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Original Article

Hepatoprotective and Nephroprotective Potentials of Aqueous Leaves Extract of *Limonia acidissimain* Phenylhydrazine-Induced Anaemic wistar Rats

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A B S T R A C T

Bioactive composition of *Limonia acidissima* leaf and liver enzyme levels and kidney status of phenyl hydrazine (phz)-induced anaemic wistar rats were investigated. A total of forty two (42) wistar rats weighing between 150-200g were used. The animals were randomly divided into seven (7) groups of six rats each. Folic acid and vitamin B_{12} were used as reference drugs. Aqueous extract of *Limonia acidissima* leaves was administered to the rats for four (4) weeks period. Qualitative phytochemical analysis on the dry leaf samples revealed the presence of the following in low abundance: alkaloid, saponins, cardiac glycoside, steroids, phenols, terpenoids and proteins while flavonoids were in high abundance. A significant increase ($p<0.05$) was observed in the activities of serum alanine transaminase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) in thephenylhtdrazine-inducedanaemic non- treated groups in comparison to normal control. There was a significant increase ($p<0.05$) in urea, creatinine, potassium and sodium levels in the phenyl hydrazine-induced anemic non- treated rats compared to the normal control. The other groups treated with aqueous leaf extract *of Limonia acidissima* subsequently ameliorated these effects on the liver and kiney markers. Vitamine B_{12} and folic acid also reversed these effects on the liver enzymes and kiney markers. This outcome illustrates that the aqueous extract of the leaf of *Limonia acidissima* possess a protective potentials on the liver and kidney of phenyl hydrazine- induced wistar rats.

Keywords: Hepatoprotective, Nephroprotective, Phenlyhydrazine, *Limonia acidissima*..

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INTRODUCTION

A medical condition that is characterized by a reduction in the amount of haemoglobinin circulation, less than 13g/dl in males and 12g/dl in females is called Anaemia.[1]Anaemia account for serious health problems in many tropical countries as a result of the dominancy of different forms of parasitic infections, including malaria.[2]There are numerous causes and types of anaemia, these include: sickle-cell anaemia, iron deficiency anaemia, vitimin B_{12}deficiencyanaemia, drug induced anaemia, disease induced anaemia, etc.[3]Hence, factors contributing to anaemia may be linked not only to malnutrition and poverty, but also from the free radicals due to the disproportionate consumption of drugs and other xenobiotics as well as viral and parasitic infections.[4][5]In developed countries, iron deficiency is the major cause of anemia and results in insufficient red blood cell production. In some individuals, infections such as peptic ulcers may cause blood loss and anemia. In developing countries, iron deficiency affects all vulnerable groups. Malaria, which can contribute to excessive red blood cell destruction, and helminthes infections, a cause of excessive red blood cell loss, are geographically specific. Other infectious diseases also may be at play. There are contributing causes of anaemia which include inadequate knowledge of the problem of anemia, environmental factors, lack of access to medical services, and poverty.[6]

In the tropics, due to prevalence of malaria and other parasitic infections, between10 to 20% of the population

is reported to have less than 10 g/dl of hemoglobin in the blood.[7]Hemolytic anemia refers to a condition in which erythrocytes have a shortened life-span. Phenyl hydrazine is used for the induction of hemolytic anaemia and the study of its mechanism in many species is documented. Phenyl hydrazine is used worldwide mainly as a chemical intermediate in the pharmaceutical, agrochemical, and chemical industries.[8]It is toxic by single exposure through the oral route with LD_{50} of 80–188 mg/kg body weight and is expected to be toxic by the inhalation and dermal routes but data from these routes of exposure are less clear.[9]

Medicinal plants are useful worldwide in the management of diseases. Plants have provided the core traditional treatment for diverse types of disease and still offers vast possible source of new chemotherapeutic agents. In a recent report released by the World Health Organization (2006), more than 80% of the world population still depends on herbal medicines as their main source of health care. Many Africans of all ages depend on herbal medicine for primary health care.[10]The medicinal use of plants has been attributed to the presence in plant parts of bioactive compounds. These phytochemicals include glycosides, saponins, tannins, alkaloids and flavonoids.[11]Phytochemical screening of plants provides information on the chemical constituents of plants that is required, for the discovery of therapeutic agents and the information may be of value in unveiling new sources of such compounds as precursors for the synthesis of complex chemical substances and used in folkloric remedies.[12]These constituents may be necessary in preventing haemolytic diseases, such as anaemia.

Limonia acidissima is the only species within the monotypicgenus*Limonia*. It is native to the Indomalayaecozone to Bangladesh, India, Pakistan, Sri Lanka, and in Indochinese eco-region east to Java and the Malesia eco-region. Common names for the species in English include wood-apple and elephant-apple.[13]The people from the Eastern part of Nigeria, Enugu people to be precise have its local name to be "Akpuru". It is reputed for its medicinal properties. *Limonia acidissima* is a large tree growing to 9 metres (30 ft) tall, with rough, spiny bark. The leaves are pinnate, with 5-7 leaflets, each leaflet 25–35 mm long and 10–20 mm broad, with a citrus-scent when crushed.[14]The fruit is a berry 5–9 cm diameter, and may be sweet or sour. It has a very hard rind which can be difficult to crack open, and contains sticky brown pulp and small white seeds.Wood apple is a deciduous, erect tree with a few upward-reaching branches bending outward near the summit where they are subdivided into slender branchlets drooping at the tips.[10]A true multipurpose tree, it is both gathered from the wild and also cultivated for its edible fruit, plus its wide range of medicinal and other uses.

Acute renal failure refers to the sudden and usually reversible loss of renal function, which develops over a period of days or weeks. Among the causes of acute renal failure, acute tubular necrosis, which occurs due to ischemia or nephrotoxins like cisplatin and gentamicin, is most common, accounting for 85% of the incidence. Gentamicin, an aminoglycoside antibiotic, is used as an effective agent against Gram-negative infections. Its chemical stability and rapid bactericidal action has made it a first-line drug in a variety of clinical situations. However, nephrotoxicity is the major side effect of aminoglycosides accounting for 10–15% of all cases of acute renal failure. Studies have also shown that 30% of the patients treated with gentamicin for more than seven days show signs of nephrotoxicity. It has been shown that the specificity of gentamicin renal toxicity is related to its preferential accumulation in the renal convoluted tubules and lysosomes. There is a continuous search for agents which provide nephroprotection against the renal impairment caused by drugs like cisplatin and gentamicin, for which allopathy offers no remedial measures.[15] Thus, it is imperative that mankind turns towards alternative systems of medicine for treatment. Hence, the present study is an attempt to screen *Limonia acidissima* leaves for their nephroprotective and hepatoprotective activities.

In the ethnobotanical claims, *Limonia acidissima* is used for the treatment of renal and hepatic diseases. To the best of our knowledge, there is no scientific report available in support of the nephroprotectiveand hepatoprotectiveactivity of *Limonia acidissima* leaves. Therefore, to justify the traditional claims we have assessed the nephroprotective and hepatoprotective effect of *Limonia acidissima* leaves using phenylhydrazine-induced anaemicrats.

MATERIALS AND METHODS

Sample Collection and Identification

Healthy and matured leaves of *Limonia acidissima* were harvested from Obuoffia in Enugu State Nigeria. The plant was identified and authenticated at the Herbarium section of the Department of Plant Science, University of Port Harcourt, Nigeria where a voucher specimen of the collected sample was deposited in the institutional herbarium for future reference.

Animals

A total of forty two (42) Wistar rats weighing between 150-200g were purchased from the animal house of Department of Biochemistry, University of Port Harcourt and kept in well aerated laboratory cages in the animal house. The animals were allowed to acclimatize for a period of two weeks before the commencement of the experiment. They were maintained under standard laboratory conditions with rat chow (Guinea Feed Ltd. Nigeria) and water *ad libitium*. All animal experiment was carried out in line with the guidelines of Institutional Animal Ethic Committee.

Induction of Anaemia[3]

Anaemia was induced by modified method of ats were injected intraperitonially twice with 40 mg/kgbw of phenyl hydrazine. The second induction was within 24 hours from the first. Anaemia was achieved in 24 hours after the second induction. Animals were treated with the extracts and with vitamin B_{12} (100µg/kgbw) and folic acid syrup (1ml/kgbw) just after establishment of anaemia in the animals.

Animal sacrifice[16]

The method of Nwauche*et al.*, 2014 was used. All experimental animals were were anaesthetized by exposure to chloroform. While under anesthesia, they were painlessly sacrificed and blood was collected from each rat into heparin sample bottles. The heparin anti-coagulated blood samples were centrifuged at 1000 x g for 10 min, after which their plasma was collected and stored for subsequent analysis.

Extraction of leaf material

Preparation of leaf samples

The leaves were cleaned of sand and other impurities. The fresh leaf samples were put in paper bags and air-dried for one week at room temperature. The fresh air-dried leaves were grinded in an electric kitchen blender with chasis number (Philips NL 9206AD-4 Drachten). The powder was sieved with mesh size 1mm and then stored in an air-tight container for further use.

Preparation of Extract

Three portions (100mg, 200mg, and 300mg) of an aqueous extract of*Limonia acidissima* were prepared. One hundred milliliter (100ml) of hot water was used to dissolve 100mg, 200mg and 300mg of the powder of the leaves. The prepared solutions were allowed to stand for 48 hours. Each extracted solution was filtered off using a Whatman® filter paper (Cat no 1001 125) of pore size 125mm. The samples were then stored in refrigerator for subsequent use.

Note: For a long time (and also currently), drug discovery programs have typically used organic solvents such as methanol, butanol, ethyl acetate, chloroform, or hexane for extraction. Hence, most of the water-soluble compounds have escaped detection and isolation. However, most preparations used in traditional medicine are formulated in water or hot water.[16]

Administration of the Extract

Animals in groups three (3) to five (5) were administered 1ml of the aqueous leaf extract i.e. 100mg, 200mg and 300mg/kgbw respectively while those in groups one (1) and two (2) received feed and water *ad libitium*throughout the period of treatment. Two standard drugs Vitamin B_{12} (100 µg/kgbw) and Folic acid (0.5mg/kgbw) were administered to animals in groups six (6) and seven (7) respectively once daily throughout the treatment period.

EXPERIMENTAL DESIGN

The animals were randomly divided into seven (7) groups with six rats each. Aqueous extract of *Limonia acidissima* leaves was administered to the rats in three(3) groups for four (4) weeks period.

The groupings are as follows:

Experimental Design

Groups	Treatment
Group 1 (NRC)	Normal rats received normal chow and water *ad libitum*
Group 2 (ARC)	Anaemic rats were given normal chow and water *ad libitum* without treatment
Group 3 (AR + 100mg/Kgbw)	Anaemic rats were fed on normal chow and water *adlibitum* and 100 mg/Kgbw of extract
Group 4 (AR + 200mg/Kgbw)	Anaemic rats were fed on normal chow and water *ad libitum* and 200 mg/Kgbw of extract.
Group 5 (AR + 300mg/Kgbw of extract)	Anaemic rats were fed on normal chow and water *ad libitum* and 300 mg/Kg bw of extract.
Group 6 (AR+Vit.B12)	Anaemic rats were fed on normal chow and water *ad libitum*, and standard drug (100 µg/Kgbw of vitamin B_{12})
Group 7 (AR + Folic Acid)	Anaemic rats were fed on normal chow and water *ad libitum* and standard drug (0.5 mg/kgbw of folic acid)

Qualitative Analysis of Phytochemicals[17][18]

Qualitative analyses were carried out using the methods of Trease and Evans (1989) and Harborne (1998) to ascertain the presences of the different phytochemicals in the leaves.

DETERMINATION OF BIOCHEMICAL PARAMETERS

Kidney Function Biomakers

Liver Enzymes

Aspartate aminotransferase (AST), Alanine aminotransferase (ALT) and Alkaline phosphatase (ALP) activities were determined using the standard operating procedure in RANDOX test kit, Unitred Kingdom(UK) and change of absorbance measured spectrophotometrically at 340nm.

Blood urea concentration in the blood was estimated by an enzymatic method using a urease enzyme kit by the modified Berth Elot method.[15]Absorbance was read using a UV-240 Vis spectrophotometer (Shimadzu Corporation, Japan). Serum creatinine levels in serum was estimated by the alkaline picrate method using a creatinine kit.[15]Absorbance was read by a UV-240 Vis spectrophotometer.Flame photometer (Jenway PFP7, UK) was used for the estimation of sodium and potassium.[19]

Statistical Analysis of Data

All data for biochemical analysis were analyzed for statistical differences and in rat treatment groups, by

means of one-way ANOVA and post hoc LSD, on SPSS 20. In all, $p<0.05$ was considered significant. Data are presented as mean±S.D (standard deviation).

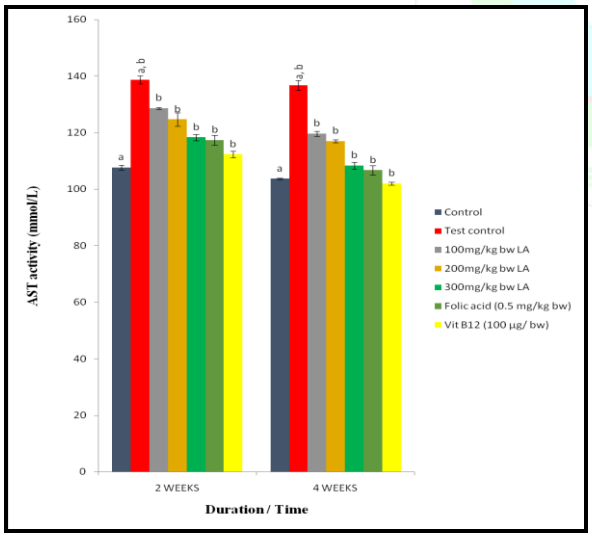
RESULTS

Qualitative Phytochemical Compositions of the aqueous extract of *Limonia acidissima* leaves are shown in table 1 below.

Table1: Qualitative Phytochemical Compositions of the aqueous extract of *Limonia acidissima* leaves.

Parameters	Inference
Alkaloids	+
Flavonoids	+ +
Tannins	-
Saponins	+
Cardiac glycosides	+
Steroids	+
Phenols	+
Resins	-
Terpenoids	+
Proteins	+

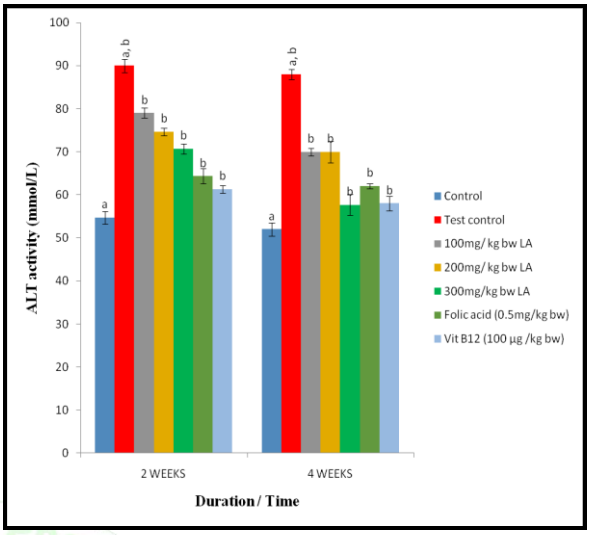
(-) = absence, (+) = present in trace amount, (+ +) = present in high amount



a shows significant difference when other bars are compared with the normal control

b shows significant difference when other bars are compared with the test control

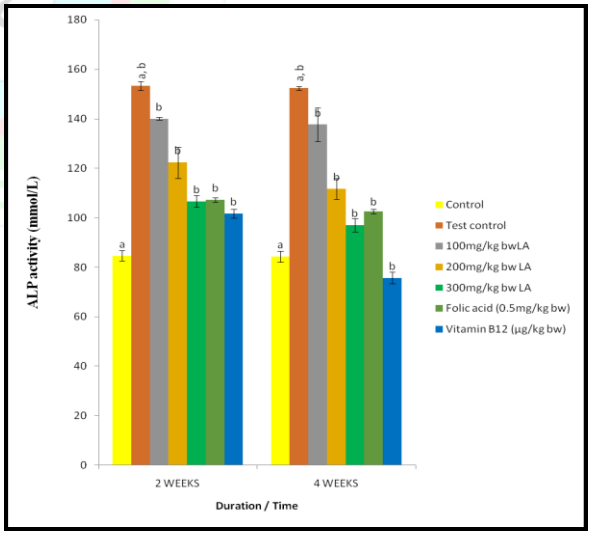
Figure. 1: Effect of aqueous leaf extract of *Limonia acidissima*, vitamin B_{12} and folic acid onAspartate aminotransaminase activity in phenylhydrazine - induced anaemia in wistar rats.



a shows significant difference when other bars are compared with the normal control

b shows significant difference when other bars are compared with the test control

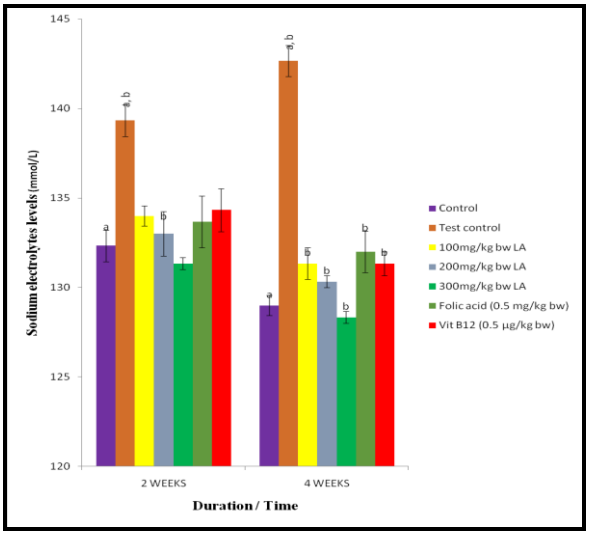
Figure 2: Effect of aqueous leaf extract of *Limonia acidissima*, vitamin B_{12} and folic acid on Alanine aminotransaminase activity in phenylhydrazine - induced anaemia in wistar rats.



a shows significant difference when other bars are compared with the normal control

b shows significant difference when other bars are compared with the test control

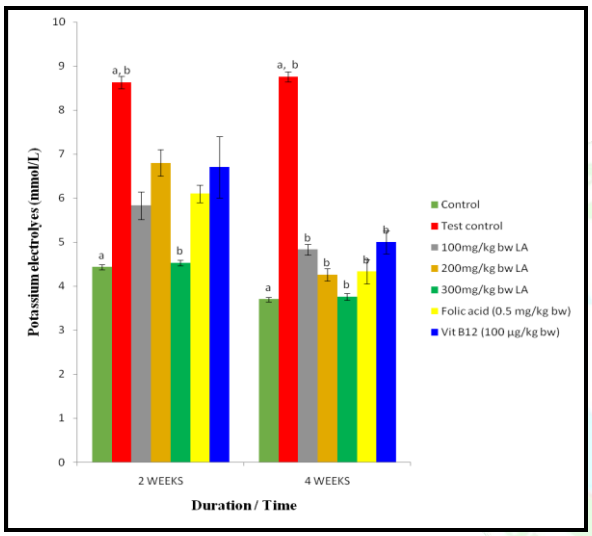
Figure 3: Effect of aqueous leaf extract of *Limonia acidissima*, vitamin B_{12} and folic acid on Alkaline phosphatase activity in phenylhydrazine - induced anaemia in wistar rats.



a shows significant difference when other bars are compared with the normal control

b shows significant difference when other bars are compared with the test control

Figure 4: Effect of aqueous leaf extracts of *Limonia acidissima*, vitamin B_{12} and folic acid on sodium electrolyte levels of phenylhydrazine - induced anaemia in wistar rats.



a shows significant difference when other bars are compared with the normal control

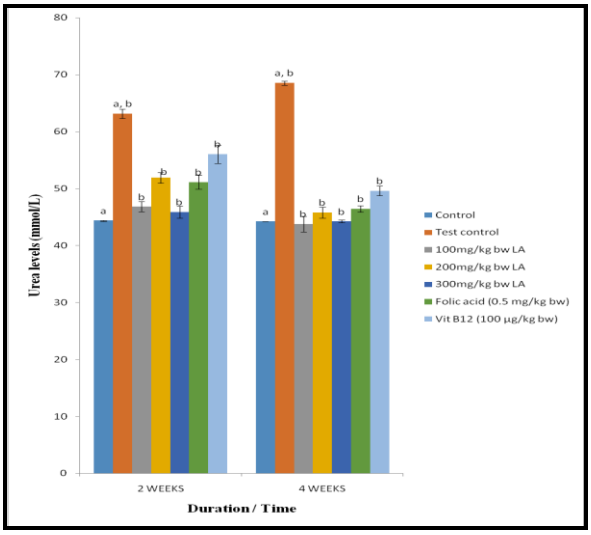
b shows significant difference when other bars are compared with the test control

Figure 5: Effect of aqueous leaf extract of *Limonia acidissima*, vitamin B_{12} and folic acid on potassium electrolyte levels of phenylhydrazine - induced anaemia in wistar rats.

DISCUSSION

Figures 1 -3 below shows the effect of aqueous leaf extract of *Limonia acidissima*, vitamin B_{12} and folic acid on liver enzyme activities in phenylhydrazine - induced anaemia in wistar rats while figures 4-7 indicates the effect of aqueous leaf extract of *Limonia acidissima*, vitamin B_{12} and folic acid on electrolyte level in phenylhydrazine - induced anaemia in wistar rats.

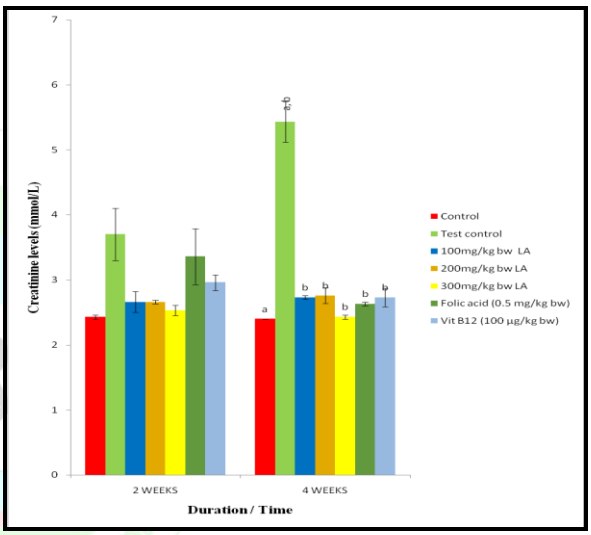
In all the figures below, it will be observed that the level of the groups administered with the leave extract of *Limonia acidissima*, vitamin B_{12} and folic acid were decreased when compared with the test control.



a shows significant difference when other bars are compared with the normal control

b shows significant difference when other bars are compared with the test control

Figure. 6: Effect of aqueous leaf extract of *Limonia acidissima*, vitamin B_{12} and folic acid on urea levels of phenylhydrazine - induced anaemia in wistar rats.



a shows significant difference when other bars are compared with the normal control

b shows significant difference when other bars are compared with the test control

Figure 7: Effect of aqueous leaf extracts of *Limonia acidissima*, vitamin B_{12} and folic acid on Creatinine levels of phenylhydrazine - induced anaemia in wistar rats.

Herbs are readily available to humans and have been explored to the maximum for their medicinal properties. Various parts of plants like roots, leaves and bark are used for their medicinal properties.[20]This is primarily because of the general belief that herbal drugs are without any side effects, besides being cheap and locally available.[21]Phytochemicals are a large group of plant-derived compounds known to be responsible for much of the disease protection conferred from diets high in fruits, vegetables, beans, cereals, and plant-basedbeverages such as tea and wine.[22]Phytochemicals in plants include steroids, triterpenes, cardiac glycosides, saponins, flavonoids,tannins, free anthraquinones, alkaloids, etc. The qualitative

phytochemical analysis of aqueous extracts of *Limonia acidissima*revealed the presence of saponins, steroids, flavonoids,terpenoids, proteins, phenols, cardiac glycoside and alkaloids with flavonoids existing in high concentrations. This result indicates the presence of a variety of phytochemicals in the extract. Each of these phytochemicals is known for various protective and therapeutic effects.[23]Flavonoids are known to possess antibacterial, anti-inflammatory, anti-allergic, antiviral and anti-neoplastic activities.[24]They have anti-oxidation effects in animals.[25]

Steroids, terpenoids, alkaloids and glycosides have demonstrated potent effects against most bacterial activities[26][27]and steroids are of immense value as a result of their links with sex hormones.[28]Terpenoids and steroids possess anti-bacterial and anti-neoplastic properties.[29]Saponins have shown immense benefits in the management of inflammation in the upper respiratory tract where it exerts expectorant action as well as anti-diabetic properties.[13]The presence of these secondary metabolites in the leaves is important as these compounds confer biological activities to the plants.[30]These phytochemicals also add up to the nutritive value of the plant and probably account for the medicinal value of these edible vegetable leaves. Medicinal herbs and plants, fruits and vegetables confer enormous benefits due to the high antioxidant abilities inherent in them as a result of their phytochemical composition.[31]Phenolic compounds have been reported to be a major contributor to the antioxidant activity of medicinal plants.[32][33]Some phenolic compounds are very reactive in neutralizing free radicals by giving an electron or hydrogen and chelating metal ions in aqueous solutions.[34]Polyphenolic molecules are able to donate a proton to a radical due to the presence of several hydroxyl groups attached to an aromatic ring thereby acting as an antioxidant or possibly, a chain breaking molecule upon secondary oxidation.[35]These earlier results supports our claim that the phytochemicals which might be responsible for the scavenging activity in the aqueous extract of *Limonia acidissima* are phenolicsand flavonoid constituents.[36][37]Flavonoids are major classes of plant polyphenols with structural requirements of free radical scavengers.[38]Decreased levels of circulatinghaemoglobin, less than 13 g/dL in male and 12 g/dLin females occurs in anemia.[1]

The assessment of enzyme levels in serum remains an essential model in clinical diagnosis based on the informations that they provide on the nature and extent of pathological tissue damage.[39] Generally, hepatic injury is often associated with alterations in the serum and liver activities of some enzymes notably alanine aminotransaminase, aspartate aminotransaminase and alkaline phosphatase[40]because hepatic damage leads to the leakage of the enzymes from the tissues to the serum.[41][42]Alanine aminotransaminase and aspartate aminotransaminase are considered precise indicators of hepatocellular injury and to an extent provides quantitative evaluation of the degree of damage to the liver.[43]ALT and AST have been established as markers of hepatocellular injury while ALP is a marker of cholestasis.[44]

The significant increase ($p<0.05$) in ALT activity and non significant increase ($p>0.05$) of AST activity in serum of anaemic control animals compared to the normal control may be due to leakage from hepatocytes leading to increased membrane permeability.[45]Elevations in ALT levels are often accompanied by elevations in the level of AST.[46]The significant increase in ALT provides a biochemical evidence of significant liver damage caused by phenylhydrazine.[44]

In conclusion, the results of this study demonstrate that aqueous leaves extract of *Limonia acidissima*in wistar rats has hepatoprotective and neproprotective activities against Phenylhydrazine-Induced toxicity in rats indicating a promising role in the treatment of acute hepatic and renal injury induced by drugs and chemicals

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