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Phyllanthus amarus possesses malarial curative and pancreatic tonic potentials in experimental mice

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ABSTRACT

Phyllanthus amarus has been observed to be protective against malarial-induced free radical generation and oxidative damage to the RBCs, liver and brain by inhibiting plasmodial growth. In this study, the curative and pancreatic tonic potentials of P. amarus in experimental mice were undertaken by assessing antiplasmodial activity and associated mice survival rate (curative), and pancreatic antioxidant capacity, oxidative damage, beta-cell function and microstructural changes (tonic). Results show that P.berghei malarial parasite infection caused oxidative damage to the pancreas. P. amarus malarial curative ability compared well with the standard chloroquine treatment. It significantly (P<0.05) enhanced pancreatic antioxidant defense capacity and beta-cell function but reduced oxidative damage when compared with the control, malarial infected and chloroquine-treated groups. P. amarus also invigorated the pancreas of the malarial infected mice as evidenced by the microstructural analysis. P. amarus malarial curative ability is associated with pancreatic cell replenishment.

Key words: *Phyllanthus amarus, Plasmodium berghei,* Malaria, Chloroquine, Pancreas

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INTRODUCTION

Phyllanthus amarus Schum and Thonn (Euphorbiaceae) is a perennial herbal plant which is widely spread throughout the tropical and subtropical areas including Nigeria. It is one of the most important medicinal plants that had been used in the Indian system of medicine for over two thousand years [1]. The different plant parts are used to treat and cure various diseases and disorders in different places [2, 3, 4, 5]. 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 [6]. The antiplasmodial activity of *P. amarus* has been consistently reported [1, 7, 8, 9]. The aqueous extract of the leaves and stems have been reported to possess a dose-dependent schizonticidal activity on *P. berghei* when administered to mice in both early and established infections [10].

The use of *P. amarus* herbal plant in traditional medicine for the treatment of various ailments particularly malarial infection is becoming increasingly popular especially in rural areas of Nigeria because of the following reasons: poverty, high cost of the approved standard drug, adulteration and chemotherapeutic failure of commonly used drugs, and lack of access to good medical facilities [11]. The phytochemicals present in *P. amarus* are alkaloids, flavonoids, hydrolysable tannins, polyphenols and lignans, and these phytocompounds have been linked to the chemoprotective and medicinal properties of the plant [7, 8, 12].

Malaria presents a public health challenge. About 300-500 million people experience clinical episodes and 1.4 – 2 million deaths occur annually [13]. It was reported that in 2010 there were 219 million cases of malaria worldwide and 660 thousand associated deaths especially in very young African children [14]. In Nigeria, malaria accounts for 30%- 50% morbidity and 25% mortality in infants [11, 15].

Malarial infection induces oxidative stress by the generation of free radicals. Recent studies suggest that the generation of reactive oxygen species associated with oxidative stress, plays a crucial role in the development of systemic complications caused by malaria. Malarial infection induces the generation of free radicals in the liver, which most probably is the main reason for the induction of oxidative stress and apoptosis [16]. Additionally, Atamna*et al*. [17] observed that erythrocytes infected with *Plasmodium falciparum* produced free radicals about twice as much compared to normal erythrocytes. A potential source of free radical generation in malarial infection is the host's haemoglobin molecule. The parasite uses this molecule as a source of amino acid for its own nutrition during the erythrocytic stage of the infection. This utilization results in the liberation of large amounts of circulating heme which are able to induce intravascular oxidative stress, causing changes in erythrocytes and endothelial cells, and facilitating the internalization of the parasite in tissues such as the liver and brain [18].

Malarial-induced remodeling and parasite internalization in liver and hepatic tissues have been reported[19]. This parasite sequestration has been associated with enhanced oxidative stress in RBCs, liver, brain [19] and kidney [8]. Whether such stress extends to the pancreas is not clearly known.

In this study therefore, the *Plasmodium berghei* malarial parasite-induced oxidative stress in the pancreas of experimental mice and the role of *Phyllanthus amarus* extract were undertaken. In order to understand the concept of this investigation, the malarial curative (evaluated by *in vivo* antiplasmodial activity and mice survival rate) and pancreatic tonic (assessed by the pancreatic antioxidant capacity, beta cell function and microstructural changes) potentials of *P. amarus* were studied.

EXPERIMENTAL SECTION

2.1 Harvesting and preparation of plant extract

Fresh leaves of *Phyllanthus amarus* were harvested from an uncultivated farmland in Abraka community in Ethiope East Local Government Area of Delta State, Nigeria. The leaves were identified to the species level at the Forest Research Institute of Nigeria, Ibadan, where a voucher specimen (NO: FHI 109728) has been deposited.

The leaves were washed, air-dried and ground using a laboratory mill (Kenwood Ltd, Hertfordshire, UK) to produce a fine powder which was extracted sequentially with ethanol (Analar Grade, BDH Chemicals, Poole, England) using a soxhlet apparatus (Corning, USA). The extract was evaporated to dryness using rotary evaporator (Buchi R-210, Hana, China) under reduced pressure. The percentage yield was 3.6%. The volume administered (i.e. equivalent dose studied) was then calculated thus:

V (ml) = D x P/ C. D= Dose studied (g/kg b.wt), P= Body weight (kg), C= Concentration of the extract (g/ml), V= Volume of extract (ml) administered.

2.2 Experimental Mice

The mice used in this study were Swiss Albino mice of mixed sexes weighing between 0.022kg (22g) to 0.027kg (27g). They were maintained at the Laboratory Animal Centre of the Faculty of Basic Medical Sciences, Delta State University, Abraka, Nigeria.

2.3 Animal Care and Handling/Ethical Approval

The selected mice were kept in plastic cages under controlled condition of 12hr light/12hr dark cycle and allowed access to standard mouse feed and water. The mice were fed on growers' mash (Top Feeds Flour Mill Limited, Sapele, Delta State, Nigeria) and given clean drinking water in bottles *ad libitum.* The animals were maintained in accordance with the guidelines approved by the Animal Ethics Committee of the Faculty of Basic Medical Sciences, Delta State University, Abraka, Nigeria, who also approved the study.

2.4 Animal Grouping, Innoculation and Extract Administration

Forty-five (45) Swiss Albino mice of mixed sexes were divided into nine (9) groups of five (5) mice each. Group 1 was uninfected but given placebo-normal saline (normal control), Group 2 was infected with *P. berghei and* also treated with same placebo (malaria control), Group 3 was infected with *P. berghei and* treated with 100mg/kg/d of *Phyllanthus amarus* ethanolic leaf extract, Group 4 was infected with *P. berghei and* treated with 200mg/kg/d of the *P. amarus* ethanolic leaf extract, Group 5 was infected with *P. berghei and* treated with 300mg/kg/d of the *P. amarus* ethanolic leaf extract, Group 6 was infected with *P. berghei and* treated with 5mg/kg of chloroquine (standard control), Group 7 was uninfected but treated with 100mg/kg/d of *P. amarus* ethanolic leaf extract, Group 8 was uninfected but treated with 200mg/kg/d of *P. amarus* leaf extract, and Group 9 was uninfected but treated with 300mg/kg/d of *P. amarus* ethanolic leaf extract.

The mice were infected by obtaining a parasitized blood (3-4 drops) from the cut tail tip of an infected (donor) mouse. Then, 0.1ml of infected blood was diluted in 0.9ml of phosphate buffer, pH 7.2. The mice were inoculated with 0.1ml of the parasitized suspension which contained about 12,000 parasites. Parasitaemia was done using thin blood films from cut tip of the infected mice's tail and stained with Giemsa stain, then viewed under the microscope (TH-9845, Serico, China) at x100 magnification. The percentage parasitaemia was determined by counting the number of parasitized red blood cells out of 1,000 blood cells in ten randomly selected microscopic fields. The percentage suppression of parasitaemia was calculated thus:

$$\text{Suppression (\%)} = \frac{\text{mean parasitaemia in untreated} - \text{mean parasitaemia in treated}}{\text{mean parasitaemia in untreated}} \text{x } 100 \text{ [7]}$$

The *Phyllanthus amarus* ethanolic leaf extract and chloroquine doses were administered as single daily dose via oral route using intragastric canula for a period of seven (7) days.

2.5 Animal Sacrifice and Collection of Specimen

On day seven (7) of the experiment, the mice were fasted overnight and sacrificed the next morning in chloroform saturated chamber (anaesthesia). Whole blood was collected by heart puncture and centrifuged (Centrifuge 80D, Serico, USA) to obtain serum which was used for the analysis of glucose and insulin, and hence, beta-cell function. The beta-cell function was calculated using homeostasis model assessment-beta (HOMA-β)

$$\text{HOMA-}\beta = \frac{360 \text{ x Insulin } [\mu\text{U/ml}]}{\text{Glucose } [\text{mg/dl}] - 63} \text{ [20]}$$

The mice were dissected and the pancreas from each mouse was carefully excised, rinsed in cold normal saline and then preserved in 10% formalin for microstructural examination. Also, part of the excised pancreas was homogenized and used for the assay of SOD, CAT, GPx, GSH and MDA.

2.6 Analysis of Specimen

The biochemical investigation was carried out to determine the activities of antioxidant enzymes, associated lipid peroxidation index and beta-cell function in the pancreas of *Plasmodium berghei* infected mice treated with *Phyllanthus amarus*.

Glutathione peroxidase (GPx) was determined using the method described by Moron *et al* [21], malondialdehyde (MDA) was determined using the method described by Ohkawa*et al* [22], reduced glutathione (GSH) was determined using the Ellmans method [23], superoxide dismutase (SOD) was assayed using the method described by Misra and Fridovich [24], and catalase (CAT) was determined using colometric method [25]. All the chemicals used were of Analar grades, supplied by BDH Chemicals, Poole, England.

2.7Histopathological Examination

The impact of *Phyllanthus amarus* ethanolic leaf extract on pancreatic tissue architecture of the treated mice was evaluated by histopathological examination of tissue sections. The isolated organ was fixed in 10% formalin for a period of twenty-four hours before the examination. Thereafter, the tissue was solidified in paraffin wax and sectioned using a microtome (HM340E, Leica, Germany). The tissue sections were then fixed on slides with haemotoxylin and eosin (H & E) stains. The stained slides were mounted with Canada balsam (Sigma, USA), allowed to dry and viewed under a binocular microscope (DM1000, Leica, Germany) connected to a computer (HP 655, USA).

2.8 Statistical Analysis

The results were expressed as Means ± Standard Deviation. Level of significance was assessed by one-way Analysis of Variance (ANOVA). A *P*-value less than 0.05 ($P < 0.05$) were considered statistically significant.

RESULTS

The results obtained from the investigation into the malarial curative and pancreatic tonic potentials of *Phyllanthus amarus* are shown in Tables 1 and 2. Histological studies on the pancreas are shown in Figures 1-9.

From Table 1, the results indicate that *P. amarus* ethanolic leaf extract showed a chemo-suppression effect comparable with the standard chloroquine drug and higher when given at 300mg/kg. The post treatment survival rate of the mice was dose-dependent. This implies that *P. amarus* can be used in the cure of malarial especially at 300mg/kg/d.

Table 1: *Phyllanthus amarus*-induced plasmodial chemosuppression and associated malarial curative potential in experimental mice

	In vivo Antiplasmodial Activity	Malarial Curative Potential of *P. amarus*	
	Plasmodial chemosuppression (%)	Post treatment survival rate (%) of mice	
		14 days	28 days
1	-	100	80
2	0.0	0	0
3	52.3±3.6	60	40
4	68.6±4.9	80	60
5	81.3±5.2	100	80
6	77.5±4.6	80	60
7	-	100	80
8	-	100	80
9	-	100	100

1= Normal control (uninfected and untreated); 2= Malarial control (infected with P. berghei but untreated); 3= infected and treated with 100mg/kg of P. amarus extract; 4= infected and treated with 200mg/kg of P. amarus extract;5= infected and treated with 300mg/kg of P. amarus extract; 6= Standard control (infected and treated with 5mg/kg of chloroquine; 7= uninfected but treated with 100mg/kg of P. amarus extract; 8= uninfected but treated with 200mg/kg of P. amarus extract; 9= uninfected but treated with 300mg/kg of P. amarus extract.

Table 2: Antioxidant defense capacity and associated pancreatic β-cell function in *P. berghei* malarial parasite infected mice treated with *Phyllanthus amarus*

Groups	SOD (µM/Min)	CAT (µM/Min)	GPx (µM/Min)	GSH (µM)	MDA (µM)	β-Cell function (%)
1.	0.82 ± 0.44^{a}	13.30 ± 1.57^{a}	5.18 ± 2.28^{a}	10.32 ± 4.49^{a}	27.18 ± 11.28^{a}	58.3 ± 5.8^{a}
2.	0.07 ± 0.02^{b}	12.52 ± 1.74^{a}	0.46 ± 0.26^{b}	0.60 ± 0.26^{b}	59.94 ± 4.17^{b}	1060.4 ± 32.6^{b}
3.	1.52 ± 0.13^{c}	16.12 ± 0.82^{b}	8.60 ± 0.37^{c}	17.58 ± 1.24^{c}	0.76 ± 0.37^{c}	117.3 ± 10.9^{c}
4.	1.56 ± 0.28^{c}	18.14 ± 0.78^{b}	9.30 ± 0.29^{c}	18.54 ± 0.59^{c}	0.58 ± 0.95^{c}	103.0 ± 15.1^{c}
5.	1.65 ± 0.10^{c}	19.34 ± 2.12^{b}	8.64 ± 0.13^{c}	18.10 ± 1.57^{c}	0.76 ± 0.19^{c}	80.2 ± 7.3^{d}
6.	0.12 ± 0.02^{b}	12.96 ± 1.50^{a}	6.22 ± 1.99^{c}	13.78 ± 2.29^{a}	60.92 ± 4.19^{b}	517.0 ± 20.4^{b}
7.	1.31 ± 0.03^{c}	18.62 ± 2.06^{b}	8.92 ± 0.44^{c}	17.28 ± 0.81^{c}	0.77 ± 0.18^{c}	92.6 ± 16.3^{a}
8.	1.65 ± 0.24^{c}	17.98 ± 2.37^{b}	9.80 ± 1.80^{c}	19.40 ± 2.13^{c}	0.88 ± 0.17^{c}	106.0 ± 14.6^{c}
9.	1.81 ± 0.11^{c}	23.09 ± 2.16^{c}	15.86 ± 3.11^{d}	25.74 ± 4.69^{d}	0.61 ± 0.05^{c}	76.6 ± 10.4^{d}

Values are expressed as Means ± SD, n=5.
Values that bear another superscript on a column differ significantly (P<0.05)
1= Normal control (uninfected and untreated)
2= Malarial control (infected with P. berghei but untreated)
3= Experimental (infected and treated with 100mg/kg of P. amarus extract)
4= Experimental (infected and treated with 200mg/kg of P. amarus extract)
5= Experimental (infected and treated with 300mg/kg of P. amarus extract)
6= Standard control (infected and treated with 5mg/kg of chloroquine)
7= Experimental (uninfected but treated with 100mg/kg of P. amarus extract)
8= Experimental (uninfected but treated with 200mg/kg of P. amarus extract)
9= Experimental (uninfected but treated with 300mg/kg of P. amarus extract)
SOD= Superoxide dismutase, CAT= Catalase, GPx= Glutathione peroxide, GSH= Reduced glutathione, MDA= Malondialdehyde, β-cell= Pancreatic beta cell

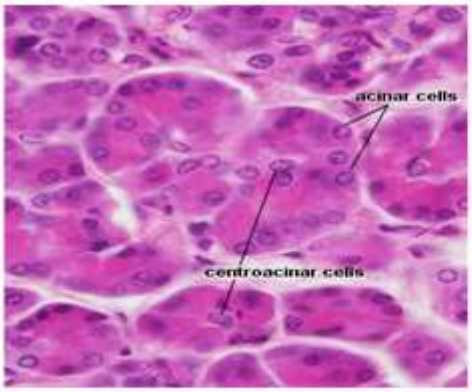

Fig.1: Microstructure of the pancreas for the control (Group 1) mice. Features show the histology of normal pancreatic cells. Microscopic magnification=x100 (H & E staining).

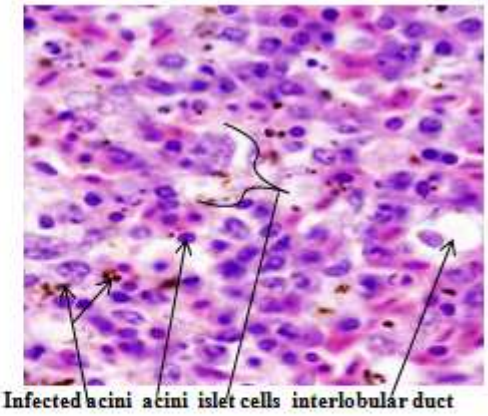

Fig.2: Histological appearance of the pancreas for the P. berghei (Group 2) mice. Pathological features show acute inflammation of the acini cells, the islet cells and the interlobular duct. Microscopic magnification=x100 (H & E staining)

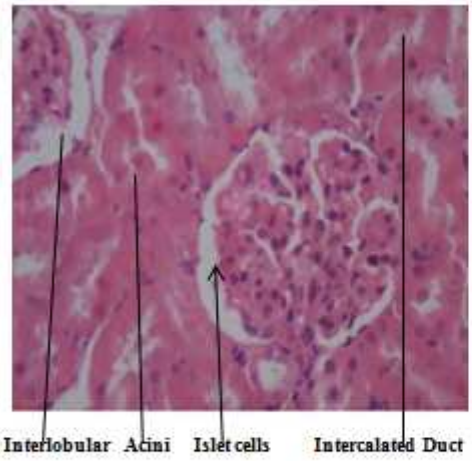

Fig.3: Features show histology of pancreatic cells for mice infected with P. berghei and treated with 100 mg/kg/d of P. amarus for 7 days (Group 3). Acini cells, islet cells and the interlobular duct show very mild inflammation. Microscopic magnification= x100 (H & E staining)

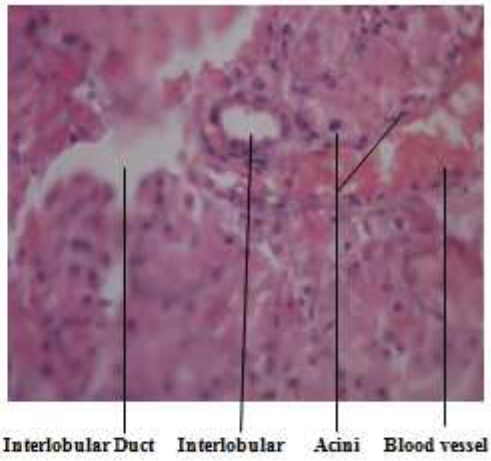

Fig.4: Features show the histology of pancreatic cells for mice infected with P. berghei and treated with 200 mg/kg/d of P. amarus for 7 days (Group 4). The pancreatic cells are recovering but not fully. Microscopic magnification= x100 (H & E staining)

From Table 2, it can be seen that the levels of antioxidant enzymes for the parasitized untreated mice (Group 2) reduced significantly ($P<0.05$), while the lipid peroxidation (MDA) of same group increased significantly ($P<0.05$) when compared with the treated groups. Antioxidant depletion by *P. berghei* in the pancreas was restored by treatment with *P. amarus*. Also, it is shown that there is elevated activity of β-cell function in both the parasitized untreated group and the chloroquine treated group when compared with the *P. amarus* treated groups.

The histopathological micrographs show that *P. berghei* malarial infection damaged the microstructural features of the pancreas. Treatment with *P. amarus* defended and replenished the tissues better than the chloroquine treatment. The 300mg/kg dose invigorated the pancreatic cells.

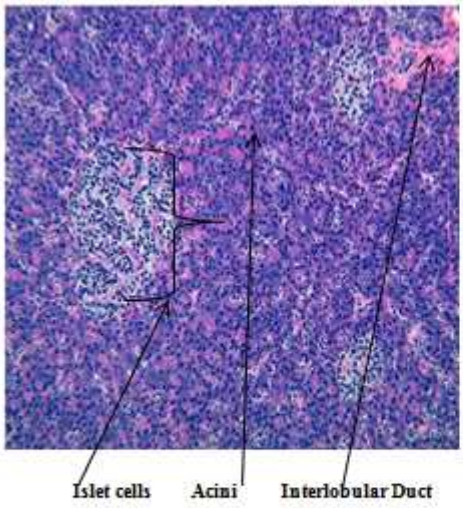

Fig 5: Features show the histology of pancreatic cells for mice infected with P. berghei and treated with 300 mg/kg/d of P. amarus (Group 5). Pancreatic cells show full recovery to normal. Microscopic magnification= x100 (H & E staining).

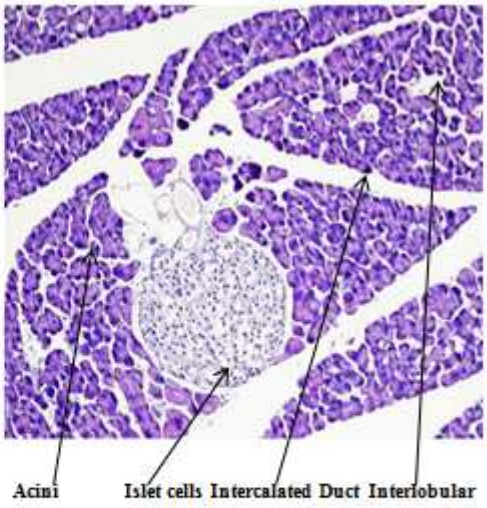

Fig. 6: Features show the histology of pancreatic cells infected with P. berghei and treated with 5mg/kgld of chloroquine for 7 days (Group 6). Cells are recovering but not as healthy as for the mice treated with P. amarus. Microscopic magnification= x100 (H & E staining).

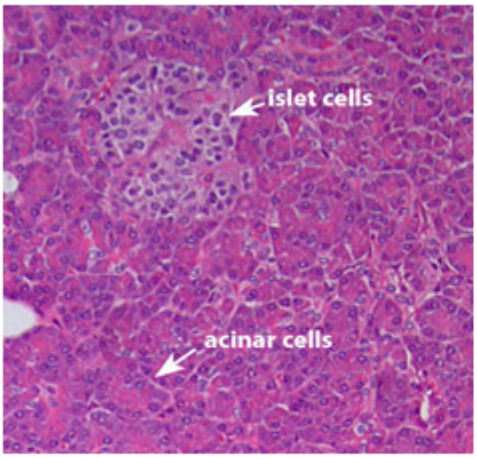

Fig 7: Histology of pancreatic cells from mice not infected with P. berghei but administered with 100mg/kg/d of P. amarus for 7 days (Group 7). Features show no difference to the control (Group 1). Microscopic magnification=x100 (H & E staining)

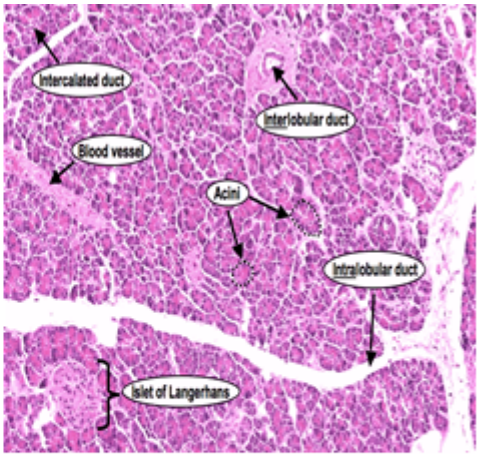

Fig 8: Histology of pancreatic cells from mice not infected with P. berghei but administered with 200mg/kg/d of P. amarus for 7 days (Group 8). The acini cells, islet cells, intralobuler and intercalated ducts appear replenished compared with the control features. Microscopic magnification= x100 (H & E staining).

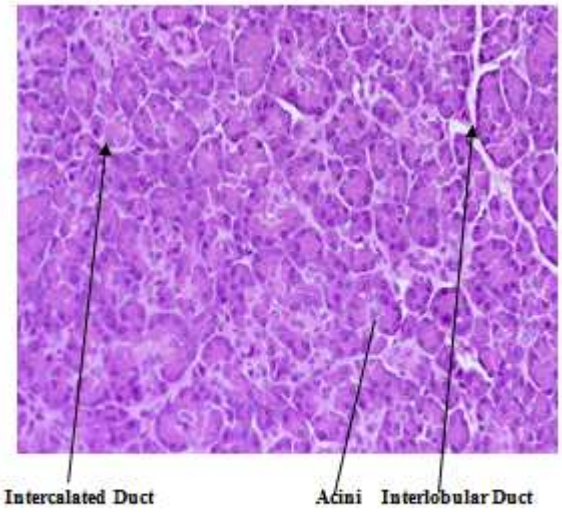

Fig.9: Histology of pancreatic cells from mice not infected with P. berghei but administered with 300mg/kg/d of P. amarus for 7 days (Group 9). This dose of P. amarus invigorated the cells. Microscopic magnification=x100. (H & E staining)

DISCUSSION

Malarial is a debilitating infection to especially humans. The infection is associated with generation of large amounts of free radicals or reactive oxygen species which cause oxidative damage in tissues such as the RBCs, liver and brain [8, 26]. Furthermore, this investigation extends the malarial-induced oxidative damage to the cells of the pancreas (Table 2, Fig. 2). Effective treatment and cure of malaria is currently challenged by proper vector (mosquito) control, lack of vaccine, high cost and non-availability of recommended (ACT) drugs, poverty, parasite resistance to cheaper and available chemotherapeutic agents and their increasing level of adulteration. So, the use of herbal medication is high in rural areas in especially sub-Saharan Africa including Nigeria. *Phyllanthus amarus* is one of such common traditional herb.

The antimalarial activity of *P. amarus* has been reported [1, 7, 8, 9]. While confirming the antiplasmodial property of the ethanolic leaf extract of *P. amarus*, we have further demonstrated the malarial curative ability of the plant (Table 1). The 300mg/kg *P. amarus* treatment displayed higher antimalarial and curative ability when compared with the standard chloroquine treatment. *P. amarus* has been shown to preserve renal function [19] and to be hepatoprotective [27]. We have in addition observed in this study that the ethanolic leaf extract of *P. amarus* enhanced pancreatic antioxidant defence capacity (Table 2) and replenished pancreatic cells (Figures 1-9). These observations were found to improve pancreatic beta cell function (Table 2). Although chloroquine treatment possessed antimalarial effect, it does not however significantly defend the pancreas from oxidative damage, study suggests (Table 2, Fig. 6).

The phytochemicals found present in the leaf extract of *Phyllanthus amarus* include saponins, tannins, flavonoids, glycosides, terpenes and alkaloids [28].

The malarial curative and plasmodial chemo-suppressive effect (Table 1) of the *P. amarus* plant extract might be due to some identified phytochemicals in the plant. This suggestion agrees with earlier reports on the antiplasmodial activity of the plant [1, 7, 8, 9].Alkaloids, saponins and flavonoids are suggested as being responsible for the antimalarial activities of the plant [29]. It has been shown to mediate its antimalarial potency by blocking protein synthesis in *Plasmodium falciparum* [30]. Triterpenoids and saponins have been found to be detrimental to several infectious protozoans such as *Plasmodium falciparum* [31]. Flavonoids are known to chelate nucleic acid base pairing of malaria parasites [32]. Flavonoids and tannins are phenolic compounds, and plant phenolics are group of major compounds that act as primary antioxidants or free radical scavengers [33]. Tannins and saponins have been found to be effective antioxidants and antimicrobial agents [34].

The observed antioxidant boost and associated reduced malondialdehyde levels (oxidative stress) following treatment with *P. amarus*(Table 2) could be due to the antioxidant phytochemicals previously identified [28, 35, 36]. The antioxidants defence, protected and invigorated pancreatic cells and enhanced beta cell function. This finding agrees with earlier report on the antioxidant activity of *Phyllanthus amarus* [37].

The elevated levels of HOMA-β observed in the chloroquine treated and the *P. berghei* infected groups could be due to hyperactivity of insulin secretion by the beta cells possibly caused by damage to the pancreatic cells by *P. berghei*[38]. As discussed above, antioxidant activities of *P. amarus* averted the damage on the pancreatic cells caused by *P. berghei* infection thus, improved β-cell function was observed following treatment with *P. amarus* extract.

Histological examination of pancreas isolated from the *P. berghei* infected mice without treatment revealed acute inflammation of the acini cells, islet cells and interlobular duct (Fig.2). In the *P. amarus* treated groups without infection, the architecture of the pancreas appeared intact and invigorated (Figs.7-9). Inflammation of the pancreatic cells as seen in the*P. berghei* infected group may be as a result of oxidative stress which occur secondary to free radical generation typical in malarial infection [26]. Results from histological studies on the pancreas isolated from malaria infected mice treated with *P. amarus* extract showed that the extract could repair pancreatic cells damaged by *P. berghei*(Figs. 3-5) and even revitalise the cells (Figs. 7-9).

Malarial infection and its treatment with chloroquine could cause oxidative damage to pancreas and disturbed beta cell function but treatment with ethanolic leaf extract of *P. amarus* especially at 300mg/kg/d yielded high curative ability, augmented antioxidant defense capacity, refreshed the pancreas and improved beta cell function. So, *P. amarus* has both malarial curative and pancreatic tonic potentials. These potentials should be further explored.

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