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Anti-Plasmodial Activity of the Aqueous Syzygium *aromaticum* (clove) Flower bud extract against *Plasmodium falciparum*

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Abstract

The major health concern in subtropical Africa is malaria and there are few effective treatment options on it. This study investigated the qualitative pharmacological analyses and in vitro anti-plasmodial activity of Syzygium aromaticum aqueous extract against Plasmodium falciparum. Phytochemical screening, anti-malarial activity and acute toxicity studies of the extract were carried out using standard procedures. The phytochemical screening of the extract showed the presence of alkaloids, flavonoids, saponins, tannins and anthraquinones; steroids were absent. The extract showed strong anti-plasmodial activity only at concentration of 50mg/ml (IC_{50} is ≥ 50mg/ml) with percentage growth inhibition and mean infected Red Blood Cells count (Standard Deviation) of 81.1(1.7 ± 0.6) at $P < 0.0002$.This study therefore assessed the acute toxicity, the results of phases I and II of the extract showed no mortality in any of the experimental groups of rats after 24hours and up to four weeks after oral administration of concentrations of 5000mg/kg or less of the extract; also, no signs of delay toxicity was observed. Thus, from results obtained, it can be concluded that the most potent extract concentration is 50mg/ml at $P < 0.05$. It can also be concluded that oral administration of the extract at doses of less than or equal to 5000mg/kg is experimentally safe. It is thus recommended that further studies be carried out to identify the actual chemical entity (ies) responsible for the anti-malarial activity of the extract, so that new anti-malarial agent(s) could be developed, to avoid development of resistance by the malarial parasite. Histophathological studies should be carried out to show whether the extract can cause problems on the liver and kidney or not, in order to advice accordingly on the safe dosage to people who use it locally for the treatment of clinical signs associated with malaria.

Keywords: Acute toxicity, Anti-plasmodial activity, Phytochemicals screening, *Syzygium aromaticum*.

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INTRODUCTION

The serious tropical disease caused by a protozoan parasitic infection transmitted by Anopheles mosquitoes is malaria. In humans, this disease is caused by one of the five species of *Plasmodium; P. falciparum, P. vivax, P. malariae P. ovale*, and *P. knowlesi.* (Visser, 2014).

Plasmodium falciparum recorded the high number of infections, and almost all deaths from malaria are due to this species and the World Health Organization quotes 228 million per year and nearly half of the world's population was at risk of malaria (WHO, 2019).
Malaria is one of the major tropical diseases that cause massive death of children under five years. More than two third (70%) of all malaria deaths occur in this age group. In 2017, about 435, 000 Africans died before their fifth birthdays (WHO, 2018).

Pregnant women are at high risk of dying from the complication of severe malaria. Malaria is also a cause of spontaneous abortion, premature delivery, still birth and severe maternal anemia and is responsible for about one third preventable low birth weight babies (WHO, 2017). Moreover, the malaria parasites are developing resistance to the current therapies. This study investigated the phytochemicals and *in vitro* anti-malarial activity of *Syzygium aromaticum* flower bud aqueous extract against *Plasmodium falciparum*.

MATERIALS AND METHODS

Collection and Identification of Plant Material

Fresh flower buds of *Syzygium aromaticum* (clove) were obtained from Kasuwar Rimi Kano. The plant was identified and authenticated in the Herbarium of the Department of Plant Biology, Bayero University Kano, Nigeria with the accession numbers of BUKHAN 342 (figure: 1).

Figure 1: *Syzygium aromaticum* (clove) flower buds

Extraction of Plant Material

The protocol used by Basniwal (2005) was adopted, the *Syzygium aromaticum* (clove) flower buds were separated and washed thoroughly and gently with tap water at room temperature to facilitate drying. The plant materials were dried under shade for two weeks. They were then pounded using pestle and mortar to obtain a fine powder.

One hundred grams of the plant material was percolated in 1000ml of sterile distilled water for five days with constant shaking at regular intervals. The percolate was then filtered and the solvent was evaporated using Thermostat water bath at 40°C, while extract was stored in a refrigerator at 4°C until used.

Phytochemical Screening of the Aqueous*Syzygium aromaticum* (clove) flower bud Extract

Phytochemical screening of the extract was carried out on the dried extract using simple chemical tests to detect the presence of alkaloids, tannins, flavonoids, saponins, anthraquinones and steroid. The following methods were used:

Test for Alkaloids

Exactly 0.5g of the plant extract was dissolved in 5ml of 1% HCl on steam bath. A milliliter of the filtrate was treated with drops of Dragendorffs reagent. Turbidity or precipitation indicates the presence of alkaloids (Evans, 1996).

Test for Tannins

About 1g of the extract was dissolved in 20ml of distilled water and filtered and 2 to 3 drops of 10% of $FeCl_3$ was added to 2ml of the filtrate. The production of a blackish-blue or blackish-green coloration indicates the presence of tannins (Evans, 1996).

Test for Flavonoids

About 0.2g of the extract was dissolved in 2ml of methanol and heated. A chip of magnesium metal was added to the mixture followed by the addition of a few drops of concentrated HCl. The occurrence of a red or orange coloration indicates the presence of flavonoids(Evans, 1996).

Test for Saponins

The extracts were subjected to frothing test, in which about 10ml of distilled water were added to portion of extracts and were shaken vigorously for 30seconds. The tubes were allowed to stand in a vertical position and were observed for 30mins. A honeycomb froth that persist for 10-15mins indicates the presence of saponins(Harborne, 1998).

Test for Anthraquinines

About 1.0g of the plant extract was dissolved in petroleum ether and filtrated. Aqueous ammonia was then added to the filtrate, formation of pink coloration indicates the presence of anthraquinones in the plant extract (Evans, 1996).

Test for Steroids
Five drops of concentrated H_2SO_4 was added to 1ml of each extract, a red coloration indicates the presence of steroids (Sofowora, 1993).

Collection of the Test Organisms
The test organisms (*Plasmodium falciparum*) were obtained from the subject confirmed of malaria case in the parasitological laboratory of Sir Muhammad Sunusi Specialist Hospital and stored in a refrigerator at 4^0C before carrying the bioassay.

Preparation of serum and erythrocytes
Blood was collected in a plain container and kept at 4^oC and then centrifuged at 10,000rpm for 20mins, at 4^oC. The serum was separated aseptically and kept in aliquots. It was then inactivated by keeping it at 56^oC water bath for half an hour. After inactivation the serum could be stored in deep freezer until used (Trager and Jensen, 1976).

Preparation of Infected Erythrocytes (RBCs) for Culture
The infected blood was collected into centrifuge tubes containing anticoagulant (EDTA). These were then centrifuged at 1500rpm for 10min at room temperature. Plasma and buffy coat was removed with the sterile Pasteur pipette. After this washing media was added, centrifuged at 1500rpm for 10min and the supernatant was removed. This washing process was repeated three times and equal amount of complete medium was added to the sediments (erythrocytes) and stored at 4^0C (Fairlamb, 1985).

Continuous Culture of Malarial Parasites
For initiation of culture, suspension (50%) of infected cells with complete medium (with 15% serum) was prepared. Appropriate amount of uninfected cells was added to get an initial parasitaemia of 3 to 5% and diluted with complete medium to get 5% cell suspension (5% hematocrit). The culture was kept in CO_2 Candle Jar incubator at 37^oC (Jensen *et. al.,* 1979).
The growth culture medium was monitored after every 24hrs, the medium was removed using a sterile Pasteur pipette without disturbing the cells that settled down. The cells were then mixed without frothing and a drop of blood was placed on the slide and a thin film was made. Fresh complete media (with 10% serum) was added, mixed properly and kept back in the incubator (Jensen *et. al.,* 1979) and the film was stained and examined microscopically following the methods describe by (Cheesbrough, 1987).

Preparation of Different Concentrations
Following the method adopted by Ekwenye and Elegalam (2005) the concentration of 100mg/ml of aqueous extract was prepared by dissolving 0.1g of extract in 1ml of dimethyl sulfoxide (DMSO) to formed the stock solution of 100mg/ml. Concentrations of 50, 25, 12.5, 6.25 and 3.125 mg/ml were then prepared from the stock concentration (100mg/ml) by double dilution procedure (Elegalam, 2005).

Anti-plasmodial Activity of the Plant Extract against *Plasmodium falciparum*

The anti-plasmodial activity of *Syzygium aromaticum* (clove) flower bud aqueous extract was evaluated *in vitro* on their ability to inhibit the *Plasmodium falciparum* growth on RPMI 1640 medium at various concentrations of the extract. The amount of the medium, aqueous extract and infected blood (test organisms) was in ratio of 8:1:1 in the culture vials (WHO, 2015). The DMSO was used as negative control (drug free well) accordingly, and artemether at a concentration of 100mg/ml was used as positive control. All vials were kept in a Candle Jar incubator and were incubated at 37°C. The percentage parasitemia was determined microscopically on thin blood smear at 72 hours intervals, after the aqueous extract and parasites contact and the percentage parasitemia levels of the aqueous extract in various concentrations were recorded. Lastly the %GI were calculated using percentage parasitemia of negative control and that of the tests vials at various concentrations (Benoit, 1996 & WHO, 2005).

Acute Toxicity Studies (LD_{50})

The LD_{50} of the extract was determined using Lorke method (1983). The study was carried out in two phases and animals (mice) were deprived of food for 16-18hours prior to administration of the *Syzygium aromaticun* aqueous extract. In phase 1, three group of three animals per group were used. The extract was administered orally in geometrically increasing doses (10mg/kg, 100mg/kg and 1000mg/kg). The treated animals were observed for four hours post administration for signs of toxicity. After 24hours, when no mortality occurred, phase 2 was initiated. In phase 2, four groups of one animal each were given the extract orally in geometrically increasing doses (1500mg/kg, 2250mg/kg, 3250mg/kg and 5000mg/kg). The animals were then observed for signs of toxicity for the first four hours and mortality for 24hours. The arithmetic mean of the lowest dose that killed an animals and the highest dose that did not kill was taken as the mean lethal dose (LD_{50}) of the extract.

STATISTICAL ANALYSES

The percentage of parasitemia was determined using the method adopted by (Kalra et al., 2006). From the tests vials, thin smears were prepared on the slides. The slides were allowed to dry and then fixed with absolute methanol/ethanol. After fixing, the slides were allowed to dry and then stained with 10% Giemsa in methanol for 30mins. After 30mins the slides were rinsed with water and then allowed to air dry. To estimate the percentage of red blood cells infected with Plasmodium falciparum, the slides were carefully observed under microscope using ×100 oil immersion objective lens in five different fields on each slide. The percentage parasitemia was calculated using the following formula.

% Parasitemia = Number of parasitized R B C/Total number of R B C× 100

The percentage of growth inhibition (%GI) of each concentrations of the crude extract was determined using the following equation:

$\% GI = a - b \div a \times 100$

Where "a" stands for mean % parasitemia of negative control vials and
"b" stands for mean % parasitemia of treated vials.

For comparing GI in treated vials and drugs free well (negative control) vials statistical values and tests such as mean, standard deviation, and analyses of variance (ANOVA) was used. One way-ANOVA was used to analyze significance difference across all concentrations at 72 hours of plant crude extract as computed using R version 3.4.0 Statistical software (WHO, 2015).

RESULTS

The mass, percentage yield and appearance of the aqueous extract revealed that, the *Syzygium aromaticum* (clove) flower bud extract had an extract of 8.6g, 21.5% yield and appears as dark brown in coloration.

Mass, Percentage Yield and Extract appearance of the Aqueous *Syzygium aromaticum* (clove) Flower Bud Extract

Plant	Mass (g)	Percentage Yield (%)	Aqueous Extract Appearance
S. aromaticum	8.6	21.5	Dark brown in color

Preliminary phytochemical screening of the aqueous *Syzygium aromaticum* (clove) flower bud extract revealed the presence of alkaloids, flavonoids, saponins, tannins and anthraquinones; only steroids were absent.

Phytochemical Screening of the Aqueous*Syzygium aromaticum* (clove) Flower bud Extract

Plant	Alkaloids	Flavonoids	Saponins	Tannins	Anthraquinones	Steroids
S. aromaticum	+	+	+	+	+	-

+ = Present
- = Absent.

The anti-plasmodial activity of *S. aromaticum* flower bud aqueous extract at different concentrations against *P. falciparum*revealed lower mean infected Red Blood Cells (SD) of 1.7 ± (0.6)at concentration of 50mg/ml than at concentration of 3.125mg/ml 9.0 ± (1.0),while higher mean infected RBC was recorded from drug free wells, which requires no treatment (negative control) 11.0 ± (1.0).The percentage growth inhibition as calculated using mean infected RBC count and mean RBC count shows a significant difference at different concentrations at $p< 0.05$ with the highest percentage growth inhibition at 50mg/ml (81.1%) and this indicates the effectiveness of the extract against the test organism.

Anti-plasmodial Activity (IC_{50}) of *S. aromaticum* Aqueous Extract against *P. falciparum*

RBC = Red Blood Cells

Parameters	Concentration (mg/ml)						
	50	25	12.5	6.25	3.125	Control	P value
S. aromaticum							
Mean RBC (SD)	100 ± (14.2)	109 ± (15.0)	117 ± (8.6)	114 ± (22.7)	117 ± (13.3)	128 ± (9.7)	
Mean infected RBC (SD)	1.7 ± (0.6)	5.3 ± (0.6)	6.3 ± (1.5)	8.7 ± (0.6)	9.0 ± (1.0)	11.0 ± (1.0)	0.0002
Mean % Parasitemia	1.63	4.98	5.42	7.91	7.85	8.63	
% Growth inhibition	**81.1**	42.3	37.2	8.3	9.0	-	

SD = Standard Deviation

Acute Toxicity Studies of *Syzygium aromaticum* (clove) Flower bud aqueous Extract

The acute toxicity studies of *Syzygium aromaticum* (clove) flower bud aqueous extract showed no death was recorded in the first phase of the study in rats. In the second phase, doses of 1500, 2250, 3250 and 5000mg/kg were used and no death was also recorded. The oral median dose (LD_{50}) for the aqueous extract of the *Syzygium aromaticum* (clove) flower bud was therefore estimated to be greater than 5000mg/kg and no sign of behavioral changes were also observed up to four weeks.

Acute Toxicity Studies of the aqueous *Syzygium aromaticum* (clove) Flower bud Extract

S. aromaticum	Group	Number of Animals	Dose (mg/kg)	Mortality recorded after 24hrs
Phase 1	I	3	10	0/3
	II	3	100	0/3
	III	3	1000	0/3
Phase 2	I	1	1500	0/1
	II	1	2250	0/1
	III	1	3250	0/1
	IV	1	5000	0/1

Numerator = Number of animals died and Denominator = Number of animals tested.

DISCUSSION

The drug resistance developed by malaria parasites is a big challenge for malaria control, elimination and eradication campaigns in sub tropic Africa. Therefore, the search for more effective anti-malarial agents from natural products is highly required as in many malaria-endemic countries of the world, natural and traditional products (plants and insects/products) are commonly used (Gupta *et. al.,* 2013).

The phytochemical screening of the aqueous *Syzygium aromaticum* flower bud extract revealed the presence of alkaloids, flavonoids, saponins, tannins, anthraquinones; however, only steroids

were absent. These phytochemicals result corroborate the work of Ayoola (2008) who also reported the presence of alkaloids, flavonoids, saponins, tannins and anthraquinones in the crude extract of *S. aromaticum*. Scrutiny of past research on *S. aromaticum*, shows that not much has been reported on the anti-malarial activity. Literature related to anti-malarial activity and phytochemical constituent of *S. aromaticum* is scanty. This might be attributed to the fact that *S. aromaticum* is a tree indigenous to West Africa and therefore research on the plant is scanty and claims by traditional herbalists on the usefulness of the plant as medicine is mostly centered on the use of the stem bark, root and leaf not the flower bud.

The result obtained from the anti-plasmodial activity of the clove flower bud aqueous extract (*S. aromaticum*) showed strong activity against *P. falciparum* only at concentration of 50mg/ml, this is in agreement with the result of the research made by Oshomoh (2012) who reported that extract from the clove flower bud (*S. aromaticum*) has appreciable activity against *P. falciparum* at concentration of 50mg/ml with % GI of 85.1%. Similarly the finding of this recent study corroborated with the report of Ayoola (2008) who equally reported that *S. aromaticum*is effective on *P. falciparum* at higher concentration of 100mg/ml. However according to the investigation carried out by Oshomoh (2015) again *S. aromaticum* aqueous extract was found to be potent on *P. falciparum* at various concentrations of 50, 25, 12.5 and 6.25mg/ml respectively, this is in disagreement with the result of the recent study. Furthermore Bruce, 2008 and Evans, 2009 describes that alkaloids are the most synthesized by plants which is the pioneer products that exhibits anti-plasmodial activity. In fact, quinines the first anti-plasmodial drugs belong to this class (Oliveira *et. al*., 2008), flavonoids showed potent *in vitro* anti-plasmodial activity against *P. falciparum.* Edeoga *et al.* (2005) and Avwioro (2010) in their studies present that saponins, tannins, anthraquinones have a greater anti-plasmodial activity against malarial parasites *in vitro* and *in vivo*. Thus; all the phytochemical mentioned were confirmed in the extract investigated in this study. However the variation of the efficacy occurred in this study might be as a result of using different solvent in extraction of the plant materials (water) or due to the absent steroids a strong secondary metabolite with anti-malarial property (Carvalho *et. al*., 2010).

The oral median lethal dose value for the flower bud aqueous extract of *Syzygium aromaticum* obtained in rats was found to be above 5000mg/kg. This suggests that the aqueous extract is non-toxic as no death was recorded. Acute toxicity are usually carried out to determine the dose that will cause death or serious toxic manifestations when administered singly or severally at few doses in order to establish doses that should be used in subsequent studies (Wanda et. al., 2002). The Organization for Economic Cooperation and Development (OECD), Paris, France, recommended chemical labelling and classification of acute systemic toxicity based on oral median lethal dose values as: very toxic if ≤ 5g/kg, toxic if > 5mg/kg but ≤ 50mg/kg, harmful if > 50mg/kg but ≤ 500g/kg and non-toxic or not harmful if > 500mg/kg or ≤ 2000mg/kg (Walum, 1998) Based on this classification, the oral median lethal dose obtained for rats found to be above 5000mg/kg, is respectively safe orally, but possibly the situation may not the same on repeat dose experiment in sub-chronic toxicity studies.

CONCLUSION

The research result obtained from the extraction confirmed that *Syzygium aromaticum* (clove) flower bud has a dark brown aqueous extract and shown positive to the phytochemical screening which indicates the presences of alkaloids, flavonoids, saponins, tannins, anthraquinones; however, steroids were absent.

This research work confirms the inhibitory effects of *Syzygium aromaticum* (clove) flower aqueous extract against *P. falciparum* at concentration of only 50mg/ml of *S. aromaticum.* The presence of phytochemicals in this aqueous extract might have been responsible for the activity possessed by the extract, which supports the use of this plant to treat malaria associated symptoms in local or traditional settings. Thus, new anti-plasmodial agent could be developed from this extract to avoid developing resistance by malarial parasites and the result shown the concentration of 50mg/ml is more potent (effective) than other concentrations. Also, the safety profile study result, shows *Syzygium aromaticum* (clove) flower bud aqueous extract is experimentally harmless.

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