World Journal of Biology Pharmacy and Health Sciences
eISSN: 2582-5542
Cross Ref DOI: 10.30574/wjbphs
Journal homepage: https://wjbphs.com/

(RESEARCH ARTICLE)

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Synergistic effects of ethanolic leaf extract of *Justicia carnea* and recombinant human erythropoietin on hematological indices in Phenylhydrazine-induced anemic Wistar Rats

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World Journal of Biology Pharmacy and Health Sciences, 2026, 27(01), 161–166

Publication history: Received on 13 June 2026; revised on 25 July 2026; accepted on 27 July 2026

Article DOI: https://doi.org/10.30574/wjbphs.2026.27.1.0407

Abstract

Background: Anaemia remains a significant public health challenge, particularly in sub-Saharan Africa. *Justicia carnea* is a traditionally used anti-anaemic plant, while recombinant human erythropoietin (rHuEPO) is a potent erythropoietic agent. This study investigated the synergistic haematological effects of their combined administration in phenylhydrazine (PHZ) induced anaemic Wistar rats.

Methods: Thirty female Wistar rats were randomly assigned to five groups (n = 6): Group A (control), Group B (PHZ alone), Group C (PHZ + ELJC 250 mg/kg), Group D (PHZ + rHuEPO 10 mg/kg), and Group E (PHZ + ELJC + rHuEPO). PHZ was administered for two weeks to induce haemolytic anaemia, followed by three weeks of treatment. Haematological parameters, including RBC count, haemoglobin (Hb), packed cell volume (PCV), MCV, MCH, MCHC, WBC, platelet count, and body weight, were assessed using a Sysmex XT-2000i automated analyzer. Data were analyzed by one way ANOVA with post hoc LSD and paired t test at $p \leq 0.05$.

Results: PHZ administration significantly reduced RBC, Hb, and PCV in Group B ($p \leq 0.05$). ELJC treatment (Group C) partially restored these indices. rHuEPO monotherapy (Group D) produced the most pronounced individual improvements in RBC, Hb, and PCV. The combined treatment (Group E) demonstrated synergistic benefits, with significant increases in RBC, Hb, and PCV compared to the PHZ group. MCV was elevated in Groups B, D, and E relative to control. WBC changes were non-significant across groups. Platelet counts were significantly elevated in Group B and significantly reduced in Groups D and E. All treatment groups showed significant body weight gains.

Conclusion: Both ELJC and rHuEPO effectively mitigated PHZ induced haematotoxicity. rHuEPO demonstrated the strongest erythropoietic effect individually, while their combination produced superior synergistic outcomes. These findings support the potential integration of *J. carnea* extract with erythropoietic agents in the management of haemolytic anaemia.

Keywords: *Justicia Carnea*; Recombinant Erythropoietin; Phenylhydrazine; Haemolytic Anaemia; Haematological Indices; Wistar Rats

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1. Introduction

Anaemia is one of the most prevalent haematological disorders worldwide, affecting approximately one third of the global population and imposing substantial morbidity, particularly among women and children in low and middle income countries [1,2]. Haemolytic anaemia, characterised by accelerated erythrocyte destruction beyond the compensatory capacity of the bone marrow, represents a clinically significant subtype with multiple aetiologies [3].

Phenylhydrazine (PHZ) is a well-established experimental haemolytic agent that induces oxidative injury to erythrocytes through lipid peroxidation of the red blood cell membrane, generating reactive oxygen species (ROS) that destabilize haemoglobin, degrade spectrin, and ultimately trigger haemolysis [4,5]. This model has been widely used to assess the haematinic properties of both synthetic and plant derived therapeutic agents.

Justicia carnea Lindl. (Family: Acanthaceae), locally known as 'Hospital Too Far', 'Blood Leaf', or 'Blood Tonic' in Nigeria, is an ethnomedicinal plant used extensively in West and Central Africa for the treatment of anaemia [6]. It is rich in diverse phytochemicals including flavonoids, alkaloids, saponins, and polyphenols, which are reported to exhibit antioxidant, anti-inflammatory, and cardioprotective activities [7,8]. Prior studies have documented the ability of *J. carnea* ethanolic leaf extract (ELJC) to reverse PHZ induced haematological deficits [9,10].

Recombinant human erythropoietin (rHuEPO) is a biologically engineered glycoprotein hormone that mirrors the action of endogenous erythropoietin in stimulating erythroid progenitor proliferation and differentiation via the anti-apoptotic Bcl-xL pathway [11]. Its clinical application in anaemia management is well established, though its co-administration with plant extracts in experimental models remains underexplored.

The present study aimed to evaluate and compare the individual and synergistic haematological effects of ELJC and rHuEPO in a PHZ induced anaemia rat model, with specific focus on erythrocyte indices, leukocyte and platelet dynamics, and body weight outcomes.

2. Material and methods

2.1. Ethical Approval

Ethical approval was obtained from the Animal Ethics Committee, Faculty of Basic Medical Sciences, Chukwuemeka Odumegwu Ojukwu University, Uli Campus. All animal handling and experimental procedures conformed to the National Institute of Health guidelines for laboratory animal care and use [12].

2.2. Plant Material and Extraction

Justicia carnea leaves were purchased from Nkwo Market, Nnewi, Anambra State, Nigeria. Botanical authentication was performed at the Herbarium of the Department of Botany, Nnamdi Azikiwe University, Awka. Leaves were washed under running tap water, air dried at ambient temperature, and milled into a coarse powder. Approximately 250 g of powdered leaf material was macerated in 1000 ml of 95% absolute ethanol for 48 hours, filtered sequentially through muslin cloth and Whatman No. 1 filter paper, concentrated using a rotary evaporator, and further dried in a laboratory oven at 45°C to obtain a gel like ethanolic extract. The extract was stored in an airtight container at 4°C until use. The method followed modifications described in established protocols [13,14].

2.3. Experimental Animals

Thirty female Wistar rats (weighing 140 g to 150 g) were obtained from the Animal House Unit, Faculty of Basic Medical Sciences, Chukwuemeka Odumegwu Ojukwu University, Uli Campus. Animals were housed in standard plastic cages at 27 ± 2°C under a 12 hour light/dark cycle and allowed free access to standard laboratory chow (Grower feed) and water ad libitum. A two week acclimatization period preceded all experimental interventions.

2.4. Experimental Design

A quasi experimental design was employed. Rats were randomly allocated to five groups (n = 6 per group):

- Group A: Untreated control (feed and distilled water only)
- Group B:Phenylhydrazine (PHZ)-induced anaemia only (0.1 ml PHZ, intraperitoneal, for 2 weeks)
- Group C: PHZ induction + ethanolic leaf extract of *J. carnea* (ELJC)250 mg/kg/day orally for 3 weeks
- Group D: PHZ induction + rHuEPO 10 mg/kg/day orally for 3 weeks

- Group E: PHZ induction + ELJC 250 mg/kg/day + rHuEPO 10 mg/kg/day orally for 3 weeks

All oral administrations were performed via gavage. The LD50 of ELJC was previously determined to exceed 5000 mg/kg using an established method [15], confirming its safety profile.

2.5. Acute Toxicity Assessment

Acute toxicity of ELJC was assessed using a modified two phase protocol [15]. In Phase I, nine rats received 10, 100, or 1000 mg/kg ELJC (three rats per dose) and were monitored for 24 hours. In Phase II, four rats received 1200, 1600, 2900, or 5000 mg/kg (one rat per dose). No mortality was observed at any dose level, establishing the LD50 as greater than 5000 mg/kg.

2.6. Blood Collection and Haematological Analysis

At study completion, animals were anaesthetized using chloroform in an enclosed chamber 24 hours after the final dose. Blood was collected via retro-orbital puncture using heparinized capillary tubes into EDTA ant-coagulated tubes as described previously [16]. Haematological parameters (RBC, Hb, PCV, MCV, MCH, MCHC, WBC, platelet count) were determined using an automated Sysmex XT-2000i analyser (Sysmex Corp., Kobe, Japan) following an established method [17].

2.7. 2.7 Statistical Analysis

All data were analyzed using SPSS version 25 (IBM Corp., USA). Haematological indices were compared across groups using one way ANOVA followed by post hoc Least Significant Difference (LSD) multiple comparison. Body weight changes were evaluated using paired t test. Statistical significance was set at $p \leq 0.05$. Values are expressed as Mean ± SEM.

3. Results

3.1. Effect on RBC, Haemoglobin, and PCV

Table 1 presents the erythrocyte indices across treatment groups. PHZ administration in Group B produced a statistically significant reduction in RBC count, Hb concentration, and PCV compared to the untreated control (Group A; $p \leq 0.05$). ELJC treatment (Group C) significantly reversed all three parameters compared to Group B ($p \leq 0.05$). rHuEPO monotherapy (Group D) produced the greatest individual improvements, with RBC, Hb, and PCV values significantly higher than both Group A and Group B. The combined treatment group (Group E) also demonstrated significant increases in these indices versus Group B, with values approaching those of Group D.

Table 1 Effect of ELJC and rHuEPO on RBC count, Haemoglobin concentration, and Packed Cell Volume

Group	RBC ($\times 10^{12}$/L) Mean ± SEM	Hb (g/dL) Mean ± SEM	PCV (L/L) Mean ± SEM
A (Control)	5.89 ± 0.15	15.13 ± 0.23	0.45 ± 0.00
B (PHZ)	3.78 ± 0.15#	11.06 ± 0.07#	0.33 ± 0.01#
C (PHZ+ELJC)	5.99 ± 0.34*	16.80 ± 0.15*	0.49 ± 0.02*
D (PHZ+rHuEPO)	6.78 ± 0.31#*	22.53 ± 1.32#*	0.61 ± 0.01#*
E (PHZ+ELJC+rHuEPO)	6.62 ± 0.19*	20.86 ± 1.53#*	0.60 ± 0.02#*
F ratio	23.46	25.03	56.06

*$p \leq 0.05$ vs. Group B; #$p \leq 0.05$ vs. Group A. ELJC = Ethanolic leaf extract of *J. carnea*; rHuEPO = Recombinant human erythropoietin; PHZ = Phenylhydrazine.

3.2. Effect on MCV, MCH, and MCHC

Table 2 shows erythrocyte indices related to cell size and haemoglobin content. MCV was significantly elevated in Groups B, D, and E compared to Group A ($p \leq 0.05$), while Group C showed a non significant reduction compared to Group B. MCH was significantly elevated only in Group D compared to both Group A and Group B ($p \leq 0.05$). MCHC differences across groups were not statistically significant, though Group D showed the highest numerical value.

Table 2 Effect of ELJC and rHuEPO on MCV, MCH, and MCHC

Group	MCV (fL) Mean ± SEM	MCH (pg) Mean ± SEM	MCHC (g/L) Mean ± SEM
A (Control)	77.60 ± 1.54	25.67 ± 0.32	331.33 ± 0.00
B (PHZ)	87.50 ± 2.36#	29.30 ± 1.45	335.33 ± 0.01
C (PHZ+ELJC)	83.10 ± 3.25	28.17 ± 1.49	339.33 ± 0.02
D (PHZ+rHuEPO)	90.56 ± 2.43#	33.17 ± 0.43#*	367.00 ± 14.0#
E (PHZ+ELJC+rHuEPO)	91.03 ± 0.57#	31.40 ± 1.47#	345.00 ± 14.42
F ratio	6.42	6.15	1.631

*p ≤ 0.05 vs. Group B; #p ≤ 0.05 vs. Group A.

3.3. Effect on WBC Count, Platelet Count, and Body Weight

Table 3 shows WBC and platelet data. WBC changes across all groups were not statistically significant. Platelet count was significantly elevated in Group B compared to Group A ($p \leq 0.05$), and was significantly reduced in Groups D and E compared to Group B ($p \leq 0.05$). Table 4 presents body weight data. All groups showed statistically significant gains in body weight from initial to final measurement ($p \leq 0.05$).

Table 3 Effect of ELJC and rHuEPO on WBC Count and Platelet Count

Group	WBC ($\times 10^9$/L) Mean ± SEM	Platelet Count ($\times 10^9$/L) Mean ± SEM	F ratio
A (Control)	5.15 ± 0.32	325.00 ± 23.63	
B (PHZ)	5.05 ± 0.28	508.33 ± 47.81#	
C (PHZ+ELJC)	4.22 ± 0.53	401.67 ± 80.89	
D (PHZ+rHuEPO)	5.12 ± 0.23	266.67 ± 31.14*	
E (PHZ+ELJC+rHuEPO)	6.28 ± 0.52	316.67 ± 77.53*	
F ratio	3.35	2.71	

*p ≤ 0.05 vs. Group B; #p ≤ 0.05 vs. Group A.

Table 4 Effect of ELJC and rHuEPO on Body Weight

Group	Initial Weight (g) Mean ± SEM	Final Weight (g) Mean ± SEM	p value / t value
A (Control)	122.20 ± 1.49	175.00 ± 2.46	0.001* / 18.28
B (PHZ)	134.40 ± 1.86	148.25 ± 3.98	0.012* / 3.388
C (PHZ+ELJC)	135.40 ± 2.06	167.60 ± 3.29	0.001* / 8.281
D (PHZ+rHuEPO)	135.40 ± 2.06	163.00 ± 3.68	0.001* / 6.531
E (PHZ+ELJC+rHuEPO)	122.00 ± 1.14	157.00 ± 3.00	0.001* / 10.91

*p ≤ 0.05 initial vs. final weight within group (paired t test).

4. Discussion

This study demonstrated that PHZ induced haemolytic anaemia, evidenced by significant decrease in RBC, Hb, and PCV in Group B, which was substantially reversed by treatment with ELJC, rHuEPO, or their combination. The haemolytic mechanism of PHZ involves metabolite driven lipid peroxidation of the erythrocyte membrane, generating ROS that disrupt the phospholipid bilayer, degrade spectrin, and increase membrane fragility, culminating in haemolysis [4,5]. This aligns with findings from multiple previous investigations [18,19,20,21].

Treatment with ELJC (Group C) significantly restored RBC, Hb, and PCV levels. This recovery is mechanistically attributable to the antioxidant phytochemicals present in the extract, particularly myricetin, which neutralises ROS at the membrane level and restores erythrocyte structural integrity. These findings are consistent with prior reports documenting haematological improvement following *J. carnea* extract treatment in PHZ anaemia models [7,9,10].

rHuEPO monotherapy (Group D) produced the highest individual recovery in RBC, Hb, and PCV. The underlying mechanism involves rHuEPO mediated upregulation of Bcl-xL, an anti-apoptotic protein that prevents premature death of erythroid progenitors and promotes their maturation into functional RBCs [11]. The synergistic combination in Group E demonstrated improvements that exceeded those of ELJC alone, suggesting complementary mechanisms of action: the antioxidant mediated membrane protection by ELJC and the anti-apoptotic erythropoietic stimulation by rHuEPO.

MCV elevation in Group B may reflect reticulocytosis, a compensatory release of immature erythrocytes, as the bone marrow responds to haemolytic stress [22,23]. The persistence of elevated MCV in Groups D and E likely reflects continued reticulocyte presence during active erythropoietic recovery. Non significant WBC changes suggest that the haematotoxic effect of PHZ under these experimental conditions was primarily restricted to the erythroid lineage. The significant elevation of platelet count in Group B, reversed in Groups D and E, may reflect oxidative stress mediated reactive thrombocytosis following PHZ exposure.

Significant body weight gains across all treatment groups, including the PHZ only group (Group B), were noted. The anabolic and appetite stimulating properties of bioactive compounds in ELJC, particularly myricetin, limonene, and robinetin, may contribute to weight gains in treated groups. The divergence from some reports of PHZ associated weight loss [24,25] may reflect strain specific or dose dependent differences.

5. Conclusion

Justicia carnea ethanolic leaf extract and recombinant human erythropoietin individually and synergistically ameliorated PHZ induced haemolytic anaemia in Wistar rats. rHuEPO demonstrated the strongest individual erythropoietic effect, while the combination of ELJC and rHuEPO yields superior haematological outcomes compared to either agent alone. These findings validate the ethnomedicinal use of *J. carnea* and support its potential integration with erythropoietic therapies for the management of oxidative stress associated haemolytic anaemia. Future studies should focus on dose optimization, molecular signaling pathways, and translational clinical trials.

Compliance with ethical standards

Disclosure of conflict of interest

The authors declare no conflict of interest.

Statement of ethical approval

Granted by the Animal Ethics Committee, Faculty of Basic Medical Sciences, Chukwuemeka Odumegwu Ojukwu University, Uli Campus.

Funding

This research received no external funding.

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