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In vitro Anti-malarial Potential of Chloroform Fruit Crude Extract of *Ficus sycomorus* L. against Blood-stage *Plasmodium Falciparum*

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ABSTRACT

Malaria accounts for a huge number of deaths and morbidity globally. In Sub-Saharan regions of the world, particularly developing countries like Nigeria, malaria kills thousands of people annually, due to emerging and spread of parasitic resistance to well-known anti-malarial drugs. This study reported the preliminary phytochemicals and *in vitro* antiplasmodial activity of chloroform fruit extract of *Ficus sycomorus* (*CFEFS*) sourced from Northern Nigeria. Using standard procedures the extract was tested for the presence of phytocompounds. The crude extract was analyzed at different concentrations of 10 mg/ml, 5 mg/ml, 2.5 mg/ml and 1.25 mg/ml for *in vitro* antiplasmodial activity against *Plasmodium falciparum* in different age groups of malaria-infected individuals. Phytochemical analysis revealed the presence of alkaloids, flavonoids, phenols, diterpenes, tannins, and phytosterols. The antiplasmodial activity revealed that *CFEFS* was effective against the malaria parasite. Group I (0-10 years old, IC_{50} 4.87mg/ml) has the high ($p<0.05$) percentage elimination of parasites compared to group II (21-30 years old Adults; IC_{50} 4.64mg/ml) at 24, 48 and 72 hrs period. The extract also showed a good anti-plasmodial activity among the age group of 11-20 year old; with IC_{50} of 3.95mg/ml. At higher concentration and time, the percentage parasitic elimination increases whereas; it decreases with a decrease in concentrations and time in all the age groups. From this study, *CFEFS* is likely to contain other chemical component(s) which are acting individually or in synergy, additive or antagonistic mechanism. They can be used when isolated as effective plant-based agent(s) for the treatment of malaria.

Keywords: Anti-plasmodium, *Ficus sycomurus*, Malaria, *Plasmodium*, Phytocompounds

INTRODUCTION

Malaria, one of the major health problems throughout the tropics is a tropical disease caused by parasites of the genus *Plasmodium*. It is one of the leading infectious diseases in African

countries, such as Nigeria, where transmission occurs all year. It is a mosquito-borne infectious disease caused by parasitic *Protozoan* belonging to the *plasmodium* type [1, 2]. It is transmitted when bitten by female *anopheles* where it releases microscopic parasites that survive in salivary glands of the mosquitoes which later enter human bloodstream [3]. Of the four malaria parasites that affect humans, *Plasmodium falciparum* is the most common and most deadly in Africa. Malaria usually causes sign and symptoms which usually involve a high fever, shaking chills, flu-like illness, and sometimes death [2]. It is predominant in many tropical and sub-tropical regions of the world accounting to more than 90% of the malaria cases most of them children [4]. The development of massive spread of multidrug-resistant strains (chloroquine resistant parasite strains) of *plasmodium* represents the biggest Nigeria problem nowadays [5]. Spread of multidrug-resistant strains of *Plasmodium*, prevention, and treatment is now the cause of primary control failure. This led to raising an alarming situation which requires urge in drug discovery to produce new, affordable and accessible anti-malarial agents against the resistant strains.

The plant family, *Moraceae* has *Ficus* as one of the main important genera, with many reported biological activities such as anti-pyretic activity [6], gastro-protective property [7], antioxidant potentials reported by Phan *et al*. [8], anticancer [9], antimicrobial activity [10] and antiulcer property [11]. *Ficus spp latex* was exploited in South and Central America for its anti-helmintic potential as reported by De-Amorin and colleagues [12]. The parasiticidal or antiprotozoal property of this genus has been linked to the occurrence of a compound called *Ficin* [13]. *Ficus sycomorus* L (fig), thus, serves as good sources of bioactive compounds such as triterpeniods, alkaloids, flavonoids, tannins, stilbenes, xanthones etc [14].

The aim of this study is to evaluate the phytochemicals and preliminary anti-plasmodium potential of fig (*Ficus sycomorus*) fruits chloroform crude extract sourced from northern Nigeria.

MATERIALS AND METHODS

Chemicals used

Methanol (98%), DMSO (Dimethyl sulfoxide), Geimsa's stain, chloroform, dilute Hydrochloric acid, Mayer's reagent, ferric chloride, sodium hydroxide, conc. Nitric acid, copper acetate, Conc. Sulphuric acid, acetic anhydride, lead acetate solution and RPMI 1640. All the chemicals and solvents used were of analytical grade.

Collection of Plant fruits, Identification and Processing

Plant samples of *Ficus sycomorus* fruits were collected from Kano, north-western Nigeria. The fruits were identified at Faculty of life sciences, Bayero University, Kano. The herbarium Accession number of specimen *BUKHAN 109* was assigned. It was chopped into small pieces and shade dried, then ground manually into powder using mortar and pestle.

Extraction of Plant Fruits

Powdered sample (250g) of *Ficus sycomorus* fruits was extracted using chloroform by soaking for 48 hrs in 500 mL of chloroform using maceration method. To allow the separation and extraction of a wide range of components present in the sample, the mixture is regularly shaken at a time interval of 2-3 hrs. It was then extracted through Muslin clothes and Whatman filter paper. The extract was evaporated using a water bath set at 40-60 °C. The extract was weighed and was transferred to a container until further analysis. The extract was dried in an oven as a means of preservation for about 60 mins [15].

Phytochemical Analyses

Qualitative Phytochemical screening

Qualitative screening of the phytochemicals; alkaloids, tannins, flavonoids, saponins, glycosides, phenols, terpenoids, diterpenes, carbohydrates, phytosterol was conducted as described by Ugochukwu *et al* [16].

Anti-plasmodium Activity of the extracts

Screening for Malaria Parasites and Groupings

Using a capillary tube, small drop of each sample was placed on a clean glass slide. A cover slip was placed at 45 degrees in front of the dropped blood which was gently pushed forward until it was in contact with the blood to make a thin smear. The smear was air dried and deepened in absolute methanol for 15 min. Several drops of Giemsa's stain were added on the smear and kept for 15 min. The slide was air dried and using spirit lamp, the cells were fixed. The slide was observed under a microscope using oil immersion. Malaria parasite was identified by the ring shape of immature *trophozoites* which retained the blue colour of the stain. Parasitaemia levels and species parasites were recorded. A sample with 5% parasitaemia was used for the anti plasmodium assay. The samples were collected from patients (who had not received anti-

malarial drugs and with AA genotypes/ Non-sickle cell patients), and were grouped into three based on age range of samples (G-1: 0-10; G-2: 11-20; G-3: 21-30 years old). The percentage of the parasitized red blood cells was counted as follows:

a. 100 x objective lens was used. An area of the thin film was selected where the total number of red cells was approximately 150 per field
b. The number of parasitized red cells was counted in 8 fields
c. Percentage of the parasitized cells was calculated by dividing [17].

Separation of the Erythrocytes

Blood sample (with 5% parasitaemia collected from individuals aged groups from Hospitals in Kano) was centrifuged at 2500 r/m for 15 min. After centrifugation, the supernatant (plasma) was discarded while the sediments (erythrocytes) were further centrifuged with normal saline at 2500 r/m for 5 min. The supernatant was then discarded and the erythrocytes were suspended in normal saline.

Preparation of the Test Concentrations

An electronic digital balance (model FA2104A, Gulfex Medical and Scientific Company, England), was used to measure 1gram of the extract and then dissolved in 1ml of DMSO in separate Bijou bottles (stock solution). Using serial doubling dilution, four different concentrations (10mg/ml, 5mg/ml, 2.5mg/ml and 1.25mg/ml) of each extract were prepared

Preparation of Culture Media (RPMI 1640)

The media was prepared by dissolving 10.4g of the powdered culture material (RPMI 1640) into 1L of distilled water and then autoclaved at 121ºC for 15 minutes as instructed by the manufacturers.

Anti-plasmodium Assay of the Activity of Extracts on *Plasmodium falciparum* Culture

Equal volume of the extract solution (0.5ml) and the culture media were transferred into flat bottomed test tubes and labeled accordingly (10mg/ml, 5mg/ml, 2.5mg/ml and 1.25mg/ml). For each concentration of the extract, 0.1ml of the malaria positive erythrocytes was added and shaken gently to ensure even distribution of the erythrocytes. The test tubes were transferred into a bell jar containing a burning candle. The cover of the bell jar was then replaced until the flame of the candle stopped burning. This supplied about 95% nitrogen, 2% oxygen, and 3% carbon

dioxide as described by Trager and Jensen [18]. The whole set up was transferred into an incubator maintained at 37^{o}C for 24 – 72hrs. A control group consisting of culture media plus positive erythrocytes (negative control) and culture media plus positive erythrocytes and anti-malarial agent *Athemeter* (positive control) was incubated along with the test concentrations. After 24 hrs of incubation, a thin smear from test tube was made on clean glass slides and fixed in absolute methanol, then stained with Giemsa's stain. Each smear was observed under a microscope using oil immersion to count the number of infected erythrocytes.

Determination of Anti-malarial Activity

At the end of the incubation periods, 24, 48 and 72 hours, a drop of a thoroughly mixed aliquot of the culture medium was smeared on microscopic slides and stained by Giemsa's staining techniques. The mean number of erythrocytes appearing as blue discoid cells containing life rings of the parasite (that appeared red pink) was estimated and the average percentage elimination of the samples was determined. The activity of the tested samples was calculated as the percentage elimination of the parasites after an incubation period of 24, 48 and 72 hours using the formula:

% = N/Nx X 100

Where % = Percentage activity of the extracts

N = Total number of cleared RBC

Nx = Total number of parasitized RBC

RBC = Red Blood Cells [19].

Statistical Analysis

Values are expressed as mean ± standard deviation (SD). The Data was analyzed by ANOVA using SPSS version 20, where the values of $p< 0.05$ were considered statistically significant. The IC_{50} values calculated Using Curve Fitting Method with Graphpad Prism Statistical Software.

RESULTS AND DISCUSSION

The phytochemical screening and *antiplasmodium* activities of *Ficus sycomorus* fruit chloroform fruit extract (CFEFS) were evaluated. The physical properties of the extract are indicated below:

Table 1: Physical Parameters of chloroform crude Fruit Extract from *Ficus sycomorus*

Properties	**Chloroform extract**
Weight of plant extract (g)	250g
Weight of extract (g)	32g
Percentage yield (%)	14%
Colour of extract	Brown
Texture of extract	Gummy

Phytochemical Analysis

The result obtained shows the presence of phytochemicals: alkaloids, flavonoids, diterpenes, phenols, tannins, and phytosterols. However, saponins were absent in the fruit extract as presented in (Table 2)

Table 2*:* Phytochemical analyses of *Ficus sycomorus* fruit chloroform extract (CFEFS)

Phytochemicals	**CFEFS**
Alkaloids	+
Flavonoids	+
Phenols	+
Saponins	-
Diterpenes	+
Phytoserols	+
Tannins	+

Key: (+) present and (-) absent.

The anti-plasmodium activities of *Ficus sycomorus* chloroform fruit extract result were shown in the Figures 1-3 below. The extract displayed a significant activity, exerting an anti-plasmodium effect at most of the concentrations with few ones exempted. The highest anti-plasmodium activity was obtained in group I after the microscopic view of the stained slides at 10mg/ml with percentage elimination of 75% after 72 hr incubation. The least elimination of 33% was observed in group II at the concentration of 1.25mg/ml. In both groups, the concentration at 10mg/ml at 72 hrs shows the highest elimination of the parasites. The extract may disrupt the parasite membrane thereby killing the parasite and thus reduces the percentage parasitaemia (Plate A, B, and C).

Figure 1: Anti-plasmodium activity of the G-1 (0-10 years of age).

Their negative control value at 72 hours incubation indicates rapid growth of the parasite. At 72 hours incubation, the extract was able to kill the parasite considerably as the concentration increases at several time intervals.

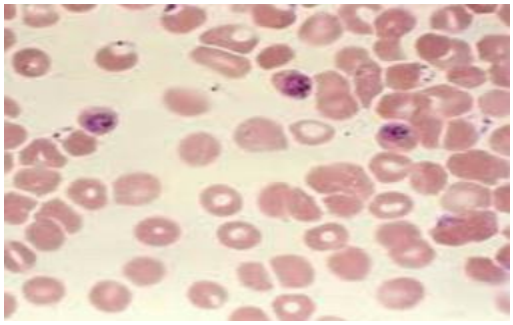
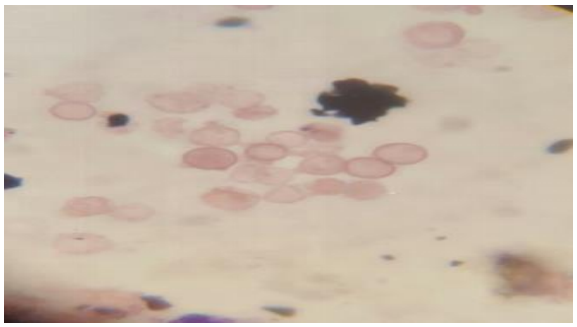


Plate A: 1. Initial parasite count for the G-1 before incubation with chloroform extract showing 5% parasitemia viewed under a microscope using ×100 magnifications.



2. Final incubation for G-1 with chloroform fruit extract showing a reduction in 5% parasitemia viewed under a microscope using × 100 magnifications.

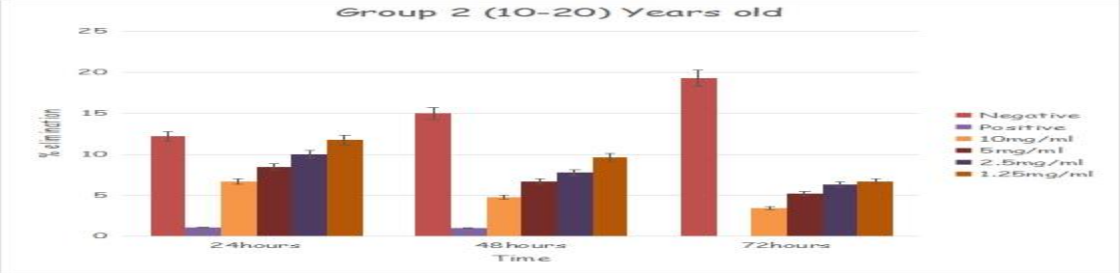


Figure 2: Antiplasmodial activity of G-2 (11-20 years old) at 24 hours incubation.

The extract concentration at 1.25mg/ml varies slightly from the negative value which shows that at 24 hrs incubation at that concentration the parasitic elimination by the extract was very low. However, there was complete parasitic destruction at 72 hrs of incubation by the positive control (arthemeter).

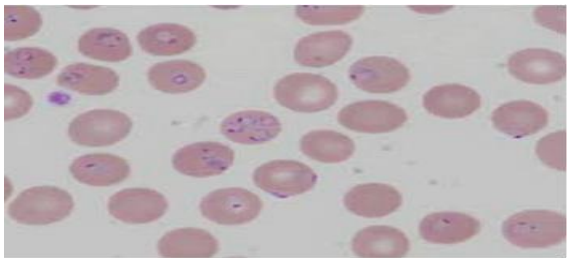


Plate B: 1-Initial parasite count for G-2 before incubation with chloroform extract showing 5% parasitemia viewed under a microscope using ×100 magnifications.

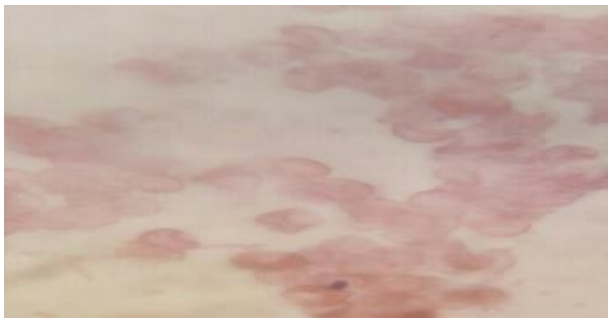


Plate B: 2-At 72hrs after incubation with chloroform extract showing a reduction in parasitemia, viewed under a microscope using × 100 magnifications.

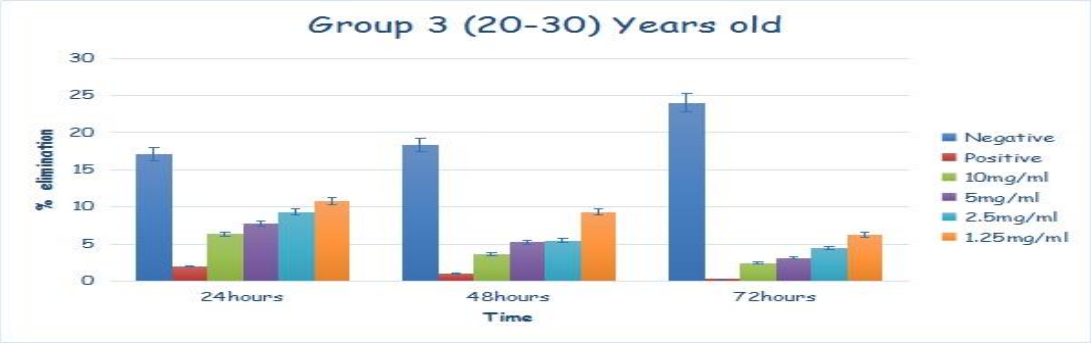


Figure 3: Antiplasmodial activity for G-3 (21-30years old)

At 24 hr of incubation, the values are much higher wherein after 72hours of incubation the parasite number decreased drastically. The negative control value tends to increase during the incubation because of the absence of the extract. However, the positive value was found to be lower after the incubation which indicates the parasite elimination by *Arthermeter* combination therapy.

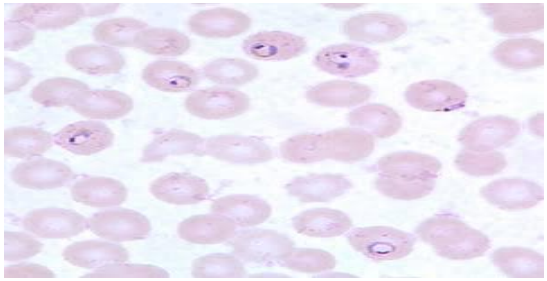


Plate C: 1-Initial parasite count before incubation for G-3 with chloroform showing 5% parasitemia using ×100 magnifications.

Plate C: 2-Final incubation for G-3 with chloroform extract showing a reduction in parasitemia, viewed under a microscope using ×100 magnifications.

Table 3: The IC_{50} for Antiplasmodial Activity of Each Age Group

Age Group	**Concentration (mg/ml)**	**% Elimination**	**IC_{50} (mg/ml)**	**R^2**
Group 1 (0-10)	10	75	4.87	0.9528
	5	68		
	2.5	52		
	1.25	56		
Group 2 (11-20)	10	65	3.95	0.9743
	5	52		
	2.5	42		
	1.25	33		
Group 3 (21-30)	10	70	4.64	0.9945
	5	62		
	2.5	54		
	1.25	52		

For promising crude anti-plasmodium plant extracts, IC_{50}, values should be below 100mg/mL as reported by Cos *et al*. [20], although most promising anti-malarial plant extracts exhibit IC_{50} values under 10mg/mL [21]. The CFEFS in this study revealed an IC_{50} value lower than 10µg/mL against blood-stage *Plasmodium spp* in all the age groups of infected patients (Table 3). This shows that the extract is a good anti-plasmodium candidate and warrant for further bioassay-guided fractionation, isolation and structural elucidation of the active agent(s).

The qualitative phytochemicals screening of CFEFS showed the presence of alkaloids, phenols, tannins, diterphenes, phyteserols, and flavonoids. These compounds produce definite physiological activity on human body serving as non-nutritive chemical compounds [22]. A comparative study on phytochemical screening of conducted by Bello *et al.* [23] using *ficus sycomorus* (Linn) stem bark with different solvents such as aqueous, chloroform, ethyl acetate and n-hexane root extracts shows the presence of tannins, alkaloids, saponins, flavonoids which were all found present in the present study with the exception of saponins which was found to be absent in the chloroform fruit extract. A similar study reported by Alsiddig and Sufyan [24] which was conducted on *F. sycomorus* using n-hexane, chloroform, ethyl acetate, butanol and aqueous, the bark extract displayed the distribution of secondary metabolites such as alkaloids, phenols, saponins, diterpenes, flavonoids and tannins which where all found present in the current study expect saponins which were found absent in the fruit extract. However, diterpenes was found to be absent in bark extract using different solvents but present in fruit extract. A

study conducted by Oumar and colleagues [25] reported the presences of saponins, alkaloids, tannins, flavonoids which were all reported in the present study except saponins.

Alkaloids can act as defense compound in plants and effective against pathogens and predators due to their toxicity. It plays a protective role, has a bitter taste, disrupts protein function after ingestion and alters the central nervous system [26]. Alkaloids exhibit toxicity against foreign cells which are widely used in cancer therapy [27] and anti-plasmodium activity [28]. Flavonoids are beneficial to human due to their biological properties [29]. It functions as anti-tumor, anti-inflammatory, antioxidant, antibacterial, antiviral compound and for the treatment of neurodegenerative diseases [30, 31]. It has been previously reported to have anti-plasmodium activities against different strains of malaria parasites by Oliveira *et al* [32], Kim *et al* [33] and Monbrisome *et al* [34].

The mechanism of exact antiplasmodial action of plant flavonoids is yet to be established, but some have been reported by Elford [35] to inhibit the influx of L-glutamine and myoinositol into *P. falciparum*-infected RBCs, while others such as a flavones glycoside from *Phlomis brunneogaleata* and iridoid from *Scrophularia lepidota* have been shown inhibiting Fab I enzyme of *P. falciparum* [36, 37]. Phenols were also determined in *CFEFS*. Terpenoids such as taraxeryl acetate and betulinic acid isolated from this genus, are revealed to have anti-protozoal properties [38]. The Flavonoids may be acting in synergy, additive or antagonistic mechanism with other phytochemicals to exert the anti-malarial activity observed.

The antiplasmodial activity shows the parasitic elimination after the 72 hrs of incubation. The average number of parasite before incubation decreases considerably after the incubation period. It has shown various responses to the parasitic elimination when tested using *CFEFS* at different age groups and time. The extract at high concentration was found to be more effective on the parasite elimination activity; which is somehow steady at 48-hour incubation. The parasite elimination observed in Group one, that is, between the age of 0-10, the extract concentration was very effective in killing the parasite. However, the negative control in this group tends to increase rapidly which shows rapid multiplication and growth of the parasites. Malaria is mostly found to be prevalent in children and is the most vulnerable group to malaria, most malaria death cases occurred in children. A number of studies have been applied in the understanding of the dynamic of malaria infection and the impact of immunity [39]. The parasite displays many

immune evasion mechanisms which show extensive antigen diversity which is able to switch between surface antigens variants and different parasitic phases in the human host which are poorly immunogenic [40].

In high transmission area, partial immunity of the disease is acquired during childhood the majority of the disease particularly severe disease with rapid progression to death occurred in young children without acquired immunity which is seen in children than in adults [41]. In the second group observed in figure 2, that the age of 11-20 years displayed total elimination of the parasite in the positive control having *Artermeter* combination therapy. In all the groups the percentage elimination was concentration and time-dependent which increases as the concentration of the extract increases and vice versa. However, protection among adult may be presumed to be the cumulative products of several infections whereas susceptibility in children would be due to their relative lack of experience with disease [42].

Percentage parasite elimination analyses revealed that the extract produced a dose-dependent decrease in the level of parasitemia compared to negative control. At high concentrations, the extract concentrations in both the age groups displayed virtual parasitic elimination which least elimination at the lowest concentration of 33% in Group 2. However, when compared with the negative control there was a significant different at $p<0.05$ whereas at positive control there were no significant differences at $p<0.05$. These could be due to the fact that it contains important bioactive chemicals such as tannins, saponins, flavanoids, triterpenoid, etc as determined from the study phytochemistry. In view of this, it is suggested that any of the classes of phytochemical compounds detected; acting individually, additive, antagonistic or in synergy may be responsible for the observed anti-malarial activity.

CONCLUSION

The study revealed the presence of phytochemicals which was confirmed using qualitative analysis. The CFEFS was found to possessed anti-plasmodial activity and was effective in malaria elimination which is primarily due to the presence of secondary metabolites having significant anti-malarial properties that could render help when fully utilized in the development of therapeutic medicines for malaria therapy. The results also support the local acclaimed anti-malarial effect of the plant. Isolation, purification, and structural elucidation of constituents from CFEFS are warranted to support the discovery of novel anti-plasmodial compound(s).

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