Immunomodulation Capabilities of Aqueous Leaf Extract of *Phyllanthus amarus* in male Wistar Rats

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Abstract: The phytochemical and mineral analysis of aqueous leaf extract of *Phyllanthus amarus*, and its potential immunomodulation was investigated in male Wistar rats. Dry leaf powder was extracted with water and lyophilized. Thirty two male Wistar rats of average body weight of 85.5 ± 4.55 g were grouped into four (A-D). Group A received distilled water (control), while doses of 250, 500 and 1000 mg/kg body weight of extract were orally administered once daily for 84 days to animals in groups B, C and D respectively. Phytochemical and mineral analyses of the aqueous leaf extract revealed the presence of alkaloids, saponins, flavonoids, iron, nitrogen and zinc among others. The extract reduced the body weights of rats ($p<0.05$) with increasing doses and significant lowering of blood glucose ($p<0.05$) was shown in an almost similar manner. Serum interleukin-2 concentration increased ($p<0.05$), while serum interleukin-6 and tumour necrosis factor-α concentrations reduced ($p<0.05$). The total white blood cell (WBC) and lymphocytes (L) count were increased ($p<0.05$) and reductions ($p<0.05$) were presented in the neutrophil counts at 500 and 1000 mg/kg body weight. The total cholesterol, triacylglycerol, very low density lipoprotein cholesterol and low density lipoprotein cholesterol concentrations were reduced ($p<0.05$) in an almost dose dependent manner. High density lipoprotein cholesterol concentration increased ($p<0.05$) and the atherogenic index were reduced significantly ($p<0.05$) at the doses. Uric acid concentrations were reduced ($p<0.05$) in an almost dose dependent manner. Significant increases ($p<0.05$) were recorded in reduced glutathione concentrations in the liver, while liver malondialdehyde concentration was decreased significantly at 250 mg/kg body weight of extract ($p>0.05$). The result of the study established scientifically the folklore use of the aqueous leaf extract of *Phyllanthus amarus* as blood tonic for the prevention and/ or cure of infective and degenerative diseases.

Introduction

Infectious and degenerative diseases remain the leading causes of death in the human populations (Van de Perre, 2003). In urban centres, many people are overfed but undernourished and due to their social status, they indulge in foods that are high in calories but low in nutritional value. In addition, human systems are constantly assaulted by free radicals in civilized environments, resulting from stress (work pressure, inadequate rest, social activities such as smoking, alcoholism and over exercise) and toxins (air, food, drugs and metabolic waste products); whose cumulative effects result in poor immune function that account for various clinical conditions such as premature aging, infections, chronic and degenerative diseases (Van de Perre, 2003; Spelman *et al*., 2006). Therefore, toxins build up in the body faster than they can be removed, contributing to the onset of almost all infectious and degenerative disease that is preceded or accompanied by breakdown of the immune system. It is thus, critical to consider strengthening the immune system by taking preventative measures to avoid unnecessary medication. However, the exploitation of natural remedies becomes imperative, due to reported toxic effects of immunomodulatory synthetic drugs (methrotrexate, mycophenolate, cyclosporine, abatacept and rituximab), such as indiscriminate killing of other constantly dividing cells and organs, thereby increasing the risk of opportunistic infections (Goldsby *et al*., 2000; Taylor *et al*., 2005) and over stimulation of the activities of immune cells, thereby sacrificing the "self" - "not self" regulatory mechanisms (Gottlieb, 2001).

Chanca piedra is the popular name given to several small shrubs like plants in the *Phyllanthus genus* of the botanical family, "*Euphorbiaceae*" including; *Phyllanthus niruri, Phyllanthus stipulates, Phyllanthus sellowianus, Phyllanthus amarus* among others (Laupattarakasem *et al*., 2001: Igwe *et al*., 2007). Chanca piedra is a composite name, 'Chanca', meaning 'to break'in Quechua and 'piedra', meaning 'stone' in Spanish. Other common names of Chanca piedra are carry-me seed, quinine weed, hurricane weed, stone breaker (English) and enyikwonwa (Ibo in Nigeria) (Morton, 1981: Igwe *et al*., 2007). According to Foo and Wong (1992), in a number of countries, the aerial part of

Phyllanthus amarus (*Schum. et Thonn*) is highly valued in traditional medicine for its healing properties. The plant has been shown to work as antifungal, antibacterial and antiviral agent, and in the treatment of liver diseases, asthma, cardiovascular problem, dropsy and jaundice among others (Foo, 1993; Houghton *et. al*., 1996). *Phyllanthus amarus* is allerged to be a restoration herb by tradition medicine practitioners, and thus recommended for regular consumption during meals as blood tonic for the detoxification of the human systems and ultimately, the prevention and/ or cure of infective and degenerative diseases (Heyde, 1990; Fernard, 2000). However, no scientific evidence is available on the capability of the extract to modulate the immune system in the prevention and/ or cure of infective and degenerative diseases. The study, therefore, seeks to provide information on the immunomodulation activities of the aqueous leaf extract of *Phyllanthus amarus* in Wistar rats.

Materials and methods
Plant material for analysis

Fresh mature *Phyllanthus amarus* plant was collected before sun rise in July from the natural habitat around LAUTECH area in Ogbomoso, Oyo State. The plant was authenticated by Dr. A. Adesina, a Taxonomist in the Department of Crop Science, Ladoke Akintola University of Technology, Ogbomoso, Oyo State. A specimen of the plant was deposited at the herbarium. The fresh leaf was rinsed thoroughly in distilled water and dried in the shade for 14 days. The dried leaf was ground to fine powder, using a domestic electric grinder and suspended in distilled water at room temperature. The filtrates were pulled together and lyophilised using a freeze dryer. The yield of the aqueous leaf extract of *Phyllanthus amarus* was 17.57% ($^w/_w$). The lyophilised extract was stored air tight and kept in the dark till when needed.

Blood glucose glucometer

Accu-chek active glucometer and visual blood glucose test stripes, products of Roche Diagnostic Gmbh, D-68298 Mannheim, Germany were used for the fasting blood glucose level estimation.

Quantitative assay kits

The ELISA kits for the determination of rat Interleukin-2 (IL-2), Interleukin-6 (IL-6) and Tumor Necrosis Factor-alpha (TNF-α) were products of RayBiotech, Inc. USA, while those for Total Cholesterol, Triacylglyceride, High Density Lipoprotein Cholesterol (HDL-C), and Uric Acid were products of LABKIT, CHEMELEX, S.A. Pol. Canovelles-Barcelona, Spain. Reduced Glutathione assay kit was a product of BioAssay Systems, Hayward, USA.

Other reagents

All the chemicals and reagents used in the study were of analytical grade and were purchased from the Bristish Drug House (BDH) Poole England and Sigma Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A.

Laboratory animals

Eight to ten weeks old male Wistar rats of average body weight of 85.5 ± 4.55 g were obtained from the Animal Care Facility II, Ladoke Akintola University of Technology (LAUTECH), Osogbo, Osun State. The rats were fed with rat pellet (product of Bendel Feeds and Flour Mills Ltd, Ewu, Edo State, Nigeria).

Methods
Leaf analysis
Phytochemical

The chemical classes of constituents in the aqueous leaf extract of *P. amarus* were identified and quantified according to the methods described by Harborne (1973) and Trease and Evans (1983). Determinations were done in triplicates.

Minerals

The leaf sample was ashed, dissolved in 1 ml of 2M HCl and diluted to 100 ml with deionized water. The resulting mixture was used for the determination of mineral such as copper, zinc, iron, calcium, lead, arsenic, selenium, magnesium, sodium, potassium and phosphorus using standard methods of AOAC (1990).

Experimental animals and procedure

The Thirty two male Wistar rats were randomly grouped into four, comprising of eight rats per group. The rats were housed in cages made of wooden frames and metal netting, and were fed *ad libitum* with rat pellet and tap water with 12-hours light/dark cycle. The cages were cleaned every morning and disinfected at intervals of 3 days. The rats were allowed to acclimatize for 10 days before extract administration was commenced. Calculated amount of lyophilized aqueous leaf extracts of *Phyllanthus amarus* were constituted in distilled water to give doses of 250, 500 and 1000 mg/kg body weight. The groups were as illustrated:

Group A: control, received 1.0 ml distilled water

Group B: received 250 mg/kg body weight of the extract

Group C: received 500 mg/kg body weight of the extract

Group D: received 1000 mg/kg body weight of the extract

Prior to the administration of aqueous leaf extract of *Phyllanthus amarus* and every interval of 7 days, the fasting blood glucose levels and the body weights of the animals were recorded. Administration of aqueous leaf extract of *Phyllanthus amarus* was performed orally once daily between 8:30 am ± 30 minutes, using metal cannula attached to a 2 ml syringe. Administration lasted for 84 days, after which the rats were fasted for 12 hours and the blood glucose level and body weights determined.

This study was conducted in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals (1985), and the study was approved by the Ethical Committee of College of Medicine, Ladoke Akintola University of Technology, Osogbo, Osun State.

Haematological analysis

The haematological parameters were analysed by the automated haemology analyzer (SYSMEX K2X1: SYSMEX CORPORATION, JAPAN).

IL-2, IL-6 and TNF-alpha determination

The serum levels of IL-2, IL-6 and TNF-α were determined by *in vitro* enzyme linked immunosorbent assay (ELISA) kit, using colourimetric reaction method as instructed in the kit manual with cat #: ELR-IL2-001, ELR-IL6-001 and ELR-TNF alpha-001 respectively.

Blood glucose determination

The blood glucose concentration was determined by glucose oxidase reaction, using Accu-chek active glucometer and test strips. Glucose oxidase chromogen indicators and non-reactive agents are contained in the reagent pad to which about 2μl of whole blood was applied.

Lipid profile assay

The lipid profile assay was performed using reagent kits from LABKIT. The assay was carried out in the serum.

Serum and total cholesterol determination

The serum total cholesterol level was estimated by CHOD-POD enzymatic colourimetric reaction, according to the method as described by Naito (1984[a]).

Triglyceride (TAG) determination

The serum triglyceride level was estimated by GPO-POD enzymatic colourimetric reaction, according to the method as described by (Fossati *et al*., 1982).

High density lipoprotein cholesterol (HDL-C) determination

The serum HDL-C cholesterol level was estimated by precipitation and CHOD-POD enzymatic colourimetric reaction, according to the method as described by (Naito 1984[b]).

Low density lipoprotein cholesterol (LDL-C) and very low lipoprotein cholesterol (VLDL-C) determination

The VLDL-C cholesterol and LDL-C were estimated by computation, according to the methods described by (Friedewald *et al*., 1972).

Reduced glutathione determination

The levels of reduced glutathione (GSH) were determined using 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) enzymatic colorimetric reaction, according to the method described by Ellman (1959), as modified by Baker *et al*. (1990).

Uric acid determination

The serum uric acid level was determined by uricase-POD enzymatic colorimetric reaction, according to the method described by Schultz (1984).

Malondialdehyde determination

The concentration of thiobarbituric acid reactive substances, malondialdehyde (MDA) was determined in the liver using the method of Slater and Sawyer (1971), modified by Fraga *et al*. (1981).

Statistical analysis

This research work used a completely randomised design (CRD). The results were expressed as mean of 5 replicates ± standard error of mean (SEM). Results were analyzed using Prism 3.00 software. Results were subjected to one way analysis of variance (ANOVA) to test the effect of each dose level on the parameter under investigation at 95% level of confidence. The Duncan Multiple Range Test (DMRT) was conducted for the pair-wise mean comparisons, to determine the significant treatment dose at 95% level of confidence. Values were considered statistically significant at ($p<0.05$) and denoted by different alphabets (Mahajan, 1997).

Results

The results were presented in figures and tables. The values were expressed as mean ± standard error of mean (S.E.M.) of 5 replicates and significantly different ($p<0.05$) mean value were denoted by alphabets, except the results of leaf analyses.

Phytochemical and mineral constituents

The phytochemical screening of the aqueous leaf extract of *P. amarus* revealed the presence of alkaloids, saponins, flavonoids,

phenols, cyanogenic glycosides, tannins, oxalate and cardenolides, while steroids and anthraquinones were not detected (Table 1). Alkaloids were the highest in terms of concentration, while the least was cyanogenic glycosides. Mineral constituents of interest that were identified in the aqueous leaf extract of *P. amarus* are presented in Table 2. Minerals implicated in immunomodulation such as selenium, zinc, copper and magnesium were present, while lead, a heavy metal was also present. However, the level of iron was reasonably high, compared to others.

Rat behaviour and morphology

Administration of the aqueous leaf extract of *P. amarus* to Wistar rats led to the death of two rats administered 1000 mg/kg body weight after 53 days. The rats in the group were observed to be obviously lean, less active (Plate 1) and consumed less food, but more water.

Body weight

The pattern of the weekly average body weights of rats following repeated administration of the extract is shown in Figure 1. The body weight of control rats increased steadily throughout the duration of the experiment. However, the body weights of the rats administered the extract appeared to decrease significantly ($p < 0.05$) with increasing concentration of the extract.

Table 1: Phytochemical constituents of aqueous leaf extract of *P. amarus*

Phytochemical	% Composition
Akaloids	9.023 ± 0.010
Tannins	0.034 ± 0.000
Oxalate	2.722 ± 0.001
Flavonoids	0.572 ± 0.001
Cyanogenic glycosides	0.007 ± 0.001
Saponins	3.192 ± 0.002
Cardenolides	0.038 ± 0.001
Phenols	0.057 ± 0.003

Values are means ± SEM; n=3

Table 2: Some mineral constituents of aqueous leaf extract of *P. amarus*

Minerals	Concentration (mg/kg)
Lead	0.315 ± 0.004
Phosphorus	0.736 ± 0.002
Magnesium	2.694 ± 0.020
Copper	0.475 ± 0.025
Calcium	1.936 ± 0.040
Iron	3.572 ± 0.055
Nitrogen	2.770 ± 0.003
Zinc	1.495 ± 0.002
Selenium	0.205 ± 0.004
Sodium	2.125 ± 0.003
Potassium	1.172 ± 0.001

Values are means ± SEM; n=3

Blood glucose

Effect of administration of the aqueous leaf extract of *P. amarus* on weekly fasting blood glucose is presented in Figure 2. All concentrations of the extract caused a significant lowering of blood glucose ($p < 0.05$) in almost a dose dependent manner after 7 days of extract.

IL-2, IL-6 and TNF-α concentration

The effect of repeated administration of the aqueous leaf extract of *P. amarus* on interleukin-2 (IL-2), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) is presented in Table

3. The administration of the extract resulted in increased serum IL-2 concentration ($p < 0.05$), while serum IL-6 concentration decreased at doses of 250 and 500 mg/kg body weight and TNF-α concentrations reduced significantly ($p < 0.05$) at all doses.

**Haemeatological parameters**

Erythrocyte parameters and indices

As shown in Table 4, the administration of leaf extract of *P. amarus* had no significant effects ($p > 0.05$) on the packed cell volume (PCV) of rats. However, the red blood cell (RBC) count and
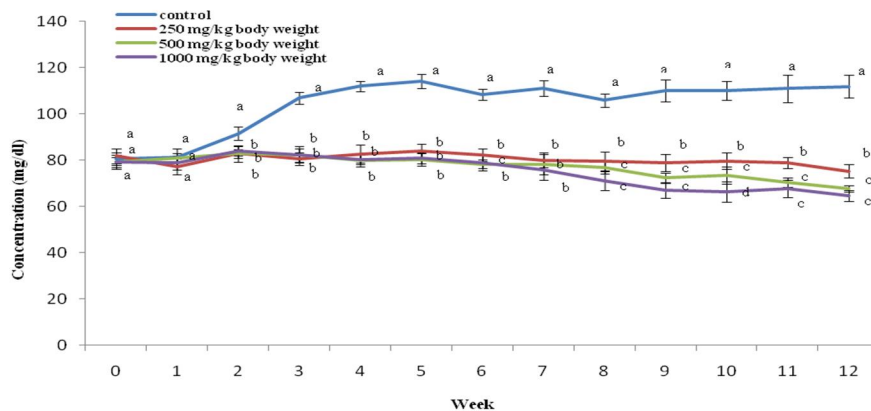
hemoglobin (Hb) count were increased significantly ($p<0.05$) at 500 and 1000 mg/kg body weight. Significant reduction ($p<0.05$) was recorded in mean corposular volume (MCV) in rats administered the leaf extract of *P. amarus* ($p>0.05$), while mean corposular hemoglobin (MCH) was reduced significantly ($p<0.05$) at 1000 mg/kg body weight only (Table 4). The mean corposular hemoglobin concentration (MCHC) of rats administered the leaf extract was reduced significantly ($p<0.05$) at 500 and 1000 mg/kg body weight. The erythrocyte sedimentation rates (ESR) was increased significantly ($p<0.05$) dose dependently in rats administered the leaf extract.
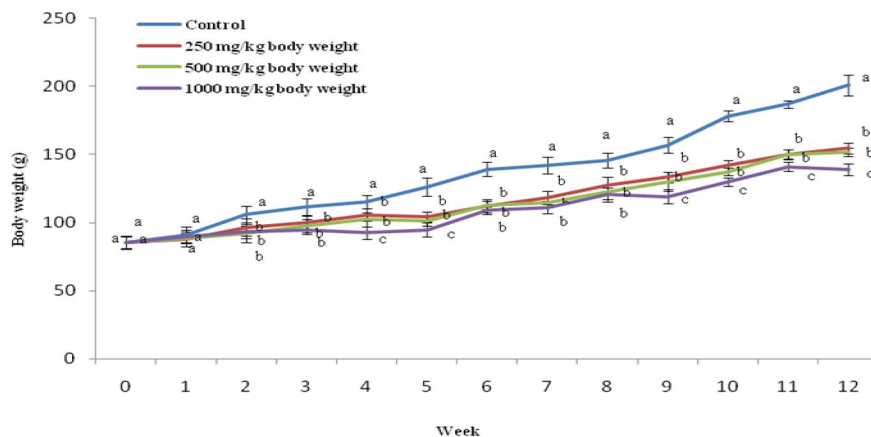
Figure 1: Body weights of rats administered aqueous leaf extract of *P. amarus*.
Values are means ± SEM; n=5. *Values bearing different alphabets are significantly different ($p<0.05$).

Figure 2: Effect of aqueous leaf extract on fasting blood.
Values are means ± SEM; n=5. *Values bearing different alphabets are significantly different ($p<0.05$).

Leukocyte parameters and platelets concentration

Table 5 depicts the concentration of leukocyte parameters in rats following the administration of the aqueous leaf extract of *P. amarus*. The total white blood cell (WBC) and lymphocytes (L) count were increased significantly ($p<0.05$) in rats administered the leaf extract. Significant reductions ($p<0.05$) were observed respectively in the neutrophil counts at 500 and 1000 mg/kg body weight of the extract. The monocyte and eosinophil of the rats were not affected ($p<0.05$) following the administration of the extract, while the basophils count was significantly increased ($p<0.05$) at 1000 mg/kg body weight only (Table 5). However, the platelet counts was decreased significantly ($p<0.05$) in an almost dose dependent manner in rats administered the aqueous leaf extract (Table 4).

Serum lipid profile

The effect of the administration of *P. amarus* leaf extract on serum lipid profile is shown in Figure 6. The total cholesterol, triacylglycerol

(TAG), very low density lipoprotein cholesterol (VLDL-C) and low density lipoprotein cholesterol (LDL- C) concentrations were reduced significantly (p<0.05) in an almost dose dependent manner. The high density lipoprotein cholesterol (HDL-C) concentration increased significantly (p<0.05) following the administration of the leaf extract, while the atherogenic index was reduced significantly (p<0.05) at the doses investigated (Table 6).

Serum uric acid concentration, liver reduced glutathione and malondialdehyde level

Table 7 presents the trend obtained in the serum uric acid, liver reduced glutathione (GSH) and malondialdehyde (MDA) concentrations following the administration of aqueous leaf extract of *P. amarus*. Uric acid concentrations were reduced significantly (p<0.05) in an almost dose dependent manner. The various doses of aqueous leaf extract of *P. amarus* resulted in significant increases (p<0.05) in reduced GSH concentrations in the liver, liver MDA concentration was decreased significantly at 250 mg/kg body weight of extract (p>0.05) only (Table 7).

Table 3: Effect of Aqueous Extract of *P. amarus* on Erythrocyte Indices in Male Wistar Rats

	Control	250 mg/kg body weight	500 mg/kg body weight	1000 mg/kg body weight
IL-2 (pg/ml)	22.19 ± 2.51^a	26.5 ± 2.04^b	27.63 ± 2.09^b	33.71 ± 3.56^c
IL-6 (pg/ml)	224.09 ± 15.82^a	138.03 ± 6.64^b	155.44 ± 8.07^b	212.51 ± 11.90^a
TNF-α (pg/ml)	376.84 ± 10.56^a	176.34 ± 8.60^b	206.63 ± 9.54^c	224.45 ± 11.45^c

Values are means ± SEM; n=6. Values bearing different alphabets are significantly different (p<0.05).

Table 4: Effect of aqueous leaf extract on erythrocyte parameters in Wistar rats

	Control	250 mg/kg body weight	500 mg/kg body weight	1000 mg/kg bodyweight
PCV (%)	38.8 ± 2.03^a	37.6 ± 1.23^a	39.1 ± 1.20^a	36.8 ± 1.36^a
RBC (10^{12})/L	6.46 ± 0.49^a	6.71 ± 0.67^a	7.03 ± 0.39^b	7.45 ± 0.44^b
Hb (g/dl)	12.88 ± 0.68^a	12.50 ± 0.45^a	12.02 ± 0.55^b	11.50 ± 0.45^b
ESR (mm^3/hr)	2.7 ± 0.50^a	3.2 ± 0.35^a	3.7 ± 0.35^c	5.1 ± 0.50^d
MCV (fL)10^{15}	52.55 ± 2.85^a	45.85 ± 3.52^b	44.50 ± 2.65^b	40.35 ± 1.15^c
MCH (10^{-12})	0.14 ± 0.01^a	0.13 ± 0.01^a	0.11 ± 0.02^a	0.07 ± 0.01^b
MCHC (g/L)	2.99 ± 0.21^a	2.78 ± 0.15^a	2.29 ± 0.11^b	2.18 ± 0.09^b

Values are means ± SEM; n=6. Values bearing different alphabets are significantly different (p<0.05).

Table 5: Effect of aqueous leaf extract on leukocytes and platelets in Wistar rats

	Control	250 mg/kg body weight	500 mg/kg body weight	1000 mg/kg body weight
WBC 10^9 (/L)	4.15 ± 0.49^a	4.5 ± 0.38^b	4.7 ± 0.47^b	6.90 ± 0.51^c
Lymphocyte (%)	32.4 ± 4.22^a	38.2 ± 4.01^b	44.5 ± 4.15^b	50.7 ± 3.54^c
Neutrophil (%)	52.9 ± 4.01^a	50.2 ± 3.51^a	40.5 ± 3.05^b	36.4 ± 3.85^b
Monocyte (%)	9.2 ± 0.97^a	10.8 ± 1.35^a	11.7 ± 0.65^a	10.6 ± 1.65^a
Eosinophil (%)	1.25 ± 0.25^a	1.25 ± 0.25^a	1.25 ± 0.25^a	1.75 ± 0.25^a
Basophil (%)	1.00 ± 0.00^a	1.00 ± 0.00^a	1.00 ± 0.00^a	2.00 ± 0.00^b
Platelets(μl)10^3	475.0 ± 8.23^a	406.6 ± 9.20^b	402.5 ± 11.01^b	429.8 ± 9.95^c

Values are means ± SEM; n=6. Values bearing different alphabets are significantly different (p<0.05).

Table 6: Effect of aqueous leaf extract on serum lipids concentrations in Wistar rat

	Control	250 mg/kg body weight	500 mg/kg body weight	1000 mg/kg bodyweight
Total cholesterol (mg/ml)	215.82 ± 6.11^a	188.90 ± 7.02^b	164.45 ± 4.92^c	145.24 ± 5.35^d
Triacylglyceride (mg/ml)	205.62 ± 4.72^a	199.75 ± 5.52^a	171.25 ± 5.11^b	162.58 ± 4.45^c
HDL-C (mg/ml)	45.21 ± 2.19^a	51.15 ± 3.05^b	58.34 ± 3.56^c	53.27 ± 2.14^b
VLDL-C (mg/ml)	43.45 ± 1.55^a	38.25 ± 1.05^b	35.95 ± 1.78^b	32.05 ± 0.95^c
LDL-C (mg/ml)	144.22 ± 5.15^a	115.64 ± 4.48^b	91.38 ± 4.0^c	69.52 ± 3.72^d
LDL-C/HDL-C	3.94 ± 0.15^a	2.36 ± 0.18^b	1.62 ± 0.09^c	1.14 ± 0.03^d

Values are means ± SEM; n=6. Values bearing different alphabets are significantly different (p<0.05).

Table 7: Effect of aqueous leaf extract on serum uric acid, liver glutathione and malondialdehyde concentrations in Wistar Rats

	Control	250 mg/kg body weight	500 mg/kg body weight	1000 mg/kg body weight
Uric acid (mg/ml)	5.25 ± 0.86^a	3.21 ± 0.42^b	4.09 ± 0.91^c	4.18 ± 0.88^c
Liver GSH (mg/ml)	36.32 ± 2.62^a	47.91 ± 2.34^b	42.22 ± 3.08^c	42.08 ± 3.51^c
Liver MDA (nmole /mg of protein)	5.19 ± 0.55^a	4.01 ± 0.33^b	4.88 ± 0.71^a	4.95 ± 0.77^a

Values are means ± SEM; n=6. Values bearing different alphabets are significantly different (p<0.05).

Discussion

Phytochemical and minerals constituents

High levels of alkaloids and saponins were present in the aqueous leaf extract of *P. amarus* (Table 1) suggesting that the extract may have marked effects on the modulation of immune system and the prevention of oxidative stress concertedly (Liu and Xaio, 1992). Some bitter alkaloids in plants are metabolised in the liver into dimethylxanthine and finally methyl uric acid by cytochrome P_{450} oxygenase systems (Yoshida *et al*., 1997). Methyl uric acid in the liver stimulates the expression of tumor necrosis factor (in the endothelia cells of the liver by macrophages), which modulates the immune system (Yoshida *et al*., 1997). In addition, saponins at low concentrations were reported to serve as adjuvant (saponins-cholesterol-phospholipid complexes) that stimulate cell mediated immune system by inducing the production of interleukins and interferons, especially by the antigen-presenting cells in mast cells (Oda *et al*., 2000; Zahid *et al*., 2007).

The high content of saponins could result in the permeabilization of plasma membranes due to the bipolar structure of saponins, in which the lipophilic components integrate easily and complex with the lipid fraction of plasma membranes, while the hydrophilic glycosidic portion forms complexes with transmembrane proteins, thereby causing irreversible disorder and disruption of the plasma membrane (Choi *et* al., 2001). Although, low concentrations of saponins were reported to enhance the absorption of dietary nutrients in the gastrointestinal tract (Choi *et* al., 2001), while high concentrations of saponins resulted in marked reduction in the absorption of dietary nutrients in the gastrointestinal tract due to 'autointoxication' or "leaky gut'(Choi *et* al., 2001; Francis *et al*., 2002; Evers, 2008). Permeabilization of erythrocyte plasma membranes results in the distortion of the outer lipid bilayer, which reduces osmostic pressure, causing the swelling and lysis of the erythrocyte (hemeolysis) (Trease and Evans, 1989; Choi *et* al., 2001).

In addition, saponins in the aqueous leaf extract could enhance the activities of antioxidant enzymes such as glutathione peroxidase, glutathione reductase and superoxide dismutase, by directly scavenging superoxides anions to hydrogen peroxide and ultimately to water and oxygen (Hu *et al*., 2002; Tirtha *et al*., 2007). The presence of phenols and flavonoids in the extract may help among others, in preventing oxidative stress by scavenging free radicals and bioactivation of carcinogens for excretion in the liver (Renaud *et al*., 1999; Khanna *et al*., 2002). Nitric oxide (NO) is constitutively produced in endothelial cells to maintain the dilation of blood vessels and relaxation of smooth muscles (Huk *et al*., 1998).

Peroxynitrite (OONO⁻), the most reactive free radical is formed by further reaction of NO with O_2^-, which is the major cause of irreversible damage to membranes and degenerative diseases (Van acker *et al*., 1995). Poly phenolic compounds are implicated to scavenge directly nitric oxide molecules, thereby preventing the oxidation of LDL-C and tissue oxidative damage (Van Acker *et al*., 1995). Flavonoids were reported to decrease also, the immobilization and adhension of leukocytes to endothelial walls, and degranulation of neutrophils without affecting superoxide production, therby regulating inflammatory responses in tissue injury and immune responses (Ferrandiz *et al*., 1996).

In addition, plants that contain alkaloids, flavonoids and saponins in substantial quantities might have good blood glucose reducing activities by reducing the absorption of dietary glucose in the gastrointestinal tract (Price *et al*., 1987; Khanna, 2002). Furthermore, the high saponins level, may reduce blood cholesterol levels by preventing cholesterol absorption in the gastrointestinal tract through the formation of complexes with cholesterol in diet (Belles *et al*., 2005) and inhibiting the production of apo B, needed for LDL-C production, transport and binding, thereby enhance the liver functions by facilitating reverse cholesterol transport and bile acid excretion (Sinclair *et al*., 2002; Turner *et al*., 2004). However, the presence of oxalates in the leaf extract may sound a note of caution in the ingestion of the plant extract. Oxalates from plant sources have been known to cause irreversible oxalate nephrosis when ingested in large doses. It is an antinutrient and prevents the absorption of some vital nutrients in food, especially divalent metals (Ca^{2+}, Mg^{2+} etc) and fatty acids by forming salts. Oxalate intoxification (high ingestion of oxalate) causes malabsorption syndromes leading to steatorrhoea, in which fatty acids are not absorbed, causing formation of insoluble calcium salt of fatty acid (Vasudevan and Sreekumari, 2000).

The concentrations of sodium, potassium and magnesium ions (Table 2) in the aqueous leaf extract of *P. amarus* may be sufficient for the maintenance of osmotic pressure, water balance and pH in the body. This could explain why the leaf preparation is used traditionally to prevent oedema, kidney problems and oliguria (Claxito *et al*., 1998). The level of calcium in the leaf may be adequate for the removal of the anti-nutritional factors (oxalate) and modulate oxalate intoxification by forming complexes with oxalate. Complex formation between calcium and oxalate makes more calcium unavailable, but ensures excretion of the oxalates.

Presence of minerals such as selenium, zinc, iron and magnesium in the leaf extract may possibly contribute to immunomodulatory action,

since these substances have been implicated in immune modulation (Prasad, 2000; Ravaglia *et al.*, 2000). This submission is strengthened by reports of De la Fuente *et al*. (1998) and Girodon *et al*. (1999) that deficiencies of selenium, copper and zinc induced attenuation of immune functions including phagocytic activity, natural killer cell activity, macrophages, antigen-specific antibody production, and the proliferative response of T cells. In addition, the presence of copper, zinc, selenium and iron may enhance the activities of antioxidant enzymes, since these elements serve as cofactors for such enzymes and ultimately modulate the immune system (Tainer *et al*., 1988; De la Fuente *et al*., 2005).

The aqueous leaf extract of *P. amarus* could enhance erythropoietin activity due to the level of iron present (Table 2). However, high iron level in blood has been reported in enhancing oxidative stress by generating reactive oxygen species via the fenton reaction (Halliway *et al*., 1992). The presence of lead may suggest possible toxicity to tissues because no safe level of lead in blood was established (WHO, 2002). Chronic exposure to lead has been implicated in kidney failure (Champe *et al*., 2005). Furthermore, lead inhibits the incorporation of iron into the protoporphyrin ring during heme biosynthesis and so, could precipitate acquired porphyria, hypochromic-microcytic anaemia and hyperuricemia (Jeremy *et al*., 2001; Champe *et al*., 2005). Paradoxically, lead has been reported to stimulate immune reponse but reduce immune function of T helper cells *in vitro* (Kamla *et al*., 2003; Zhao *et al*., 2004).

Male rat behaviour and morphology

The observation in the rats administered 1000 mg/kg body weight of the aqueous leaf extract of *P. amarus* (Plate 1) suggested that the aqueous leaf extract of *P. amarus* was toxic at the dose (Ecobichon, 1997). The ability of the extract to reduce body weight of male rats at various doses (Figure 1) suggests an immune modulating possibility of *P. amarus* leaf, since immune stimulating plants or drugs have been reported to possess body weight reducing or maintenance properties (Pond, 2005). In addition, immune disorders are frequently reported in over weight individuals (Digirolamo, 1994). However, care must be taken as chronic weight loss, except for medical reasons (obesity) may be a sign of systemic reaction.

IL-2, IL-6 and TNF-α

The trend presented in the serum IL-2 concentrations of rats administered the aqueous leaf extract (Table 2) suggested that the *P. amarus* leaf has immune modulation potential. IL-2 is normally produced by the body to regulate immune

response to environmental substances (molecules or microbes) that gain access to the body (Smith, 1988). These substances (antigens) are recognized as foreign by antigen receptors that are expressed on the surface of lymphocytes. Antigen binding to the T cell receptor stimulates the secretion of IL-2, and the expression of IL-2 receptors. The IL-2/IL-2R interaction then stimulates the growth, differentiation and survival of antigen-selected cytotoxic T cells via the activation of the expression of specific genes (Beadling *et al*., 1993; Beadling and Smith, 2002*).* As such, IL-2 is necessary for the development of T cell immunologic memory, one of the unique characteristics of the immune system, which depends upon the expansion of the number and function of antigen-selected T cell clones. IL-2 is also necessary during T cell development in the thymus for the maturation of a unique subset of T cells that are termed regulatory T cells (T-regs) (Sakaguchi *et al*., 1995). After exiting from the thymus, T-regs function to prevent other T cells from recognizing and reacting against "self antigens", which could result in "autoimmunity". Thus, IL-2 is required to discriminate between self and non-self, another one of the unique characteristics of the immune system (Thornton *et al.,* 2004). In addition, IL-2 is able to facilitate production of immunoglobulins made by B cells and induce the differentiation and proliferation of natural killer cells (Waldmann, 2006).

The result of the serum IL-6 and TNF-α concentrations in rats administered the leaf extract (Table 3) supported the immune modulation potential of the aqueous leaf extract of *P. amarus*. IL-6 and and TNF-α are important cytokines involved in the differentiation and proliferation of immune cells (Janeway *et al*., 2001).

IL-6 is one of the mediators that are released very early in an injury process. Monocytes/macrophages release IL-6 in skin burns or other tissue damage leading to inflammation (Murtaugh *et al*., 1996). It is also secreted by fibroblasts, endothelial cells, keratinocytes, mast cells, T cells and many tumor cell lines to stimulate immune response to trauma (Febbraio and Pedersen, 2005). It acts as both a pro-inflammatory and anti-inflammatory cytokine. It is one of the mediators that are released very early in an injury process and it protects against tissue damage and increase synthesis of fibrinogen as part of healing processes (Murtaugh *et al*., 1996). IL-6's role as an anti-inflammatory cytokine is mediated through its inhibitory effects on TNF-alpha (Heinrich *et al*., 2003). IL-6 has major effects on hemeatopoiesis, stimulation of acute phase reaction and tyrosine kinase, which enhance diapedisis and cell-cell communication of innate immune cells (Abbas *et al*., 1997). In addition, IL-6 regulates glucose and lipid metabolisms in the liver, adipose tissue, and

skeletal muscles and mediates the transcription of varieties of proteins through cAMP/protein kinase, signal transduction pathway and protein kinase C (Nakajima *et al*., 1995; Febbraio and Pedersen, 2005). However, the over-expression of IL-6 has been reported in some disease conditions such as atherosclerosis due to increased fibrinogen production, sepsis, liver diseases, degenerative disease (cancer), oedema, massive weight loss and inflammatory disorders (Smolen and Maini, 2006; Dubiński and Zdrojewicz, 2007). Therefore, the reduction in the serum IL-6 concentrations at 250 and 500 mg/kg body weight of the extract suggested that *P. amarus* leaf regulated the release of IL-6 in muscles and tissues, probably indicating no tissue damages and inflammatory processes. Increase in dose of the extract suggested the loss of the immunoprotective activity of *P. amarus* leaf.

TNF-α is produced by activated macrophages and other cell types including T and B cells, natural cells, endothelial cells, smooth muscle cells and some tumor cells (Aggarwal and Reddy, 1994). It regulates the growth of normal cells and induces the apoptosis of abnormal or infected cells (Janeway, 1997). It is involved in systemic inflammation and stimulates the acute phase reaction (Locksley *et al*., 2001). The primary role of TNF-α is in the regulation of immune cells by inducing apoptotic cell death, inflammation and inhibition of tumorigenesis and viral replication. TNF-α play critical roles in normal host resistance to infection and to the growth of malignant tumors, serving as immunostimulants and as mediators of the inflammatory response (Aggarwal and Vilcek, 1991). TNF-α has receptors on the surface of all organs in the body and aids in maintaining homeostasis by the regulation the body's circadian rhythms. The over-expression of TNF-α, however, has been implicated in increased risk of mortality, heart disease, septic shock, dehydration, anorexia, net catabolism, weight loss, anaemia, hepatosplenomegaly, autoimmune disorders and increased risk of cancer (Cesari *et al*., 2003; Dubiński and Zdrojewicz, 2007). Therefore, the changes observed in serum TNF-α concentration in rats administered with the extract supported the immunomodulatory potential of the extract as serum TNF-α concentration correlates with systemic inflammation and stimulation of the acute phase reaction, suggesting infection of tissue damage.

Hemeatological parameters

The administration of aqueous leaf extract of *P. amarus* might have induced anaemia as observed with red blood cell and hemeoglobin count at 500 and 1000 mg/kg body weight (Table 4). Although, packed cell volume (PCV) was not affected by the administrations of the extract at all doses, but increase in erythrocyte sedimentation rate at all doses further strengthened the anaemic capability of the extract at that dose. The increase in the total RBCs may be due to recticulocytes (although not counted in this study), that could have resulted from severe destruction of matured RBCs with a concomitant increase in erythropoiesis (Ganong, 2002). The aqueous leaf extract of *P. amarus* may have stimulated the kidney to release erythropoietin, which regulates RBC production (Sanchez-Elsner *et al*., 2004). Moreover, the destruction of mature RBCs might have caused hypoxia in tissues, resulting in the stimulation of erythropoiesis. However, prolonged or long-term hypoxia in cells or tissues could result in the death of such cells (Champe *et al*., 2005).

The trends obtained in the mean cell volume (MCV) (Table 4) signifies that the sizes of RBCs were reduced, indicating microcytic anaemia due to either iron deficiency and/ or anaemia of chronic disease (inflammation) (Topley, 1998). In addition, result of the mean cell hemeoglobin (MCH) and mean cell hemeoglobin concentration (MCHC) suggests the possibility of the extract predisposing the consumer to iron deficiency anaemia and/ microcytic hypochromic anaemia (Vasudevan and Sreekumari, 2000). Thus, the erythrocyte function indices confirmed the anaemic capabilities of administration of the aqueous leaf extract of *P. amarus* at 1000 mg/kg body weight in male rats. The anaemic tendency following administration of aqueous leaf extract of *P. amarus* in male rats may be due to the presence of saponins (Choi *et* al., 2001) and/or lead (Champe *et al*., 2005).

The administration of the aqueous leaf extract of *P. amarus* boosted the total white blood cell and lymphocyte counts at all doses (Table 5), thus, suggesting immunomodulation capability as white blood cells are involved in fighting infection and clearing off injured or dead cells and tissues in body (Jeremy *et al*., 2001). However, excessive count of white blood cells (WBC) and lymphocytes is implicated in trauma, uraemia, leukamia, myeloproliferative disorder, hemeorrhage, myocardial infarction, chronic inflammation, and tissue necrosis (Vasudevan and Sreekumari, 2000; Powers and Silberstein, 2008). Therefore, the sharp increase in WBC count at 1000 mg/kg body weight could indicate possible over–stimulation of the immune system that requires careful interpretation. In addition, the marked increase in lymphocyte count at 1000 mg/kg body weight of extract could also indicate over-stimulation of lymphocytes production (lymphocytosis) and might indicate trauma or tissue damage. This is because inflammatory responses elicit markedly increased lymphocytes count, especially T helper cells, whose production is stimulated by inflammation and help to recruit other immune cells to the site of inflammation (Jeremy *et al*., 2001). The result of

the total white blood cell and lymphocyte counts supports the reported trend in serum IL-2 concentration (Table 3).

Reduction in the blood level of neutrophils at 500 and 1000 mg/kg body weight of the extract (Table 5) may suggest tissue damage, malignant diseases, splenomegaly, megaloblastic anaemia or a decrease in the production of neutrophils due to the reduced of the expression of IL-6 and TNF-α. IL-6 and TNF-α are known to regulate the activities of innate immune cells, especially neutrophils and macrophages (Abbas *et al*., 1997). The blood basophil levels observed in this study at 1000 mg/kg body weight of the extract could trauma and/or some allergies (Topley, 1998).

The results for the leukocytes counts, therefore, provided a scientific basis for the traditional use of the leaf extract *P. amarus* as an immune modulator. The trends obtained in the leukocyte parameters are due to the serum concentrations of IL-2, IL-6 and TNF-α in rats following the administration of the leaf extract. The almost dose dependent decreases in the platelet count in the study (Table 5) supported the anaemic capability of the extract as suggested by the reduced hemeoglobin count, erythrocyte function indices and increased erythrocyte sedimentation rate (Table 4) (Champe *et al*., 2005). Anaemia has been reported in cases of reduced number of platelets (Topley, 1998).

Blood glucose

The administrations of the aqueous leaf extract of *P. amarus* had blood glucose reducing effects (Figure 9), which may be attributed to the presence of alkaloids and polyphenols (Khanna *et al*., 2002). The blood glucose reducing activity was accompanied by reduction in the body weights of rats (Figure 8). The probable mechanism of the reduction in blood glucose level-body weight loss; could be through increased insulin secretion by pancreatic stimulation and the prevention of absorption of glucose in the gut (Borhanduddlin *et al*., 1994). IL-6 mediates the absorption of dietary glucose in the gastrointestinal tract by inhibiting intake of glucose by GLUT-2. However, since IL-6 concentration was reduced in the study (Table 3), the absorption of dietary glucose could be increased and the blood glucose level is increased sharply, thereby stimulating the pancreas to release insulin that regulates the uptake/storage of glucose at the muscles by GLUT-4 (Jeremy *et al*., 2001).

In addition, the reported levels of alkaloids, saponins and flavonoids might prevent the absorption of dietary glucose in the gastrointestinal tract (Price *et al*., 1987; Khanna, 2002). The high content of saponins in the leaf extract could have permeabilize the plasma membranes of the small intestine, thereby causing irreversible disorder and disruption of the plasma membrane resulting in marked reduction in the absorption of dietary glucose in the gastrointestinal tract due to 'autointoxication' or "leaky gut" (Choi *et* al., 2001; Francis *et al*., 2002; Evers, 2008). Thus, the reported decreases in fasting blood glucose levels suggest that the aqueous leaf extract of *P. amarus* might be useful as an immune modulator regime, as supported by the report of Volk *et al*. (1993) that blood glucose value of 120 mg/dl reduced the phagocytic index of macrophage and neutrophils by 75%. In addition, Langley-Evans and Carrington (2006) reported that increased concentrations of glucose in the blood proportionally reduce the ability of cell-mediated immune cells to capture bacteria and increased the incidence of degenerative diseases (cancer).

Serum lipid profile

The dose dependent reductions in total cholesterol concentration in serum of male rats following the administration of aqueous leaf extract of *P. amarus* (Table 6) might have resulted from the presence of flavonoids, tannins and saponins that were reported to inhibit cholesterol biosynthesis in the liver (Sinclair *et al*., 2001), and the inhibition of the absorption of cholesterol via the small intestine (Gorinstein *et al*., 2006; Evers, 2008). The reduction in triacylglycerol recorded in this study could result from the reported levels of alkaloids, saponins and oxalates, which could result in the reduction of the absorption of dietary glucose in the gastrointestinal tract due to 'autointoxication' or "leaky gut" (Choi *et* al., 2001; Francis *et al*., 2002; Evers, 2008). This has to be so since the reported reduction in serum cholesterol concentration in this study was not accompanied by lipolysis, as seen in the serum TAG levels (Table 6).

The increase in serum HDL levels at all the dose of the leaf extract administered suggested a possible boost of HDL-C biosynthesis in the liver promoted by the presence of flavonoids (Renaud *et al*., 1999). Therefore, more cholesterol would be transported from peripheral tissues to the liver for excretion and could be the reason for the reported trend in the serum cholesterol concentration. The reduced serum levels of VLDL-C is consistent with the reported decrease in serum TAG, meaning that less TAG was exported from the liver to extra-hepatic tissues. However, the risk of liver fatty infiltration would be increased (Jeremy *et al*., 2001).

Dose dependent decreases in serum LDL-C levels is consistent with the serum cholesterol-lowering capability of the aqueous leaf extract that possibly enhanced reverse cholesterol transport and bile acid excretion, through the inhibition of production apo B, needed for LDL-C production, transport and binding (Turner *et al.*, 2004). The observed increase and decrease in the serum HDL-

C and LDL-C levels respectively, suggests a reduced risk of developing atherosclerosis following repeated administrations of the aqueous leaf extract of *P. amarus* (Table 6). However, complete hypolipidemia (marked decreased levels of VLDL-C, LDL-C, total cholesterol, and TAG) suggest possible malabsorpion of lipids (in small intestine) that could prevent the absorption of fat soluble vitamins, which in turn may lead to blindness or eyes defects that arise from degenerative changes in the retina, and also, physical and mental retardations (Jeremy *et al*., 2001). Therefore, the marked reduction in the serum lipids may be due to the prevention of the absorption of dietary lipid in the small intestine promoted by saponins and oxalate (Vasudevan and Sreekumari, 2000), which were reasonably present in the aqueous leaf extract of *P. amarus*.

Serum uric acid, liver GSH and MDA

The administration of the aqueous leaf extract of *P. amarus* demonstrated a good means of recovery of reduced glutathione (GSH), in line with the presented increase in liver GSH levels at all the administered doses (Table 7). However, increasing the dose of extract possibly promoted the generation of more pro-oxidants (Proctor, 1970; Cutler, 1984), or caused loss of organ function due to hepatocyte infiltrations, necrosis of chronic inflammation. GSH is the one of the most proactive endogenous antioxidants in the body, because it is involved in many detoxification processes (Beck, 1993). The levels of saponins and polyphenolic compounds in the aqueous extract (Table 1), inferably suggest antioxidant properties and thus, the recovery of reduced GSH that could prevent the development of degenerative diseases caused by oxidative stress. The trend obtained in liver GSH following administration of the leaf extract is consistent with the reports of Anderson *et al*. (1997) and Xiang *et al*. (2001) that decrease in liver GSH concentration resulted in the increase in tissue inflammation and over-expression of IL-6.

The significant decrease in serum uric acid concentration presented following the administration of the aqueous leaf extract of *P. amarus* (Table 7) was propably due to reduced tissue degradation (turn over due to trauma and high rate of catabolism as in starvation), reduced possibilties of developinging malignant tissues (lymphomas, polycethemia, luekaemia), renal injury or failure and gout (Jeremy *et al*., 2001; Champe *et al*., 2005). In addition, the concentration of uric acid in the blood was reported to be tightly regulated by the level of glutathione in the liver, in which the increase in GSH concentration in liver facilitated the excretion of uric acid in the blood, thereby reducing the risk of the formation of kidney stones and gout (Beck, 1993). However, there is increase in the demand for uric acid as an antioxidant (greater than 50% of total antioxidant pool in the body) when the concentration of GSH in the liver is low, in conditions such as oxidative stress, liver diseases and chronic inflammation (Beck, 1993; Xiang *et al*., 2001).

The increase in serum uric acid concentration with increase in doses of the extract may suggest an increase in demand for a boost in the antioxidant system since over half the antioxidant capacity of blood plasma comes from uric acid (Glantzounis *et al*., 2005; Baillie *et al*., 2007). Strong reducing substances, such as ascorbate, uric acid can also act as a pro-oxidant particularly at elevated levels (Proctor, 1970; Cutler, 1984). In addition, uric acid is an endogenous adjuvant that drives immune responses in the absence of microbial stimulation, thus modulating innate immune response by triggering interleukin-mediated inflammation (Short *et al*., 2005).

The reduced MDA concentration in the liver following administration of the aqueous leaf extract of *P. amarus* at 250 mg/kg body weight doses (Figure 40), supported the suggested antioxidative property of the aqueous leaf extract. However, the antioxidative property of the leaf extract decreased with increase in dose. The reductions in MDA concentrations in the liver at 250 mg/kg body weight doses suggest that the leaf extract at these doses reduced the induction or progression of oxidative stress, but at high doses, pro-oxidants were probably generated as indicated in reduced glutathione concentrations in the liver and increase in serum uric acid concentrations (Table 7), because elevated levels of strong reducing agents have been reported to act also as pro-oxidant (Proctor, 1970; Cutler, 1984). The result obtained in the MDA concentrations in the liver is consistent with the trends reported in the liver GSH concentrations and serum uric acid concentrations, all indicating decrease in the endogenous antioxidant capability with increase in dose of the leaf extract.

Conclusion

From the foregoing, it is logical to make the scientific submission that the aqueous leaf extract of *P. amarus* modulated the immune system of rats, thereby establishing scientifically the folklore use of the aqueous leaf extract of *Phyllanthus amarus* as blood tonic for the prevention and/ or cure of infective and degenerative diseases. However, the use of the leaf extract is recommended at doses not beyond 250 mg/kg body weight.

Disclosure Statement

"No competing financial interests exist."

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