

PRECLINICAL EVALUATION OF THE ANTIMALARIAL ACTIVITY OF *Persea americana* SEED METHANOL EXTRACT IN *Plasmodium berghei* INFECTED MICE

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ABSTRACT

Generally, avocado seeds are discarded after the consumption of the fleshy part of the fruit. Given its rich phytochemical and biochemical profile, disposal represents a huge economic loss. We thus assessed the toxicity and antimalarial potential of avocado seed methanol extract using the OECD and Peters' 4-day suppressive antimalarial testing methods, respectively. For the acute toxicity study, 2 g/kg body weight of the extract was investigated. The antimalarial study was conducted using mice infected with 1×10^5 *Plasmodium berghei* NK65. Twenty-five male mice were randomly distributed into five groups of five mice. They were all infected with *P. berghei*. Three hours after infection, groups 1 and 2 mice received normal saline and chloroquine (25 mg/kg body weight), respectively. The mice in the other groups received graded doses (100, 250 and 500 mg/kg b.wt.) of the extract. Treatment was done for a total of 4 days, while survival was assessed within 30 days. The extract was non-toxic to mice at 2 g/kg body weight and graded doses reduced parasitemia in a dose-dependent manner. Parasite killing may be due to its high antioxidative capacity. Alarmingly, treatment with higher doses of the extract resulted in increased mortality, which is suggestive of toxicity. A chronic toxicity study may reveal the potential toxicity of avocado seed methanol extract, which may have been missed in our acute toxicity study. Furthermore, as its bioactive principle(s) may be selective for the malaria parasite, their isolation may guide the development of a robust extraction method that would guarantee potency and safety.

Keywords: Avocado seed, Antioxidative capacity, Antimalarial activity, Toxicity

INTRODUCTION

Malaria is a parasitic infectious disease that is transmitted by an infected female *Anopheles* mosquito during a blood meal (Azmi et al., 2023). Despite being one of the oldest documented diseases, it remains one of the most prevalent infectious diseases affecting humans to date (Acharya et al., 2017). According to the WHO's report of

2024, an estimate of two hundred and sixty-three million cases i.e. 58.6 per 1000 persons risked contracting malaria, with about 597,000 deaths recorded in 2023 (WHO, 2024). The disease is caused by protozoan parasites of the genus *Plasmodium*. Five *Plasmodium* species are known to cause human malaria, viz; *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, *Plasmodium knowlesi* and *Plasmodium falciparum* (Fletcher et al.,

2013). Of these, *Plasmodium falciparum* is the most virulent (Acharya *et al.*, 2017). Unfortunately, most of the malaria cases in sub-Saharan Africa are due to *Plasmodium falciparum* infection (Mace *et al.*, 2018). Also, Africa is home to *Anopheles gambiae* and *An. funestus*; the most efficient vectors for malaria parasite transmission (Hadebe *et al.*, 2024). The African continent thus shoulders the greatest burden of the disease (WHO, 2024).

Several approaches are currently in use to prevent malaria. These include vector control (i.e. the use of insecticide treated nets as well as in-door residual spraying), antimalarial vaccines as well as the use of chemotherapeutic agents (Erhunse and Sahal, 2021). While the antimalarial vaccines (RTS, S and R21) in concert with other malaria prevention tools have been successful at reducing severe malaria cases and deaths in children under 5, they are only recommended for young children in high transmission areas. As both antimalarial vaccines do not cut across all age groups, individuals over 5 years of age still have to rely heavily on antimalarial medicines. However, *P. falciparum* has generated resistant mutations to all antimalarial drugs approved for the management of the disease, including artemisinin (Erhunse and Sahal, 2021). Hence, the need for the discovery of new drugs with novel scaffolds that are alien to the malaria parasite. As traditional knowledge of indigenous plants has historically guided the discovery of some of the most effective antimalarials, including quinine from *Cinchona* bark, atovaquone whose backbone was inspired by lapachol which was isolated

from *Tabebuia impetiginosa* and artemisinin from *Artemisia annua* (Achan *et al.*, 2011; De Paula *et al.*, 2014). Plant-based medicines thus present valuable opportunities for developing alternative therapeutic strategies against this devastating global health threat.

Persea americana Mill, commonly known as Avocado pear or Alligator pear, is a commercially valuable fruit tree native to Central and South America, that is widely cultivated in tropical and subtropical regions globally (Boadi *et al.*, 2015). While its leaf is used traditionally for malaria treatment (Boadi *et al.*, 2015), its seed has been largely unexplored for the same. Currently, the seed represents an under-utilized resource and a waste issue for avocado processors. There is ethno-pharmacological information on the use of seeds for the treatment of some health-related conditions. For instance, it has been reported to possess insecticidal, fungicidal, and anti-microbial activities (Chia and Dykes, 2010; Egbuonu *et al.*, 2018; Cardoso, 2016). The avocado fruit itself is botanically classified as a berry. The fruit is composed of a single big seed surrounded by a buttery pulp (Olas, 2024). Avocado seeds (Figure 1) are rich in phenolic compounds, which may play a role in the putative health effects that have been ascribed to it (Dabas *et al.*, 2013).

In the current study, we assessed the antimalarial activity of avocado seeds methanol extract in mice infected with chloroquine sensitive *Plasmodium berghei*. Prior to the antimalarial study, an acute toxicity study was first conducted for safety assessment.

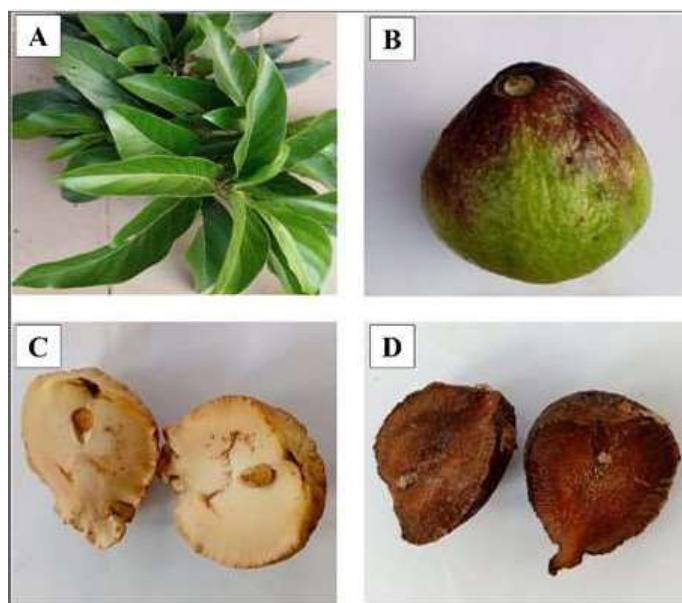


Figure 1: Leaves (A), fruit (B), fresh (C) and dried (D) seeds of *P. americana*

MATERIALS AND METHODS

Plant Sample Collection, Authentication and Preparation

Fruits and leaves of *P. americana* were collected from a private garden in Benin City, Nigeria and submitted to Prof. H.A. Akinibosun for authentication. A voucher specimen number UBH-P408 was thereafter allotted.

Fruits were allowed to ripen, after which the seeds were retrieved, cleaned and shade dried. Thereafter, 35 g of the dried sample were soaked in 2 L of methanol with constant stirring for 72 hours. At the end of the 72 hours, the methanol extract was filtered with the aid of a Muslin cloth. The filtrate (extract) was then concentrated to dryness with the aid of a rotary evaporator. By letting the extract sit in a 30 °C oven overnight, the extract was further concentrated.

Acute toxicity testing

This was done by employing the limit test dose up and down procedure of the Organisation for Economic Cooperation and Development guideline 423 (OECD, 2001). Briefly, six male mice (Wistar strain) (6–8 weeks old) were randomly placed in 2 groups of 3 mice each and fasted for 6 hr. with water *ad libitum*.

Group 1 received normal saline (and hence, served as control) while members of the remaining group were administered 2000 mg/kg b.wt. of *P. americana* seed methanol extract. After administering the sample, animals were observed for signs of toxicity such as lacrimation, stereotypies, abstinence from food, fur changes as well as death for a period of 14 days.

4-day Suppressive Antimalarial Study

The Peter's 4-day Suppressive antimalarial testing model was adopted for testing the antimalarial activity of *P. americana*. The test extracts (100, 250 and 500 mg/kg b.wt.) were prepared in normal saline. Twenty-five male mice (Wistar strain) (6–8 weeks old, weighing 20–22 g) were randomly distributed into five groups of five mice each and intraperitoneally inoculated with 1×10^5 *P. berghei* NK65 infected RBCs from blood obtained from *P. berghei* infected mice obtained from NIMR, Lagos. Three hours after infection, groups 3 to 5 mice were orally administered 100, 250 or 500 mg/kg b.wt. of *P. americana*. Group 1 (negative control group) received normal saline, whereas group 2 (positive control group) received 25 mg/kg b. wt. chloroquine. Oral feedings were done daily for a total duration of 4 days. On the 5th day, smears were

made from the lateral tail vein of all animals in the different groups, fixed in methanol and stained with Giemsa. These were thereafter subjected to microscopic determination of % Parasitemia. Furthermore, animals were kept for a total duration of 30 days to compute mean survival time (MST) (Knight and Peters, 1980).

Statistical analysis

Results have been presented as mean $\pm$ standard deviation. One-way analysis of variance between groups was done using SPSS version 24. P values < 0.01 were taken to be statistically significant.

RESULTS

Acute toxicity of *P. americana* in mice

The Organisation for Economic Cooperation and Development (OECD) guideline 423 requests that the limit test dose up and down procedure be adopted in a bid to reduce the quantity of animals used for acute toxicity testing (OECD, 2001). According to the OECD, (2001) method, should 2 out of 3 mice in a group die, testing should be repeated at lower doses (300, 50 & 5 mg/kg b.wt.) until a safe dose is obtained. Following the 14-day observation, no mice death was recorded after the extract was administered at 2000 mg/kg b.wt., and no other sign of toxicity was observed. Therefore, *P. americana* (PA) seed methanol extract can be said to be safe at 2 g/kg b.wt. in mice. As such, testing wasn't repeated at lower doses.

Table 1: Acute toxicity of mice administered 2 g/kg b.wt of *P. americana* seed methanol extract.

Group ID	Substance administered	Mortality recorded	Symptoms observed				
			0 – 1 hour	2 – 24 hours	Day 2 - 14	Day 3	Day 4 -14
Control (Grp 1)	Normal saline	0/3	None	None	None	None	None
PA group (Grp 2)	<i>P. americana</i> seed methanol extract	0/3	2/3 abstained from food	None	None	None	None

Antimalarial activity of *P. americana* seed methanol extract in mice

A dose-dependent decrease was recorded when mice infected with *P. berghei* NK65 were administered graded doses of *P. americana* seed methanol extract (Figure 2 & 3)

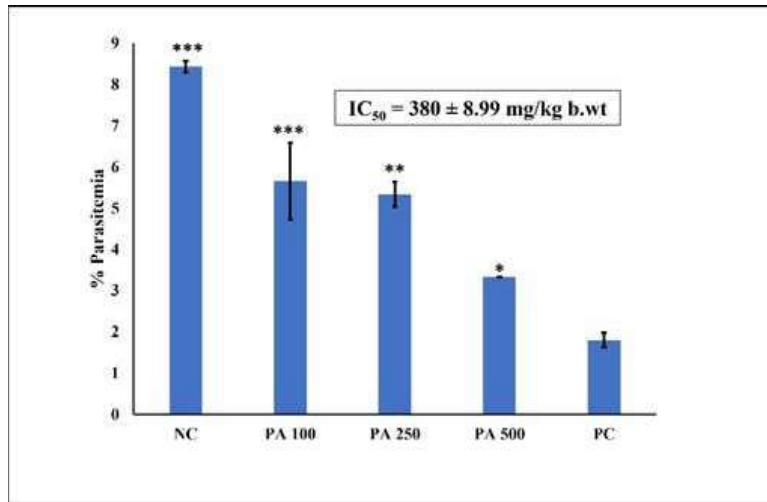


Figure 2: Treatment of chloroquine sensitive *P. berghei* infected mice with *P. americana* seed methanol extract. PC = Positive control, NC = Negative control, PA1= 100 mg/kg b.wt. *P. americana*, PA2 = 250 mg/kg b.wt. *P. americana*, PA3= 500 mg/kg b.wt. *P. americana*. Different superscripts (*p< 0.05, **p<0.01, ***p < 0.001) denote significant difference between means in comparison to positive control group.

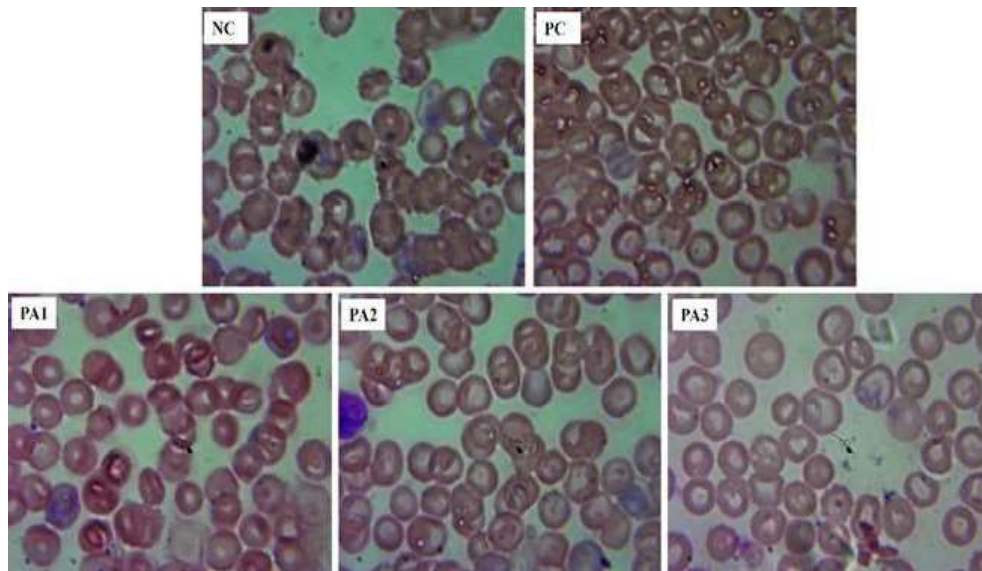


Figure 3: Representative images of red blood cells of *P. berghei*-infected mice administered graded doses of *P. americana*. PC = Positive control, NC = Negative control, PA1= 100 mg/kg b.wt. *P. americana*, PA2 = 250 mg/kg b.wt. *P. americana*, PA3= 500 mg/kg b.wt. *P. americana*.

Although the extract caused a dose-dependent decrease in parasitemia, mice administered higher doses of the extract however died ahead of those that received lower doses (Table 2).

Table 2: Survival of *P. berghei* infected mice administered graded doses of *P. americana* seed methanol extract

Sample ID	MST (Days)
PC	30 ± 0
NC	19.50 ± 7.33**
PA1	24.75 ± 3.20

PA2	21.25 ± 5.44*
PA3	21.00 ± 2.94*

Key: PC = Positive control, NC = Negative control, MST = mean survival time, PA1= 100 mg/kg b.wt. *P. americana*, PA2 = 250 mg/kg b.wt. *P. americana*, PA3= 500 mg/kg b.wt. *P. americana*. Different superscripts (*p< 0.05, **p < 0.01) denote significant difference between means in comparison to positive control group.

DISCUSSION

Due to its superior nutritional and phytochemical composition, Avocado pear is hailed as a “superfood”. Its mature flesh is consumed and often used to produce guacamole or oil, whereas, the epicarp and seed are discarded in most countries of the world (Padilla-Camberos et al., 2013) including Nigeria. However, avocado seed contains several bioactive compounds including phenolics (Saavedra et al., 2017), acetogenins (Rodríguez-Sánchez et al., 2013), procyanidins (Wang et al. 2010) and triterpenoids (Abubakar et al., 2017) which have potential health benefits. Avocado seed extracts have been reported to possess anti-hyperglycemic (Tremocoldi et al., 2018), anticancer (Lara-Marquez et al., 2020), anti-inflammation (Dabas et al., 2019), antihypercholesterolemia (Uchenna et al., 2017), antimicrobial (Villarreal-Lara et al., 2019) and antioxidant properties (Soledad et al., 2021), amongst others. When compared to the pulp, avocado seed extract possesses superior antioxidative capacity (Dabas et al., 2013). Higher levels of certain phytochemicals including catechin, (-) epicatechin, proanthocyanidins, triterpenes, furoic acid and leucoanthocyanidin (Dabas et al., 2013) have been attributed to this. For instance, in a recent study, epicatechin was isolated as the major active principle from the methanol extract of avocado seed which displayed antioxidant activity (Matsusaka et al., 2003).

The malaria parasite undergoes a 48-hr erythrocytic cycle. During this stage, the parasite feeds on its host's hemoglobin, ruptures the red blood cell membrane and invades neighboring uninfected red blood cells. During this process, toxic heme is released. This then generates reactive oxygen species (ROS) via the Fenton pathway thereby

increasing the oxidative burden of the disease (Percario et al., 2012). Furthermore, the host's immune cells are activated during malaria infection. Their activation also causes the production of ROS which kills the parasite and overwhelms oxidative defenses (Liguori et al., 2018). As malaria is an oxidative disease (Vasquez et al., 2021), and avocado seed is reported to have high antioxidative capacity, the current study explored the antimalarial potential of the methanol extract of avocado seed. To the best of our knowledge, this is the first study to assess the antimalarial potential of avocado seed. Prior to the antimalarial study, an acute toxicity study was first conducted. This was done using the OECD method which revealed the extract to be safe in mice at 2 g/kg b.wt. (Table 1). Following this, we conducted an antimalarial study using 100, 250 and 500 mg/kg b.wt. of the extract. The extract caused a dose-dependent decrease in parasitemia (Figures 2 & 3). With an IC₅₀ of 380 mg/kg b.wt. for parasite killing, avocado seed methanol extract is moderately active against *P. berghei* (Munõz et al., 2000). However, despite the extract resulting in a dose-dependent decrease in parasitemia, this did not translate to an increase in mice survival as higher doses of the extract resulted in increased mouse death (Table 2). It is not clear why this was the case. Earlier, Padilla-Camberos et al. (2013) reported the LD₅₀ of the ethanol extract of avocado seed to be 1200.75 mg/kg b.wt. with no sign of genotoxicity observed in mice administered 250 mg/kg b.wt. of the extract using the erythrocyte micronucleus formation test. Prior to the study of Padilla-Camberos et al. (2013), another group had reported the toxicity of avocado fruit and leaf extracts to human peripheral lymphocytes *in vitro* (Kulkarni et al., 2010).

Persin (15; (2R,12Z,15Z)-2hydroxy-4-oxoheneicosa-12,15-dienyl acetate), a polyketide acetogenin with antibacterial, antifungal (Chia and Dykes, 2010; Domergue et al., 2000) as well as anticancer (Domergue et al., 2000) properties, has been fingered as a toxic principle in avocado (Matsusaka et al., 2003). It has been reported to be present in various cultivars of avocado (Rodriguez-Lopez et al., 2015). Persin alongside two of its analogs; persenones A and B have been seen to reduce superoxide O_2^- and nitric oxide (NO) generation in cell culture and may thus contribute to avocado's antioxidative and anti-inflammatory properties (Dabas et al., 2019). In addition to Persin, avocado seeds also contain small quantities of hydrocyanic acid and cyanogenic glycosides (amygdalin form), which can produce toxic hydrogen cyanide (Baker et al., 2018). Furthermore, the seeds contain tannins, phytate and oxalate (Okibe et al., 2023) which act as anti-nutrients since they reduce the absorption of certain vitamins and minerals. Given avocado's elaborate phytochemical composition, isolation and purification of antimalarial agent(s) that are selective for the malaria parasite from avocado seeds is crucial. This can guide the development of robust extraction methods targeted at obtaining greater yields of the potent antioxidants while reducing toxicity.

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