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Research article

Anti-Malarial Activity of Methanol Extracts of *Anacardium occidentale* Linn. (Anacardiaceae) and *Psidium guajava* Linn. (Myrtaceae) Leaves

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ABSTRACT

The antiplasmodial activities of *Anacardium occidentale* L. and *Psidium guajava* L. have been severally reported in literature. β--hematin production is an exclusive method implemented by Plasmodium protozoan parasite to produce very high quantities of redox active free hemoglobin. The purpose of this study is to determine the effectiveness of methanol extracts of *Anacardium occidentale* L. and *Psidium guajava* L. leaves used to treat severe malaria attacks in Nigeria and their impact on the inhibition of β-hematin production. The LD50 values for the leaves of *Psidium guajava* L. and *Anacardium occidentale* L. in methanol extracts were >5000 mg/kg and 1600 mg/kg, respectively. On Day 4, *Plasmodium berghei*-infected mice (NK 65) displayed comparable chemo-suppression of parasitaemia for A. occidentale (73.88%) and P. guajava (72.75%). However, both extracts had lower activities than chloroquine (83.58 %; 20 mg/mL). A. occidentale had a higher inhibition of formation of β hematin, with IC50 of 36.1 ± 0.52 µg/mL than P. guajava with IC50 of l0.25 ± 0.07 µg/mL and chloroquine with IC50 of 2.71±0.39 µg/mL. According to the current study, methanol extracts of the leaves of *Anacardium occidentale* L. and *Psidium guajava* L. have similar antimalarial effects and are associated with a reduction in β-hematin production. The in vivo antimalarial activity of *Psidium guajava* L. and *Anacardium occidentale* L. were equivalent, but *Psidium guajava* L. possessed a more potent inhibitor of the production of β-hematin.

Keywords: *Antimalarial activity, β-hematin formation inhibition, Plasmodium berghei, Anacardium occidentale L., Psidium guajava L.*

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INTRODUCTION

It is thought that medicinal plants are significant sources from which possible malarial therapeutic actions have been identified. This prompted researchers to look for antimalarial plant components, particularly in tropical areas. Finding effective antimalarials remains a vital task because malaria is still one of the most contagious diseases in the world, with an estimated 241 million cases yearly. (WHO, 2021). It is a parasite illness caused by the Plasmodium protozoan, and it is particularly prevalent in areas with tropical and subtropical climates (Ouattara *et al*., 2006). Chemotherapy is still an effective way to treat malaria but P. falciparum resistance has led researchers to look at a range of traditional medicinal plants in search of novel antimalarial drugs due to its prevalence (Bekono *et al*., 2020). One of the most dependable ways to find new antimalarial compounds is through the traditional medical system, which is also an option (Tajudden and van Heerden, 2019).

Anacardium occidentale, sometimes known as cashew, belongs to the Anacardiaceae plant family. It is prevalent in the tropical regions of West Africa. The bark and leaves are used in traditional medicine to cure a variety of ailments (Konan and Bacchi, 2007). Cashew leaves are blended with those of Carica papaya, Cymbopogon citratus, and Azadirachta indica to create a multi-herbal concoction that is used in "steam therapy" in the southern part of Nigeria (Adebayo and Krettli, 2011). The sap or bark extract is reported to have contraceptive qualities, and the bark and leaves are traditionally used to cure malaria (Odugbemi *et al*., 2007). Its bark's antiplasmodial effects can be obtained by ethanol and aqueous extracts with activity (IC50) values of 5.69 µM and 5.39 µM respectively. Cardol triene and 2-methylcardol triene stand out among the alkyl phenols identified in the plant's nut as having potent antiplasmodial effects (Gutierrez *et al*., 2008)

Psidium guajava sometimes referred to as guava in English, is a plant that belongs to the Myrtaceae family. It is a

tropical tree that originated in Central America and is now widely farmed and may be found throughout the tropics of the world (El-Mahmood, 2009). Various conditions have been treated with guava leaves, including diabetes, malaria, rheumatism, diarrhea, dysentery, menstrual disorders, anorexia, digestive issues, gastric insufficiency, skin issues, ulcers, vaginal discharge, colds, and coughs (Kumar and Sharma, 2014). Extracts from the plant's stem bark demonstrated antiplasmodial effects on P. falciparum cultures *in vitro* (Nundkumar and Ojewole, 2002). Recent studies discovered that among numerous unrefined plant leaf extracts (Yadav *et al*., 2020).

The suppression of β-hematin production is one of the stages of the Plasmodium lifecycle that antimalarial medications target. An efficient antimalarial medicine simply stops the parasite's detoxifying process when it prevents the production of β-hematin. As a result, toxic heme builds up inside the parasite's digestive vacuole, killing it and stopping the infection from spreading further (de Villers and Egan, 2021). Antimalarial research that focuses on preventing the development of β-hematin is therefore essential in the fight against malaria as they interrupt the parasite lifecycle which leads to the eradication of the infection which spares millions of lives in malaria-endemic areas by interfering with this essential detoxifying process (Munro and McMorran 2022).
The available literature, however, could not demonstrate either the potential route of antiplasmodial action or in vivo antimalarial activity of these two well-known medicinal herbs. Although more antimalarial medicinal plants are being found in Nigeria and other regions of Africa, little is known or investigated about the potential mechanisms of antimalarial action (Adebayo and Krettli 2011, Uzor 2020). The objective of the present study was to assess the antimalarial efficacy of methanol extracts of A. occidentale and P. guajava leaves as well as how they influence the inhibition of β-hematin formation, a well-known, viable, and crucial antiplasmodial therapeutic target (de Villiers and Egan 2021).

MATERIALS AND METHODS

Reagents/instruments/equipment: Analytical grade of methanol (Sigma Aldrich), Giemsa stain (pH 7.2) (Sigma–Aldrich), immersion oil, distilled water, bovine hemin, chloroquine diphosphate (Sigma–Aldrich), sodium acetate, acetic Acid, HEPES - hydroxyethylpiperazine-N-[2-ethanesulfonicacid] (Fluka), pyridine 99%, dimethylsulfoxide, hydrochloric acid and sodium hydroxide (Merck), 96-well plates of 2 mL and sterilized pipette tips. Microscopy was performed with a light microscope (Olympus®) and colorimetry with a microplate reader (SpectraMax Plus Molecular devices).

Plant collection, authentication and preparation: The leaves of the plants *Anacardium occidentale* L. and *Psidium guajava* L. were collected in October 2019 at Akinmoorin in Oyo town of Oyo State which was located at 7.7832◦N, 3.9419◦E. The plants were authenticated, and voucher specimens deposited at the Forest Herbarium Institute. (A. occidentale, FHI 112899; P. guajava, FHI 112990). Plant samples were cleaned, air-dried for two weeks in the shade, and then dried in an oven at 40°C. They were ground in a mill and kept until needed in sealed containers.

Extraction of plant materials: Plant materials (1 kg) were macerated with intermittent stirring at room temperature for 72 hours to extract them in distilled methanol. Plant extracts were concentrated in vacuo at 40°C from the extracts until required for analysis. The plant extracts were kept in the refrigerator at 4°C.

Acute toxicity test: *Anacardium occidentale* and *Psidium guajava* leaves extract were tested for oral acute toxicity in mice using a modified Lorke's method (Lorke, 1983). Two stages of the investigation were completed. Nine mice were randomly divided into three groups of three mice each in phase one of the study, and each group received 10, 100, and 1000 mg kg-1 of the extract orally. The mice were observed for paw licking, salivation, stretching of the entire body, weakness, sleep, respiratory trouble, coma and death in the first 4 h and afterwards daily for 7 days. In the second phase of the study another fresh set of nine mice were randomized into three groups of three mice each and were given 1600, 2900 and 5000 mg kg-1 b.wt. of the extract orally based on the results of the first phase.

For the first crucial four hours and then every day for seven days, these were monitored for indicators of toxicity and mortality. After that, the geometric mean of the successive doses for which 0 and 100 percent survival rates were noted in the second phase was used to determine the LD50, which is equal to the square root of the product of the lowest fatal dose and maximum non-lethal dose. The formula below was used to get the oral median fatal dose:

$$LD_{50} = \sqrt{\text{Minimum toxic dose x Maximum tolerated dose}}$$

The chloroquine-sensitive *Plasmodium berghei* NK-65 parasite, which is frequently used in rodent malaria studies, was obtained from the Institute of Advanced Medical Research and Training (IAMRAT), University College Hospital in Ibadan, Nigeria. The study animals (Swiss albino mice) used were obtained from the animal house of IAMRAT. They were allowed to acclimatize to the laboratory environment under normal room temperature and conditions for not less than 14 days before the commencement of inoculation and observation. The experiment was carried out in calm and standardized laboratory conditions according to Peter and Anatoli (Peter and Anatoli, 1998). The treatment of the animals followed the guidelines of ARRIVE and the EU directives on animal study (EU 2020, du Sert *et al*., 2020)

***In vivo* chemo-suppressive antimalarial assay:** Using an anti-malaria mouse model test, the in vivo antimalarial activity of *Anacardium occidentale* and *Psidium guajava* leaf extracts was assessed (18-22 g of male Swiss Albino mice). Mice infected with the chloroquine-sensitive Plasmodium berghei NK 65 parasite were tested using a modified version of Peter's 4-day suppression test (Peters, 1987). Swiss albino adult male mice weighing 18 to 22 g were intraperitoneally (IP) injected with a conventional inoculum of P. berghei-infected erythrocytes (0.2 mL, 1 x 107) to induce infection. The mice

were put into 5 groups of 5 mice each and given daily oral doses of 25, 50, and 100 mg of extract/kg of body weight for 4 days straight (D0-D3). The study included two control groups; the positive control received daily treatment with 20 mg/kg chloroquine while the negative control received 0.2 mL of ordinary saline. On experiment day 5, blood was drawn from each mouse's tail and spread on a microscope slide to create a film (Akuodor 2011). The blood films were preserved with methanol, stained for 10 minutes at pH 7.2 with 10% Giemsa, and then the parasitaemia was inspected under a microscope.

Parasite density was determined by microscopically counting the parasitized red blood cells on at least 1000 erythrocytes in 10 different fields. By comparing the parasitaemia of infected controls with those of treated mice, the percentage suppression of parasitaemia was computed for each dose level.

On day four (D3) following inoculation, tail snips from each mouse were used to make thin smears of blood films (White 2011). The smears were applied to microscopic glass slides and allowed to air dry before being treated with methanol and allowed to dry. Giemsa stain was then applied to the slides to determine the parasitaemia by microscopic means. The presence of at least 1000 parasitized erythrocytes was required to determine the presence of parasitaemia. The average of three (3) sets of results was computed to determine the parasitaemia of each infected mouse. The following formula was used to compute the percentage of suppression: (Dikasso *et al*., 2006)

$$\% \text{ Suppression} = \frac{\text{Parasitaemia in negative control} - \text{Parasitaemia in study group}}{\text{Parasitaemia in negative control}} \times 100$$

β-hematin Inhibition Assay: The ability of the extracts to inhibit the formation of beta-hematin *in vitro* was determined using the method of Vargas *et al*. 2011**.** The plant extracts were produced as a stock concentration by combining 500 mg of each methanol extract with 10 mL of methanol to make 50 mg/mL. For each measurement, 6.8 mg of bovine hemin were dissolved in 10 mL of 0.1M NaOH to create a fresh bovine hematin solution (0.68 mg/mL). 238 mg of HEPES were dissolved in 50 mL of distilled water, and the pH was then raised to 7.5 to create HEPES solution (4.76 mg/mL). To prepare the 15% of pyridine that was required for the experiment, 15 mL of pyridine was mixed with 85 mL of HEPES solution. 36 g of sodium acetate were dissolved in 20 mL of distilled water and 48 mL of acetic acid to create a saturated acetate solution. 15 mL of pyridine was mixed with 85 mL of HEPES solution to create the 15% of pyridine that was used in the experiment. 36 g of sodium acetate were dissolved in 20 mL of distilled water and 48 mL of acetic acid to create a saturated acetate solution, which was then pre-warmed at 60 °C before each usage.

Each plant extract was tested at a 25 mg/mL concentration for its ability to inhibit beta-hematin production. In a nutshell, 96-well plates with columns of 10 L each of the plant extract and chloroquine were examined. Additionally, all plant extracts in the 96-well plates received 10 L of 1M HCl. A 100 L portion of freshly made hematin solution was introduced to the wells in rows A and B. For ten minutes, the test plate was shaken at 900 rpm. Then, 60 L of saturated acetate solution (pH 5.0), which had been pre-warmed at 60 °C, was added to each well. The test plate was then incubated for an additional 90 minutes at 60 °C. Thereafter, 750 µL of 15% pyridine was added to wells in row A and C while 750 µL HEPES (pH 7.5) was added to wells in row B and D. The test plate was then shaken for 10 minutes at 900 rpm before being let to settle for 15 minutes. A 100 L aliquot was transferred in triplicate to a 96-well non-sterile plate. At 650 nm, the absorbance was measured.

The pharmacological controls included chloroquine. In 96-well plates, the extracts and chloroquine were examined in triplicate. IC50 values were calculated using non-linear regression in the statistical software program Graphpad Prism 5.0®. The previous experiment was done six more times with chloroquine and each extract at doses ranging from 12.5 to 0.39 mg/mL. The extracts and chloroquine were examined in triplicates.

RESULTS

The selected plants were collected and macerated with absolute methanol resulting in yields of 10.55 g and 9.07 g for *A. occidentale* and *P. guajava*, respectively. The acute toxicity of the methanol extract of *A. occidentale* leaves showed no sign or symptom of toxicity at any of the tested concentrations of the extract given to the mice. The LD50 of *A. occidentale* is reported in this studyto be equal to or greater than 5000 mg/kg (Table 1). There was no lethality or behavioral changes in the three groups of the mice that received 10, 100, or 1000 mg/kg body weight of the extract. Increase in extract dosage to 1900, 2600 and 5000 mg/kg body weight did not induce death within 24 hours of administration Together, these results showed that the extract was relatively safe at doses equal to or greater than 5000 mg/kg body weight.

Table 1
Acute toxicity tests of *A. occidentale* and *P. guajava* leaves

Dose (mg/kg)	Mean mortality after 24 hours	
	Anacardiumoccidentale	*Psidiumguajava*
5	0	0
10	0	0
100	0	0
1600	0	3
2900	0	3
5000	0	3

Table 2
In vivo chemo-suppressive antimalarial assay

Dose (mg/kg)	% Chemosuppression of parasitaemia							
	A. occidentale				*P. guajava*			
	D0	D1	D2	D3	D0	D1	D2	D3
25	4.12	34.36	56.94	71.27	4.12	30.77	51.85	67.16
50	21.65	41.03	47.41	71.13	12.89	40.51	57.87	72.01
100	28.32	47.69	60.49	73.88	29.90	43.59	61.11	72.75

**Chloroquine (20 mg/kg): D0 (27.32 %), (D1 (46.15 %), D2 (72.22 %), D3 (83.58 %)*

A modified 4-day chemo suppressive test was carried out using male albino Swiss mice on the methanol extract of *A. occidentale* and *P. guajava* leaves. The plant extracts were tested for their antimalarial activity using chloroquine as the standard. *Plasmodium berghei* NK-65 was used as parasite for this study because the parasite is sensitive to chloroquine. The percentage of parasitaemia obtained changed significantly from those in the untreated control animals on D_0 till D_3 showing degrees of suppression for various dosages from 25 mg/kg, 50 mg/kg and 100 mg/kg which implied that the extracts produced significant ($p<0.05$) dose dependent reduction in parasitaemia levels in the extract treated groups (Table 2). Both extracts showed the highest antimalarial activity (% parasitaemia suppression) at 100 mg/kg: *Psidium guajava* (73.88 %) and *A. occidentale* (72.75 %) on day 4 (D3) compared to that of the control, chloroquine (20 mg/kg, 83.58%).

Table 3
β-hematin Inhibition Assay

Extract/ Chloroquine	IC_{50} ± SEM (mg/mL)
*A. occidentale**	36.1±0.52
*P. guajava **	10.25±0.07
Chloroquine	2.71±0.39

**p< 0.05 compared to control (2.71±0.39); SEM = Standard Error of Mean*

The chemo suppression was mildly dose-dependent at lower doses especially for *Psidium guajava* values of 71.27 % and 67.16% for *A. occidentale* and *P. guajava*, respectively recorded at 25 mg/kg dose]. The β-hematin formation inhibition of *A. occidentale* was 36.1±0.52 µg/mL while *P. guajava* had activity of 10.25±0.07 µg/mL (Table 3). The higher inhibitory activity of *P. guajava* indicated that the extract contained more potent inhibitors than *A. occidentale*. However,the comparable chemo suppressive activity of *A. occidentale* with the other extract suggested thatthe former extract had additional mode(s) of action. The activities of either extract was lower than that of reference drug, chloroquine (2.72 ± 0.39 µg/mL).

DISCUSSION

The two medicinal plants, A. occidentale and P. guajava plants have plant parts (stem bark, leaves, roots and even their oils) used in the traditional treatment of malaria (Adebayo and Krettli, 2011). The *in vitro* antiplasmodial activities of crude extracts or isolates of both plants have been reported in literature (Gimenez *et al*., 2019, Rajendran *et al*., 2014). This particular study has evaluated the methanol leaf extracts of A. occidentale and P. guajava for in vivo antimalarial activity in a murine model as well as their effect on inhibition of β-hematin formation, a major mode of action of several clinical partner drugs.

The acute toxicity study showed that the A. occidentale extract was relatively safe at doses equal to or greater than 5000 mg/kg body weight. This finding agreed with that of Sha'a and Omoboyowa *et al* that reported the non-toxicity of A. occidentale at tested doses administered (Omoboyowa *et al*., 2018, Sha'a 2014). The result of the acute toxicity of the methanol extract of P. guajava leaves in this study showed an LD_{50} of 400 mg/kg. However, Roy *et al* showed that the P. guajava leaf extract administered at a dose of 2000 mg/kg had an LD50 greater than the dose administered (Roy *et al*., 2006). The in vivo model was used for this study because it takes into account the possible positive drug effect and possible contribution of the immune system in eradication of the infection (Waako *et al*., 2005). P. berghei provides an entrenched experimental model of malaria infection in rodents whose pathological symptoms closely imitate the symptoms produced by human malaria (Syahmi *et al*., 2010). Even though the rodent malaria is not exactly similar to that of the human Plasmodium parasites, it is the first step to screen most in vivo antimalarial activities of test compounds (Kalra *et al*., 2006). Most of the currently available antimalarial medications (chloroquine, halofantrine, mefloquine, and artemisinin derivatives) have been identified by employing this model (Simwela and Waters, 2022)

When a standard antimalarial drug is used in mice infected with P. berghei, it suppresses parasitaemia to non-detectable levels (White *et al*., 2009), which is in agreement with the effects of chloroquine in this study. In untreated mice, the parasite count increased markedly from day to day until the death of the animal.

The two extracts contained constituents inhibiting formation of β-hematin formation. The formation of haemozoin (β-hematin) is recognized as an important, essential detoxification route for haem released from haemoglobin catabolism in plasmodium (Matz 2022). The pathway has other several desirable features for discovery of new antiplasmodial drugs- druggability, viability and immutability (Okombo and Chibale, 2017). Consequently, it is becoming a popular platform for antiplasmodial drug discovery leading to an increasing number of drug leads (de Villiers and Egan, 2021).

The antimalarial activity of a plant extract might be caused by a single constituent or group of compounds that have been screened for the presence of secondary metabolites (Kumar and Sharma, 2014). The presence of alkaloids, steroids, terpenoids and flavonoids in both plants acting alone or in combination are important phytoconstituents which have been purported to be responsible for antimalarial activity in medicinal plants (Uzor, 2020).

In conclusion, the methanol extract of leaves of A. occidentale and P. guajava are effective against Plasmodium berghei NK-65 strain in the 4-day suppressive test. The crude extracts have shown significant, comparable, dose-dependent suppression of parasitaemia of NK-65 strain of chloroquine-sensitive Plasmodium berghei. Both extracts also inhibited formation of β-hematin formation. However, the non-toxic and less active inhibitor of β-hematin formation (A. occidentale) possibly has additional mode(s) of action. The two samples can be further studied as antimalarial drugs or drug leads.

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