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ANTI-ANAEMIC AND IMMUNOMODULATORY POTENTIALS OF AQUEOUS, CHLOROFORM AND METHANOL LEAF EXTRACTS OF *WHITFIELDIA LATERITIA* ON 2, 4-DINITROPHENYLHYDRAZINE-INDUCED ANAEMIA IN RATS

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ABSTRACT

This study investigated the anti-anaemic and immunomodulatory potentials of the aqueous, chloroform and methanol leaf extracts of *Whitfieldia lateritia* on 2, 4-dinitrophenylhydrazine (2,4-DNPH)-induced anaemia in rats. Anaemia is a well-known life threatening disease in the society, and the leaves of *W. lateritia* are purportedly used for blood boosting in folkloric medicine. The medicinal and nutritional potential available in the plant depends on its chemical composition. The general public is becoming very interested in the plant because of its medicinal and nutritional importance; however, there are little or no documented reports to this regard, it is on this basis that this work was designed. The toxicity study, selected vitamins and mineral analyses of the extracts were carried out using standard procedures. Thirty-six Wistar rats were grouped into six (*n* =6). Group I: normal control; Group II: negative control; Group III: administered 0.6 ml/kg body weight (b.w) of astifer (standard drug); Group IV to VI were administered 400 mg/kg b. w. of the aqueous, chloroform and methanol leaf extracts, respectively. The result showed a significant ($P < 0.05$) decrease in PCV and haemoglobin (Hb) levels of group II rats compared with those of group I. The rats in groups V and VI showed significant ($P < 0.05$) increase in PCV and Hb

levels compared with those of group II. Groups IV and V showed significant ($P < 0.05$) increase in WBC count compared with those of group II. Vitamin E and C levels in groups V and VI showed significant ($P < 0.05$) increase compared with those of group II. Similarly, group II rats showed significant ($P < 0.05$) increase in interleukin 10 concentration while non-significant ($P > 0.05$) decrease was observed in serum iron concentration compared with those of group I. Serum iron concentration of group III rats showed significant ($P < 0.05$) increase compared with that of group II. In conclusion, *W. lateritia* leaf has beneficial immunological and haematological properties in Wistar rats and possesses erythropoietic potential at minimal dose that supports its use for treating anaemia.

KEYWORDS: Anti-Anaemic, Immunomodulatory, Haematology, Vitamins, Interleukin-10.

INTRODUCTION

Anaemia is a well-known life threatening disease in the society, which is often caused by excessive blood loss, excessive red blood cell destruction (haemolysis), and deficiency in the production of red blood cell as a result of iron deficiency, due to inadequate dietary intake or absorption from the digestive tract. The synthetic drugs available for its management/cure like Astifer, Chemiron, HB-12 and other haematinic synthetic drugs, are not without their side effects, they may also be cost ineffective to some patients and inaccessible. All these limitations of the synthetic drugs have necessitated researches, focusing on less toxic herbal therapies known for their anti-anaemic efficacies as claimed by ethno medicinal practitioners.

Herbal plants have been studied and used as alternative treatment for diseases, but the full potentials of plants still remain under exploited. The leaves of *W. lateritia* are purportedly used for blood boosting, and also for treating different ailments in folkloric medicine. The general public is becoming very interested in the plant because of its medicinal and nutritional importance; however, there are little or no documented reports to this regard, it is on this basis that this work was designed. Hence it is pertinent to investigate its anti-anaemic potentials and of course the safe dose.

Fig. 1 Diagram of *W. lateritia*.

MATERIALS AND METHODS

3.1 PLANT MATERIAL

The leaves of *Whitfieldia lateritia* used for this study were collected from a local farm in Unwana community, Afikpo-North Local Government of Ebonyi State, Nigeria. The leaf was authenticated by a Botanist at the Botany Department of Michael Okpara University of Agriculture; some of the leaves were deposited in the herbarium with authentication (voucher) number W0045, for reference purposes. The leaves were destalked, washed and shade dried at ambient temperature with constant turning to avert fungal growth. The dried leaves were milled to obtained the vegetable leaf meals (VLMs) using an electric blender and was stored in 4^{o}C temperature in refrigerator in well labeled air – tight containers for analysis.

3.2 EQUIPMENT AND REAGENTS USED/MODEL

Atomic Absorption Spectrometer (AA32ON), Ultraviolet Visible Spectrophotomètre (UN 72ON), Hot air oven (DHG 910), Muffle furnace (P5900), Electronic weighing balance (Scout Pro Pu 401) and Electric Blender (BN2033). All chemicals and reagents used are of standard analytical grade. Hydrochloric acid, ferric chloride, tetraoxosulphate (VI) acid, acetic anhydride, Potassium hydroxide methanol, and Ammonia solution were purchased from Jaj Chemical Ltd, China.

3.2.1 Extraction

Ground sample of *W. lateritia leaves* (400 g each) was macerated with analytically graded methanol, chloroform and water for 72 h with occasional stirring with a stirring rod. The extract was sieved with a sieving cloth; the filtrate was passed through filter paper, concentrated by rotary evaporator, and dried at room temperature.

3.3 DETERMINATION OF VITAMIN COMPOSITION

3.3.1 Determination of vitamin A

Vitamin A was determined by the calorimetric method of Kirk and Sawyer (1998).

3.3.2 Determination of vitamin E

This was determined by the Futter- mayer colometric method with association of vitamin chemist's (Kirk and Sawyer, 1998).

3.3.3 Determination of vitamin C

This was determined by the titrimetric method reported by (Kirk and Sawyer, 1998).

3.3.4 Determination of vitamin B_1 and B_2

This was determined by the titrimetric method reported by (Kirk and Sawyer, 1998).

3.3.5 Determination of vitamin B_3 (nicotinamide)

This was determined by the titrimetric method reported by (Kirk and Sawyer, 1998).

3.3.6 Determination of vitamin B_6

This was determined by the titrimetric method reported by (Kirk and Sawyer, 1998).

3.3.7 Determination of vitamin B_{12}

This was determined by the titrimetric method reported by (Kirk and Sawyer, 1998).

3.4 MINERAL COMPOSITION ANALYSIS

Calcium, magnesium, potassium, and phosphorus, were all determined using the methods of AOAC (AOAC, 2010).

3.5 ACUTE TOXICITY AND LETHALITY (LD_{50}) TEST

The acute toxicity study of all extracts of *W. lateritia* leaves were carried out using the modified method of Lorke, (1983). The test was divided into two phases. In the first phase, total of nine randomly selected adult mice were divided into three groups (n=3) and received 10, 100 and 1000 mg/kg b.w. of the extracts, and there were no signs of toxicity and death recorded after 24 hours of observation. The doses for phase two were determined based on the outcome of the phase one. Since there was no death recorded, a fresh batch of animals were used following the same procedure with higher doses of 1900, 2600, and 5000 mg/kg body weight of the extract. The animals were observed for 24 hours for signs of toxicity and

death. The LD_{50} was calculated as the geometric mean of the high nonlethal dose and lowest lethal dose (Lorke, 1983).

3.6 INDUCTION OF HAEMOLYTIC ANAEMIA WITH 2, 4-DNPH AND COLLECTION OF BLOOD SAMPLES

A modified method described by Berger, 1983 as described by Omoboyowa *et al.*, 2017 was used in this study. The animals of Groups II to VI received 2,4-dinitrophenylhydrazine (20 mg/kg body weight) once daily for seven days. On the eighth day, their blood samples were collected by tail snip of each rat into heparinized capillary tubes for haematological analysis. The tails were first sterilized by swabbing with 70% ethanol and then the tip of the tails pierced. Bleeding was enhanced by gently milking the tail from the body towards the tip. Blood of approximately 2 ml was drawn into heparinized capillary tubes containing anticoagulant for haematological parameters analysis. Rats with packed cell volume (PCV) less or equal to 30 (≤30) were considered anaemic and selected for the experimental groups. On the twenty-first day the animals were euthanized by use of chloroform and blood was collected through cardiac puncture for further laboratory experiments.

3.7 EXPERIMENTAL DESIGN

A total of 36 healthy rats were used for the experimental study, they were randomly allotted into six (6) groups (I to VI) with 6 animals per group (n=6). Appropriate solvent (10% tween 80) vehicle was used to dissolve the extracts. All test substances were administered once daily for 21 consecutive days by oral-feeding cannula. All tested substances were prepared fresh before administration through oral gavage according to design below:

Group I: Normal control: Non-anaemic rats administered 7 ml/kg b. w. of 10% tween 80.

Group II: Negative control: 2,4-dinitrophenylhydrazine-induced anemic rats administered 7 ml/kg b. w of 10% tween 80.

Group III: Positive control: 2,4-dinitrophenylhydrazine-induced anemic rats administered 0.6 ml/kg b. w of astifer.

Group IV: Test Group I: 2,4-dinitrophenylhydrazine-induced anemic rats administered 400 mg/kg b. w. of aqueous leaf extract of *W. lateritia*.

Group V: Test Group II: 2,4-dinitrophenylhydrazine-induced anemic rats administered 400 mg/kg b. w. of chloroform leaf extract of *W. lateritia.*

Group VI: Test Group III: 2,4-dinitrophenylhydrazine-induced anemic rats administered 400 mg/kg b. w. of methanol leaf extract of *W. lateritia.*

3.8 HEMATOLOGICAL ANALYSIS

On the twenty-first day the animals were euthanized by use of chloroform and blood was collected through cardiac puncture for further laboratory experiments. The hemoglobin (HB) concentration, PCV, red blood cell (RBC) count, white blood cell (WBC) count, neutrophil count, and Eosinophils count were determined according to the methods outlined by Dacie and Lewis (1997).

3. 9 IMMUNOLOGICAL PARAMETERS

Interleukin-10 and iron concentrations were determined by methods of AOAC (AOAC, 2010)

3.10 STATISTICAL ANALYSIS

The data obtained were analyzed using analysis of variance (ANOVA). The data were further subjected to Turkey test for multiple comparisons and differences between means regarded significant at $P < 0.05$. The results were analyzed using Graphpad prism.

RESULTS

Table 1: Acute Toxicity of Aqueous, Chloroform and Methanol Extract of *W. lateritia* Leaf.

	Dosage (mg/kg b.w)	Mortality		
PHASE 1		**Aqueous Extract**	**Chloroform Extract**	**Methanol Extract**
Group 1	10	0/3	0/3	0/3
Group II	100	0/3	0/3	0/3
Group III	1000	0/3	0/3	0/3
PHASE II				
Group 1	1900	0/3	0/3	0/3
Group II	2600	0/3	0/3	0/3
Group III	5000	0/3	0/3	0/3

x/y: Number of Death ratio number of animals in a group

Table 2: Vitamin Composition of *W. lateritia* leaf.

VITAMINS	COMPOSITION (mg%)
Vitamin B_1	0.033 ± 0.001
Vitamin B_2	0.032 ± 0.001
Vitamin B_3	0.546 ± 0.008
Vitamin B_6	166.250 ± 1.768
Vitamin B_{12}	0.036 ± 0.014

Two replicate (n = 2), Values represented in Mean ± SEM

Table 3: Mineral Composition of *W. lateritia* leaf.

MINERALS	COMPOSITION (ppm)
Calcium (Ca)	9.967 ± 1.299
Magnesium (Mg)	9.133 ± 0.267
Potassium (K)	3.333 ± 0.120
Phosphorus (P)	1.223 ± 0.021

Values represented in mean ±SEM (Standard Error Mean).

Table 4: Haematological Indices of 2,4-dinitrophenylhydrazine induced anaemic rats treated with extracts of *W. lateritia* leaf.

Treatment	HB Count (%)	PCV (%)	WBC Count ($\times 10^9$/L)	Neu. Count (%)	Monocyte Count (%)	Leuco Count (%)
NC	14.50 ± 1.00	43.5 ± 2.52	14.55 ± 2.12	77.25 ± 1.26	1.75 ± 0.96	21.00 ± 1.41
DT	12.25 ± 1.89$^{*\#}$	34.00 ± 1.63*	12.08 ± 1.80	70.25 ± 7.41*	3.00 ± 0.00*	20.50 ± 0.58
DA	16.50 ± 0.58*	49.25 ± 1.71$^{*\#}$	12.68 ± 0.80	76.00 ± 3.56	3.75 ± 0.50*	29.50 ± 10.34$^{*\#}$
DWLALE	13.5 ± 1.73$^{\&}$	41.00 ± 4.24$^{\#\&}$	13.60 ± 0.33	77.75 ± 2.06$^{\#}$	1.75 ± 0.50$^{\#\&}$	20.50 ± 1.73$^{\&}$
DWLCLE	15.00 ± 0.82$^{\#}$	45.25 ± 1.71$^{\#}$	15.27 ± 2.56$^{\#}$	79.75 ± 2.36$^{\#}$	2.00 ± 0.82$^{\#\&}$	18.25 ± 1.71$^{\&}$
DWLMLE	15.25 ± 1.26$^{\#}$	45.75 ± 3.77$^{\#}$	13.82 ± 3.39	78.25 ± 5.50$^{\#}$	2.00 ± 0.00$^{\#\&}$	19.75 ± 4.86$^{\&}$

Mean ± SD; *($P < 0.05$) significant compared to Normal control Rats; #($P < 0.05$) significant compared to DT; & ($P < 0.05$) significant compared to DA

NC: Normal Control Rats

DT: 2,4-dintrophenylhydrazin induced anaemic rats treated with 10% Tween 80

DA: 2,4-dintrophenylhydrazin induced anaemic rats treated with Astifer

DWLALE: 2,4-dintrophenylhydrazin induced anaemic rats treated with 400 mg/kg b.w of Aqueous extract of *W. lateritia* leaf

DWLCLE: 2,4-dintrophenylhydrazin induced anaemic rats treated with 400 mg/kg b.w of Chloroform extract of *W. lateritia* leaf

DWLMLE: 2,4-dintrophenylhydrazin induced anaemic rats treated with 400 mg/kg b.w of Methanol extract of *W. lateritia* leaf

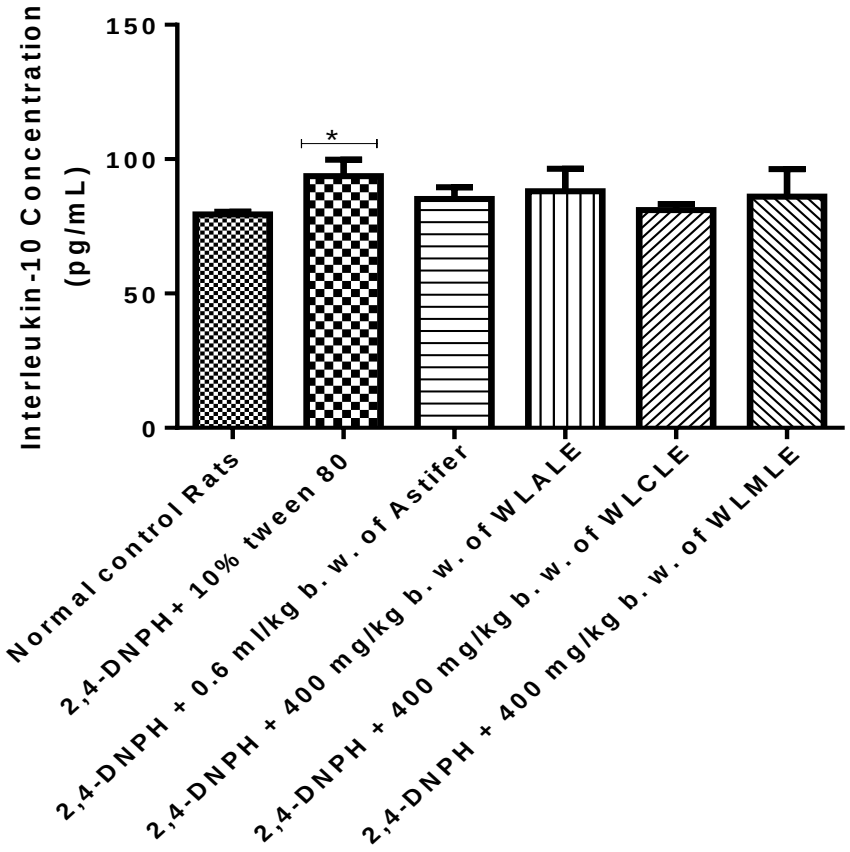

Fig. 2: Effects of *W. lateritia* Leaf Extracts on Serum Interleukin 10 Concentration.

* ($P < 0.05$) Significant compared to Normal control rats; # ($P < 0.05$) significant compare with 2,4-DNPH + 10% tween 80

From figure 2 the rats administered 10% tween 80 after anaemia induction showed significant ($P < 0.05$) increase in interleukin 10 concentration compared with normal control rats. The interleukin 10 concentration of anaemic rats treated with 0.6 ml/kg b. w of astifer showed significant ($P < 0.05$) reduction compared with anaemic rats administered 10% tween 80. The rats induced with 2, 4-dinitrophenylhydrazine and treated with 400 mg/kg b. w of aqueous and methanol extracts of *W. lateritia* leaf showed non-significant ($P > 0.05$) increase in interleukin 10 concentration compared with anaemic rats treated with astifer. Both aqueous and methanol extracts treated anaemic rats showed non-significant ($P > 0.05$) increase in interleukin 10 concentration compared with the chloroform extract treated anaemic rats.

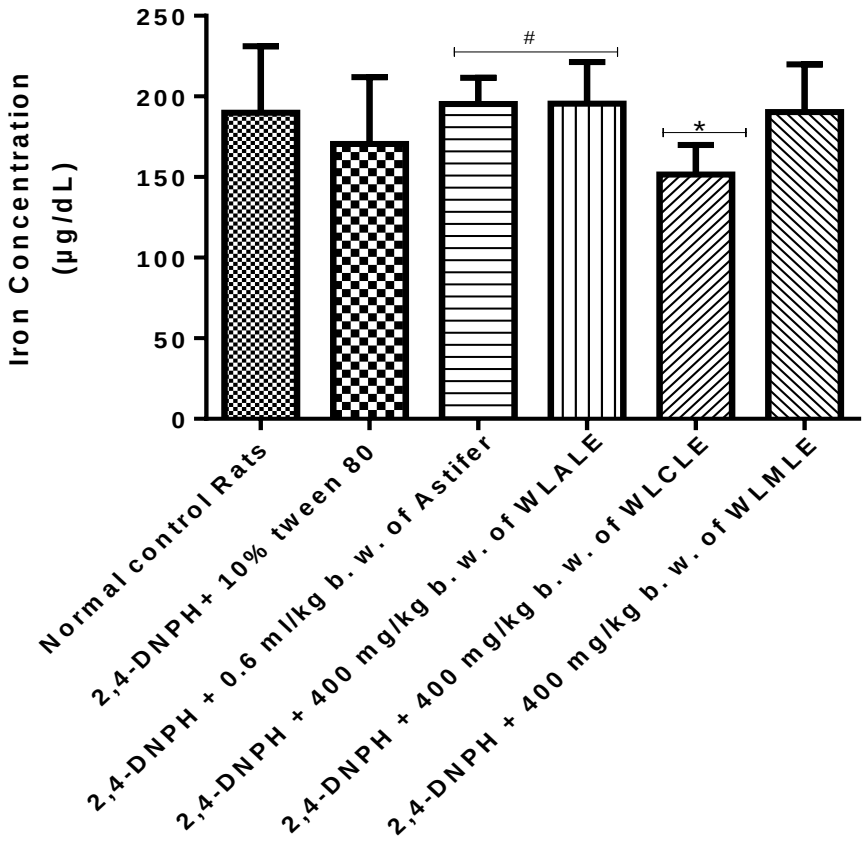

Fig. 3: Effects of *W. lateritia* Leaf Extracts on Serum Iron Concentration.

* ($P < 0.05$) Significant compared to Normal control rats; # ($P < 0.05$) significant compare with 2,4-DNPH + 10% tween 80

From figure 3 the rats administered 10% tween 80 after anaemia induction showed significant ($P < 0.05$) decrease in iron concentration compared with normal control rats. The iron concentration of anaemic rats treated with 0.6 ml/kg b. w of astifer showed significant ($P < 0.05$) increase compared with anaemic rats administered 10% tween 80. The rats induced with 2, 4-dinitrophenylhydrazine and treated with 400 mg/kg b. w of aqueous and methanol extracts of *W. lateritia* leaf showed significant ($P < 0.05$) increase in iron concentration compared with anaemic rats administered with chloroform extract.

5.1 DISCUSSION

Anaemia is a well-known life threatening condition. It may be caused by excessive blood loss, haemolysis, and deficiency associated with RBC synthesis (due to iron deficiency). The synthetic drugs available for its management/cure are not without their side effects, they may

also be inaccessible and cost ineffective to some rural sufferers. All these limitations of the synthetic drugs have necessitated researches, focusing on less toxic herbal therapies known for their anti-anaemic efficacies as claimed by ethno medicinal practitioners (Eleje and Akujobi, 2011).

The percentage vitamin content of *W. lateritia* was presented in table 2. The high value of vitamin B_6 (pyridoxine) is an indication that the *W. lateritia* leaves helps the body to turn food into energy. Vitamin B_6 also helps the body fight infections, *W. lateritia* contains an adequate amount of antioxidants (when compared with most Nigeria and European leafy vegetables) which helps in mopping up of free radical in the body and also helps to keep the skin free from certain infections (Herrero and Barbas, 2011). The values of vitamin B complex is in line with the findings of Aja *et al.*, (2016), who showed that the *W. lateritia* leaves contain medicinal values when compared with other leaf vegetables in the sense that they help to fight prostate cancer, recovering of blood/blood boosting in the body (Aja *et al.*, 2016).

The results obtained, showed that *W. lateritia* is a rich source of minerals. The mineral compositions include Calcium (Ca), Magnesium (Mg), Potassium (K) and Phosphorus (P). Calcium is required for normal growth activities of muscles and skeletal development and electrical impulses in brain and also prevents Osteoporosis (Choudhary and Bandy Opaelhyayi, 2013). The potassium ratio in the body is of great concern for prevention of high blood pressure. Hence, consumption of *W. lateritia* would probably reduce high blood pressure. Phosphorus aids in regulation of acid-base balance (Delgodia *et al.*, 2010). Magnesium aids various chemical reactions in the body, intestinal absorption and also prevents heart diseases and high blood pressure. On this premise, it may be deduced from the result that consumption of *W. lateritia* supplies these minerals which are of medicinal importance to humans. As shown in the result (tables 3), the composition of Calcium in *W. lateritia* is higher than that of magnesium, followed by potassium and phosphorus.

Acute toxicity tests on *W. lateritia* in albino rats using the method of Lorke (1983) established a high LD_{50}, which suggests that the aqueous, chloroform and methanol extracts of the leaf *W. lateritia* may be generally regarded as safe with a remote risk of acute intoxication and sedation at high dose above 5000mg/kg b. w. Signs of acute toxicity include decreased locomotor activity, decreased feed intake, tremor, change of hair colour, prostration and death (Barbosa-Ferreira *et al.*, 2005). None of these signs was noticed in the

experimental mice given the extracts. The degree of safety is also consistent with its popular use locally. Thus, since *W. lateritia* is believed to have anti-anaemic potentials by many traditional healers, the experimental determination of this good safety margin would justify the plant as relatively safe at the dose level (400mg/kg b. w) used in this study.

Assessment of haematological parameters can be used to explain haematological functions of a chemical compound or plant extracts in an organism (Yakubu *et al.,* 2007). Blood act as a pathological reflector of the status of exposed animals to toxicants and other conditions and/or agents (Olafedehan *et al*., 2010). In this study, the aqueous, chloroform and methanol leaf extracts of *W. lateritia* demonstrated varying degrees of haematological parameter changes in normal rats and induced anaemic rats at 400 mg/kgbw. The extract of *W. lateritia* significantly increased ($P < 0.05$) the levels of Hb and PCV. The observed increases in Hb and PCV levels upon administration of aqueous, chloroform and methanol leaf extracts of *W. lateritia* suggests that the extract could have stimulated erythropoietin release in the liver, which is the humoral regulator of RBC production (Degruchy, 2016). This result is consistent with the report of other researchers (Alada, 2010; and Nwinuka *et al.*, 2018); who elucidated haematological parameters using *Telifaira occidentalis* and *Magnifera indica* respectively. Wambi *et al.,* (2008), in a previous research also showed that prophylactic and therapeutic oral administration of antioxidant supplements of plant extracts significantly increased cells of hematopoietic origin in animals exposed to potentially lethal dose of radiation. The increase in these haematological parameters (table 4) suggest an increased production of majority of the cells involved in the immune system which are produced in the stem cells of the bone marrow which have been suppressed by the myelo-suppressant, pyrogallol (Egba *et al.,* 2013). It can be deduced that *W. lateritia may* possess haematinic abilities as it stimulates the activities of the bone marrow and thus has reversed experimentally induced haemolytic anaemia. Haemoglobin is a natural constituent of RBCs and biochemically adapted to carry oxygen in the lungs and deposit it at tissues for oxidative metabolism (Ologundudu *et al.,* 2009). The increase in haematological parameters investigated could be as a result of some constituents such as iron and some B-complex vitamin (especially vitamin B_{12}) that the plant extract possess, as these serves as hematopoietic factors that influence direct blood cells production in the bone marrow (Toma *et al.,* 2015 and, Ganong, 2001). The results of this study were comparable to the values reported by Toma *et al.,* (2015), and Salman *et al.,* (2008), who reported significant increase in haematological parameters of 2, 4-dinitrophenylhydrazine-induced anemic rats treated with fluted pumpkin.

From figure 2, the rats administered 10% tween 80 after anaemia induction showed significant ($P < 0.05$) increase in interleukin 10 concentration compared with normal control rats. The interleukin 10 concentration of anaemic rats treated with 0.6 ml/kg b. w of astifer showed significant ($P < 0.05$) reduction as compared with anaemic rats administered 10% tween 80. This is in agreement with Groux and Crottrez, (2003), who's worked on complex roles of interleukin-10 on autoimmunity, buttressed such. The rats induced with 2, 4-dinitrophenylhydrazine and treated with 400 mg/kg b. w of aqueous extract of *W. lateritia* leaf showed non-significant ($P > 0.05$) increase in interleukin 10 concentration compared with anaemic rats treated with astifer. Both aqueous and methanol extracts treated anaemic rats showed significant ($P< 0.05$) increase in interleukin 10 and serum iron concentration compared with the chloroform extract treated anaemic rats, as reported similarly in the study of Liorente and Richaud-Patin, (2003).

From figure 3 the rats administered 10% tween 80 after anaemia induction showed significant ($P < 0.05$) decrease in iron concentration compared with normal control rats. The iron concentration of anaemic rats treated with 0.6 ml/kg b. w of astifer showed significant ($P < 0.05$) increase compared with anaemic rats administered 10% tween 80. This is in agreement with Groux and Crottrez, (2003), who's worked on complex roles of iron on autoimmunity, buttressed such. The rats induced with 2, 4-dinitrophenylhydrazine and treated with 400 mg/kg b. w of aqueous and methanol extracts of *W. lateritia* leaf showed significant ($P < 0.05$) increase in iron concentration compared with anaemic rats administered with chloroform extract.

5.2 CONCLUSION

All the data obtained from this study showed strong preliminary evidence that *W. lateritia* leaf extracts have anti-anaemic effects against anaemia induced by 2,4-dinitrophenylhydrazin as proven by biochemical, haematological and immunomodulatory analyses. The effects of crude extract were comparable to that of multivitamin astifer used as standard anti-anaemic drug. Accordingly, the extract can be used as an effective herbal product for the prevention of anaemia and as a blood boosting product. It is believed to be due to its phytochemicals, proteins, fats, carbohydrates, vitamins and mineral contents. It is therefore recommended that *W. lateritia* leaf extract be incorporated in the treatment of anemia and boosting of blood, since it contains the active ingredients needed for blood formation. It is pertinent to equally recommend that further researches should investigate and develop safer, low concentration

and economically viable dosing protocols and guidelines for the use of *W. lateritia* leaf extract in managing anaemia. Finally, public awareness on the importance and the use of *W. lateritia* in managing anaemia is advocated.

DECLARATIONS

Ethics approval

The experimental protocol was approved by the Ethical Committee of Michael Okpara University of Agriculture, Umudike, Nigeria.

Consent for publication

Not Applicable.

Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available due protection of the findings but are available from the corresponding author on reasonable request.

Competing interests

No competing interests associated with this work.

Funding

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Authors' contributions

This study was conducted with contributions from all the authors. Authors ESI and ODA designed the study, wrote the protocol, and supervised the work. Authors AOA, VIC, and OFO performed all the laboratory work. Author ODA performed the statistical analysis.

Authors ODA and OCE managed the analyses of the study. Authors AOA, ESI, and OCE wrote the first draft of the manuscript. Authors AOA, ODA, OFO, and VIC managed the literature searches, and all authors read, edited, and approved the final draft of the manuscript.

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