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ANTIOXIDANT AND HEPATOPROTECTIVE ACTIVITIES OF *HARUNGANA MADAGASCARIENSIS* LAM. EX POIR. (HYPERICACEAE) ETHANOL LEAF EXTRACT IN PHENYLHYDRAZINE-INDUCED ANAEMIA IN WISTAR RATS

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Abstract

This study was designed to investigate the antioxidant and hepatoprotective activities of ethanol leaf extract of *Harungana madagascariensis* in phenylhydrazine-induced anaemic Wistar rats. Twenty male Wistar rats were divided into five groups of four rats each. Group 1 served as Normal control while Group 2 (Postive control) was induced with anaemia using phenylhydrazine (PHZ) at 40 mg/kg body weight intraperitoneally. Group 3 (extract alone) received ethanolic leaf extract of *H. madagascariensis* at 2000 mg/kg body weight. Groups 4 and 5 were both induced with anaemia using PHZ at 40 mg/kg and treated with the ethanolic leaf extract of *H. madagascariensis* low dose (LD) at 2000 mg/kg and high dose (HD) at 4800 mg/kg body weight respectively. After 28 days of treatment, blood samples from each animal were collected for the estimation of the biochemical parameters. There was a significant increase in the level of Superoxide dismutase (SOD), Catalase (CAT) and Glutathione peroxidase (GPx) ($p<0.001$) and a significant reduction ($p<0.001$) in lipid peroxidation in the extract treated groups compared with the anaemic control group. Also, the extract significantly reduced the levels of liver enzymes when compared with the anaemic control group ($p<0.001$). Oral administration of ethanol leaf extract of *Harungana madagascariensis* can be said to have antioxidant and hepatoprotective potentials.

Keywords: *Harungana madagascariensis (HM), Anaemia, Phenylhydrazine (PHZ), Antioxidant Enzymes, Lipid peroxidation, Liver Enzymes*

Introduction

Over the years, medicinal plants have been recognized to be of great importance to the health of individuals and communities [1]. These medicinal plants are said to contain biologically active compounds such as carbohydrates, proteins, enzymes, fats and oil, minerals, vitamins, alkaloids, anthraquinones, terpenes, flavonoids, anthocyanins, carotenoids, sterols, simple phenolic glycosides, tannins, saponins, polyphenols, to mention a few which have medicinal activities that are either of therapeutic importance or are precursors for the synthesis of useful drugs [2]. *Harungana madagascariensis* is one of such traditional medicinal plants used in the treatment of anaemia [3,4,5].

The plant is native to Madagascar, Mauritius and Tropical Africa (i.e. Sudan, Kenya, Tanzania, Rwanda, Cameroon, Burundi, Equatorial Guinea, Congo, Ethiopia, Nigeria, and Malawi) [6]. *H. madagascariensis* occurs at medium to low altitudes in evergreen forest, at forest margins and along river and stream banks [6]. Its common names are: Dragon blood tree, haronga, harungana, orange-blood, orange-milk tree, praying hands bush, or praying-hands [7]. It has several names among various tribes in Nigeria. Among the Efiks, Hausa, Yoruba and Igbo people in Nigeria, it is referred to as Oton, Alillibar, elepo, and uturu (ururtu) respectively [7]. *Harungana madagascariensis* plant belongs to the genius that is composed of single species belonging to the family of Hypericaceae.

Anaemia is a common blood disorder that affects people of all ages, although the people at greater risk are the elderly, young women of childbearing age and the infants [8]. It is a condition that has multiple origins and one of such origin is destruction of red blood cells membrane induced by free radicals. It is well known that phenylhydrazine induces haemolytic anaemia. This is thought to result from the reaction of phenylhydrazine with haemoglobin [9]. The accompanying oxidation of phenylhydrazine leads to the formation of a number of products, including benzene, nitrogen, hydrogen peroxides, superoxide anion and the phenyl-radical [9].

Therefore, the aim of this study was to investigate the antioxidant and hepatoprotective activities of ethanolic leaf extract of *Harungana madagascariensis* (HM) in phenylhydrazine (PHZ)-induced anaemic Wistar rats by mimicking ethno-medicinal practices.

Methods

Drugs and Chemicals

The following drugs and reagents were used; dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA), phenylhydrazine chloride, EDTA, chloroform and ethanol (Sigma-Aldrich, Germany). All drugs and reagents were of analytical grade.

Collection of plant material

Fresh leaves of *Harungana madagascariensis* (HM) were collected from the deciduous forest in Calabar, Nigeria. The plant was identified at the Herbarium in Botany Department, University of Calabar, Nigeria. The leaves were thoroughly rinsed in tap water and thereafter dried in open-air for about 2 weeks, away from direct sunlight. It was then cut into small pieces and pulverized into coarse powder using an electric blender.

Extraction

The preparation of the extract was done according to the method described by Adeneye et al. [7]. A 1000 g of the powdered leaves of HM was soaked in 80 % ethanol/water (v/v) in a glass jar, stirred and kept for 48 h. The mixture was then filtered first with a white cotton material, then with Whatman filter paper into a beaker. Thereafter, this was concentrated to dryness using a rotary evaporator (Büchi, Switzerland). The HM ethanol leaf extract yield was 108.43 g.

Acute toxicity test

Twenty-four (24) Wistar rats of both sexes (135 – 147 g) were purchased from the Department of Agriculture, University of Calabar, Nigeria. They were randomly grouped into 6 groups of 4 animals each. The rats were allowed to acclimatize for 7 days. Five doses of the extract (300 mg/kg, 600 mg /kg, 1200 mg/kg, 2400 mg/kg and 4800 mg/kg), each dissolved in distilled water was administered *per os* to the rats in different groups respectively. The first group served as the control. Animals were monitored closely within the first 24 h post treatment for behavioural changes and death due to

toxicity. The median lethal dose (LD50) was determined according to the method of Kengni et al. [10].

Experimental animals

A total of twenty (20) male albino Wistar rats (150-230 g) were used for this experiment. The animals were obtained from the rat colony of the animal houses of Department of Agriculture, University of Calabar, Nigeria. The rats were acclimatized for 7 days and maintained on standard rat feed (growers feed) and tap water which was made available ad libitum. The rats were maintained at an ambient temperature between 28 – 30 °C, humidity of 55 ± 5 %, and standard (natural) photoperiod of approximately 12 h of light (06:30 h – 18:30 h) alternating with approximately 12 h of darkness (18:30 h - 06:30 h). The conduct of experiment was approved and in accordance with the approved research guidelines on laboratory animal use of the Faculty of Basic Medical Sciences, University of Calabar, Calabar where the animal studies were carried out. The rodents were handled in accordance with the internationally accepted principles for laboratory animal use and care as found in the European Community guide lines (EEC Directive of 1986; 86/609/EEC).

Determination of haemoglobin (Hb) concentration

Before the induction of anaemia using Phenylhydrazine (PHZ), Hb concentration of the animals was determined using Sahli's apparatus. After the determination of Hb concentration, anaemia was induced by intraperitoneal administration of phenylhydrazine (PHZ) at 40 mg/kg at 48 h interval for two (2) consecutive times [11]. After the induction of anaemia, rats with Hb concentration <11.5 g/dl were declared anaemic.

Experimental Design

The animals were allowed to acclimatize for 7 days. Thereafter, they were randomly divided into 5 groups of 4 rats kept in different cages. The Experiment lasted for 28 days and the groups were as follows:

Group 1 - Normal Control group received distilled water only.

Group 2- Anaemic Control group was challenged with a single dose of 40 mg/kg phenylhydrazine (PHZ) intraperitoneally and received distilled water only.

Group 3 - Extract Treated Group: received HM extract at 1500 mg/kg/day orally alone.

Group 4 Anaemia + Extract Treated Group (Low Dose) was challenged with a single dose of 40 mg/kg phenylhydrazine (PHZ) intraperitoneally and received HM extract at 1500 mg/kg daily after challenge.

Group 5 - Anaemia + Extract Treated Group (High Dose) was challenged with a single dose of 40 mg/kg phenylhydrazine (PHZ) intraperitoneally and received HM extract at 3000 mg/kg daily after challenge.

Collection and analysis of blood samples

The animals were starved for overnight but had water and were anesthetized using chloroform in an inhalation chamber with 4 % isoflurane (IsoFlo, Abbott Laboratories, Berkshire, UK) regulated with a calibrated vaporizer. Blood samples from each rat were collected via retrobulbar plexus into plain sample bottles. Blood samples were collected into anti-coagulant-free plain sample bottles were allowed to clot.

The resultant serum was collected into pre-labelled eppendorf tubes on ice after centrifugation at 3000 rpm for 10 min and used for determination of Superoxide dismutase (SOD), Catalase (CAT) and Glutathione peroxidase (GPx). These enzymes were assayed by methods described by Marklund [12], Aebi [13] and Rice-Evans [14] respectively, lipid peroxidation (MDA) was assayed using the method described by Jiang et al [15] and the following liver enzymes, Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) were measured by the method of Reitman and Frankel [16] as described by Random laboratories, United Kingdom using Random kits while Alkaline phosphatase (ALP) was assayed based on the method described by King and Armstrong [17].

Statistical analysis

Data were presented as mean ± SEM. Experimental data were analysed using Analysis of

variance (ANOVA) followed by a post HOC test (least square difference {LSD} test) to determine significant differences between means. The analysis was done with an SPSS 18 statistical package. $p<0.05$ was accepted as statistically significant.

Results

Acute toxicity test

After 24 h of administration of the extract orally, A total of five (5) Wistar rats mortality occurred. Two (2) Wistar rats at the dose of 2400 mg/kg and three (3) Wistar rats at the dose of 4800 mg/kg. Figure 1 shows an LD50 graph plotted and its value was found to be 4639.80 mg/kg.

Comparison of the serum antioxidant enzymes and Malondialdehyde (MDA) concentrations

Table 1 shows the comparison of the serum antioxidant enzymes and Malondialdehyde (MDA) concentrations in the different experimental groups where it showed a significant ($p<0.001$) reduction in the antioxidant enzymes (SOD, CAT, GPx) concentrations and a significant ($p<0.001$) elevation in MDA concentration in the anaemic control group when compared to the different experimental groups while there was a significant ($p<0.001$) elevation in the extract alone groups of theses antioxidant enzymes.

Comparison of the liver enzymes concentrations

Table 2 shows the comparison of the liver enzymes concentrations in the different experimental groups. The liver enzymes (AST, ALP and ALT) concentrations in the anaemic untreated group showed a significant ($p<0.001$) increase when compared to other experimental groups.

Discussion

Results from this study shows that oral administration of *H. madagascariensis* ethanol leaf extract is non-toxic and can be consumed by humans since the lethal dose is very high (Figure 1). We also observed that there was a significant decrease in antioxidant enzymes (SOD, GPx and CAT) level in the anaemic control group compared with the normal control group (Table 1). This result suggests that there is a continuous generation of reactive oxygen species (ROS) which is a steady state cellular event in respiring cells [18] and their production can be grossly amplified in response to a variety of pathological conditions such as inflammation, immunologic disorders, hypoxia, metabolism of drugs or alcohol, exposure to UV and deficiency in antioxidant enzymes [19] but in this study is due to the administration of PHZ in the anaemic control group. Whenever the balance between ROS production and antioxidant defence is lost, 'oxidative stress' results which through a series of events deregulates the cellular functions leading to various pathological conditions [18, 20].

The result also showed a significant increase in these antioxidant enzymes in the extract treated groups compared with the anaemic control group and this could be suggested that ethanol leaf extract of *Harungana madagascariensis* has the ability to protect cell membrane from free radical activity hence helps to reduce or prevent oxidative stress due to its high content of antioxidants phytochemicals like flavonoids and phenols. This finding is in agreement with earlier finding that the phytochemicals screening of *Harungana madagascariensis* leaf contains saponin, tannin, phenols, oils, sterols and flavonoids [21]. Phenolic compounds can protect the human body from free radicals [14]. This clearly indicates that *Harungana madagascariensis* ethanol leaf extract exerted an antioxidant effect. The function of antioxidant enzymes such as CAT and SOD is to protect cells from toxic reactive oxygen species (superoxide anion, hydroxyl radical, hydrogen peroxide etc.) generated during cellular metabolism, biotransformation and xenobiotics [22]. The increase in these antioxidant enzymes in the extract treated groups reduces the damage of cellular macromolecules (DNA, protein and lipids) [23], scavenges the free radicals produced by PHZ in the low and high dose anaemic treated groups, SOD catalyses the dismutation of the highly reactive superoxide anion to O_2 and to the less reactive species Hydrogen Peroxide (H_2O_2). Hydrogen Peroxide is in turn destroyed by CAT or GPX reactions [24, 25, 26].

Therefore, increase antioxidant enzymes may preserve vascular function and protect vascular injuries from ROS and perhaps from other oxidant species, including phenylhydrazine (PHZ) radicals. Another possible mechanism is that antioxidants

could stimulate erythropoiesis process [27] as seen in the above result where the level of RBC, PCV, Hb concentration increased remarkably in the extract treated groups compared with the anaemic control group. Increased levels of these antioxidant enzymes are at particularly high levels in the liver and also serve in detoxification metabolism [28].

The level of Malondialdehyde (MDA) which is a product of lipid peroxidation was measured in all the experimental groups. The production of MDA is used as a biomarker to measure the level of oxidative stress in an organism [29]. Result showed a significant increase in MDA level in the anaemic control group compared with the normal control group. This could be suggested that due to a high generation of reactive oxygen species (ROS) by the auto-oxidation of PHZ, free radicals are produced such as phenyl-hydrazyl radical, phenyl-diazene and benzenediazonium ions [30]. These free radicals form a chain reaction which will lead to oxidative stress, this is understood as an imbalance situation with increased oxidants or decreased antioxidants [31, 32] and then lipid peroxidation will occur. Currently, lipid peroxidation is considered as the main molecular mechanisms involved in the oxidative damage to cell structures and in the toxicity process that lead to cell death [33].

The result also showed a significant decrease in the level of MDA in the extract treated groups when compared with the anaemic control group. Since little or no literature review has been cited on this study, it could be suggested that this inhibitory effect of the extract on MDA production in these extracts treated groups may be attributed to the high phenol content of *Harungana madagascariensis* leaf extract [29]. In addition, flavonoids, terpenes and saponins phytochemicals contained in this extract reduce the level of hydrogen peroxide (H_2O_2)- induced stress through the reduction of MDA concentration [34]. Thus, *Harungana madagascariensis* ethanol leaf extract has the potentials to protect cell membranes from lipid peroxidation (oxidative stress) by inhibiting or reducing the production of free radicals because it contains phytochemicals like flavonoids and saponins as reported by Henneberg et al. [35]. There was a significant increase in AST, ASP, ALT levels in the anaemic control group compared with the normal control group. Physiologically, every organ in the body is exposed to a degree of oxidative stress by oxygen free radicals generated during metabolism and the plasma membranes are the majorly attacked by these free radicals. More so, the activities of liver enzymes take place exclusively in the plasma membranes of the hepatocytes [36]. Thus, under normal conditions, if the plasma membrane is disrupted by oxidative stress then, there should be a rise of the liver enzymes in the plasma. The increase of these liver enzymes in the anaemic control group could suggest that the liver cell is damaged and the enzymes leaked into the blood where they were measured. This dramatic rise could be associated with acute liver damage due to the administration of PHZ which generates free radicals that are toxic to the liver. Hence could lead to the occurrence of hepatocellular damage. In addition to these markers of hepatocellular injury, measurement of the serum levels of alkaline phosphatase and bilirubin are also considered adjunct markers of acute or chronic hepatic damage. Serum aminotransferases are reflective of hepatocellular injury while alkaline phosphatase and bilirubin are reflective of hepatobiliary damage or obstruction [37, 38]. A useful biochemical index of bile duct damage is a sharp elevation in serum activities of enzymes localized to the bile ducts, primarily alkaline phosphatase [37, 38].

This result also showed a decrease in ALT, ASP and ALP levels in the extract treated groups compared with anaemic control group (Table 2). This could be due to the administration of *H. madagascariensis* which had reversed this deteriorating effect. The reduction observed in the level of these liver enzymes is suggestive of a cellular membrane/hepatocellular membrane protective effect of this plant extract. Again, this reduction indicates a reduced peroxidation of cell membrane [39] as observed in this study. This is in line with the study conducted by Adeneye et al [7] which reported that aqueous root extract of *Harungana madagascariensis* confers hepatocellular protection in rats. This positive effect may be attributed to the presence of flavonoid in the plant extract. Flavonoids have been reported to possess antioxidant activity [40] and thus capable of protecting cell membrane as of the extract of

Harungana madagascariensis. Flavonoids are known to have antioxidant activities [21]. In this study, they may have contributed to the reduction or prevention in the rise of liver alkaline phosphate. Hence, the leaf extract of *Harungana madagascariensis* can be said to have hepatoprotective potentials. With correlation to the results obtained from antioxidant enzymes which were significantly high in the extract treated groups, it is in accordance with a study that reported that antioxidant enzymes are particularly at high levels in the liver to serve in detoxification metabolism [28]. Saponin which is also a possible phytochemical constituent of this leaf extract has been successfully used in the management of liver inflammation, as tonic sedative formulas, to promote and vitalize blood circulation [41, 42].

In conclusion, the oral administration of ethanol leaf extract of *H. madagascariensis* can be said to have antioxidant and hepatoprotective potentials.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Table 1. Serum antioxidant enzymes and MDA concentrations of Wistar rats treated with ethanol leaf extract of *Harungana madagascariensis* in phenylhydrazine-induced anaemic

	CAT (U/ml)	GPx (nM/min/mg protein)	MDA (nmol/ml)	SOD (U/g tissue)
Normal control	0.64±0.01	76.66±0.37	3.08±0.02	226.86±1.79
Positive control	0.33±0.01***	42.79±0.75***	4.75±0.02***	176.48±0.65***
Extract treated	0.74±0.01***,c	86.88±0.46***£	2.34±0.02***,c	284.66±1.60***£
Ane + Ext. LD	0.59±0.00***,c,z	53.81±0.60***£,z	3.41±0.02***,c,z	220.36±2.30*,c,z
Ane + Ext. HD	0.66±0.01c,z,+++	64.06±0.54***£,z,+++	3.19±0.01***,c,z,+++	256.96±2.70***£,z,+++

Values are expressed as mean ±SEM, n = 8. *** = significantly different from normal control at p<0.001; * = significantly different from normal control at p<0.05; c = significantly different from positive control at p<0.001; z = significantly different from extract treated at p<0.001; +++ = significantly different from anaemic + Ext. (LD) at p<0.001

Table 2. Liver enzymes concentrations of Wistar rats treated with ethanol leaf extract of *Harungana madagascariensis* in phenylhydrazine-induced anaemic

	ALT (IU/L)	AST (IU/L)	ALP (IU/L)
Normal control	37.89±0.63	57.03±0.85	175.47±0.90
Anaemic control	190.57±3.63***	127.34±0.43***	279.13±1.01***
Extract treated	115.36±11.03***,c	95.490.56***£	226.88±1.58***£
Anaemia + Ext. (LD)	128.68±0.91***£	111.07±1.12***£,z	186.53±1.61***£,z
Anaemia + Ext. (HD)	109.46±0.67***£,+	81.77±1.02***£,z,+++	228.29±0.89***£,+++

Values are expressed as mean ±SEM, n = 8. *** = significantly different from normal control at p<0.001; c = significantly different from positive control at p<0.001; z = significantly different from extract treated at p<0.001; + = significantly different from anaemic + Ext. (LD) at p<0.05; +++ = significantly different from anaemic + Ext. (LD) at p<0.001

Figure 1. Lethality studies showing the effects of administering graded doses (300 – 4800 mg/kg orally in mice) of *H. madagascariensis* extract against the percentage mortalities (converted probits)