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Anti-Anaemic Potentials of Folic Acid, Vitamin B12 and Aqueous Extract of *Limonia acidissima* Leaves on Phenylhydrazine-Induced Anaemic Wistar Rats

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Abstract: **Qualitative phytochemical composition of *Limonia acidissima* leaf and heamatological indices of phenylhydrazine (phz)-induced anaemic wistar rats were investigated. A total of forty two (42) wistar rats of both sexes weighing between 150-200g were used. The animals were randomly divided into seven (7) groups of six rats each. Folic acid and Vitamin B12 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. Induction of anaemia significantly ($p<0.05$) decreased Haemoglobin (Hb) levels, Red Blood Cell (RBC) concentration, Mean Corpuscular Volume (MCV) and White Blood Cell Count (WBC) of Phenylhydrazine-Induced non- treated rats compared to normal control. In the groups treated with aqueous extract of *Limonia acidissima* at the doses of 100mg/kgbw, 200mg/kgbw and 300mg/kgbw, Hb, MCV,WBC and RBC were significantly ($p<0.05$) elevated in the first two weeks of the investigation while a further significant ($p<0.05$) increase occurred in the fourth week in comparison to the Phenylhydrazine-induced anemic non- treated rats. A significant increase ($p<0.05$) in the haematological parameters was also observed in the groups treated with folic acid and vitamin B_{12} when compared to the Phenylhydrazine-induced anaemic non- treated groups at the first two weeks and the fourth week.This outcome illustrates the anti-anaemic potential of aqueous extract of the leaf of *Limonia acidissima and its* possession of hematopoietic properties and that at a dose of 300mg/kgbw, it has the most anti-anemic potentials.**

Keywords: **Anaemia, phenlyhydrazine, *Limonia acidissima*, Haemoglobin.**

1. INTRODUCTION

Anaemia, one of the most common and widespread disorders in the world, is a public health problem in both industrialised and non-industrialised countries (Bhutta *et al.*, 2013). According to the World Health Organization (WHO, 2011), anaemia is a condition in which the number of red blood cells or their oxygen-carrying capacity is insufficient to meet physiologic needs, which vary by age, sex, altitude, smoking, and pregnancy status. Anemia is a disease condition characterized by a reduction in the amount of haemoglobin in circulation, less than 13g/dL in males and 12g/dL in females (Okochi *et al.*, 2004). Anaemia is a medical condition that account for serious health problems in many tropical countries as a result of the dominancy of different forms of parasitic infections, including malaria (Dacie and Lewis, 1995). There are numerous causes and types of anaemia; these include: sickle-cell anaemia, iron deficiency anaemia, vitamin B_{12} deficiency anaemia, drug induced anaemia as side effect of drug therapy, disease induced anaemia, etc

(Iwalewa *et al*.,2009). 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 (WHO, 2009; Biapa *et al*., 2011). There are three main categories of the causes of anemia:

1) Poor, insufficient, or abnormal red blood cell production;

2) Excessive red blood cell destruction; and

3) Excessive red blood cell loss. 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 helminth 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 about the problem of anemia, environmental factors, lack of access to medical services, and poverty (Janz *et al*., 2013).

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 haemoglobin in the blood (Diallo *et al*., 2008). Haemolytic anemia refers to a condition in which erythrocytes have a shortened life-span. Phenyl hydrazine is used for the induction of haemolytic anaemia and the study of its mechanism in many species is known. Phenyl hydrazine is used worldwide mainly as a chemical intermediate in the pharmaceutical, agrochemical, and chemical industries (Burkhard *et al*., 2001). 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 (WHO, 2006).

Limonia acidissima is the only species within the monotypic genus *Limonia*. It is native to the Indomalaya ecozone 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 (Abdlrahman *et al*., 2010). 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 (Abolaji *et al*.,2007). 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 (Abubakar *et al*.,2005).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.

Medicinal plants are useful worldwide in the management of diseases. Plants have provided the core traditional treatment for diverse types of disease and still offer a 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. Millions of Africans of all ages rely on herbal medicine for primary health care (Abubaka r*et al.,* 2005). The medicinal use of plant has been attributed to the presence in plant parts of phytochemicals. These phytochemicals include glycosides, saponins, tannins, alkaloids and flavonoids (Giral and Hidalgo, 1983).

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 substance and used in folkloric remedies (Farnsworth, 1966). These constituents may be necessary in preventing haemolytic diseases, such as anaemia.

2. 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.

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Animals:

A total of forty two (42) Wistar rats of both sexes weighing between 150-200g were purchased from the animal house Department of Biochemistry, University of Port Harcourt and kept in well aerated laboratory cages in the animal house, Department of Biochemistry, Faculty of Science, University of Port Harcourt. The animals were allowed to acclimatize to the laboratory environment 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 libitum*. All animal experiment was carried out in line with the guidelines of Institutional Animal Ethics Committee.

Induction of Anaemia:

Anaemia was induced by modified method of Iwalewa *et al.*, (2009). Rats were injected intraperitonially twice with 40 mg/kgbw of phenyl hydrazine. The second induction was within 24 hours from the first. Anaemia was established within 24 hours after the second induction. Animals were treated with the extracts and with vitamin B_{12} (100 µg/kgbw) and folic acid syrup (1ml/kgbw) just after establishment of anaemia in the animals.

Animal sacrifice:

All experimental animals were anaesthetized using chloroform fumes 24 hours after the last administration of the extract. Blood samples were collected in EDTA anticoagulant tubes for hematological studies and heparin bottles for biochemical assays.

Extraction of leaf material:

Preparation of leaf samples:

The leaves were cleaned and made free from 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 powdered 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.

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.

3. 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:

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 (Trease and Evans (1989) and Harborne (1998):

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.

Haematological assay:

The concentration of red blood cell, haemoglobin, mean corpuscular volume and white blood cell, neutrophils and lymphocytes were analyzed using BC 5300 Mindray Hematology Auto – Analyzer.

Procedure:

The samples were properly mixed in a blood mixer and introduced in the sample probe inherent in the auto analyzer for aspiration. The machine automatically analyzed the sample after aspiration and revealed the result in the computer screen which automatically printed the result from the printer.

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$\pm$S.D (standard deviation).

4. RESULTS

Table 4.1: Qualitative Phytochemical Compositions of the aqueous extract of *Limonia acidissima* leaf

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

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

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Results of Heamatological Indices:

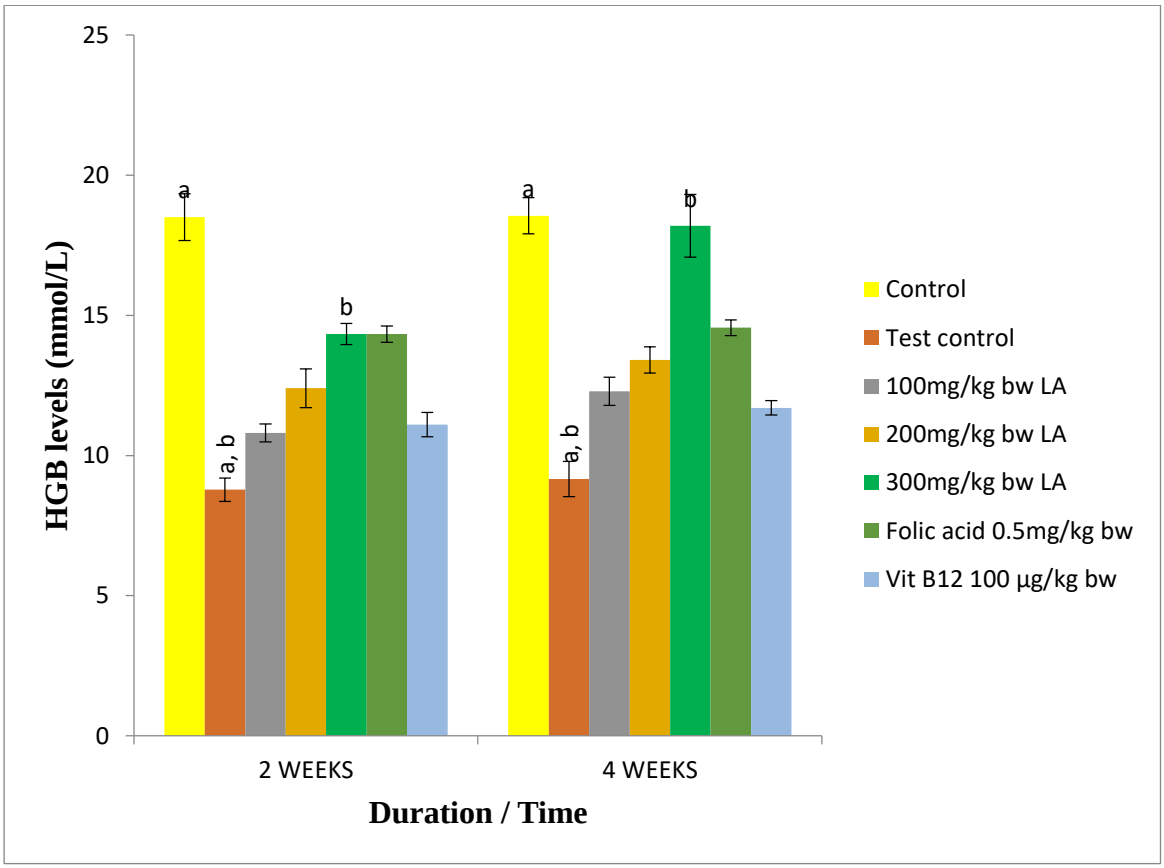

Fig 4.1: Haemoglobin levels of wistar rats treated with different doses of *Limonia acidissima* aqueous leaf extract.

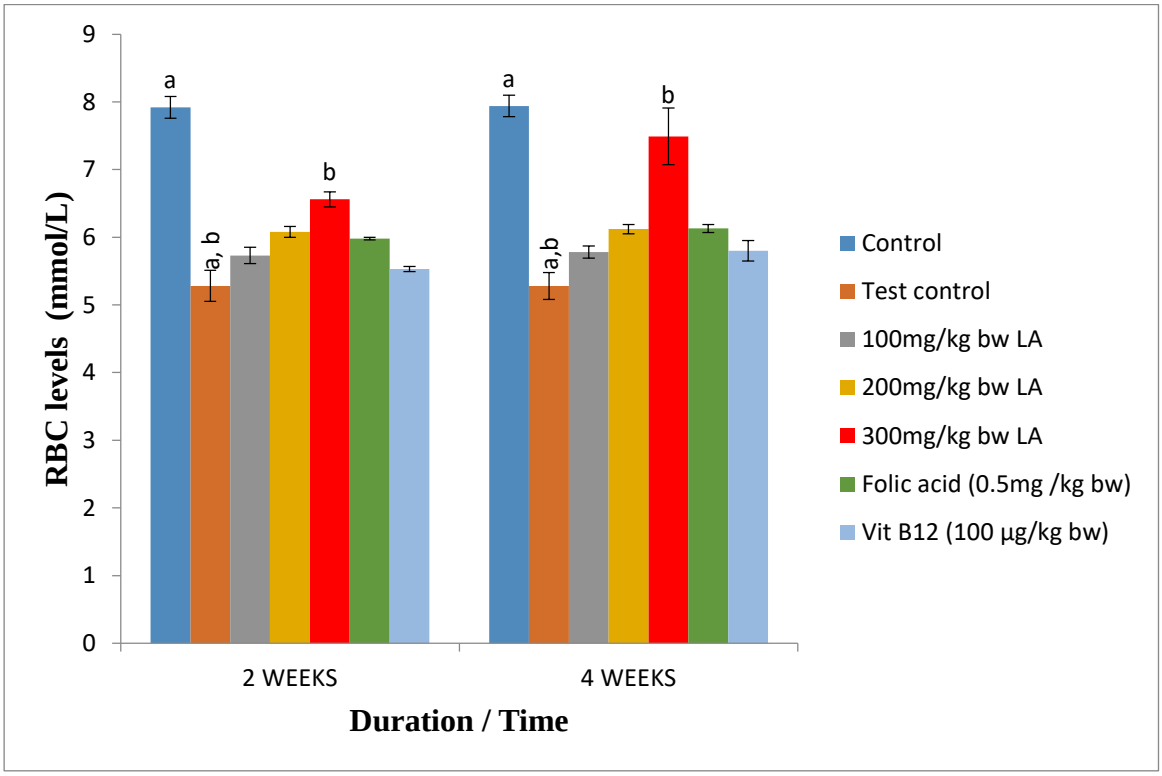

Fig 4.2: Red Blood Cell levels of wistar rats treated with different doses of *Limonia acidissima* aqueous leaf extract.

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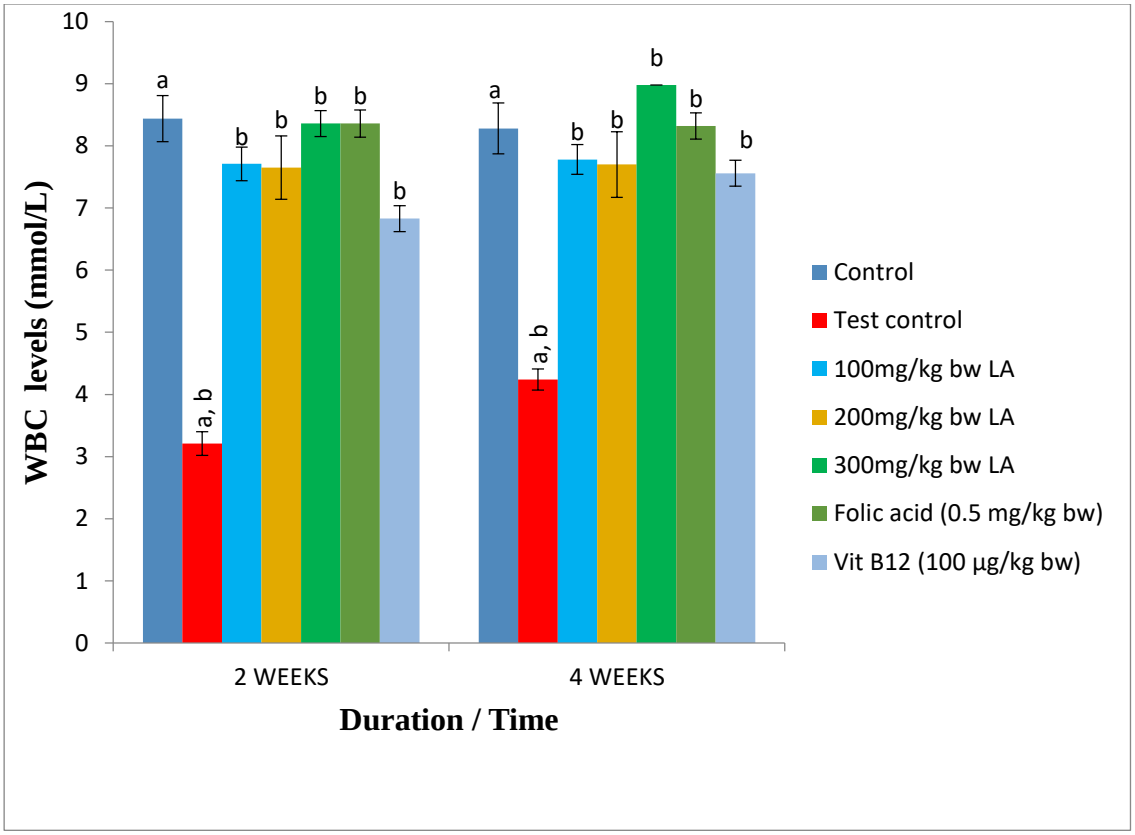

Fig 4.3: White blood cell count of Wistar rats treated with different doses of *Limonia acidissima* aqueous leaf extract.

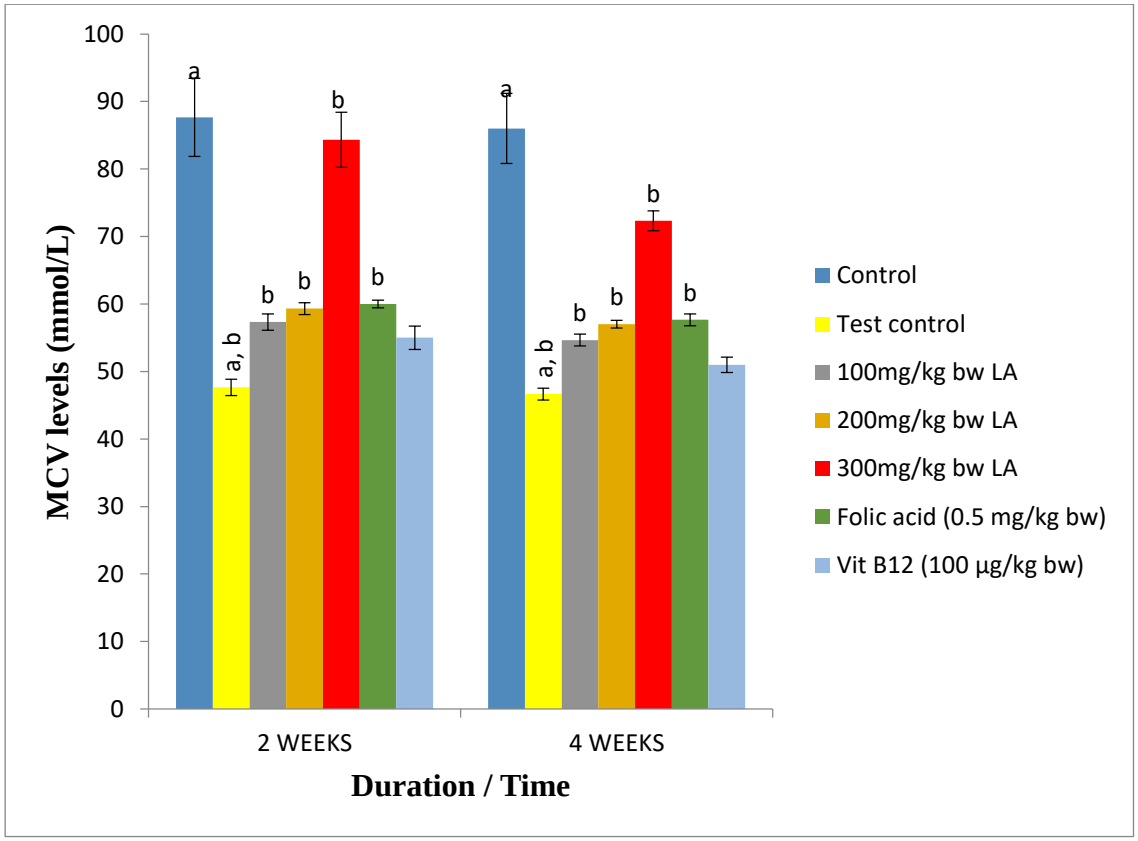

Fig 4.4: Mean corpuscular volume of wistar rats treated with different doses of *Limonia acidissima* aqueous leaf extract.

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5. DISCUSSION

The leaf, barks and roots of different plants have been used over the years for medicinal purposes as a consequence of their availability and lesser side effects (Achinewu and Aniena, 1995; Perumal and Gupala, 2007). Some phytochemicals have exhibited potent benefits against infections and diseases (Arts and Hollman, 2005). They include steroids, triterpenes, cardiac glycosides, saponins, flavonoids, tannins, free anthraquinones, alkaloids, etc. The qualitative phytochemical analysis of aqueous extracts of *Limonia acidissima* leaves revealed the presence of saponins, steroids, flavonoids, terpenoids, proteins, phenols, cardiac glycosides and alkaloids with flavonoids existing in high concentrations. This result demonstrates the availability of a variety of phytochemicals in the extract. Each of these phytochemicals is known for various protective and therapeutic effects (Asaolu *et al.*, 2009). Flavonoids have exhibited antibacterial, anti-inflammatory, anti-allergic, antiviral and anti-neoplastic activities (Alli, 2009). They have anti-oxidation effects in animals (Enwere, 1998).

Steroids, terpenoids, alkaloids and glycosides have demonstrated potent effects against most bacterial activities (Camacho-Corona *et al.*,2008; Al-Bayati and Sulaiman, 2009) and steroids are of immense value as a result of their links with sex hormones (Okwu, 2001). Terpenoids and steroids possess anti-bacterial and anti-neoplastic properties (Oduro *et al*., 2009). 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 (Abdulrahman *et al*., 2010). The presence of these secondary metabolites in the leaves is important as these compounds confer biological activities to the plants (Corthout and Kotra, 1995). 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 (Jastrzebski *et al*., 2007). Phenolic compounds have been reported to be a major contributor to the antioxidant activity of medicinal plants (Dragland *et al*., 2003; Cai *et al*., 2004). Some phenolic compounds are very reactive in neutralizing free radicals by giving an electron or hydrogen and chelating metal ions in aqueous solutions (Petti and Scully, 2009). 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 (Franco *et al*., 2008) 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 phenolics and flavonoid constituents (Rohman*et al*., 2010; Masoumeh *et al*., 2011). Flavonoids are major classes of plant polyphenols with structural requirements of free radical scavengers (Jayathilakan *et al*., 2007). Decreased levels of circulating haemoglobin, less than 13 g/dL in male and 12g/dL in females occurs in anemia (Okochi *et al*., 2004). Phenylhydrazine decreases haemoglobin level, red blood cell level, and white blood cell count. Okonkwo *et al.*, (2004) suggested that precise laboratory assessment of blood parameters remains the only sensitive and dependable foundation for ethical and rational research, diagnosis, treatment and prevention of anaemia. Researchers have shown that ingestion of medicinal compounds or drugs can alter the normal range of haematological parameters (Ajagbonna *et al.*, 2001). The major focus of the scientific world in relation to therapeutic plants and haematological studies is based on the measures that can maintain a normal haematological state and normalize any negative haematological status related with different anaemic conditions (Alexander and Griffiths, 1993). Intraperitoneal administration of phenylhydrazine generated a decrease that is significant ($p<0.05$) in PCV, Hb, WBC, RBC and MCV. This is in line with the findings of Turaskar *et al*., (2013), who showed that phenylhydrazine disrupted the function of red blood cells through haemolysis illustrated by a considerable decrease in red blood cells (74.06%), haemoglobin (48.17%) and packed cell volume (41.8%). Studies have illustrated the decrease in haemoglobin concentration, red blood cell and haematocrit as a consequence of the intraperitoneal administration of phenylhydrazine (O'Riordan *et al*., 1995 and Criswell *et al*., 2000). Also, Agbor *et al.*, (2005) suggested that the oral administration of 10 mg/kgbw phenylhydrazine for 8 days considerably decreased haematological indices by 50%. The significant increase ($p<0.05$) in RBC of anaemic treated rats compared to the normal control after the four (4) weeks of administration possibly points to an imbalance existing in the rate of production (erythropoiesis) and destruction of the blood corpuscles in favour of the destruction caused by phenylhydrazine. This significant ($p<0.05$) reduction in Hb, RBC and WBC confirms induction of haemolytic anaemia using phenylhydrazine. Decrease in Hb, RBC and WBC causes a corresponding decrease in MCV. Orally administered aqueous extracts of *Limonia acidissima* to anaemic animals demonstrated haematopoietic potentials of the extracts within two weeks as Hb of treated animals were raised beyond the

threshold for anaemia (11.0g/dL) whereas the Hb of the anaemic rat control group was below this threshold. Haematopoietic effect of aqueous extract of *Limonia acidissima* leaves, folic acid and vitamin B_{12} was demonstrated by an increase that is significant ($p<0.05$) in RBC and Hb concentration of phenylhydrazine-induced anaemic rats receiving extract (300mg/kg bw), folic acid and vitamin B_{12} compared to the anaemic control group on week two of administration.

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