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Antiplasmodial and Haem Transition Activities of Tannin and Glycoside Fractions of *Phyllanthus amarus*

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Abstract

Malaria remains a major public health threat, particularly in Nigeria, amid rising resistance of *Plasmodium falciparum* to existing therapies, underscoring the need for new, effective, and affordable antimalarials. This study aimed to evaluate the antiplasmodial activity of *Phyllanthus amarus* tannin and glycoside extracts and to assess their effects on haem-related mechanisms. Standard procedures were used to prepare crude extracts, which were tested against sensitive *P. falciparum* 3D7 strains; parasite growth inhibition and haem transition inhibition assays were conducted, and performance was benchmarked against chloroquine. Key findings show that the tannin extract reduced parasite growth (albeit less than chloroquine, $p < 0.05$) with IC_{50} = 3.09 µg/mL, whereas the glycoside extract exhibited minimal activity (IC_{50} = 14.45 µg/mL) compared with chloroquine (IC_{50} = 0.41 µg/mL). Neither extract significantly inhibited haem transition, in contrast to chloroquine, suggesting possible interference at early stages of haem oligomerisation while haem remains in solution. The study concludes that the tannin extract demonstrates promising antiplasmodial activity, while the glycoside extract does not. The contribution and implication are that the antimalarial properties of *P. amarus* observed *in vitro* and *in vivo* may

be partly attributable to its tannin content, warranting further investigation as a candidate for antimalarial development.

Keywords: *Phyllanthus amarus*; *Plasmodium falciparum*; Tannins; Glycosides; Antiplasmodial Activity; IC_{50}; Haem Oligomerisation

Introduction

Malaria is a parasitic disease caused by protozoan parasites *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae, Plasmodium ovale* and *Plasmodium knowlesi* (Bethel *et al.,* 2019). Malaraia has posed to be one of the most life-threatening infectious diseases in the tropics. Malaria is endemic in all tropical regions of the world (Oronsaye *et al.*, 2017). About 300-500 million people experienced clinical episodes and 1.4 – 2 million deaths occurred in 2009 (WHO, 2009). In 2018, *Plasmodium falciparum* accounted for 99.7% of estimated malaria cases in the WHO African region 50% of cases in the South-East Asia region, 71% of cases in the Eastern Mediterranean and 65% in the Western Pacific (WHO, 2020). In 2019, the World Health Organisation (WHO) attested that Nigeria had the highest cases (62 million) and death (94 thousand) (WHO, 2020). This observation could be due to the growing resistance of the *Plasmodium* parasite to drugs known to be previously active.

Medicinal plants have been recognized as veritable therapeutic agents since they house a cocktail of secondary metabolite (phytochemicals) with vast medicinal benefits. Herbalism could be traced to early man who perhaps acquired skills of healing through deliberate or unintentional selection of plants and their parts (Onyesom *et al.*, 2015). In the identification of new medicinal plants or focusing on those earlier reported which have bio-active phytochemical constituents, herbalism (ethno-medicinal study) is regarded as the most viable and feasible method (Adjanahoun, *et al.,* 1991). Traditional medicine, a major sociocultural heritage in Africa for several decades, was once believed to be primitive and challenged by foreign religion and orthodox or conventional medical practitioners (Elujoba, 2005). However, as a result of increasing chemotherapeutic failure in recent times, natural herbs are becoming of interest in providing remedy. Medicinal plants have posed to be the backbone of traditional medicine since the medicinal values lie in their bioactive phytochemical constituents which produce definite physiological effects on the human

body (Onyesom, 2021). *Phyllanthus amarus* has been shown to exhibit haem transition activities and protection against malarial-induced free radical generation and oxidative damage to the Red Blood Cells (RBCs), liver and brain by inhibiting plasmodial growth (Onyesom and Adu, 2015).

Phyllanthus amarus is a small, erect, perennial herbal plant having large numbers of phytochemicals that are attributed to its roots which is widely spread throughout the tropical and subtropical areas including Nigeria (Onyesom and Adu, 2015). It is one of the most important medicinal plants that had been used in medicine for over two thousand years (Ajala *et al.* 2011). The different plant parts are used to treat and cure various diseases and disorders in different places (Nchinda, 2007). *Phyllanthus amarus* is considered a good tonic, diuretic and antipyretic (Ameen *et al.,* 2021). In Southern Nigeria, the whole plant is washed and chewed for menstrual pain, stomach and toothache. Also, the ethanolic root extract is taken for the treatment of malaria (Green, 2007).

Phytochemicals are biologically active, naturally occurring chemical compounds found in plant, which provide health benefits for human as medicinal ingredients and nutrients (Hasler and Blumberg, 1999). The phytochemicals present in plants are responsible for preventing and treating several diseases and promoting health. The phytochemicals that have been found to be present in *Phyllanthus amarus* are alkaloids, saponins, flavonoids, tannins, glycoside, polyphenols and lignans (Faremi *et al.,* 2008). The antiplasmodial and haem transition activity of the tannin and glycoside extracts of *Phyllanthus amarus* has been consistently reported (Oyewole *et al.,* 2013). Therefore, these therapeutic actions of the secondary metabolites can be of great importance in the development of more effective drugs for malaria treatment.

In this study, the antiplasmodial and haem transition activities of tannin and glycoside fractions from *Phyllanthus amarus,* used in treating malaria were undertaken. In order to understand the concept of this investigation, *in vitro* antiplasmodial activity, host survival rate and haem transition activities of *Phyllanthus* amarus were studied.

Statement Of Problem

As a result of the chemotherapeutic failure in recent times, natural herbs are becoming of interest in providing remedy and cures. Several parts of the plant, *Phyllantus amarus,* has been investigated for its antimalarial activity. Though, there is little thesis of knowledge on the haem transition and antiplasmodial activity of its glycoside and tannin

fractions. This present study, surges to investigate the antiplasmodial activity and haem transition activity of the tannin and glycoside fractions from *Phyllanthus amarus*.

Aim:

The aim is to evaluate the antiplasmodial and haem transition activities of the tannin and glycoside fractions of *Phyllanthus amarus*.

Objectives: To:

i. Harness and validate the plant of interest – *Phyllanthus amarus* plant;

ii. Determine the presence of the phytochemicals (tannin and glycoside) in *Phyllanthus amarus*; and

iii. To prepare the tannin and glycoside extracts from the validated plant of study (*Phyllanthus amarus)*.

Hypothesis

The tannin and glycoside fractions of *Phyllanthus amarus* possess antiplasmodial and haem transition activity against malaria.

Significance of Study

Being one of the most important medicinal plants, *Phyllanthus amarus* has been used in particularly rural areas to treat and cure various diseases. the ethanol-water leaves extracts has shown to be scientifically efficacious in treating malaria. The phytochemicals of focused attention in this study are tannin and glycoside are under investigation for the antiplasmodial and haem transition activities in *Phyllanthus amarus*. The information obtained from this study will be of utmost importance in future drug design and as an information source for future research.

Justification of Study

Yearly, more research works are being done on attempting to pin-point a lifelong breakthrough on malaria due to the high morbidity and mortality mostly among pregnant women and young children. Also, it is frightening the degree at which *Plasmodium* parasites has developed resistance towards conventional drugs available which is why most individuals in developing countries use herbs (such as *Phyllanthus amarus*) in treating malaria. This study attempts to comprehend the antiplasmodial and haem transition activity of *Phyllanthus amarus* with anticipation of unraveling the mechanisms responsible for observed therapeutic activities which could be used in developing a malaria drug.

Literature Review

Malaria

Malaria presents a public health challenge. The etymology of the word 'Malaria; can be traced to an 18th century Italian origin; mala meaning 'bad' and aria meaning 'air'. It is a mosquito-borne infectious disease that affects humans and other animals. Malaria is one of the most life-threatening infectious diseases in the tropics. Malaria is endemic in all tropical and subtropical regions of the world. Malaria is associated with symptoms that typically include fever, vomiting and headaches, in some serious cases, it can cause seizures, coma or death (Caraballo and King, 2014). This disease can be caused by protozoan parasites: *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae, Plasmodium ovale* and *Plasmodium knowlesi* (Bethel *et al.,* 2019). Occasionally, Asians from Brunei, Darussalam, Cambodia, China, Indonesia, Lao People's Republic, Malaysia, Myanmar, Philippines, Singapore, Thailand and Vietnam become infected with *Plasmodium knowlesi* that normally infects other primates such as monkey. The symptoms of caused by the *Plasmodium knowlesi* may be a typical of malaria. *Plasmodium falciparum* is the most common specie identified (75%), followed by *Plasmodium vivax*. Although *Plasmodium falciparum* traditionally accounts for the majority of deaths (Senior, 2008). *Plasmodium falciparum* is the most prevalent malaria parasite in the WHO Africa region, accounting for 99.7% of estimated malaria cases in 2020 (WHO, 2020). Over 80% of malaria deaths occur in the sub-Saharan Africa, where the disease is estimated to kill one child every 30 seconds (WHO, 2020). Malaria is an acute febrile illness. In a non-immune individual, symptoms usually appear 10 – 15 days after the infective mosquito bite. The first symptoms – fever, headache, and chills – may be mild and difficult to recognize as malaria. If not treated with 24 hours, it can progress to severe illness, often to death. Children with malaria often develop severe anemia, respiratory distress in relation to metabolic acidosis. In adults, multi-organ failure is also frequent. The WHO (2020) documented that young children and pregnant woman are highly at risk to this disease which could be fatal to them due to their low resistance and weak defense mechanisms against the disease. In non-immune pregnant women have increased risk of maternal death, miscarriage, stillbirth and neonatal death.

Transmission

Malaria is transmitted through the bite of female *Anopheles* mosquitoes, which bite mainly between dusk till dawn. There are more than 400 different species

of *Anopheles* mosquitoes; around 30 are malaria vectors of major importance. All of the important vector species bite between dusk and dawn. The intensity of transmission depends on factors related to the parasite, the vector, the human host, and the environment. *Anopheles* mosquitoes lay their eggs in water, which hatch into larvae, eventually emerging as adult mosquitoes. The female mosquitoes seek a blood meal to nurture their eggs (WHO, 2020). Each species of *Anopheles* mosquito has its own preferred aquatic habitat; for example, some prefer small, shallow collections of fresh water, such as puddles and hoof prints, which are abundant during the rainy season in tropical countries.

Life Cycle of Malaria Parasite

Infection in human begins with the bite of an infected female Anopheles mosquito. The infective stage called sporozoites released from the salivary glands through the proboscis of the mosquito enter the bloodstream during feeding. The mosquito saliva contains antihemostatic and anti-inflammatory enzymes that disrupt blood clotting and inhibit the pain reaction. Typically, each infected bite contains 20-200 sporozoites (Garcia *et al.,* 2006). The immune system clears the sporozoites from the circulation within 30 minutes. But a few escapes and quickly invade liver cells (hepatocytes) (Gerald *et al.,* 2011). Entering the hepatocytes, the parasite loses its apical complex and surface coat, and transforms into a trophozoite. Within the parasitophorous vacuole of the hepatocyte, it undergoes 13-14 rounds of mitosis and meiosis which produce a syncytial cell (coenocyte) called a schizont (contains tens of thousands of nuclei). This process is called schizogony. From the surface of the schizont, tens of thousands of haploids (1n) daughter cells called merozoites emerge. The liver stage can produce up to 90,000 merozoites (Vaughan *et al*., 2017), which are eventually released into the bloodstream in parasite-filled vesicles called merosomes (Strum, 2006). Within the erythrocyte, the parasite metabolism depends on the digestion of hemoglobin. The parasite can also alter the morphology of the erythrocyte, causing knobs on the erythrocyte membrane. Infected erythrocytes are often sequestered in various human tissues or organs, such as the heart, liver and brain. After invading the erythrocyte, the parasite loses its specific invasion organelles (apical complex and surface coat) and de-differentiates into a round trophozoite (ring stage) located within a parasitophorous vacuole. At the schizont stage, the parasite replicates its DNA multiple times and multiple mitotic divisions occur asynchronously. Each schizont forms 16-18 memerozoites. The red blood cells are ruptured by the merozoites. The liberated merozoites invade fresh erythrocytes. A free merozoite is in the bloodstream for roughly

60 seconds before it enters another erythrocyte (Cowman *et al.*, 2012). The duration of each blood stage is approximately 48 hours. This gives rise to the characteristic clinical manifestations of falciparum malaria, such as fever and chills, corresponding to the synchronous rupture of the infected erythrocytes (Trampuz *et al.*, 2003). Not all of the merozoites divide into schizonts; some get differentiated into sexual forms, male and female gametocytes. These gametocytes take roughly 7–15 days to reach full maturity, through the process called gametocytogenesis. These gametocytes are taken up by a female Anopheles mosquito during a blood meal (Talman *et al.*, 2004).

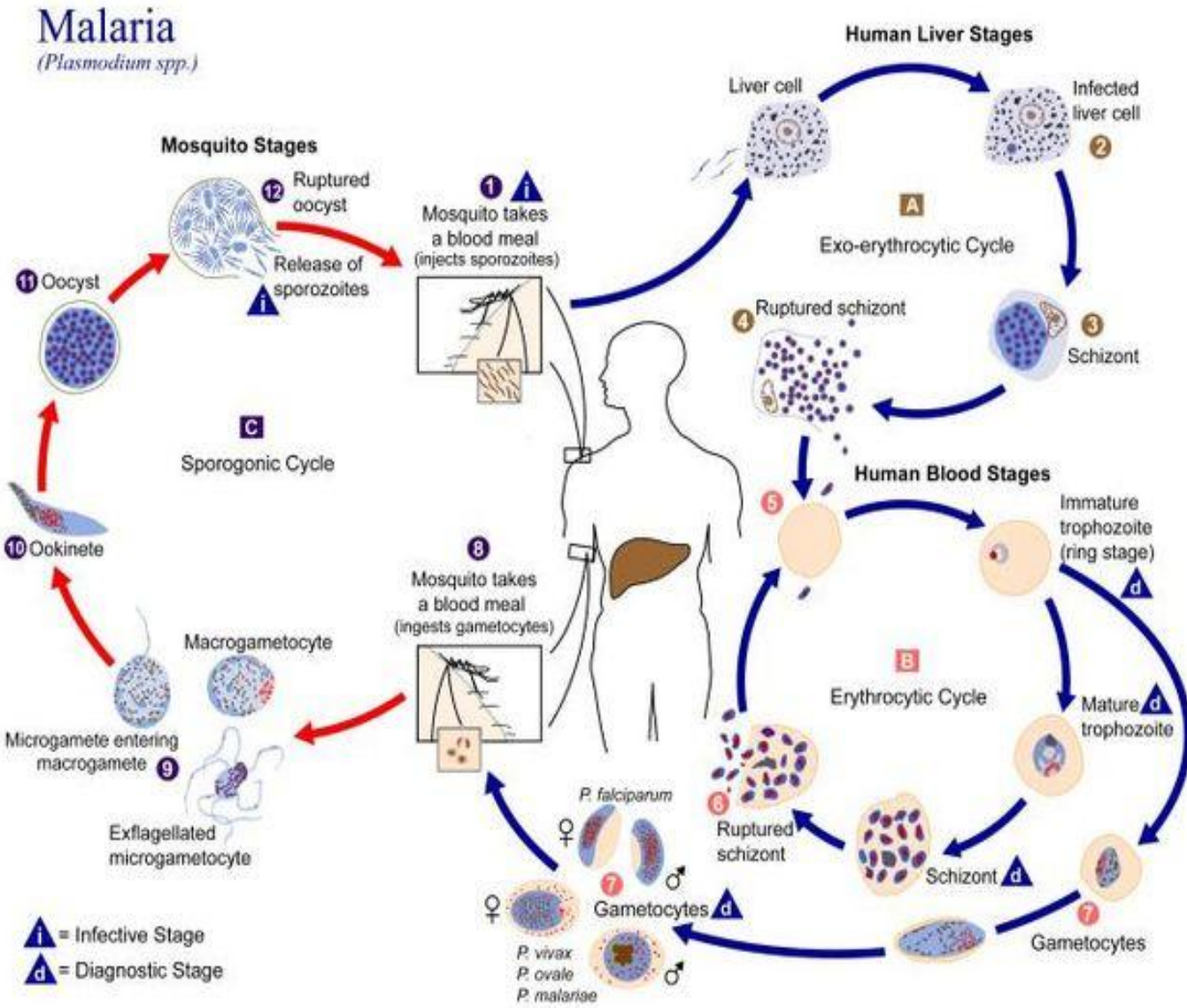

Fig 1: Diagram showing the life cycle of *Plasmodium falciparum*

Source: Adapted from: Centers for disease control and prevention, CDC. (https:// www.cdc.gov/malaria/about biology/index/html)

Accessed: November 1, 2021.

The metabolically active trophozoite digests large quantities of RBC haemoglobin (Sherman, 1998). Several enzymes are involved in this process, releasing ferroprotoporphyrin IX into the parasite's digestive vacuole (DV). Subsequently, this must be oxidized to ferriprotoporphyrin IX since the Fe in the crystalline malaria pigment (hemozoin) is indisputably in the ferric state based on spectroscopic evidence. Since parasites lack a haem oxygenase function, its disposal is primarily via sequestration as hemozoin (Sigala *et al.,* 2012), comprising cyclic μ-propionato dimers of haem (Fig. 1). While several antimalarials, including components of ACT, have been shown to inhibit the formation of synthetic hemozoin (β-hematin) (Pagola *et al.,* 2000), their mechanisms of drug action remain poorly understood (Egan *et al.,* 1994).

Disease Burden

Globally, malaria deaths have reduced steadily over the period 2000-2019, from 736,000 in 2000 to 409,000 in 2019. It has been attested by the World Health Organization (2019) report that the malaria problem has been of a huge burden in sub-Saharan Africa, predominantly Nigeria where the highest mortality (23%) due to malaria has been recorded. In other areas of the world, malaria causes substantial morbidity, especially in the rural areas of some countries in Asia and South America. In 2019, WHO (2020) documented an estimate 229 million cases and 409,000 deaths of malaria in 87 malaria-endemic countries with Nigeria contributing the highest of 27% cases. Due to the emergence of the COVID-19, the global progress of the eradication of malaria was stunted (WH0, 2020). Due to progressive evolution, in has been discovered that the vector (female *anopheles* mosquito) and parasite (*Plasmodium*) have developed certain resistance and defense mechanisms against available anti-malarial drugs and the vectors developing resistance against insecticides (WHO, 2020). Resistance to antimalarial medicines is a recurring problem. Resistance of *Plasmodium falciparum* malaria parasites to previous generations of medicines, such as chloroquine became widespread in the 1950s and 1960s, undermining malaria control efforts and reversing gains in child survival.

Diagnosis

In the laboratory, malaria is diagnosed using different techniques, e.g., conventional microscopic diagnosis by staining thin and thick peripheral blood smear (Ngasala *et al.*, 2008), Other concentration techniques, e.g., quantitative buffy coat (QBC) method and molecular diagnostic methods, such as polymerase chain reaction (PCR).

Malaria Parasite Resistance To Drugs

Drug resistance poses a growing problem in 21st-century malaria treatment (Sinha *et al.,* 2014). Resistance is now common against all classes of antimalarial drugs apart from artemisinins. Treatment of resistant strains became increasingly dependent on this class of drugs. The cost of artemisinins limits their use in the developing world (White *et al.,* 2008). Malaria strains found on the Cambodia–Thailand border are resistant to combination therapies that include artemisinins, and may, therefore, be untreatable (Wongsrichanalai *et al.,* 2008). Exposure of the parasite population to artemisinin monotherapies in subtherapeutic doses for over 30 years and the availability of substandard artemisinins likely drove the selection of the resistant phenotype (Dondorp *et al.,* 2010). Resistance to artemisinin has been detected in Cambodia, Myanmar, Thailand, and Vietnam, (WHO*,* 2013) and there has been emerging resistance in Laos (Ashley *et al.,* 2014).

Prevention and Treatment of Malaria

So far, vector control has been an effective way of preventing and reducing the malaria transmission. WHO recommends protection for all people at risk of malaria with effective malaria vector control. Two forms of vector control: insecticide-treated mosquito nets and indoor residual spraying (WHO, 2020). They are effective in a wide range of circumstances.

Mosquito nets help keep mosquitoes away from people and reduce infection rates and transmission of malaria. Nets are not a perfect barrier and are often treated with an insecticide designed to kill the mosquito before it has time to find a way past the net. Insecticide-treated nets (ITNs) are estimated to be twice as effective as untreated nets and offer greater than 70% protection compared with no net (Furnival-Adams, 2020).

There are a number of medications that can help prevent or interrupt malaria in travellers to places where infection is common. Many of these medications are also used in treatment. In places where Plasmodium is resistant to one or more medications, three medications— mefloquine, doxycycline, or the combination of atovaquone/proguanil (Malarone)—are frequently used for prevention.

Aboriginally, use of medicinal plants such as *Phyllanthus amarus* have been employed in the treatment of malaria.

Haem

Haem is a porphyrin ring complexed with ferrous iron and protoporphyrin IX. Haem is an essential prosthetic group in proteins that is necessary as a subcellular compartment to perform diverse biological functions like hemoglobin and myoglobin (Yuan *et al.*, 2016). Other enzymes which use haem as a prosthetic group includes cytochromes of the electron transport chain, catalase, and nitric oxide synthase. The major tissues for haem synthesis are bone marrow by erythrocytes and the liver by hepatocytes. Haem synthesis occurs in the cytosol and mitochondria; haem acquisition also occurs through intestinal absorption and intercellular transport. (Chung, 2012). Haem is a component of different biological structures mainly, hemoglobin, others include myoglobin, cytochromes, catalases, haem peroxidase, and endothelial nitric oxide synthase. There are different forms of biological haem. The most common type is haem b, found in hemoglobin leads to a derivative of other haem groups. Haem a exists in cytochrome a and haem c in cytochrome c; they are both involved in the process of oxidative phosphorylation.

The metabolically active trophozoite digests large quantities of hemoglobin (Sherman, 1998). Several enzymes are involved in this process, releasing ferroprotoporphyrin IX into the parasite's digestive vacuole (DV). Subsequently, this must be oxidized to ferriprotoporphyrin IX since the Fe in the crystalline malaria pigment (hemozoin) is indisputably in the ferric state based on spectroscopic evidence. Since parasites lack a haem oxygenase function, its disposal is primarily via sequestration as hemozoin (Sigala *et al.,* 2012), comprising cyclic μ-propionato dimers of haem. While several antimalarials, including components of ACT, have been shown to inhibit the formation of synthetic hemozoin (β-hematin) (Pagola *et al.,* 2000), their mechanisms of drug action remain poorly understood (Egan *et al.,* 1994).

Phyllanthus amarus and Its Description

Fig. 2.. *Phyllanthus amarus*

Source: http://www.naturelovesyou.sg/Phyllanthus%20amarus/Main.html

Accessed: October 29, 2021.

Taxonomic Classification of *Phyllanthus amarus*

Kingdom: Plantae

Division: Angiospermae

Class: Dicotyledoneae

Order: Tubiflorae

Family: Phyllantaceae

Genus: *Phyllanthus.*

Species: *Amarus*

Phyllanthus amarus is a member of the family Phyllanthaceae with about 800 species of which 200 are American, 100 African, 70 from Madagascar and the remaining Asian and Australasian (Neeraj *et al.*, 2003). It grows up to 10 – 60cm. Some common names of *Phyllanthus amarus* in North, Central and South America are black catnip, carry-me seed, egg woman, hurricane weed, quinine creole, quinine weed, seed-under-leaf, stone breaker

among others (Bello and Ibaba, 2020). In Nigeria, it is called "Oyomokeisoamankedem" in Efik, "Iyin Olobe" in Yoruba and "Ebebenizo" in Bini (Etta, 2008). Because of the phytochemicals in the plant extracts of *Phyllanthus amarus* target the biochemical pathway, it is now implying that herbal medicines are safer than synthetic medicines (Zaidan *et al.*, 2005).

Traditional Uses

Phyllanthus has been used in Ayurvedic medicine for over 2,000 years and has a wide number of traditional uses. This includes employing the whole plant for jaundice, gonorrhea, frequent menstruation and diabetes and using it topically as a poultice for skin ulcers, sores, swelling and itchiness. The plant is bitter, astringent, cooling, diuretic, stomachic, febrifuge and antiseptic. It is useful in dropsy, jaundice, diarrhoea, dysentery, intermittent fevers, diseases of urino-genital system, scabies ulcers and wounds. The young shoots of the plant are administered in the form of an infusion for the treatment of chronic dysentery. Its efficacy in the field of gastro intestinal disorders like dyspepsia, colic, diarrhoea, constipation and dysentery is undisputed. In females it is used as a galactogogue, in leucorrhoea, menorrhagia and mammary abscess. In skin conditions, especially scabby or crusty lesions, bruises, wounds, scabies, offensive ulcers and sores, oedematous swellings, tubercular ulcers and ringworm, it has been utilized with good effect since many years. It is applied effectively in intermittent fevers and gonorrhoea as well as in ophthalmia and conjunctivitis. It has a urolithic property, dissolving renal calculi. Also, used in cough, asthma and other bronchial affections. It's antifungal, antiviral and anticancerous properties have also been demonstrated in experimental animals. The powdered leaves of *Phyllanthus Amarus* (Bahupatra) were used in clinical studies evaluating its usefulness in patients suffering from chronic damage to the liver due to the protracted hepatitis B virus infection. This type of infection results in inability of the body's immune system to eliminate the virus from the liver cells. This condition is described as a carrier state, because a continuously harbors the virus. Some of the components of the virus detectable in the carrier state in the blood are: HBsAg or the surface antigen of the virus and HBeAg or the envelope antigen of the virus. In addition, the carrier state may be confirmed by the presence of antibodies directed against the core of the virus or the anti-HBc antibodies. The powdered leaves of *Phyllanthus amarus* were given in form of capsules to the patients with chronic viral hepatitis B in a dose of 200 mg three times a day for 30 days. *Phyllanthus amarus* treated patients tested negative for the viral antigen 15-20 days after the end of the

treatment. Due to its antiseptic, styptic, carminative, deobstruent, coolant, febrifugal, stomachic, astringent and diuretic properties of this plant it is very much utilized in traditional medicine.

Phytochemicals in *Phyllanthus amarus*

Phytochemicals are biologically active, naturally occurring chemical compounds found in plants, which provide health benefits for humans further than those attributed to macronutrient. These compounds are known as secondary plant metabolites and have biological properties such as antioxidant activity, antimicrobial effect, decrease of platelet aggregation and modulation of hormone metabolism and anticancer property (Kaur, 2021). The phytochemicals present in *Phyllanthus amarus* are alkaloids, flavonoids, tannins, polyphenols and lignans, and these phytocompounds have been linked to the chemoprotective and medicinal properties of the plant (Onyesom and Adu, 2015).

The quantitative and qualitative assessment of phytochemicals in methanolic extracts of *Phyllanthus amarus* has been demonstrated in addition to cytotoxicity and antiplasmodial activity of alkaloid extract prepared from eight African medicinal plants used in Nigeria (Onyesom *et al*., 2020).

Tannin

These phytochemicals are naturally occurring complex organic compounds possessing nitrogen free polyphenols of high molecular weight (Kaur, 2021). Tannins are used as astrigents, diuretics, anti-inflammatory, antiseptic, antioxidant and haemostatic pharmaceuticals (Dolara *et al.,* 2005).

Glycosides

They are secondary metabolites that comprise of a sugar portion that is linked to a non-sugar moiety. They can be activated by enzyme hydrolysis (Brito-Arias, 2007), which causes the sugar part to be broken off, making the chemical available for use, Glycoside play numerous roles in living organism including protective, regulatory and sanitary roles. They are used as medications (Gleadow and Moller, 2014).

Tannin and glycoside have been isolated from the *Phyllanthus amarus* specie and have demonstrated to show antiplasmodial and haem transition activities although the evidence remains rare. Thus, it is imperative to investigate the effects of *Phyllanthus amarus* ethanol-water extracts on *Plasmodium falciparum* parasite. This study therefore efforts to

assess the antiplasmodial and haem transition activities of tannin and glycoside fractions of *Phyllanthus amarus* on *Plasmodium falciparum* parasite.

MATERIALS AND METHODS

Materials

Phyllanthus amarus (dried leaves), RPMI 1640 medium (Sigma, USA). 96-well microliter plate (PTR-853 Techmel and Techmel, USA). Incubator (Buchi R210, Hana, China), 0.4% sodium bicarbonate (NaHC03) (Sigma, USA), 0.72% N-2hydroxyethylpiperazine-N-2 ethanesulfonic acid (HEPES) (Sigma, USA), Giemsa stain, Human ORh+ red blood cells, Choloquine, Spectrophotometer (Spec 20D, Techmel and Techmel, USA), Centrifuge (Cent 80D, Serico, China), Water bath (TW20, Seelbach, Germany), Analytical balance (JA3003; Citizen Electronics, Tanaka, Japan), Glasswares (Pyrex, MBL, Germany), Rotary evaporator (Buchi R210, Hana, China), Soxhlet apparatus (Corning, USA), Refrigerator (HTF319; Haier Thermocool, Japan), Ethanol (AnalaR Grade, BDH Chemicals, Poole, England).

Collection of plant material

Fresh mature whole plants of *Phyllanthus amarus* were collected from their natural habitat in Abraka community located in Ethiope East Local Government Area of Delta State Nigeria. The plants were collected cautiously to evade contamination with other plants. The leaves of the plants were taken, washed and air-dried for two weeks at a temperature of 28–32℃. The dried leaves where then grinded using laboratory blender (Nulek, Japan).

Methods

Qualitative screening of the phytochemical constituents of tannin and glycoside fractions of *Phyllanthus amarus*

The qualitative phytochemical screening of *Phyllanthus amarus* leaf fractions were carried out using standard procedures to determine the presence of tannin and glycoside.

Determination of Tannin: $FeCl_3$ was diluted with little amount of deionized distilled water in a test tube and mixed. Little amount of the powdered leaves were collected and added into another test tube. Then, 2ml of the diluted $FeCl_3$ was pipetted into the test tube

containing the grinded leaves and then mixed. After allowing to stand for a few minutes, an evident dark-green coloration depicts the presence of tannin.

Determination of Glycoside: Little amount of the powdered leaves were collected and added into a test tube. $FeCl_3$ was diluted with little amount of deionized distilled water in another test tube and mixed. Few drops of concentrated H_2SO_4 were added to the dilute $FeCl_3$ mixture. 1ml of the mixture was added to the test tube containing the grinded leaves and was mixed properly. After allowing to settle, a greenish-blue coloration depicts the presence of glycoside in the plant.

Preparation of Tannin extracts

The extraction of tannins was done using the old extraction process already described by Ukoha *et al.* (2011), although, with some modifications. About 100g of the dried, methanol and water (1:10 v/v) at 80℃ for 2 hours to obtain crude extract. The resultant mixture was filtered and the residue was re-extracted. Then, the methanol and water were removed using a rotatory evaporator to obtain the solid tannins.

Preparation of Glycoside Extract

Glycoside extracts were obtained from the three plants as described by Sharma *et al.* (2014), but with some alterations. About 100g of the powdered plants' leaves we're extracted with 80% methanol with stirring at room temperature and then, filtered to obtain the crude extracts. The filtrate was then distilled in methanol at 60°C using vacuum. The remaining aqueous extract was then extracted with n-hexane. The n-hexane fraction was thereafter distilled out under vacuum and remaining aqueous extract phase rich in mixture of glycosides was exchanged with *n*-butanol for three (3) times. The remaining *n*-butanol phase was distilled out under vacuum at 80°C. A brownish mass was obtained which was rich in glycosides. The glycoside-rich brownish mixture was further subjected to column chromatography and eluted with $CHCl_3$:CH_3OH:H_2O [65:40:12], and further with methanol to obtain the glycoside fraction.

Percentage Yield of Extract was calculated using the formula:

$$\text{Yield\%} = \frac{\text{Weight of dry e}}{\text{Weight of dry plant}} \times 100$$

Dilutions were prepared from each phytochemical extract as required for the study.

Investigation of antiplasmodial activity

Plasmodium falciparum culture was maintained according to the method described by Trager and Jensen (1976). Chloroquine sensitive *Plasmodium falciparum* 3D7 were maintained at 5% hematocrit (human type O- positive red blood cells) in complete RPMI 1640 (supplemented with 10% Albumax II, 50 μg mL^{-1} gentamycin, 1% L-glutamine) medium and kept in a 37°C incubator. *In vitro* antiplasmodial activity was determined by parasite growth inhibition assay as described by Uzuegbu *et al.* (2020), with some modifications. The *in vitro* antiplasmodial assay was performed in duplicates. The concentrations of standard drug (chloroquine 98% purity) were prepared by dissolving in DMSO. Concentrations of extracts and chloroquine used, were between the ranges 5.0-80.0μg/ml. Then, 96-well microtiter plates already containing multiple concentrations of the extracts were used to incubate the culture (parasitemia 1.5% and 3% hematocrit) for 48h at 37°C in CO_2 condition. Positive control wells were also prepared by incubating culture with different concentrations of chloroquine under the same conditions. Wells incubated, under the same conditions, without extracts or standard drug served as negative control. After incubation, the upper part of the suspension was removed and transferred to a clean microscopic slide to form a series of thick blood smears. The films were stained with 10% Giemsa stain (pH 7.3). The smear was then viewed under the microscope at 100× magnification. Parasites were counted in 10 microscopic fields and the mean calculated. The percentage parasite suppression was calculated using the formula of WHO (2001) and Ngemenya *et al.* (2006), as stated below:

$$\frac{\%\text{Parasitemia in negative control wells} - \%\text{Parasitemia in test wells}}{\%\text{ Parasitemia in negative control wells}} \times 100$$

Parasite growth inhibition curves were plotted to determine parasite growth suppression at 50% (IC_{50}) i.e., parasite suppression versus log concentration graphs.

Investigation of Haem transition activity

The haem transition activity was investigated based on the Basilico method with minor adjustment in the concentration of hematin solution and the sample used. The Basilico method is an *in vitro* spectrophotometric micro assay of haem transition. A 96-well Ubottomed microplates was used in this assay. The relative amounts of polymerized and unpolymerized hematin were determined using an ELISA reader. The final concentration of the extract samples ranged from 2 to 0.01 mg/ ml. A 100 μl of 1 mM hematin solution

was mixed with 0.2 M NaOH and 50 µl be of the test extract. A 50µl of glacial acetic acid solution (pH 2.6) was then added to this mixture. This test was carried out at 37°C for 24 h. The microtube was then centrifuged at 8000 rpm for 10 min, the sediment was then washed with 200 µl of DMSO three times at 8000 rpm for 10 min. The β-hematin crystalline precipitated was dissolved in 200 µl of NaOH 0.1 M to form alkaline hematin. A 100 µl of the alkaline hematin solution was transferred to 96-well microplates and the absorbance was read by ELISA reader at a wavelength of 405 nm. The effects of each test substance on β-hematin production were calculated and compared with negative controls.

Statistical Analysis

Level of significance was assessed by student's T - Test. A P-value less than 0.05 ($P < 0.05$) were considered statistically significant.

RESULTS

The results obtained from the determination of the antiplasmodial and haem transition activity of *Phyllanthus amarus* phytochemical (tannin and glycoside) extract are presented in Fig. 3 – 4 and Table 1 – 4. Data are represented as in triplicate determinations.

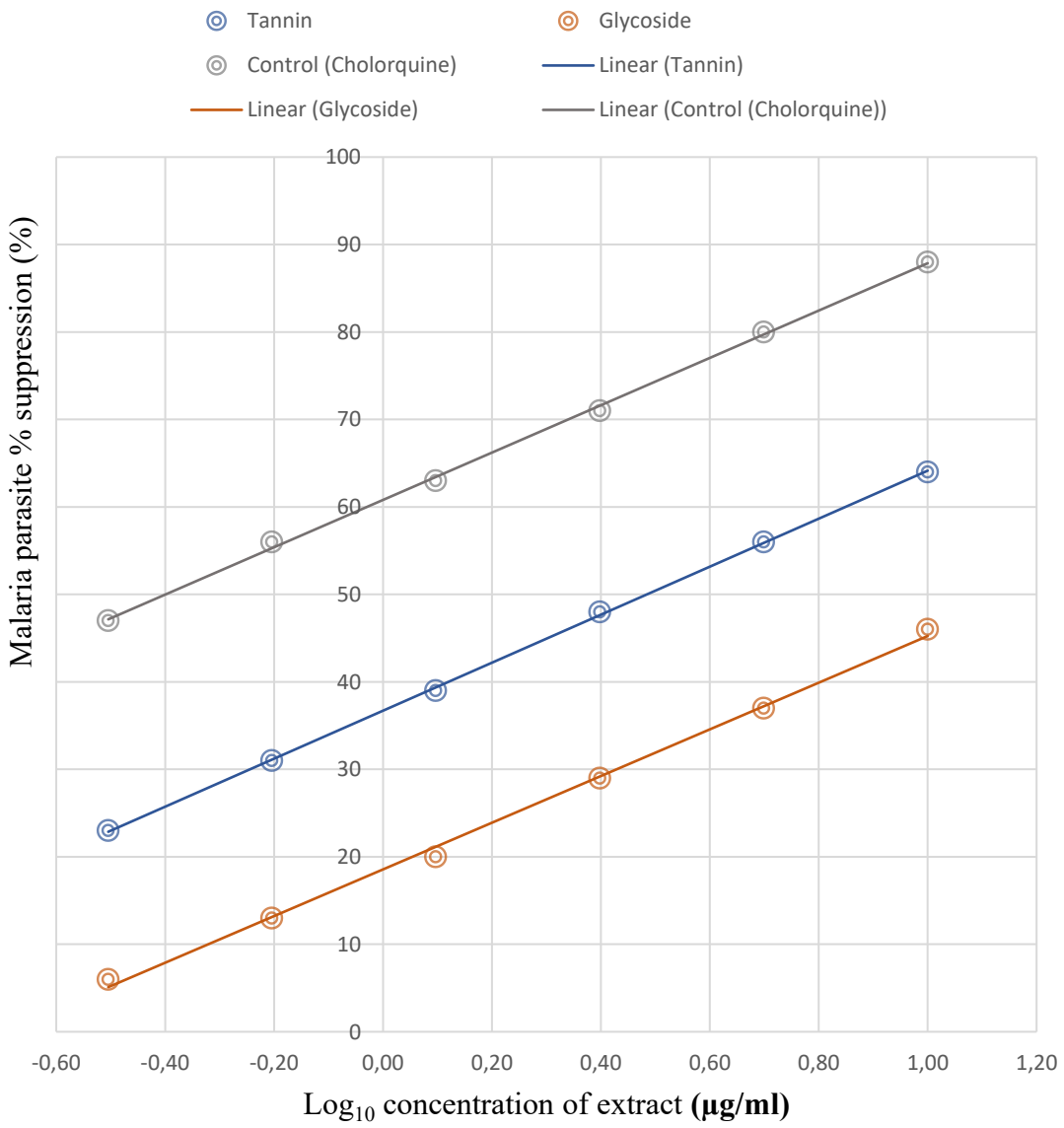

Fig. 3. Line graph for the plot of malaria parasite % suppression against concentration of the *Phyllanthus amarus* phytochemical extracts (Tannin and Glycoside).

Table 1: IC_{50} values obtained with control (chloroquine) and extracts (Tannin and Glycoside) of *Phyllanthus amarus*.

Phytochemical Extracts	**IC_{50} (µg/ml)**
Tannin	3.09
Glycoside	14.45
Control (Chloroquine)	0.41

IC_{50} (Inhibition Concentration) value of **≤ 5µg/ml** (very active), IC_{50} value of **≤ 10µg/ml** (moderately active) and an IC_{50} value of **> 10µg/ml** (inactive) (Ouattara *et al.*, 2014).

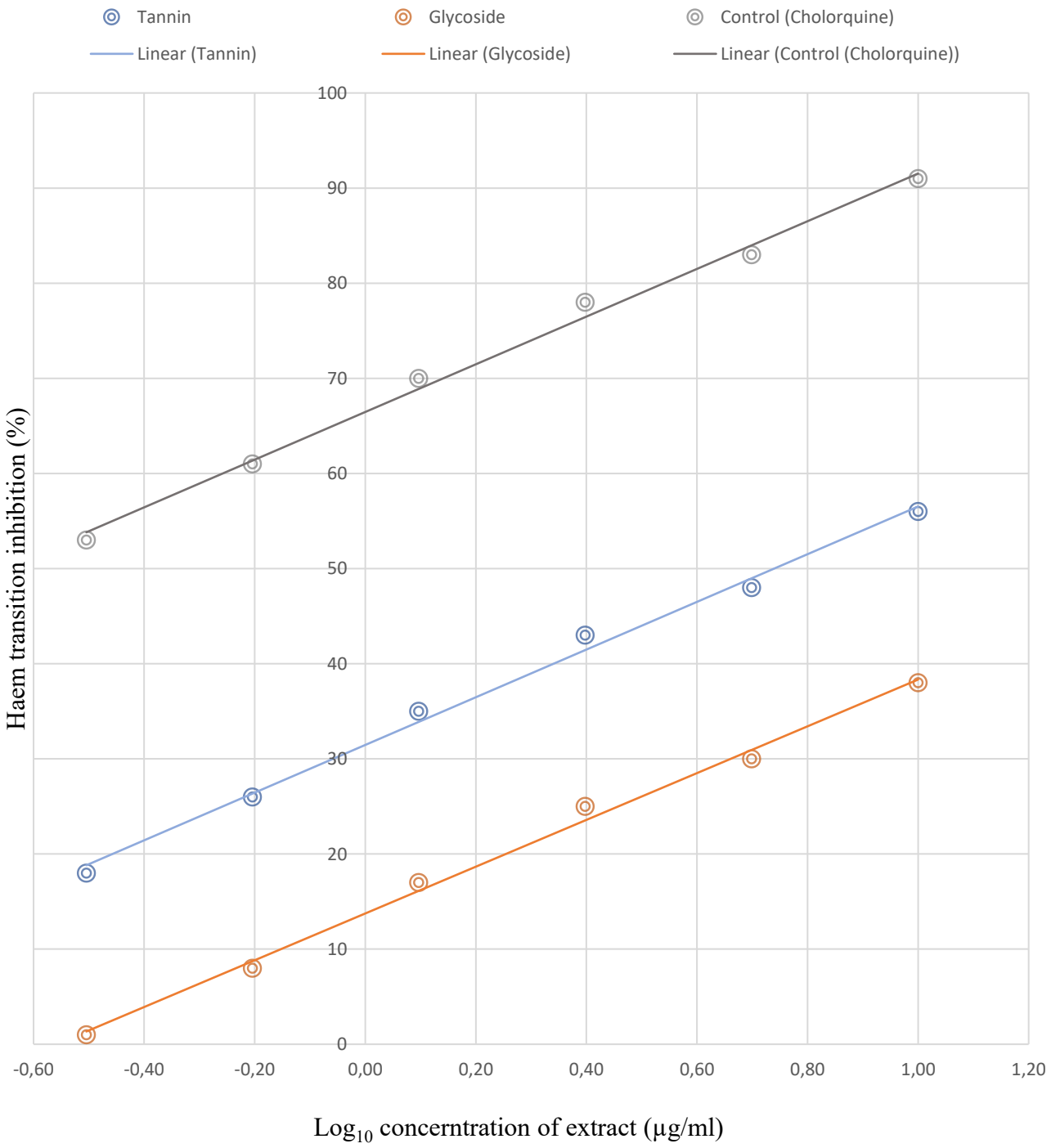

Fig. 4. Line graph for the Plot of Haem Transition Inhibition against Log_{10} Concentration of the *Phyllanthus amarus* phytochemical extracts (Tannin and Glycoside) and control (Chloroquine).

The IC_{50} values for haem transformation prevention activity induced by chloroquine and the extracts (tannin and glycoside) of *Phyllanthus amarus,* obtained from Figure 4 are given in Table 2.

Table 2: IC_{50} values obtained with standard chloroquine and extracts (tannin and glycoside) of *Phyllanthus amarus*

Phytochemical Extracts	**IC_{50} (µg/ml)**
Tannin	4.5
Glycoside	28.18
Control (Chloroquine)	0.25

Table 3. Malaria Parasite suppression induced by *Phyllanthus amarus* phytochemicals (Tannin and Glycoside) extracts.

		Malaria Parasite Suppression		
Concentration of Extract (µg/ml)	**Log_{10} Concentration of Extract (µg/ml)**	**Tannin**	**Glycoside**	**Chloroquine (Control)**
10.00	1.00	64	46	88
5.000	0.70	56	37	80
2.500	0.40	48	29	71
1.250	0.10	39	20	63
0.625	-0.20	31	13	56
0.313	-0.50	23	6	47

Table 4. Haem transition inhibition activities induced by *Phyllanthus amarus* phytochemicals (Tannin and Glycoside) extracts.

		Haem Transition Inhibition (%)		
Concentration of Extract (µg/ml)	**Log_{10} Concentration of Extract (µg/ml)**	**Tannin**	**Glycoside**	**Chloroquine (Control)**
10.00	1.00	56	38	91
5.000	0.70	48	30	83
2.500	0.40	43	25	78
1.250	0.10	35	17	70
0.625	-0.20	26	8	61
0.313	-0.50	18	1	53

Data from Table 1 shows that, Tannin extract of *Phyllanthus amarus* with IC_{50} value of 3.09 µg/ml possess very active antiplasmodial activity, while, glycoside extract with an IC_{50} of 14.45 µg/ml was inactive when compared to chloroquine with an IC_{50} of 0.41 µg/ml as judged by the suggestion of Ouattara *et al.* (2014).

Also, the control (chloroquine) showed excellent haem transition inhibition activity with an IC_{50} value of 0.25µg/ml from Table 2. However, both tannin and glycoside extracts of *Phyllanthus amarus* with an IC_{50} of 4.5 µg/ml and 28.18 µg/ml respectively, possessed no haem transformation inhibition activity which means they were inactive.

DISCUSSION

Malaria is a mosquito-borne infectious disease that affects humans and other animals (WHO, 2014). It is a dangerous parasitic disease caused by the intracellular, protozoan parasites of Plasmodium species that are transmitted to people through the bites of infected female *Anopheles* mosquitoes.

This present study evaluates the antiplasmodial and haem transition activity of tannin and glycoside extract of *Phyllanthus amarus* against malaria. The tannin and glycoside

extract of *Phyllanthus amarus* were tested for their *in vitro* antiplasmodial and haem transition activities by assessing their ability to inhibit haem formation and *Plasmodium falciparum* with chloroquine as the control.

The results obtained from this from this study presented that the tannin extract of *Phyllanthus amarus* showed very active inhibition of malaria parasitemia/antiplasmodial activity and the glycoside extract showed no active antiplasmodial activity.

IC_{50} (half maximal inhibitory concentration) which is the concentration of the extract or chemical compound required to produce 50% inhibition was extrapolated from a graph of malaria parasite % suppression against the logarithm of the concentrations used. The IC_{50} of the tannin and glycoside extracts of *Phyllanthus amarus* as seen in this study were 3.09 μg/ml and 14.45 μg/ml, respectively, while that of the control (Choloroquine) was 0.41 μg/ml (Table 4.1). Low values of IC_{50} usually correlates with high antiplasmodial activity of a compound. Based on reviewed literature, an IC_{50} greater than 10 μg/ml shows an inactive compound. An IC_{50} between 5 μg/ml and is moderately active, while an IC_{50} less than 5 μg/ml is very active (Ouattara et al., 2014). Hence, the tannin extract of *Phyllanthus amarus* was very active against Plasmodium falciparum, the glycoside extract was inactive, while the control (Chloroquine) was very active against *Plasmodium falciparum* as would be expected. Although, tannins are not particularly popular for having antimalarial activities, results from this study show that they possess good activity against malaria and could be further exploited for treatment of malaria.

From the analysis, it was also obtained that both extracts of *Phyllanthus amarus* showed no active inhibition of haem polymerization/transition activity. This suggests that tannin and glycoside can only interfere with the initial formation of haem oligomers when haem molecules are still in solution. Under these experimental conditions, addition of antimalarial compounds after the start of the reaction could not efficiently terminate chain extension (Basilico *et al*., 1998).

CONCLUSION

On the whole, the tannin extract of *Phyllathus amarus* showed high antiplasmodial activity, while the glycoside extract was inactive against *Plasmodium falciparum*. Both the tannin and glycoside extract of *Phyllanthus amarus* showed no inhibition of haem

formation/transition/polymerization. Also, this study does not support the further study of glycosides from *Phyllanthus amarus* as antimalarial agent.

Recommendation

Elaborate research should be conducted to investigate the antiplasmodial and haem transition activities of other phytochemicals from this plant in order to determine which active compounds are responsible for the known antimalarial activity of the plant.

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