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Evaluation of blood boosting potentials of combined administration of *Vigna unguiculata* and *Citrus limon* ethanol extracts using Wister rat models

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Abstract

The use of traditional herbal medicines started in ancient times. It is estimated that 80% of world population depend on it for solutions to their health challenges. Currently, there has been enormous increase in the use of herbal medicines attributable to their readily availability, affordability, efficacy and good safety profile. This study evaluated the blood boosting potentials of combined administration of *Vigna unguiculata* and *Citrus limon* in rat models. Wister rats (135 – 140 g) were used and *Vigna unguiculata* and *Citrus limon* were extracted in ethanol. Apart from some preliminary tests, hematology studies were done using standard methods. The parameters tested included red blood cells (RBCs), hemoglobin (Hb), Packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) using hematological analyzer (DxH 560 AL). In the RBC test, combination of *Vigna unguiculata* and *Citrus limon* recorded a count of $8.07 \pm 0.20 \times 10^6$ on day 15 of treatment. This was highest when compared with the individual administrations which attained 4.04 ± 0.18 and $3.57 \pm 0.07 \times 10^6$ respectively. The combination treatment approached that of the normal control which had RBC count of $8.29 \pm 0.13 \times 10^6$. This trend was applicable to other tested parameters. In conclusion, *V. unguiculata* and *C. limon* had blood boosting potentials which was more remarkable in the group treated with combination of the two herbs and this was attributed to their phytochemicals such as iron, protein and citric acid as well as their synergistic herb-herb interaction.

Keywords: Blood boosting; *Citrus limon*; Hematology parameters; *Vigna unguiculata*

1. Introduction

The use of traditional herbal medicines started in ancient times. It is estimated that 80% of world population depend on it for solutions to their health challenges. Currently, there has been enormous increase in the use of herbal medicines attributable to their readily availability, affordability, and efficacy with good safety profile [1]. The efficacy of herbal medicines is a result of their numerous phytochemical components which exhibit synergistic actions such as anti-inflammatory, antiviral, antibacterial, anti-protozoa, and antioxidant among other properties. Owing to their cost effectiveness, traditional medicines are in great demand for primary health care. In a particular study, the effective use of Indian medicinal plants for the treatment of various types of infectious diseases ranging from COVID 19, gastrointestinal infection, viral diseases, and skin and wound infections among others was reported [2]. Hematology is the study of blood and blood disorders and hematological tests can help diagnose anemia, infection, hemophilia, blood-clotting disorders, and leukemia. Common hematological tests include: Complete blood count (CBC) which includes: White blood cell count (WBC), Red blood cell count (RBC), Platelet count, Hematocrit red blood cell volume

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(HCT), Hemoglobin concentration (HB), Differential white blood count and Red blood cell indices (measurements). These tests help in the diagnosis of anemia, polycythemia, bleeding and clotting disorders. Anemia is a condition in which the body does not have enough healthy red blood cells. In this condition, the blood cannot carry enough oxygen to the body's organs and as a result, the individual feels cold and symptoms of tiredness or weakness. There are many different types of anemia, but the most common type is iron-deficiency anemia. Consequently, treatment of anemia involves supplementing with iron, vitamin B12 and folate. Due to paucity of orthodox drugs that are effective in the treatment of anemia, researches are now been diverted to blood boosting herbal medicines. In a study conducted in Tanzania via questionnaire, twenty eight plant species were reported to be effective in treating anemia. Some of these plants include: *Hibiscus sabdariffa* which was the most mentioned species. Others include: *Lawsonia inermis*, *Aloe sp*, *Uvaria acuminata*, *Parinari curatellifolia*, *Ozoroa reticulata*, *Manihot esculenta*, *Canthium sp* and *Azelia quanzensis* [3]. In a recent study, anemia was defined as a decrease of the hemoglobin level in the blood and a reduction in carrying capacity of oxygen; it is also regarded as a major public health problem which affects people of all ages. According to the researchers, the methods used to treat anemia are blood transfusion and oral administration of iron-based supplements. These treatments they said are associated with a number of side effects, such as nausea, vomiting, constipation, and stomach pain, which limit its long-term use. In addition, oral iron supplements are poorly absorbed in the intestinal tract, due to overexpression of hepcidin, a peptide hormone that plays a central role in iron homeostasis. In the study, the workers conducted an analysis of active compounds and plant extracts used in the treatment of various types of anemia. The advantage of using these plant based materials are their availability from natural sources and relatively low toxicities when compared with orthodox drugs [4]. This study therefore evaluated the blood boosting activities of combined administration of ethanol extracts of *Vigna unguiculata* and *Citrus limon* using Wister rat model.

The circulatory system is made up of blood vessels that carry blood away from and towards the heart. Arteries carry blood away from the heart and veins carry blood back to the heart. Blood is a specialized body fluid. It has four main components: plasma, red blood cells, white blood cells, and platelets. Blood has many different functions, including: transporting oxygen and nutrients to the lungs and tissues, forming blood clots to prevent excess blood loss, carrying cells and antibodies that fight infection, bringing waste products to the kidneys and liver, which filter and clean the blood, and regulating body temperature. Hemoglobin is a protein found in red cells that carries oxygen from the lungs to all other organs in the body. Anemia is a problem of not having enough healthy red blood cells or hemoglobin to carry oxygen to the body's tissues. Having anemia can cause tiredness, weakness and shortness of breath. Treatment of anemia should be directed at the cause of the anemia, and may include: blood transfusions, corticosteroids or other medicines that suppress the immune system, erythropoietin, a medicine that helps your bone marrow make more blood cells, and supplements of iron, vitamin B12, folic acid, or other vitamins and minerals. This study therefore aimed at evaluating the anti-oxidative, anti-inflammatory and blood boosting activities of combined administration of ethanol extracts of *Vigna unguiculata* and *Citrus limon* using Wister rat model. This is justified by the rich iron content of *Vigna unguiculata* as well as the abundant vitamin C (citric acid) that occur in *Citrus limon*. In a certain study, eight improved haricot bean varieties were studied for their proximate composition. The nutrition related parameters studied were moisture, ash, crude protein, Crude lipid, crude fiber, crude fat, total carbohydrates, total energy, and mineral content. The beans were shown to contain high amount of protein among other nutritional components and calcium and vitamin C were among the minerals contained in the beans [5]. In another study, *Citrus limon* (L.) Burm was noted to contain many important natural bioactive compounds, such as ascorbic acid, essential oils, and antioxidant substances. In the study, an experiment was carried out on four lemon cultivars (*Ovale di Sorrento*, *Sfusato Amalfitano*, *Femminello Cerza* and *Femminello Adamo*) from Southern Italy to study the changes in physico-chemical properties of juice during fruit ripening. Morphological characteristics of fruits, total soluble solids, pH and titratable acidity, ascorbic acid (AsA), and antioxidant activity (TEAC) of juice were tested. The study supported the fact that *Citrus limon* contain high concentration of ascorbic acid in the lemon juice which showed significant differences among cultivars and ripening stages [6].

2. Material and methods

3. Materials

3.1.1. Animals

Wister rats (135 – 140 g) were used for this study. All the animals were obtained from the Animal House of the Department of Pharmacology, Faculty of Pharmaceutical Sciences, Enugu State University of Science and Technology, Enugu State, Nigeria. The animals were housed in standard laboratory conditions of 12 hours light, room temperature, and 40 - 60% relative humidity and fed with rodent feed (Guinea Feeds Nigeria Ltd). They were allowed free access to food and water. Maintenance and care of all animals were carried out in accordance with EU Directive 2010/63/EU for animal experiments. Guide for the care and use of Laboratory Animals, DHHS Publ. # (NIH 86-123 were strictly adhered

to. Ethical approval was obtained from the Animal Ethical Committee of the Enugu State University of Science and Technology. There was additional approval by the Nnamdi Azikiwe University's Ethical Committee for the use of Laboratory Animals for Research Purposes.

3.1.2. Chemicals and Reagents

Wash Buffer Concentrate (Sigma Aldrich Germany), Assay Buffer (Alpco USA), TMB Substrate (Cayman Chemical USA), Stop Solution (Cayman Chemical USA), Hydrochloric acid (Prime laboratories, India); Dragendoff reagent (Sigma Aldrich, United States of America); Ammonia (Shackti Industrial Gases, India), sodium hydroxide (Treveni Chemical Pvt., India); Ferric chloride (AkashPurochem. Pvt., India); Fehling's solution (Lab care Diagnostics, India); Million reagent (Interlab Chemical Pvt., India); Ethanol (TAJ Pharmaceutical Ltd., India); Acetic anhydride (Ashok Organics Industries, India); Concentrated sulfuric acid (Navin Chemical Pvt., India), Acetic acid (Kayla Africa Suppliers, South Africa); Molisch reagent (Interlab Chemical Pvt., India); alcoholic alpha naphatol (Prat Industry Corcopation, India).

3.1.3. Equipment

Glass column, flasks, beakers, test tubes, measuring cylinders, surgical blade, forceps, scissors, graph paper, white transparent paper, rotary evaporator, Analytical Weighing Balance (Metler H30, Switzerland), Electric Oven (Gallenkamp, England), Spectrophotometer (B. Bran Scientific & Instrument Company, England), Water Bath (Techmel & Techmel, Texas, USA), National Blender (Japan), Micropipette (Finnipipette® Labsystems, Finland), Plethysmometer (Biodevices, New Delhi, India) and Intubation tubes. Precision pipettes (25, 50, 100 and 300 µl, 1,000 µL) (Labcompare USA); Disposable pipette tips (Labcompare USA); Distilled or deionized water (SnowPure Water Technologies USA); Plate shaker (Biocompare USA); Microwell plate reader (BioTek India); Centrifuge (Sharplex Filters Pvt., India); Vortex mixer (Bionics Scientific Technologies (P) LTD, India); Graduated cylinder for 500 ml (Boenmed Healthcare Co. Ltd, Hong Kong); Stop watch (Avi Scientific India); EDTA containers (Sure Care Corporation), heparinized capillary tube (Thomas Scientific, USA), disposable hand gloves (Supermax Malaysia), toilet tissue.

3.1.4. Drugs

Iron supplement was purchased from a Pharmacy shop in Enugu state of Nigeria.

3.1.5. Plant materials

Vigna unguiculata and *Citrus limon* were procured from a market in Enugu state.

3.1.6. Extraction

Vigna unguiculata 1000 g seed powder was weighed using a weighing balance (Camry EK5350 Model, China) and extracted using cold maceration in ethanol for 72 h with intermittent shaking. The resulting solution was filtered using Whatman filter paper and the filtrate concentrated to dryness *in vacuo* using rotary evaporator (RE300 Model, United Kingdom) at 40 °C. The extract was stored in refrigerator between 0-4 °C.

3.2. Methods

3.2.1. Determination of citric acid content of the lemon juice

Citric acid content of the lemon juice was determined using standard method [7].

3.2.2. Proximate analysis

Proximate analysis was done using standard methods described by Association of Official Analytical Chemists (AOAC 1990) [8].

3.2.3. Total Phenolic Content by Folin Ciocalteu's Assay

The total phenolic content of the extract and fruit juice were determined using the method described by Kim *et al.*, (2003) [9].

3.3. Acute toxicity studies (LD50) of co-administration of *Vigna unguiculata* and *Citrus limon*

3.3.1. Acute Toxicity Studies

Acute toxicity analysis of the combined extract of *V. unguiculata* and *C. limon* was performed using Lorke's method (1983) [10].

3.3.2. Induction of anemia

Anemia was induced by intraperitoneal injections of phenylhydrazine (40 mg/kg) for 2 consecutive days [11]. Rats were considered as anemia-induced when RBC level as well as haemoglobin content of the blood reduced by 30% [12].

3.3.3. Experimental design

Rats were divided into 7 groups containing five rats in each group (n = 5). Group I was normal control and did not receive any treatment. Remaining rats were treated with phenylhydrazine to induce anemia. Group II was anemic control that received 10 ml/kg of distilled water. Group III received *V. unguiculata* (250 mg/kg, orally daily). Group IV received *C. limon* juice (5 ml/kg). Group 5 received combination of *V. unguiculata* and *C. limon* (250 mg/kg + 5 ml/kg). Group 7 was a standard drug treatment group and received ferrous ascorbate (9 mg of iron/kg, once daily). Blood samples were tested on days 3, 7 and 15 after first injection of phenylhydrazine by oral route.

3.3.4. Hematology study

Blood (0.5 ml) was collected from retro-orbital sinus on day 0-before phenylhydrazine administration, and on days 3, 7 and 15 after first injection of phenylhydrazine using a heparinized tube to avoid blood clotting. Blood was analyzed for red blood cells (RBCs), hemoglobin (Hb), and packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) using hematological analyzer (DxH 560 AL). Separate blood sample was collected on a plain tube on the 15th day, allowed to clot and centrifuged to obtain serum for the analysis of lipid peroxidation.

3.3.5. Statistical analysis

Results were presented as Mean \pm Standard Error of Mean (S.E.M). Means were analyzed using one way analyses of variance (ANOVA) followed by post hoc Turkey's test for multiple comparisons. P < 0.05 was set to be statistically significant. Results analysis was conducted using Statistical Package for Social Science, SPSS- version 20.

4. Results and discussion

4.1. Extraction

The yield of the extract was 19 g representing about 1.9% yield [7].

4.2. Acute toxicity study

No mortality or obvious sign of toxicity was observed at both phases of the acute toxicity of the combined extract and juice. The LD₅₀ is therefore estimated to be above 5000 mg/kg in combination with 10 ml/kg of the juice [7].

4.3. Citric acid content determination of *Citrus limon*

The citric acid content of *Citrus limon* was 54.2 \pm 0.003 mg/ml [7].

4.4. Proximate analysis and total phenolic content

Both *Vigna unguiculata* and *Citrus limon* contained protein, fat, carbohydrate, dietary fibre, and moisture and the total phenolic content was 15.3 mgGAE/g and 93 mgGAE/L respectively [7].

4.5. Hematology assays

Table 1 Results of red blood cell (RBC) concentration

Red blood cell (RBC) concentration ($\times 10^{-6}$)						
Days	Normal control (naive)	Anemia induced control	<i>Vigna unguiculata</i>	<i>Citrus limon</i>	<i>V. unguiculata</i> + <i>C. limon</i>	<i>Ferrous ascorbate</i> (9 mgFe/kg)
Day 0	8.57 \pm 0.12	8.38 \pm 0.08	8.45 \pm 0.07	8.40 \pm 0.11	8.45 \pm 0.07	8.46 \pm 0.66
Day 3	8.64 \pm 0.12	2.68 \pm 0.07	2.68 \pm 0.12	2.61 \pm 0.12	2.52 \pm 0.14	2.06 \pm 0.03
Day 7	8.47 \pm 0.15	2.81 \pm 0.07	3.21 \pm 0.14	2.68 \pm 0.09	3.97 \pm 0.16	5.10 \pm 0.11
Day 15	8.29 \pm 0.13	3.44 \pm 0.17	4.04 \pm 0.18	3.57 \pm 0.07	8.07 \pm 0.20	8.61 \pm 0.10

Table 2 Results of haemoglobin concentration

Haemoglobin concentration (g/dL)						
Days	Normal control (naive)	Anemia induced control	<i>Vigna unguiculata</i>	<i>Citrus limon</i>	<i>V. unguiculata</i> + <i>C. limon</i>	<i>Ferrous ascorbate</i> (9 mg Fe/kg)
Day 0	13.89 \pm 0.32	13.44 \pm 0.23	13.70 \pm 0.24	14.40 \pm 0.31	13.37 \pm 0.24	13.31 \pm 0.19
Day 3	13.89 \pm 0.26	7.18 \pm 0.34	7.04 \pm 0.24	7.74 \pm 0.27	6.71 \pm 0.33	6.97 \pm 0.27
Day 7	13.84 \pm 0.15	7.53 \pm 0.29	8.70 \pm 0.33	8.34 \pm 0.25	10.77 \pm 0.36	12.77 \pm 0.34
Day 15	13.44 \pm 0.32	9.28 \pm 0.35	11.50 \pm 0.51	11.54 \pm 0.50	12.71 \pm 0.58	13.77 \pm 0.29

Table 3 Results of packed cell volume (PCV)

Packed cell volume (PCV) (%)						
Days	Normal control (naive)	Anemia induced control	<i>Vigna unguiculata</i>	<i>Citrus limon</i>	<i>V. unguiculata</i> + <i>C. limon</i>	<i>Ferrous ascorbate</i> (9 mg Fe/kg)
Day 0	44.6 \pm 0.93	43.4 \pm 0.66	44 \pm 0.71	43.4 \pm 0.93	43 \pm 0.71	42.8 \pm 0.58
Day 3	45.4 \pm 0.93	24.6 \pm 1.03	24 \pm 0.71	23.4 \pm 0.81	23 \pm 1.00	23.8 \pm 0.80
Day 7	44.4 \pm 1.17	25.6 \pm 0.87	29 \pm 1.00	25.2 \pm 0.74	35.2 \pm 1.07	44.2 \pm 0.62
Day 15	43.4 \pm 0.97	39.8 \pm 1.07	37.4 \pm 1.56	34.8 \pm 1.50	41 \pm 1.73	44.2 \pm 0.86

Table 4 Results of Mean cellular volume (MCV)

Mean cellular volume (MCV) (ft)						
Days	Normal control (naive)	Anemia induced control	<i>Vigna unguiculata</i>	<i>Citrus limon</i>	<i>V. unguiculata</i> + <i>C. limon</i>	Ferrous ascorbate (9 mg Fe/kg)
Day 0	52.04±0.41	51.80±0.39	52.05±0.43	51.65±0.47	50.89±0.05	50.58±0.38
Day 3	52.52±0.36	91.69±1.35	89.62±1.73	89.59±0.99	91.20±1.00	91.47±3.18
Day 7	52.41±0.51ign	90.98±1.38	90.29±1.30	93.96±0.95	88.75±1.49	80.78±0.62
Day 15	52.14±0.48	89.74±1.09	92.53±1.57	97.53±2.40	50.82±0.91	51.34±0.48

Table 5 Results of mean cellular hemoglobin (MCH)

Mean cellular haemoglobin (MCH) (pg)						
Days	Normal control (naive)	Anemia induced control	<i>Vigna unguiculata</i>	<i>Citrus limon</i>	<i>V. unguiculata</i> + <i>C. limon</i>	Ferrous ascorbate (9 mg Fe/kg)
Day 0	16.21±0.16	16.04±0.15	16.21±0.15	17.14±0.16	15.83±0.17	15.73±0.13
Day 3	16.06±0.19	26.74±0.46	26.28±0.44	29.62±0.32	26.59±0.42	26.80±1.05
Day 7	16.33±0.19	26.76±0.52	27.10±0.35	31.08±0.31	27.16±0.45	25.05±0.22
Day 15	16.72±0.17	27.02±0.28	28.46±0.49	32.33±0.81	15.75±0.33	16.00±0.17

Table 6 Results of mean cellular haemoglobin concentration (MCHC)

Mean cellular haemoglobin concentration (MCHC) (g/dL)						
Days	Normal control (naive)	Anemia induced control	<i>Vigna unguiculata</i>	<i>Citrus limon</i>	<i>V. unguiculata</i> + <i>C. limon</i>	Ferrous ascorbate (9 mg Fe/kg)
Day 0	31.14±0.06	30.96±0.12	31.14±0.04	33.19±0.00	31.10±0.04	31.09±0.03
Day 3	30.59±0.38	29.17±0.19	29.32±0.12	33.06±0.01	20.16±0.18	29.30±0.13
Day 7	31.17±0.05	29.40±0.17	30.01±0.12	33.08±0.01	30.61±0.09	31.00±0.06
Day 15	31.11±0.05	30.11±0.11	30.76±0.10	33.15±0.01	30.99±0.10	31.16±0.04

5. Discussion

The hematology assay involved estimation of the concentrations of red blood cell (RBC), hemoglobin (HB), packed cell volume (PCV), mean corpuscular volume (MCV), mean cellular hemoglobin (MCH) and mean cellular hemoglobin concentration (MCHC). In the RBC test, the normal control group maintained high level of RBC up till day 15. Expectedly, the anemia induced group had reductions in RBC levels. However, the quantity of RBC increased with time signaling a possible recovery from anemia induction. *V. unguiculata* and *C. limon* recorded sharp drop in RBC levels from day 0 to day 3. But as the days increased, RBC concentration rises gradually and attained 4.04 ± 0.18 and $3.57 \pm 0.07 \times 10^{-6}$ respectively. In the group treated with combined administration of *V. unguiculata* and *C. limon*, the sharp decrease in RBC level observed on day 3 was overcome more quickly with time. The group recorded RBC concentration of $8.07 \pm 0.20 \times 10^{-6}$ on day 15 which was not significantly ($p > 0.05$) smaller than day 0 value. This gave an insight to the nature of interaction between the two herbs which might be regarded as synergistically additive. The group treated with

ferrous ascorbate (a reference standard blood booster containing 9 mg Fe/kg) offered a confirmatory outcome (a day 15 RBC concentration of $8.61 \pm 0.10 \times 10^{-6}$), suggesting that the combination therapy had blood boosting potentials. This was also buttressed with the fact that *V. unguiculata* contains high amount of iron (Fe) coupled with the high content of citric acid in *C. limon* which is renowned for helping in Fe absorption. In a study aimed at establishing short-term effects of iron supplementation on the red blood cells (RBC) in case of suspected iron deficient erythropoiesis (IDE) in the third trimester of pregnancy, reticulocyte counts increased after supplementation from $0.061 \pm 0.015 \times 10^{12}/L$ to $0.079 \pm 0.026 \times 10^{12}/L$ and RBC-He increased from 26.9 ± 1.9 pg to 27.4 ± 1.8 pg (not significant, NS) and Ret-He/RBC-He ratio increased from 0.97 ± 0.06 towards 1.07 ± 0.05 ($P < 0.001$) [13]. In another study, citrate lyase treatment inhibited the ferric iron reduction, solubilization, and uptake with a consequent increase in iron absorption. These demonstrated that citric acid solubilizes the ferric iron leading to increased uptake in intestinal cells [14].

In the hemoglobin (HB) assay, the normal control group maintained approximately similar levels of HB; the anemia induced control had decreased HB level that improved with time; while the ferrous ascorbate treated group, a positive control group, increased the HB level as expected reaching the level of 13.77 ± 0.29 g/dL on day 15. When compared with the three control groups (normal, anemia induced and ferrous ascorbate), *V. unguiculata* and *C. limon* given separately had gradual increase in HB concentration after initial drop on day 3. By the day of 15, they had attained HB levels of 11.50 ± 0.51 and 11.54 ± 0.50 g/dL respectively. This enhancement of HB level was remarkable in the group treated with the combination of the two herbs which recorded 12.71 ± 0.58 g/dL on day 15. This again suggested additive herb-herb interaction. However, none of the groups experienced abnormally high HB level rather the increment tilted towards normal values. High hemoglobin level is most often caused by low oxygen levels in the blood (hypoxia), present over a long period of time. Common reasons include: Bone marrow disease that causes abnormal increase in red blood cells (polycythemia vera) Congenital heart disease among others.

The PCV assay also followed similar trend. While the normal control maintained %PCV, anemia induced had initial drop in %PCV which increased with elongation of treatment while the ferrous ascorbate treated group had enhanced %PCV on day 15, reaching 44.2 ± 0.86 %. The individual herbs had initial drop in %PCV to 24 ± 0.71 and 23.4 ± 0.81 % respectively on day 3. These increased to 37.4 ± 1.56 and 34.8 ± 1.50 % respectively on day 15. The combination of the two herbs showed greater increment in %PCV of 41 ± 1.73 on day 15. This was comparable to that of ferrous ascorbate reference standard which was 44.2 ± 0.86 % on day 15. It was observed that none of the groups had PCV value above the baseline value. However, the combination therapy increased the PCV most quickly than the individual herbs monotherapy. A packed cell volume (hematocrit) level that is higher than normal can be a sign that the body is making too many red blood cells. That can be caused by lung disease, congenital heart disease, heart failure, and polycythemia.

The normal control showed approximately constant MCV levels from days 0-15 while the positive control, after initial increase, returned to normal MCV level on day 15 (day 0 = 50.58 ± 0.38 and day 15 = 51.34 ± 0.48 ft). The combination of the herbs recorded similar trend and had almost equal MCV levels on days 0 and 15 (50.89 ± 0.50 and 50.82 ± 0.91 ft respectively). The anemia induced, *V. unguiculata* and *C. limon* treated groups had elevated MCV on day 15 as follows: 89.74 ± 1.09 , 92.53 ± 1.57 and 97.53 ± 2.40 respectively as compared to the day 0 values of 51.8 ± 0.39 , 52.05 ± 0.43 and 51.65 ± 0.47 ft respectively. Elevated MCV levels signified macrocytic anemia. In a certain study, high mean corpuscular volume (MCV) was reported to be implicated in various health problems, such as anemia, liver disease, and thyroid disease [15]. This implied that although *V. unguiculata* and *C. limon* could increase RBC, HB, and PCV levels, only the combination of the two herbs prevented the rats from macrocytic anemia. MCH levels was not significantly varied ($p > 0.05$) in groups treated with combination of *V. unguiculata* and *C. limon*; ferrous ascorbate; and the normal control. Levels of MCH was elevated in groups that received monotheapy of *V. unguiculata*, *C. limon* and anemia induced group. The most common reason for high MCH is macrocytic anemia, which is a blood disorder in which the body fails to produce enough red blood cells. In MCHC assay, all the groups maintained similar MCHC levels on days 0-15 with the exception of the group treated with herbal combination which had significant drop of MCHC on day 3 (20.16 ± 0.18 g/dL) compared to day 0 MCHC of 31.10 ± 0.04 g/dL). Mean corpuscular hemoglobin concentration (MCHC) measures the average hemoglobin concentration in a given volume of red blood cells. A higher or lower than normal MCHC value may indicate a type of anemia. A **low MCHC** (hypochromia) may mean that there is a lower concentration of hemoglobin within a given volume of red blood cells and, hence, a reduced capacity to carry oxygen to the body's tissues. A **high MCHC** (hyperchromia) can mean that there is a higher hemoglobin concentration in red blood cells than usual. However, MCHC value may be normal with many types of anemia; a condition called normochromic anemia.

6. Conclusion

In conclusion, both *Vigna unguiculata* and *Citrus limon* separately exhibited remarkable blood boosting potentials which might be attributed to their protein and iron contents in the case of *Vigna unguiculata* as well as citric acid content of

Citrus limon which is a natural antioxidant and helps in iron absorption. However, the combination of the two herbs recorded a superior blood boosting activity attributable to their synergistically additive herb-herb interaction.

Compliance with ethical standards

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Disclosure of conflict of interest

All authors declared no conflict of interest.

Statement of ethical approval

Maintenance and care of all animals were carried out in accordance with EU Directive 2010/63/EU for animal experiments. Guide for the care and use of Laboratory Animals, DHHS Publ. # (NIH 86-123) were strictly adhered to. Ethical approval was obtained from the Animal Ethical Committee of the Enugu State University of Science and Technology. There was additional approval by the Nnamdi Azikiwe University's Ethical Committee for the use of Laboratory Animals for Research Purposes.

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