JOURNAL OF COMPLEMENTARY MEDICINE RESEARCH, 2020
VOL 11, NO. 1
10.5455/jcmr.2020.11.01.12

RESEARCH ARTICLE

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The effect of *Justicia insularis* ethanol leaf extract on haematological parameters in Phenylhydrazine-induced anaemic Wistar rats

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Abstract

Aim: Medicinal plants have been globally used as alternative to synthetic drugs in treating different disease conditions. This study investigated the effect of oral administration of ethanol leaf extract of *Justicia insularis* in Phenylhydrazin induced anaemia in Wistar rats.

Methods: A total of 30 male Wistar rats including 24 anaemic and 6 normal rats were used for experiment. Anaemia was induced in rats by intraperitoneal administration of phenylhydrazine at a dose of 40 mg/kg for 48 hours. After being confirmed anaemic, rats were orally treated with distilled water and ethanol extract of *Justicia insularis* at doses of 200 mg/kg, 400 mg/kg and 600 mg/kg respectively for three weeks. The haematological parameters including the red blood and white blood cells and their functional indices were investigated in anaemic treated rats compared with the control rats. Phytochemical analysis and acute toxicity test of the extract were also carried out.

Results: Administration of the ethanol leaf extract of *J. insularis* daily for three weeks significantly increased the haematological parameters which conclude that it exhibits antianaemic activity. Phytochemical test on *J. insularis* indicated the presence of alkaloid, saponins, tannins, flavonoids, terpenoids, steroids, glycosides, phenol, resins and reducing sugar. The extract was also found to be non-toxic in rats.

Conclusion: The implication of our findings is that ethanol leaf extract of *J. insularis* possesses potent antianaemic activity and thus, could be considered a potential candidate for the development of new drug in the treatment of anaemic conditions.

ARTICLE HISTORY

Received January 22 2020, Accepted February 12, 2020
Published 25 June, 2020

KEYWORDS

Anaemia; Haematological parameters; Phenylhydrazine; *Justicia insularis*; Leaf extract; Wistar rats

INTRODUCTION

Anaemia is said to be known as a crucial health challenge across the globe; also seen as a condition where there is depletion on red blood cells and haemoglobin in the blood, which can cause paleness, fatigue, exhausted and even lack of interest or excitement. Three quarter of the world population exhibit some type of symptomatic anaemic condition

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in their life time. Prevalence of anaemia is seen mostly in an under developed than developed countries [1]. This condition of anaemia can also develop due to factors like pregnancy, malnutrition, blood parasite like helminthes infection trypanosome and plasmodium, long usage of some drugs like NSAIDs, long exposure to (poisonous) harmful chemicals, like phenylhydrazine. There are other causes of anaemia such as non-access to balance diet, folate and Vitamin B12 deficiencies, chronic inflammation and inherited disorders [2]. Anaemia has been considered to be among the important contributing factors to the global burden of diseases [3]. Although, there are variety of drugs used for the treatment of anaemia, but they are not affordable to numerous poor people in developing countries especially the Sub-Saharan Africa. More so, the rural communities in different parts of the world do not have access to quality drugs for the treatment of anaemia, so rely more on plant products for the treatment of anaemia. Since there is increase in anaemia there should also be a serious desire to establish preventive and curative plans too for the disease.

A number of plants have been reportedly used in traditional management of anaemia and have received ample of scientific validation [4, 5, 6]. One of such plants is *J. insularis*. *Justicia insularis* is one of the plants commonly used by traditional medical practitioners in Nigeria for the management of some health problems. *J. insularis* T. Andeson (family, Acanthaceae) is an herbaceous and perennial plant 30 - 75cm high with opposite ascending branches. Its leaves are simple, opposite, and the flower white, pink or purple [7]. It can be found in variety of habitats from moist forest to dry savannah region. You can find it in cultivated land, refuse heaps, grasslands and forest edges. It requires humus-rich, fertile soil for optimum growth, but can succeed in sandy or loamy soil. It propagates in the wild and the seed remains dormant during dry season, germinating with the return of the rains. *J. insularis* is cultivated in home gardens in west and central African, especially in Guinea, Sierra leone, Ghana, Togo, Benin, Nigeria, Cameroon and DR Congo [8]. They are edible leaves that are gathered from the wild for local use. It is a traditional vegetable in Benin Republic [9]. The leaves are used traditionally in Edo State South South, Nigeria to treat and prevent anaemia. In Akwa Ibom State also in South South, Nigeria, the people uses the leaves to cook soup and it is known there as isepe-akera. *J. insularis* is used as a laxative, to aid digestion and as weaning agent [10]. The aqueous mixture of *J. insularis* has also been proven to induce ovarian steroidogenesis and folliculogenesis in female rats [11]. (Telefo et al., 1998). In recent studies carried out by Akuodor *et al* [12], the leaf extract of this plant proved to be non-toxic in experimental animal.

However, there is no data on scientific literature to justify its folkloric usage. Thus, this study investigated the antianaemic beneficial effect of ethanol extract of *J. insularis* in Phenylhydrazine induced anaemic Wistar rats.

Materials and Methods

Plant collection

Fresh leaves of *Justicia insularis* were collected in the month of July, 2018 from Iguelaiho village, Ova South West Local Government of Edo State, Nigeria. The leaves were identified and authenticated by a taxonomist in Herbarium unit of Department of Botany, University of Nigeria, Nsukka.

Preparation of the extract

The leaves were washed, shade dried at room temperature in the Department of Pharmacology and Therapeutics Laboratory, Ebonyi State University (EBSU), Abakaliki and subjected to size reduction to a coarse powder by using mortar and pestle. Four-hundred grams (400 g) of the powder was macerated in 2.5 L of ethanol for 24 hours with frequent agitation until the soluble matter has dissolved. The mixture was strained, the marc pressed, and the filtrate concentrated on water bath at reduced temperature of 45^{0}c to recover the extract. The gathered yield was 8.5% w/w semi-solid green powder. The concentrated extract was dissolved in distilled water and used for the *in vivo* study.

Phytochemical analysis

Phytochemical screening of the leaf extract of *J. insularis* was carried out for various secondary metabolites such as alkaloid, carbohydrates, saponins, tannins, flavonoids, terpenoids, steroids, glycosides, phenol, resins and reducing sugar using standard method [13, 14].

Acute toxicity test

Oral acute toxicity test was performed using the Organization of Economic Cooperation and Development OECD) [15] guideline 423 with little modification. Male rats weighing 170-200 g were used for this study and conducted in two phases. In the first phase, three groups of 3 rats in each cage, were administered with 500, 1000 and 2000 mg/kg of the leaf extract orally. These rats were observed continuously for the 4 hours and every hour for signs of toxicity and mortality for 24 hours. This was followed by administration of 3000, 4000 and 5000 mg/kg to the next three groups of 3 rats and also observed for signs of toxicity such as hyper activity, salivation, paw-licking, writhing, distress and mortality. The number of deaths recorded in each group and the final LD50 values were calculated.

Maintenance of animals

The animals were kept in clean and dry polypropylene cages with stainless steel top grill having facilities for pelleted food and water. The animals were maintained in a well-ventilated animal house in 12 hours day and 12 hours dark cycle at a temperature of 28 ^{o}C ± 2 ^{o}C and they were acclimatized to laboratory conditions for 10 days prior to the commencement of the experiment. Saw-dust was used as bedding material and changed twice a week.

Experimental animals

Healthy male Wistar albino rats with average weight of 170 to 200 g were obtained from the animal house, Department of Anatomy, Faculty of Medicine, Ebonyi State University. The animals were fed with standard pellet and water *ad libitum*. The study protocol was carried out as per the rules and regulations of the Institutional Animal Ethical Committee (IAEC), Faculty of Medicine, Ebonyi State University, Abakaliki (EBSU/DRIC/UREC/04/053) as well as the international guidelines on the Use and Handling of Experimental Animals [16].

Collection of sample before and after induction

Samples were collected in three batches of baseline, after the onset of anaemia on day 3 and post treatment [17] by orbital plexus of the eye with micro haematocrit capillary tube for determination of haematological parameters. The samples were collected in EDTA sample bottles and analyzed (automated haematology analyzer; Mythic 18 by Orphee, Switzerland) in less than 2 hours after collection.

Induction of anaemia

Anaemia was induced in rats by intraperitoneal administration of Phenylhydrazine (Sigma Chemical Co., St. Louis MO, USA.) for 48 hours at the dose of 40 mg/kg [18]. All the animals were weighed and divided into six groups, each group containing five animals.

Experimental Design

Following induction of anaemia with Phenylhydrazin, the animals were randomly grouped into 6 with 5 rats (n=5) in each cage. Group 1: non-anaemic control given 10 mL/kg distilled water, Group 2: anaemic control received 10 mL/kg distilled water, Group 3, 4 and 5 anaemic rats treated with 200 mg/kg, 400 mg/kg and 600 mg/kg of ethanol leaf extract of *J. insularis* respectively. Group 6: anaemic rats treated with vitamin B_{12} (100 µg/kg). All administrations were through oral by gastric intubation for 21 days.

Determination of haematological parameters

At the end of the treatment (21 days), blood samples were collected following the method as described above in EDTA and analyzed (automated haematology analyzer; Mythic 18 by Orphee, Switzerland). The haematological components including haemoglobin (Hb), packed cell volume (PCV), red blood cells(RBC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), white blood cells (WBC), platelet count (PLC), differential count (neutrophils, lymphocytes, monocytes, eosinophils and basophils) were determined. Thin blood films were also smeared and used for RBC morphology studies.

Statistical analysis

The results of the haematological estimations were presented as mean ± S.E.M and analyzed with statistical package for social sciences (SPSS version 20) using one way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. A $P<0.05$ was considered statistically significant.

Results

Phytochemical analysis

The phytochemical screening of the ethanol leaf extract of *J. insuaris* contains the following secondary metabolites; alkaloid, saponins, tannins, flavonoids, terpenoids, steroids, glycosides, phenol, resins and reducing sugar.

Acute toxicity test

The ethanol leaf extract of *J. insularis* did not produce any lethality or significant signs of toxicity in rats up to 5000 mg/kg body weight for 24 hours post treatment.

Pharmacological activities

Table 1 and 2 shows the baseline of red blood cells and its differentials as well as white cells with its differentials in before challenging rats with Phenylhydrazine (PHZ).

Intraperitoneal administration of PHZ for 2 days caused a significant ($P<0.05$) decrease in the levels of Red blood cells, Haemoglobin, Packed cell volume, Mean cell volume, Mean cell haemoglobin, Mean cell haemoglobin concentration and Red cell distribution width (Table 3). The levels of white blood cells, Neutrophils, Monocytes, Lymphocytes, Eosinophils, Basophils and Platelet were also significantly ($p<0.05$) reduced in wistar rats (Table 4).

The levels of these parameters (RBC, Hb, PCV, MCV, MCH and MCHC, and the levels of White blood cells, Neutrophils, Monocytes, Lymphocytes, Eosinophils, Basophils and Platelet were significantly ($p<0.01$) increased near normal level, as well as the reference (Vitamin B_{12}) treated group after administration of *J. insularis* ethanol leaf extract for 21 days (Table 5 and 6).

Table 1: Haematological parameters (red cells and its differentials) in Wistar rats before induction of anaemia

Parameters	Normal Control	Anaemic Control	100 µg/kg vit. B12	200mg/kg *J. insularis*	400mg/kg *insularis*	600mg/kg *insularis*
RBC ($x10^{12}$/L)	8.48± 0.21	8.28± 0.22	7.50±0.18	7.66± 0.51	8.18± 0.31	7.86± 0.15
Hb (g/dL)	13.96± 0.53	14.14±0.10	13.85±0.11	13.00±0.59	13.66± 0.42	13.52± 0.53
PCV ((%))	38.62±1 .62	38.96± 1.51	38.55±1.49	38.52± 0.92	39.66± 1.44	39.12± 0.63
MCV (fl)	56.30±1.57	54.96±1.69	55.82±1.45	53.92±1.20	55.40±1.07	55.50±0.96
MCH (pg)	16.36±1.59	16.72±0.34	15.80±1.08	17.56±1.58	16.92±3.71	15.78±0.31
MCHC (g/dL)	27.52±1.26	28.28±0.2	27.76±0.11	27.78±3.80	29.86±2.89	28.60±0.20

Table 2: Haematological parameters (white blood cells and its differentials) in Wistar rats before induction of anaemia

Parameters	Normal Control	Anaemic Control	100 µg/kg vit. B12	200mg/kg *J. insularis*	400mg/kg *insularis*	600mg/kg *insularis*
WBC ($x10^9$/L)	11.30±1.38	10.18± 1.8	11.25±1.6	12.72±2.39	11.86±1.45	11.90± 0.62
Neutrophil (%)	29.56±1.33	30.10±1.00	32.10±1.22	28.78±1.18	27.70±1.13	29.90±1.45
Lymphocyte (%)	64.51±2.12	68.68±1.11	66.49±1.22	66.40±1.35	64.32±3.10	63.39±3.40
Monocytes (%)	19.29±4.30	17.22±1.33	18.79±0.48	17.48±0.12	16.54±0.52	17.22±0.58
Eosinophil (%)	6.47±1.65	6.85±0.64	5.43±0.19	5.39±0.50	6.22±0.50	5.18±0.22
Basophils (%)	0.32±0.10	0.33±0.03	0.33±0.03	0.34±0.02	0.34±0.03	0.33±0.18
Platelet ($x10^9$/L)	370.20±3.42	337.60±8.62	369.57±6.77	410.40±51.89	440.20±71.53	411.60±35.95

Table 3: **Haematological parameters (red cells and its differentials) in Wistar rats after induction of anaemia**

Parameters	Normal Control	Anaemic Control	100 µg/kg vit. B12	200mg/kg *J. insularis*	400mg/kg *insularis*	600mg/kg *insularis*
RBC ($x10^{12}$/L)	8.89± 0.66	6.88± 0.21	5.54±1.91	5.64±0.24	6.78±0.45	5.54± 1.91
Hb (g/dL)	13.78± 0.40	9.76± 0.19 [a]	10.50±0.31[a]	9.72± 0.37[a]	9.28± 0.90[a]	8.62± 0.37[a]
PCV ((%))	38.82± 2.24	32.38± 1.26[a]	33.82± 2.243[a]	3.58± 1.26[a]	30.60± 1.11[a]	34.44± 1.51[a]
MCV (fl)	59.30±0.84	48.84±0.87[a]	48.46±4.38[a]	47.48±4.39[a]	46.96±0.63[a]	46.68±6.85[a]
MCH (pg)	28.46±0.61	22.32±0.29[a]	24.08±0.38[a]	26.56±1.58[a]	25.62±0.20 [a]	23.74±0.74[a]
MCHC (g/dL)	27.06±0.35	23.32±0.88[a]	22.72±0.31[a]	23.82±0.23[a]	24.28±0.09[a]	24.64±0.37[a]

Values are expressed as mean ± SD (n=5). [a] P<0.05 compared to the control group; [b] P<0.01 compared to control group

Table 4: **Haematological parameters (white blood cells and its differentials) in Wistar rats**

after induction of anaemia

Parameters	Normal Control	Anaemic Control	100 µg/kg vit. B12	200mg/kg *J. insularis*	400mg/kg *insularis*	600mg/kg *insularis*
WBC ($x10^9$/L)	11.02±0.75	9.22± 0.88[a]	8.24±1.67[a]	9.62±3.35[a]	8.18± 0.76 [a]	7.78± 1.77[a]
Neutrophil (%)	30.06±2.54	23.10±1.00[a]	23.80±1.22[a]	25.18±1.18[a]	24.70±1.13[a]	25.90±1.45[a]
Lymphocyte (%)	64.51±2.12	55.68±1.11[a]	53.49±1.22[a]	54.40±1.35[a]	54.32±3.10[a]	53.39±3.40[a]
Monocytes (%)	19.29±4.30	14.22±1.33[a]	13.79±0.48[a]	13.48±0.12[a]	13.54±0.52[a]	14.22±0.58[a]
Eosinophil (%)	6.47±1.65	4.85±0.64[a]	3.43±0.19[a]	4.39±0.50[a]	4.22±0.50[a]	3.18±0.22[a]
Basophils (%)	0.32±0.10	0.23±0.03[a]	0.22±0.03[a]	0.23±0.02[a]	0.21±0.03[a]	0.20±0.18[a]
Platelet ($x10^9$/L)	370.60±72.56	350.60±71.38[a]	353.00±92.68[a]	348.80±71.53[a]	355.80±71.53[a]	349.80±62.51[a]

Values are expressed as mean ± SD (n=5). [a] P<0.05 compared to the control group; [b] P<0.01 compared to control group.

Table 5: The effect of ethanol extract of *Justicia insularis* leaf on haematological parameters (red cells and its differentials) in Wistar rats after 3 weeks treatment

Parameters	Normal Control	Anaemic Control	100 µg/kg vit. B12	200mg/kg *J. insularis*	400mg/kg *insularis*	600mg/kg *insularis*
RBC ($x10^{12}$/L)	9.30±0.19	4.41±0.15[a]	9.43±0.37[b]	8.50±0.27[b]	8.77±0.12[b]	8.87±0.36[b]
Hb (g/dL)	14.40±0.37	6.45±0.66[a]	14.85±0.99[b]	13.63±0.38[b]	14.50±0.30[b]	14.54±0.53[b]
PCV ((%))	41.74±0.68	28.50±2.10[a]	50.74±1.10[b]	46.57±1.16[b]	48.86±0.88[b]	49.30±0.61[b]
MCV (fl)	58.50±1.84	38.13±2.24[a]	71.44±0.03[b]	61.80±2.22[b]	68.38±1.28[b]	69.68±0.05[b]
MCH (pg)	30.80±0.81	20.05±0.70[a]	34.92±0.61[b]	27.00±0.47[b]	28.72±0.33[b]	29.42±0.64[b]
MCHC (g/dL)	31.16±0.47	25.03±0.16[a]	41 .42±4.32[b]	35.90±0.36[b]	37.10±0.31[b]	38.60±0.19[b]

Values are expressed as mean ± SD (n=5). [a] P<0.05 compared to the control group; [b] P<0.01 compared to anaemic control group.

Table 6: The effect of ethanol extract of *Justicia insularis* leaf on haematological parameters (white blood cells and its differentials) in Wistar rats after 3

weeks treatment

Parameters	Normal Control	Anaemic Control	100 µg/kg vit. B12	200mg/kg *J. insularis*	400mg/kg *insularis*	600mg/kg *insularis*
WBC ($x10^9$/L)	11.80±1.44	6.88±0.70[a]	10.72±0.71[b]	11.67±0.89[b]	10.82±1.65[b]	9.70±0.82[b]
Neutrophil (%)	32.34±1.66	16.05±0.17[a]	32.27±0.44	30.53±0.45[b]	31.52±0.67[b]	33.68±0.22[b]
Lymphocyte (%)	75.48±5.66	30.89±1.23[a]	73.77±3.05[b]	71.88±2.56[b]	72.87±3.45[b]	73.88±5.16[b]
Monocytes (%)	29.59±4.30	14.22±1.33[a]	28.79±0.48[b]	27.48±0.12[b]	27.54±0.52[b]	28.22±0.58[b]
Eosinophil (%)	8.47±1.65	2.85±0.64[a]	7.43±0.19[b]	6.59±0.50[b]	7.22±0.50[b]	8.18±0.22[b]
Basophils (%)	0.40±0.10	0.20±0.03[a]	0.39±0.03[b]	0.36±0.02[b]	0.38±0.03[b]	0.39±0.18[b]
Platelet ($x10^9$/L)	410.60±2.88	320.75±2.24[a]	450.40±3.50[b]	443.00±2.19[b]	450.40±3.92[b]	460.00±3.27[b]

Values are expressed as mean ± SD (n=5). [a] P<0.05 compared to the control group; [b] P<0.01 compared to anaemic control group.

Discussion

Herbs are readily available to humans and have been explored greatly for their medicinal properties. Herbal medicines are recently being sought for as alternatives to synthetic pharmaceutical products, and this has led to increase in their demand as natural medicine [19].

Justicia insularis ethanol leaf extract was investigated for its beneficial effect on haemolytic anaemia induced by Phenylhydrazine in Wistar rats. Anemia has brought serious economic consequences and obstacles to national development by affecting over 30% of the world population especially in children [20, 21]. The incidence of anaemia is higher in developing than in developed countries [22]. Anaemia affect normal development in children, and it constitutes a major health problem in young children in developing countries [23]. Therefore, this study was relevant to discover an effective agent to manage the condition in a cost-effective fashion.

Phenylhydrazine (PHZ) is known for its capacity to cause hemolysis *in vivo* by the formation of aryl and hydroxyl radicals due to its interaction with erythrocytes [20]. It has been reported that intraperitoneal administration of this agent (40 mg/kg PHZ) for 2 days reduces hematological indices [24]. Phenylhydrazine-induced anaemia is one of the experimental models for the study of haematinic effects of drugs [25, 26].

Treatment with different doses of *J. insularis* ethanol leaf extract speedily and progressively increased the values of these parameters (Hb, RBC, PCV, MCV, MCH, and MCHC) in anaemic rats within 21 days compared to the negative control group of animals.

An important correlation with diagnostic values has been shown between RBC, Hb, PCV, and red cell indices (MCV, MCH, and MCHC) in both humans and rats [27]. Animals are similar to humans in that reduction in Hb, RBC, and PCV is indicative of anemia [28]. Administration of *J. insularis* suggest that the leaf extract has the ability to stimulate erythropoietic factors capable of influencing the production of blood in the bone marrow. Erythropoietin is a maturation factor of RBC synthesis, which increases the number of erythropoietin-sensitive committed stem cells in the bone marrow that is converted to RBCs and subsequently to mature erythrocytes.

It was also observed that the recovery of the treated groups was dose related with the highest dose of 600 mg/day affecting the highest change.

Hematological parameters is a widely use markers in assessing the toxicity or safety of a drug/extracts as well as assessing the health status of animal [29].The significant increase in the white blood cells count in the rats treated with *J. insularis* leaf extracts could be an indication of leucopoetic potentials and possible immunomodulatory properties of the leaf extract which enhances the production of more white blood cells [30]. This will enhance the antibodies generating potential of the animals via phagocytosis and will have high resistance to infection and diseases [31].

Similarly, the significant increase in erythrocytic indices including HGB, PCV, MCH, MCHC, in all the treatment groups when compared with the control is an indication of stimulation of erythropoiesis by the plant extract. The ethanol extract of *J. insularis* must have enhanced the release of erythropoietin in the kidney, a humoral mediator of RBC production [32].

The increase in haematological parameters exhibited by *J. insularis* might be connected with the phytoconstituents present in the leaf. Phytoconstituents are known for various protective and therapeutic effects [33]. Most importantly, the ethanol leaf extract appears safe hence the LD50 of the extract was greater than 500 mg/kg in rats.

The blood film morphology of the Wistar rats treated with PHZ showed that the normal control groups had 100% normocytic normochromic red blood cells and 100% anisopiokilocytosis and Heinz bodies for other groups. Different sizes and shapes of red blood cells showed evidence of haemolysis. Exposure of albino wistar rats to PHZ has been associated with inclusion bodies on erythrocytes [34]. White blood cells were normal and adequate for the normal control group whereas the other groups that were induced showed 20% leucopenia, moderate monocytosis and 80% moderate leukocytosis, moderate monocytosis. Platelets showed normal and adequate cells for the normal control group and 20% thrombocytopenia and 80% normal and adequate cells for the induced group. There where deformities in the physical characteristics of the WBC and platelets.

Conclusion

The present study therefore, supports the therapeutic use of *J. insularis* in traditional medicine for the treatment of anaemia. The displayed antianaemic activity and the lack of toxic effect render *J. insularis*a candidate for the bioassay-guided isolation of active compounds which could develop into new lead structures and candidates for drug development programs against anaemia.

Acknowledgements

The authors wish to thank Mr. Simon Eze Nwibo, Chibueze Nwonu and other technical staff for their technical assistance and continuous support.

Disclosure of conflict of interest

The authors declare no conflict of interest

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