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Changes in CD_{36} and Correlation with Hepatic Insulin Resistance Index and Triacylglycerol Level in Liver of Malarial Infected Mice Treated with *Phyllanthus amarus*

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Abstract

Phyllanthus amarus is an herb for the treatment of various ailments including malaria. Its antiplasmodial properties and antioxidant capacity have been reported, but very little is known about its effect on CD_{36} in relation to hepatic insulin resistance and triacylglycerol level. Hence, this study was undertaken to evaluate the changes in soluble CD_{36} concentrations and correlation with hepatic insulin resistance index and triacylglycerol levels in liver of *Plasmodium berghei* malarial parasite infected mice treated with graded doses of *Phyllanthus amarus* ethanolic leaf extract. Thirty (30) adult Swiss albino mice of both sexes weighing between 20-30g were assigned into six (6) groups (n=5/grp). Crude ethanolic leaf extract of *Phyllanthus amarus* was administered at 150, 300 and 450mg/kg/d as single daily dose for 7 days. On the 8th day of study, the mice were sacrificed under chloroform anaesthesia after an overnight fast. Blood sample was collected by cardiac puncture and centrifuged to obtain serum which was used for the assay of serum CD_{36}, glucose and insulin, while, liver of each mouse was excised and processed for the assay of hepatic triacylglycerol, using documented methods. Results indicate that infection of mice with *Plasmodium berghei* malarial parasite yielded significant increase in level of CD_{36} (5.40±0.70pg/ml) and liver triacylglycerol, TAG (259.00±7.21mg/dl), but abnormal reduced (p<0.05) hepatic insulin resistance, HIR index (0.37±0.05) when compared with the control mice (CD_{36} = 3.47±0.46pg/ml, liver TAG = 161.00±10.00, HIR index = 0.68±0.18) at the 5% probability level. However, administration of varying doses (150, 300 and 450mg/kg/d) of *Phyllanthus amarus* crude ethanolic leaf extract to experimental mice infected with *P. berghei* malarial parasite for seven days maintained serum soluble CD_{36} (3.40±0.52pg/ml, 3.47±0.46pg/ml and 3.37±0.46pg/ml), hepatic insulin resistance index (0.85±0.10, 0.77±0.17 and 0.82±0.13) and liver triacylglycerol (134.33±15.95mg/dl, 174.00±11.27mg/dl and 163.67±11.02mg/dl) levels, respectively, in trends that compared well with the Control and chloroquine-treated data (CD_{36} = 3.03±0.12pg/ml, liver TAG = 139.33±12.01, HIR index = 0.64±0.07). Correlation of CD_{36} with liver triacylglycerol for the malarial infected group without treatment was strongly positive, while, that with hepatic insulin resistance index was strongly negative. The control and chloroquine treated groups produced similar correlation pattern, but those for the extract-treated were weaker. Malarial infection in experimental mice significantly (p<0.05) increased serum soluble CD_{36} and this impacted HIR index and liver TAG. However, extract and chloroquine treatments ameliorated the malaria-induced level of serum soluble CD_{36} and association with HIR index and liver TAG in manners that were similar to the control trend.

Keywords: *Phyllanthus amarus*, *Plasmodium berghei*, Soluble CD_{36}, Hepatic Insulin Resistance, Liver, Triacylglycerol.

Introduction

Traditionally, medicinal plants are therapeutic resource used by the population of the continent specifically for health care. Herbs may also serve

as starting materials for drugs (Sofowora, 1993). Plants have provided an alternative strategy in research for new drugs. It is likely that plants will continue to be a valuable source of new molecules which may after possible chemical manipulations provide new and improved drugs (Shan *et al*., 2006). One of such plants is *Phyllanthus amarus*.

Phyllanthus amarus belongs to the family Euphorbiaceae (the spurge family) from which the largest genus is the Euphorbia. It has about eight hundred (800) species which are found in tropical and subtropical countries of the world (Mazumder *et al*., 2005). Phytochemicals identified in the leaf extract include alkaloids, flavonoids, tannins, saponins, anthraquinone and glycosides (Onyesom *et al*., 2015). The antimalarial activity of *P. amarus* has been observed (Onyesom and Adu, 2015).

Malaria is a mosquito borne infectious disease of humans caused by eukaryotic protists of the genus *Plasmodium*. It is widespread in tropical and subtropical regions of the world including much of the sub-Saharan Africa. In Nigeria, malaria is mostly caused by *Plasmodium falciparum*. The female *Anopheles* mosquito transmits these parasites to humans. Malaria has great morbidity and mortality than any other infectious disease of the world. They infect red blood cells to cause characteristic symptoms (WHO, 2011). CD_{36} contributes significantly to the uptake of infected red blood cells (IRBCs) and pro-inflammatory cytokine responses by dendritic cells (DCs), and the ability of DCs to activate natural killer (NK) and T cells to produce IFN-γ. DCs respond to malarial parasites during the early stage of infection, and so, CD_{36} contributes substantially to cytokine production by DCs, NK and T cells, suggesting that CD_{36} plays an important role in malarial immunity. Thus, the effect of CD_{36} on malarial immunity is imprinted during the early stage of infection when parasite load is low (Gowda *et al*., 2013).

CD_{36} (Cluster of Differentiation) is a multiligand scavenger receptor expressed on a variety of cell types including adipocytes, monocytes, platelets, hepatocytes and vascular epithelial cells (Silverstein and Febbraio, 2009; Su and Abumrad, 2009). CD_{36} functions in the uptake of fatty acids and oxidized lipoproteins. Signal transduction triggered by the binding of CD_{36} and other ligands contributes to multicellular effects of lipoproteins in pathways related to lipid utilization, insulin resistance, inflammation, atherosclerosis and thrombosis (Rahaman *et al*., 2006; Collot-Teixeira *et al*., 2007; Heilbronn *et al*., 2007; Kashyap *et al*., 2009; Park *et al*., 2009; Silverstein and Febbraio, 2009; Su and Abumrad, 2009). CD_{36} may also contribute to oral fat perception and intestinal chylomicron formation (Drover *et al*., 2005; Langerette *et al*., 2005; Selafani *et al*., 2007). In humans, deficiency of CD_{36} results in defective myocardial fatty acid (FA) uptake measured by non-invasive scintigraphy (Tanaka *et al*., 2001), and a link with hyperthropic cardiomyopathy has been proposed (Tanaka *et al*., 1997).

Several independent factors, including (i) increased free fatty acid (FFA) uptake, (ii) *de novo* lipogenesis, (iii) decreased FA oxidation, and (iv) reduced VLDL secretion, may contribute to hepatic fat accumulation (Bradbury, 2006; Postic and Girard, 2008; Birkenfeld and Shulman, 2014). The fatty acid transporter CD_{36} (also known as FA translocase) functions as an important mediator of hepatic FA uptake (Buqué *et al*., 2012). CD_{36} mRNA levels are drastically increased in livers of murine models of obesity and Type II diabetes (T2D) (Memon *et al*., 1999), and CD_{36} expression correlates with liver TG accumulation, insulin resistance, and hyperinsulinaemia in human (Greco *et al*., 2008; Miquilena-Colina *et al*., 2011). Moreover, under normal, non-metabolically challenged conditions, forced expression of CD_{36} alone increases FA uptake in mouse hepatocytes *ex vivo* and mouse liver TG content *in vivo* (Koonen *et al*., 2007). Nonetheless, the factors provoking increased hepatic CD_{36} expression remain unknown.

This study therefore, attempts to evaluate the changes in soluble CD_{36} concentrations and correlation with hepatic insulin resistance index and levels of triacylglycerol, TAG in livers of *Plasmodium berghei* malarial parasite infected

mice treated with graded doses of *Phyllanthus amarus* ethanolic leaf extract, since malarial infection is known to modify hepatic lipids (Onyesom and Agho, 2011).

Materials and Methods

Harvesting and Preparation of Plant Extracts: *Phyllanthus amarus* plants, growing freely in uncultivated land space in Abraka, Ethiope East Local Government Area of Delta State, Nigeria, were uprooted and authenticated (Voucher No: FHI 109728) in the Herbarium Unit, Forestry Research Institute of Nigeria, Ibadan. Crude ethanolic leaf extract was prepared as already documented (Onyesom *et al*., 2015).

Experimental Mice: The mice procured for this study were Swiss albino BALB/c mice of mixed sexes weighing between 20-30g. They were maintained at the Laboratory Animal Centre, Faculty of Basic Medical Sciences, Delta State University, Abraka, Nigeria. Three (3) *Plasmodium berghei* (NK 65 strain) infected (donor) mice were obtained from the Department of Parasitology, Nigerian Institute of Medical Research (NIMR), Yaba, Lagos, Nigeria, and used to prepare inoculum.

Animal Care and Handling: The selected mice were kept in plastic cages under controlled condition of 12h light/12h dark cycle and allowed free access to standard mouse feed (Top Feeds Flour Mill, Limited, Sapele, Delta State, Nigeria) and drinking water *ad libitum*. The animals were handled in compliance with the guidelines approved by our Faculty's Ethics Committee.

Animal Grouping and Extract Administration: The already acclimatized mice were assigned into six (6) groups (n=5/grp). Group 1: Control (no infection, no treatment), Group 2: Infected mice, but no treatment, Groups 3-5 were also infected, but treated with 150, 300 and 450mg/kg/d of the crude ethanolic leaf extract, while, Group 6 was infected, but treated with 5mg/kg/d chloroquine. The volume equivalent to the dose administered was calculated (Onyesom, *et al*., 2015) and administered as single daily dose using intragastric canula for a period of seven days having established parasitaemia.

Animal Sacrifice and Collection of Specimen: On the 7th day of the experiment, the animals were fasted overnight and sacrificed (n=5 mice/grp) the next morning under chloroform anaesthesia. Whole blood was collected by heart puncture and centrifuged (Cent 80D, Serico, China) to obtain serum which was used for the determination of serum soluble $CD_{36,}$ glucose and insulin. Then, liver of each mouse was excised and processed for the assay of hepatic TAG.

Parasite Count: Count was determined by the Ochei and Kolhatkar (2010) method, using Geimsa-stained thin blood smear prepared with blood sample obtained from the cut tail tip.

Analysis of Serum Specimen: Serum soluble CD_{36} was determined by the method described by Anderson *et al.* (2006). Blood glucose was estimated by the oxidase method (Marks and Dawson, 1965). Hepatic triacylglycerol (TAG) levels were determined using the GPO – PAP method (Tietz, 1994), while, insulin was evaluated by the immune-enzymometric assay (Bangham *et al*., 1988).

Determination of Insulin Resistance: Hepatic insulin resistance index was calculated by the Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) as expressed HOMA-IR = Glucose (mg/dL) x Insulin (μIU/mL)/405 (Matthew *et al*., 1985).

Result

Table 1: Biochemical and derived parameters showing correlation with hepatic insulin resistance index and triacylglycerol level in liver of malaria infected mice treated with *Phyllanthus amarus*

Group	Insulin (µu/mL)	Glucose (mg/dL)	CD_{36} (pg/ml)	HIR Index	*r*-value	*P*-level	Hepatic TAG (mg/dL)	*r*-value	*P*-level
1.Positive control (no infection, no treatment	4.03±0.60[a]	85.67±4.07[a]	3.47±0.46[a]	0.68±0.18[a]	-0.998	0.467	161.00±10.00[a]	0.866	0.333
2.Negative Control (*P. berghei* infected, no treatment) Treatment of *P. berghei* infection	2.70±0.17[b]	56.33±6.09[b]	5.40±0.70[b]	0.37±0.05[b]	-0.970	0.156	259.00±7.21[b]	0.911	0.027*
3. 150mg/kg/d of *P. amarus* leaf extract	3.17±0.38[a]	91.67±7.42[a]	3.40±0.52[a]	0.85±0.10[a]	-0.454	0.700	134.33±15.95[a]	0.724	0.485
4. 300mg/kg/d of *P. amarus* leaf extract	2.80±0.25[b]	93.00±5.02[a]	3.47±0.46[a]	0.77±0.17[a]	-0.711	0.136	174.00±11.27[a]	0.538	0.638
5. 450mg/kg/d of *P. amarus* leaf extract	4.20±0.71[a]	94.00±8.13[a]	3.37±0.46[a]	0.82±0.13[a]	-0.500	0.667	163.67±11.02[a]	0.577	0.609
6. 5mg/kg/d of chloroquine	4.17±0.64[a]	85.33±4.75[a]	3.03±0.12[a]	0.64±0.07[a]	-0.924	0.249	139.33±12.01[a]	0.841	0.364

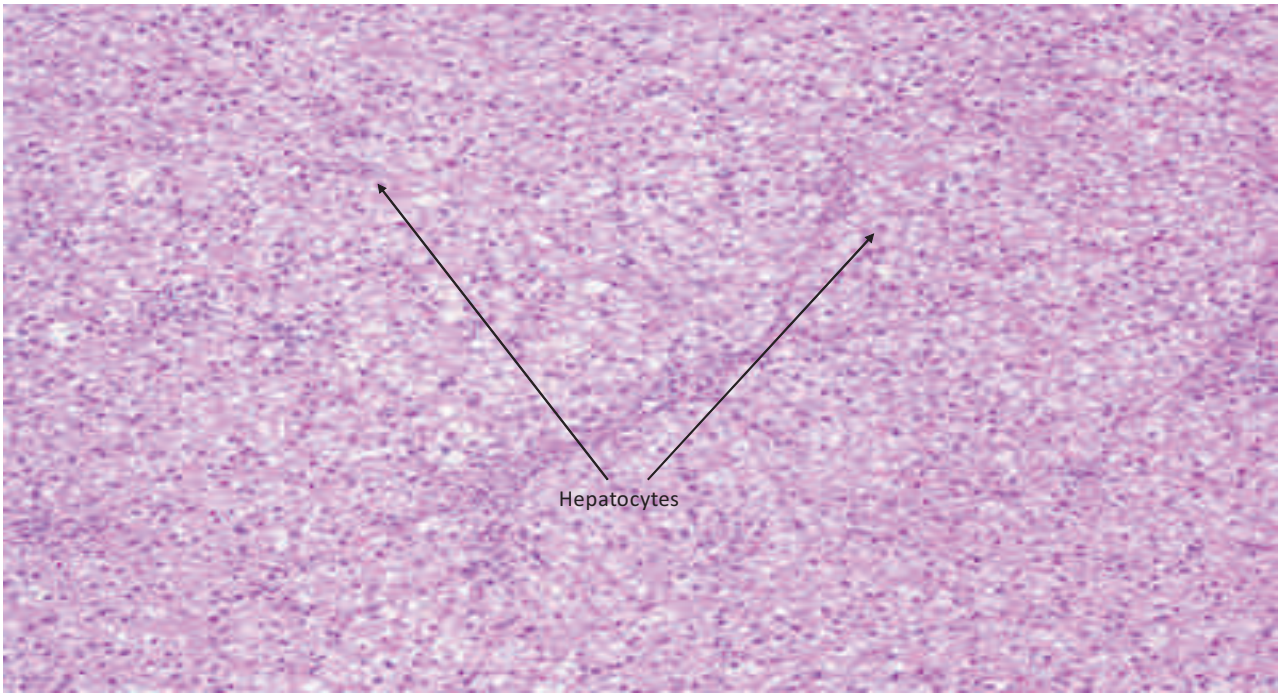

Figure 1: ***Photomicrograph of liver tissue from control mouse showing normal hepatocytes. Magnification ×100 (H & E stain).***

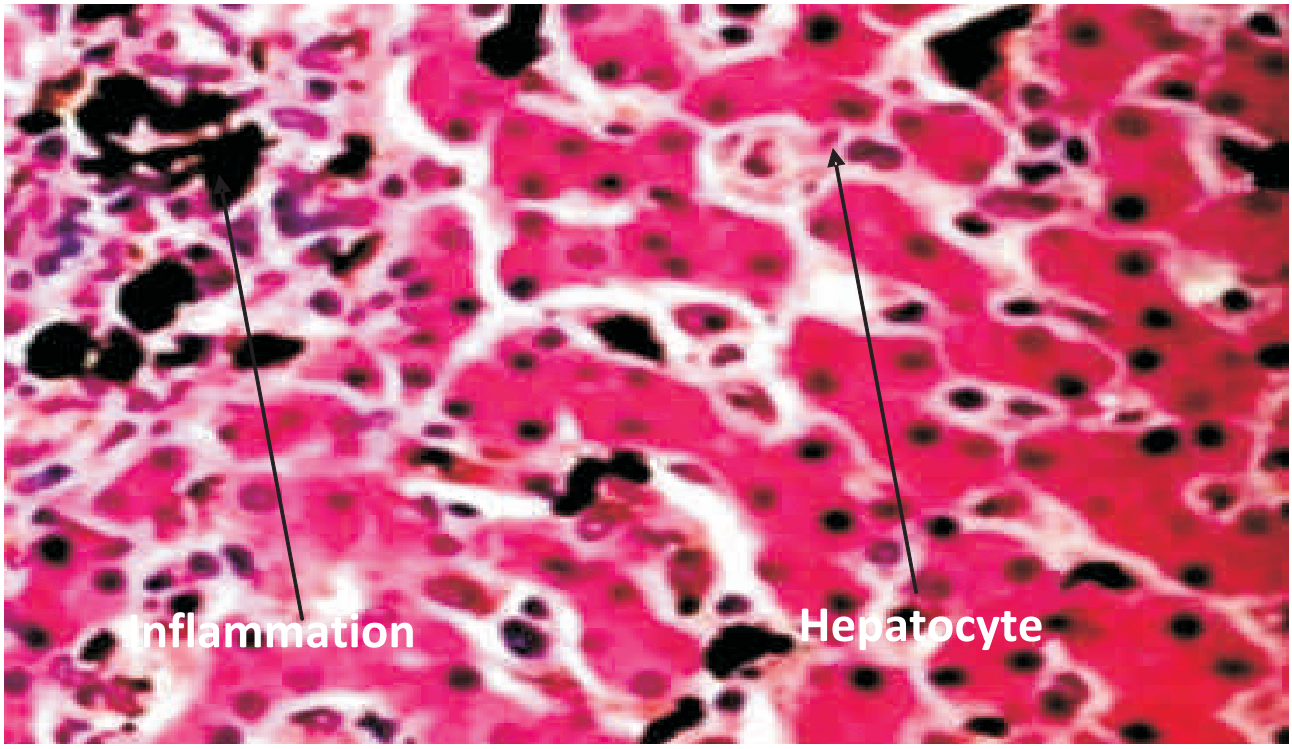

Figure 2: ***Histo-architecture of liver tissue infected with P. berghei showing abnormal features, structural irregularities and acute inflamation of the hepatocyte.***

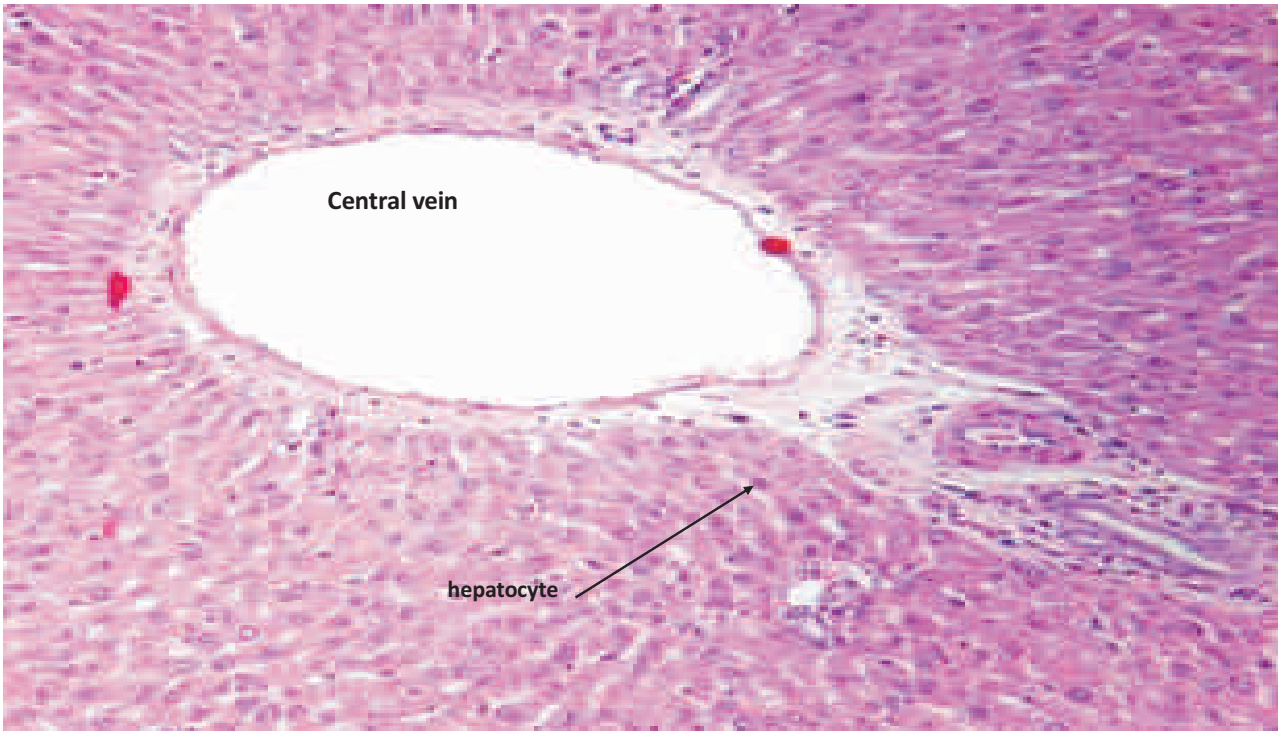

Figure 3: ***Photomicrograph of liver tissue obtained from mouse administered 300mg/kg body weight of crude ethanol leaf extract of P. amarus, indicating normal histological features of invigorated hepatocytes and central vein. Magnification × 100 (H & E stain).***

Discussion

Before now, studies have shown that CD_{36} functions as pattern recognition receptors on phagocytic cells, which are able to recognize specific classes of molecular model presented by pathogen or by pathogen-infected cells and so, involved in innate immune response (Silverstein and Febbraio, 2009). Several independent factors, including (i) increased free fatty acid (FFA) uptake, (ii) *de novo* lipogenesis, (iii) decreased FA oxidation, and (iv) reduced VLDL secretion, may contribute to hepatic fat accumulation (Bradbury, 2006; Postic and Girard, 2008; Birkenfeld and Shulman, 2014). The fatty acid transporter CD_{36} (also known as FA translocase) functions as an important mediator of hepatic FA uptake (Buqué *et al*., 2012). *CD_{36}* mRNA levels are drastically increased in livers of murine models of obesity and Type II diabetes (T2D) (Memon *et al*., 1999), and *CD_{36}* expression correlates with liver TAG accumulation, insulin resistance, and hyperinsulinaemia in human (Greco *et al*., 2008; Miquilena-Colina *et al*., 2011).

Table 1, indicates that infection of experimental mice with *Plasmodium berghei* yielded significant increases in levels of CD_{36} and liver TAG, but significant and abnormal reduction in HIR index when compared with positive control (Group 1) at the 5% probability level. However, administration of varying doses (150, 300 and 450mg/kg/d) of *Phyllanthus amarus* crude ethanolic leaf extract to experimental mice infected with *P. berghei* malarial parasite for seven days-maintained serum soluble CD_{36}, HIR and hepatic TAG levels in trends that compared well with the control (Group 1) and chloroquine-treated (Group 6) data. Correlation of CD_{36} with hepatic TAG for the malarial infected group without treatment (Group 2) was strongly positive, while, that with HIR was strongly negative. The control and chloroquine treatment produced similar correlation pattern, but those for the extract-treated were however, weaker (Table 1). Microstructural changes of the liver tissue obtained from *P. berghei* infected mice (Figure 2) show pathological features of acute inflamation of the liver (hepatitis), called steatohepatitis. *Plasmodium berghei* causes inflamation of the hepatocyte of infected mice. More deposition of histopathological examination of the liver (figure 3), however, shows that *P. amarus* administration invigorated liver cells that experienced inflammation from malaria infection.

Infection with malarial parasite is characterized by inactivation of infected erythrocytes with mature forms of the parasite, and CD_{36} has been shown to be a major inactivation receptor on microvascular endothelial cells. This hypothesis is supported by the work of Newbold and Colleagues (1999) who have reported that significantly higher binding to CD_{36} occurs in cases of non-severe disease. The alterations of CD_{36} can be attributed to the presence of fisetin in flavonoid of *P. amarus*. Conjugated diene formation analyses by Lian *et al*. (2008) have shown that fisetin of flavonoid inhibits Cu^{2+} mediated LDL oxidation stronger than morin and myricetin. Binding of CD_{36} (Class B scavenger receptor) to oxidized LDL causes the formation of atherosclerotic lesion. Fisetin of flavonoid blocks macrophages oxidized LDL uptake by altering the CD_{36} expression on the macrophages (Lian *et al*., 2008).

Infection of mice with *P. berghei* and treatment with *P. amarus* led to an increase in insulin levels (Table 1). This report agrees with earlier work done by Shetti and Kaliwal (2015). They observed that the hypoglycaemic effect of *P. amarus* extracts could be linked to more than one mechanism. The possible mechanisms include the stimulation of β cells and subsequent release of insulin, and activation of the insulin receptors. The plants anti- hyperglycaemic action may be by potentiating of pancreatic secretion of insulin. In this context, a number of other plants have also been reported to have anti-hyperglycaemic and insulin release stimulatory effect (Pulok *et al*., 2006; Leila *et al*., 2013). The observed increase in insulin levels as a result of the administration of *P. amarus* leaf extract could be a factor that led to the expression of CD_{36}. CD_{36} being a free fatty acid transporter, when its expression is enhanced, possibly due to increase in insulin levels, subsequently caused changes in hepatic insulin resistance index. This hypothesis is well

corroborated by the findings of Pär *et al*. (2015), who suggested that elevated insulin levels, via enhanced CD_{36} expression, provoked fatty liver development that in turn leads to hepatic insulin resistance. Their data provides evidence for a direct role for hyperinsulinaemia in stimulating hepatic CD_{36} expression and thus, the development of hepatic insulin resistance.

Infection with malaria is characterized with increased TAG concentration. This observation is consistent with reports from earlier studies which showed increased serum lipoprotein fractions in malarial patients compared with apparently healthy control subjects (Onyeneke *et al.*, 1997; Onongbu and Onyeneke, 1983). The observed increases in hepatic TAG content are also in agreement with the report of Lombard *et al*. (1998). According to Lombard *et al.* (1998), increase in hepatic lipid (TAG) in malarial subjects is consistent with the degree of parasitaemia (Lombard *et al*., 1998). Treatment with ethanolic extract of *Phyllanthus amarus* had significant hepatic lipid-lowering effect on the increased level of TAG caused by the infection with *Plasmodium berghei*. The observed low TAG effect may be attributed to the gut intra-lumenal interactive effect of saponins. Saponins are known antinutritional factors which reduce the uptake of certain nutrients including glucose and lipids especially triacylglycerol at the gut through intra-lumena physicochemical interaction. Hence, saponins have been reported to have hypolipidaemic effect (Price *et al*., 1987). Presence of saponins has been reported by Chidi *et al*. (2007) in aqueous extract of *Phyllanthus amarus* and this saponin may explain the antilipidaemic effect observed in this study. Recall, that a strong positive correlation was observed in malarial infected mice between CD_{36} and TAG, and this correlation was seen to be significant in this study. The correlation between CD_{36} and TAG in malarial infection is as a result of the higher expression or increase in CD_{36} level in malaria condition which led to a drastic increase in TAG. Results by Koonen *et al*. (2007) observed that during diet induced obesity (DIO), CD_{36} protein levels in the liver are significantly elevated, as in the case of malaria, and these elevated levels correlate with increase hepatic triglyceride storage and secretion. These alterations in liver lipid storage and secretion were also observed upon forced expression of hepatic CD_{36} in the absence of DIO and were accompanied with a marked rise in hepatic fatty acid uptake *in vivo*, demonstrating that increase CD_{36} expression is sufficient to recapitulate the aberrant liver lipid handling.

Conclusion

Infection of experimental mice with *Plasmodium berghei* yielded significant increase in levels of CD_{36} and liver TAG, but produced abnormal reduction in HIR index. However, administration of varying doses of *Phyllanthus amarus* ethanolic leaf extract ameliorated the changes observed. Correlation between CD_{36} and hepatic TAG in malarial infection is strongly positive. Study, therefore, establishes association between CD_{36} and liver TAG to be strongly positive and significant during malarial condition, but weak and insignificant when the condition is treated with *P. amarus* leaf extract or chloroquine, just like the association in the control group of mice.

Recommendation

The chemical fractions of *P. amarus* leaf extract that ameliorated the changes observed in $CD_{36,}$ liver TAG and HIR index should be identified for further study.

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