al.* 2010; Philip, 2020; Tajjuddeen *et al*., 2021), there are reported studies indicating antiplasmodial activity with saponins (Akanbi *et al*., 2018; Nafiu *et al*., 2021) and glycosides (Yun *et al*., 2021).

A number of alkaloids such as oxyacanthine, alstonerine isolated from *Dehaasia incrassate*, *Alstonia angustifolia* respectively had been reported to possess antiplasmodial activity (Kirby, 1997). Others such as saponins, flavonoids, cardiac glycosides, steroids etc are reported to be responsible for the antiplasmodial activity of many medicinal plants (Olugbade *et al*., 2000). Moreover, alkaloids are also known to show antimalaria property by blocking protein synthesis in *Plasmodium falciparum* (Nergiz and Otles, 1993; Asanga *et al*., 2017), whereas saponins, flavonoids and tannins have been reported to act as primary antioxidants or free radical scavengers that can counteract the oxidative damage induced by the malaria parasite (David *et al*., 2004). However, (Kirby, 1997) had reported that some plant extracts exerts their antiplasmodial action by either causing elevation in red blood cell oxidation or by the inhibition of their protein synthesis. Furthermore, cardiac glycosides have been implicated in prophylactic activity of extracts as they attack the broadest age range of parasites from the tiniest rings that have invaded the erythrocytes to more mature stages of parasites such as developing trophozoites and schizonts (Yoo *et al*., 2008). Their relative broad stage specificity of action had been reported to extend the ability to impede developments of gametocytes (Asanga *et al*., 2017). Therefore, the relative availability of alkaloids, flavonoids, cardiac glycosides and saponins in the methanol leaf extract and residual aqueous fraction of *Ficus asperifolia* could be responsible for their antiplasmodial activity.

Administration of *Ficus asperifolia* leaf extract and its residual aqueous fraction did not exhibit any signs of toxicity or lethality during both phases of the study. Acute systemic toxicity assessment involves investigating the adverse effects that may occur when organisms are exposed to a single or multiple doses of a test substance within 24 hours through a known pathway (Subramanian *et al*., 2018). It is essential to evaluate the acute toxic potential of substances to identify potential harmful effects resulting from unintentional or intentional short-term exposure (Clemedson *et al*., 2000).

The Organization for Economic Cooperation and Development (OECD), has recommended chemical labeling and classification of acute systemic toxicity based on oral median lethal dose values. According to this classification, substances are categorized as very toxic if the median lethal dose is ≤ 5 mg/kg, toxic if > 5 mg/kg but ≤ 50 mg/kg, harmful if > 50 mg/kg but ≤ 500 mg/kg, and non-toxic or not harmful if > 500 mg/kg or ≤ 2000 mg/kg (Walum, 1998). In this context, the oral median lethal dose obtained for mice was found to be above 5000 mg/kg, indicating relative safety when

administered orally. An alternative toxicity scale, as proposed by Hodge and Sterner (1943), who suggests that compounds with an oral LD_{50} range of 500-2000 mg/kg should be considered practically non-toxic.
Haem is a crucial metabolic factor that is derived primarily from the parasite's haem biosynthesis pathway (Bonday *et al*., 2000) at the early ring stage and from haemoglobin digestion at later stages (Goldberg *et al*., 1993). Malaria parasites ingest more than 75% of the host cell's haemoglobin within a short period due to their nutritional requirements in the blood stage (Goldberg *et al*., 1993; Ridley *et al*., 1996). The haemoglobin is digested in the cell's food vacuole to generate amino acids, releasing the toxic haem moiety (Olliaro and Goldberg, 1995). Since haem is toxic, excess haem is stored as the pigment haemozoin, a biocrystallized form of haem aggregates (Sigala and Goldberg, 2014). Chloroquine and other drugs that exploit similar mechanism, bind to the toxic haem metabolites, thereby preventing their conversion to and deposition as the inert haemozoin (Sullivan *et al*., 1996). Formation of malaria pigment or hemozoin is the major route of heme detoxification in the malarial parasite (Ravikumar *et al*., 2012).
Malaria parasites proteases break down the hemoglobin of the host for survival (Juan-Ricardo *et al*., 2006). By this act, these parasites (*Plasmodium* species), despite the absence of heme oxygenases, are able to detoxify heme which is harmful to them, and convert it to hemozoin (β-hematin is the homologue of hemozoin), also known as malaria pigment (Juan-Ricardo *et al*., 2006). This implies that, substances or compounds which are able to inhibit the synthesis of this malarial pigment (β-hematin or hemozoin) and the degradation of haemoglobin might be useful anti-malarial agents. Moreover, inhibition of heme biocrystalization has been used as one of the major target for drug discovery in the fight against malaria (Rathore *et al*., 2006; Sashidhara *et al*., 2013). Inhibition of the conversion or polymerization of heme to β-hematin (hemazoin or malaria pigment) will lead to accumulation of heme in the parasite food vacuole thereby causing the death of the parasites (Meshnick *et al*., 2002; Mpiana *et al*., 2007; Ravikumar *et al*., 2012).
The β-hematin inhibition assay also serves as an *in vitro* antiplasmodial activity screening model as well as a means of determination of possible antiplasmodial mechanism. In this study, the MEFA showed a very good antiplasmodial activity (IC_{50} 4.373 ug/ml) which was comparable to that of the standard drug, chloroquine (IC_{50} 5.334 ug/ml). The RAF equally showed very good activity (IC_{50} 4.768 ug/ml) that was comparable to the standard drug. The ability of the extract to inhibit the formation of β-hematin might be due to the presence of some bioactive secondary metabolites that were detected in the extract and the fractions, as the antimalarial effect of flavonoids, saponins alkaloids and steroids have been reported by previous researchers (Saxena *et al*., 2003; Ayoola *et al*., 2008).

CONCLUSION

The antimalarial activity of methanol leaf extract of *Ficus asperifolia* and the residual aqueous fraction was observed through the inhibition of β-hematin synthesis. The methanol extract of *Ficus asperifolia* leaf and its residual aqueous fraction have demonstrated significant *in vitro* antimalarial activity. These findings confirm its traditional use in the management of malaria, it is further recommended that the active compounds be identified and isolated for refinement into effective pharmaceutical formulation.

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