

Protective Effect of *Justicia carnea* Leaf Extract Against Cyclophosphamide-Induced Megaloblastic Anaemia in Rats: An Experimental Study

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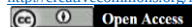
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ABSTRACT

Megaloblastic anaemia is a frequent haematological complication of cytotoxic chemotherapy, and safe adjunctive agents are needed. *Justicia carnea* Lindl. (Acanthaceae) is widely used in Nigerian folk medicine as a “blood tonic”, but its effect on megaloblastic anaemia has not been characterised. To evaluate the protective effect of the ethanol leaf extract of *J. carnea* against cyclophosphamide-induced megaloblastic anaemia in rats. Qualitative and quantitative phytochemical screening and acute oral toxicity testing (Lorke’s method) were performed. Rats were allocated to five groups: normal control; cyclophosphamide 30 mg/kg; and extract at 250, 500 or 1000 mg/kg plus cyclophosphamide 30 mg/kg. Treatments were administered orally every 48 h for 28 days. Body weight, serum folate (vitamin B₉) and cobalamin (vitamin B₁₂), haematological indices, erythrocyte osmotic fragility, relative liver weight, liver function markers, antioxidant status and pro-inflammatory cytokines (IL-1 β , TNF- α) were assessed. The extract was rich in phenols (22.52 $\pm$ 0.25), flavonoids (15.86 $\pm$ 0.12) and saponins (12.04 $\pm$ 0.08), and its oral LD₅₀ exceeded 5000 mg/kg. Cyclophosphamide reduced RBC count, haemoglobin and PCV by 53.7%, 46.8% and 38.6%, respectively, increased MCV by 32.8%, and lowered serum vitamin B₉ and B₁₂ by 60.4% and 56.7%. The extract attenuated these changes dose-dependently: at 1000 mg/kg, vitamin B₉ rose from 1.63 $\pm$ 0.03 to 3.21 $\pm$ 0.04 and vitamin B₁₂ from 2.83 $\pm$ 0.03 to 5.98 $\pm$ 0.07 (p ≤0.05), while MCV fell to 66.43 $\pm$ 0.08 fl. Median corpuscular fragility decreased from 0.638% to 0.490% NaCl; MDA, IL-1 β , TNF- α and aminotransferase activities decreased, whereas SOD, CAT, GPx and GSH recovered towards control values. *J. carnea* leaf extract provides dose-dependent haematological, membrane-stabilising, hepatoprotective, antioxidant and anti-inflammatory protection in cyclophosphamide-induced megaloblastic anaemia, supporting its traditional use and further development as an adjunctive phytotherapeutic agent.

1. Introduction

Anaemia, defined as a reduction in haemoglobin concentration or red blood cell (RBC) mass below physiological limits, is one of the most prevalent health problems worldwide [1]. Among its morphological types, megaloblastic anaemia occupies a distinct place. It results from impaired DNA synthesis in the bone marrow, which produces nuclear–cytoplasmic asynchrony, large immature erythroid precursors (megaloblasts), ineffective erythropoiesis and macrocytosis in the peripheral blood [2, 3].

The principal causes of megaloblastic anaemia are deficiencies of folate (vitamin B₉) and cobalamin (vitamin B₁₂). These vitamins are closely linked in one-carbon metabolism: cobalamin is the cofactor of methionine synthase, which transfers a methyl group from 5-methyltetrahydrofolate to homocysteine, while tetrahydrofolate derivatives are required for thymidylate and purine synthesis [2, 4]. A deficiency of either vitamin therefore slows DNA replication in rapidly dividing cells, above all in

erythroid precursors. In addition to nutritional deficiency, megaloblastic changes may be induced by drugs that directly disturb nucleotide metabolism, including antimetabolites and alkylating cytotoxic agents [2].

Cyclophosphamide is a nitrogen-mustard alkylating agent widely used in cancer chemotherapy and in the treatment of autoimmune diseases [5]. It is activated in the liver by cytochrome P450 enzymes to 4-hydroxycyclophosphamide and then to the active metabolites phosphoramidate mustard and acrolein. Phosphoramidate mustard forms interstrand DNA cross-links and arrests cell division, whereas acrolein promotes the generation of reactive oxygen species, depletes glutathione and triggers lipid peroxidation [5, 6]. Consequently, cyclophosphamide causes myelosuppression, haematological toxicity, hepatotoxicity and immune dysregulation [7]. These properties make cyclophosphamide a convenient tool for modelling anaemia associated with impaired DNA synthesis and oxidative stress under experimental conditions.

The conventional management of megaloblastic anaemia relies on folate and cobalamin replacement. However, in drug-induced anaemia, the main pathogenic factors — direct cytotoxicity and the associated oxidative stress and inflammation — persist, so vitamin replacement alone may be insufficient [8]. Natural products that can simultaneously supply micronutrients and exert antioxidant and anti-inflammatory activity are therefore of particular interest.

Medicinal plants have been used in traditional medicine since antiquity and remain an important source of new bioactive compounds in modern pharmacology [9, 10]. According to the Royal Botanic Gardens, Kew, at least 17,810 plant species are used for medicinal purposes worldwide [11]. A large proportion of the population in developing countries depends on traditional remedies for primary health care because they are affordable, accessible and culturally acceptable [12, 13]. Nevertheless, the pharmacological efficacy and safety of many such plants remain insufficiently substantiated [14, 15].

Justicia is the largest genus of the family Acanthaceae, comprising more than 600 species distributed across the tropical and subtropical regions of the Americas, Africa and Asia [16]. Members of the genus are used in folk medicine to treat anaemia, inflammation, fever, respiratory and gastrointestinal disorders, arthritis and other conditions, and their extracts have shown antioxidant, antimutagenic, anticancer, antimicrobial and antidiabetic activities [16, 17]. Secondary metabolites identified in the genus include alkaloids, steroids, tannins, terpenoids, lignans and flavonoids [16].

Justicia carnea Lindl. (Brazilian plume, flamingo flower) is a perennial shrub native to the Atlantic Forest of eastern Brazil and is now widely cultivated in tropical countries, including Nigeria. In south-eastern Nigeria, the leaves are boiled for 10–15 minutes and the resulting purplish-red, blood-like decoction is drunk as a “blood booster” [18, 19]. *Justicia* species are also used for anaemia in the Republic of Congo [20]. Scientific studies have shown that *J. carnea* leaves are rich in micronutrients (iron, vitamins) and natural antioxidants [19]; that their extracts increase RBC count, haemoglobin and packed cell volume (PCV) in phenylhydrazine-induced anaemia [21, 22]; that the ethanol extract is safe in healthy rats on prolonged administration [23]; and that it possesses antidiabetic activity [24].

However, previous work has been conducted mainly in the phenylhydrazine model, i.e. in haemolytic anaemia driven by oxidative destruction of erythrocytes. The effect of *J. carnea* extract in megaloblastic anaemia — characterised by impaired DNA synthesis and folate and cobalamin deficiency — and its influence on vitamin status, erythrocyte membrane stability, liver function and pro-inflammatory cytokines have not been examined in an integrated manner. Filling this gap is important both for the scientific validation of the plant’s traditional use and for the development of new phytopharmaceuticals.

The **aim** of this study was to evaluate the protective effect of *Justicia carnea* leaf extract against cyclophosphamide-induced megaloblastic anaemia in rats. The specific objectives were: (1) to determine

the qualitative and quantitative phytochemical composition of the extract; (2) to evaluate its acute oral toxicity; (3) to assess its effect on body weight changes in cyclophosphamide-treated rats; (4) to determine its effect on serum vitamin B₉ and B₁₂ concentrations; (5) to evaluate its effect on anaemia parameters and red cell indices; (6) to assess its effect on erythrocyte osmotic fragility; (7) to determine its effect on relative liver weight and liver function; and (8) to evaluate its effect on antioxidant status and serum pro-inflammatory cytokines.

2. Materials and Methods

2.1. Plant material and extract preparation

Fresh leaves of *Justicia carnea* were collected, washed under running water and dried in a hot-air oven. The dried leaves were ground into a fine powder and extracted with ethanol. The extract was filtered and concentrated in a rotary evaporator to remove the solvent; where required, it was further purified by column chromatography. The dry residue was used for phytochemical analysis and for administration to animals, and dosing solutions of the required concentration were freshly prepared before each administration.

2.2. Phytochemical analysis

Qualitative screening was performed using standard phytochemical tests: alkaloids were detected with Wagner's reagent, tannins with ferric chloride, and other classes of compounds with the corresponding colour reactions. Results were graded semi-quantitatively as "+" (present in small amounts), "++" (present in moderate amounts) or "+++" (present in high amounts). Quantitative analysis was carried out by gas chromatography with flame-ionisation detection (GC-FID), gas chromatography–mass spectrometry (GC-MS), high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC). Each parameter was determined in triplicate and expressed as mean $\pm$ standard error of the mean (SEM).

2.3. Experimental animals

Healthy adult albino rats of both sexes were used. The animals were housed in cages bedded with sawdust in the animal house of Michael Okpara University of Agriculture, Umudike, Nigeria, under standard conditions (natural light/dark cycle, room temperature). Before the experiment, the rats were acclimatised for two weeks. They had free access (*ad libitum*) to standard pelleted feed and tap water. At the start of the experiment, body weight ranged from 113 to 123 g. All procedures were conducted in accordance with internationally accepted principles for the care and use of laboratory animals.

2.4. Acute oral toxicity

Acute toxicity was determined in two phases using Lorke's method [25]. In phase I, rats were divided into three groups of three animals and given a single oral dose of the extract at 10, 100 or 1000 mg/kg. Because no deaths occurred in phase I, phase II was performed with doses of 1600, 2900 and 5000 mg/kg (three rats per dose). The extract was administered with a 1 ml syringe at a constant volume. The animals were observed for the first 24 h for signs of toxicity (agitation, piloerection, depression, writhing, lethargy, tremor, behavioural changes) and mortality; body weight and food and water intake were then monitored for 28 days to detect delayed effects.

2.5. Experimental design and induction of megaloblastic anaemia

The rats were randomly assigned to five groups, each consisting of two males and two females. Megaloblastic anaemia was induced with cyclophosphamide at 30 mg/kg. Cyclophosphamide and the extract were given orally with a 1 ml syringe every 48 h (morning and evening of every second day) for 28 days. The groups and treatments are shown in Table 1. Extract doses (250, 500 and 1000 mg/kg) were selected in the light of the acute toxicity results, the highest dose being one fifth of the LD₅₀ limit (>5000 mg/kg).

Table 1

Experimental groups and treatment schedule

Group	Designation	Treatment (oral, every 48 h, 28 days)
1	Normal control (NC)	Standard feed and water; no treatment
2	Megaloblastic anaemia control (CP)	Cyclophosphamide 30 mg/kg
3	JC250 + CP	<i>J. carnea</i> extract 250 mg/kg + cyclophosphamide 30 mg/kg
4	JC500 + CP	<i>J. carnea</i> extract 500 mg/kg + cyclophosphamide 30 mg/kg
5	JC1000 + CP	<i>J. carnea</i> extract 1000 mg/kg + cyclophosphamide 30 mg/kg

Note: n = 4 per group (2 males, 2 females). All treatments were given with a 1 ml syringe at a constant volume.

2.6. Body weight and relative liver weight

Body weight was recorded regularly throughout the experiment on a Zhengya electronic balance. Weight gain was calculated as the difference between final and initial body weight, and percentage weight gain as $(\text{weight gain} / \text{initial body weight}) \times 100$. At the end of the experiment, the animals were sacrificed and the liver was excised and weighed; relative liver weight (%) was calculated as $\text{liver weight} / \text{body weight} \times 100$.

2.7. Blood sampling and haematological analysis

At the end of the experiment, blood was collected by exsanguination. Samples for haematology were collected into EDTA tubes, samples for osmotic fragility into heparinised tubes, and samples for biochemistry into plain tubes; serum was separated by centrifugation. RBC count, haemoglobin (Hb) and packed cell volume (PCV) were measured with an automated haematology analyser. Red cell indices were calculated as follows [26]: $\text{MCV (fl)} = \text{PCV} \times 10 / \text{RBC}$; $\text{MCH (pg)} = \text{Hb} \times 10 / \text{RBC}$; $\text{MCHC (g/dl)} = \text{Hb} \times 100 / \text{PCV}$.

2.8. Serum vitamin B₉ and B₁₂

Serum folate (vitamin B₉) and cobalamin (vitamin B₁₂) concentrations were determined with standard biochemical assay kits according to the manufacturers' instructions.

2.9. Erythrocyte osmotic fragility

Erythrocytes were isolated from heparinised blood by density-gradient centrifugation and resuspended in isotonic saline. A series of sodium chloride (NaCl) solutions ranging from 0% to 0.85% was prepared. Equal volumes of the erythrocyte suspension were incubated in each solution, centrifuged, and the haemoglobin released into the supernatant was measured spectrophotometrically at 540 nm [26]. Percentage haemolysis was plotted against NaCl concentration to obtain the osmotic fragility curve, and the median corpuscular fragility (MCF), i.e. the NaCl concentration producing 50% haemolysis, was determined by linear interpolation between adjacent points.

2.10. Liver function tests

Serum total protein, albumin and total bilirubin, as well as the activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP), were determined by standard colorimetric and kinetic methods. Globulin was calculated as the difference between total protein and albumin.

2.11. Antioxidant status and lipid peroxidation

Serum reduced glutathione (GSH), the activities of glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT), and malondialdehyde (MDA), the end product of lipid peroxidation, were determined by standard spectrophotometric methods [6].

2.12. Pro-inflammatory cytokines

Serum interleukin-1 β (IL-1 β) and tumour necrosis factor- α (TNF- α) concentrations were measured using rat-specific enzyme-linked immunosorbent assay kits according to the manufacturers' instructions.

2.13. Statistical analysis

Data are expressed as mean $\pm$ SEM. Differences between groups were analysed by one-way analysis of variance (ANOVA) followed by a post-hoc multiple comparison test. Differences were considered statistically significant at $p \leq 0.05$. In the tables, values in the same column bearing different superscript letters (a–e) differ significantly ($p \leq 0.05$), whereas values sharing a letter do not. To quantify the degree to which cyclophosphamide-induced changes were reversed by the extract, a protection index was additionally calculated: $PI (\%) = (X_{JC} - X_{CP}) / (X_{NC} - X_{CP}) \times 100$, where X_{JC} , X_{CP} and X_{NC} are the group means of the extract-treated, cyclophosphamide control and normal control groups, respectively. $PI = 0\%$ indicates no recovery and $PI = 100\%$ indicates complete normalisation.

3. Results

3.1. Phytochemical composition of the extract

The results of qualitative and quantitative phytochemical analysis are presented in Table 2. All eight classes of secondary metabolites investigated were detected in the ethanol leaf extract of *J. carnea*. Phenolic compounds were the most abundant (“+++”; 22.52 ± 0.25), followed by flavonoids (15.86 ± 0.12), saponins (12.04 ± 0.08) and alkaloids (9.49 ± 0.06), all of which were present in moderate amounts (“++”). Terpenoids (5.77 ± 0.02), tannins (3.42 ± 0.02), steroids (1.81 ± 0.05) and cardiac glycosides (0.94 ± 0.01) were present in small amounts (“+”). Polyphenols with high antioxidant potential (phenols and flavonoids) thus accounted for approximately 53% of the total quantified metabolites.

Table 2

Qualitative and quantitative phytochemical composition of *J. carnea* leaf extract

No.	Class of compounds	Qualitative test	Quantitative test, mean $\pm$ SEM
1	Phenols	+++	22.52 ± 0.25
2	Flavonoids	++	15.86 ± 0.12
3	Saponins	++	12.04 ± 0.08
4	Alkaloids	++	9.49 ± 0.06
5	Terpenoids	+	5.77 ± 0.02
6	Tannins	+	3.42 ± 0.02
7	Steroids	+	1.81 ± 0.05
8	Cardiac glycosides	+	0.94 ± 0.01

Note: “+” — present in small amounts; “++” — moderate amounts; “+++” — high amounts. Compounds are listed in descending order of quantitative value.

3.2. Acute oral toxicity

No mortality occurred in either phase of the acute toxicity test, even at the highest dose of 5000 mg/kg (Table 3). At 10–1600 mg/kg, the animals remained active and physically stable, with no signs of toxicity such as agitation, piloerection, depression or writhing. At 2900 mg/kg the animals were calm and hypoactive for about 25 minutes, and at 5000 mg/kg for about 3 hours, after which normal activity was fully regained. The oral median lethal dose (LD_{50}) of the extract was therefore greater than 5000 mg/kg.

Table 3

Acute oral toxicity of *J. carnea* leaf extract (Lorke's method)

Phase	Dose, mg/kg	Mortality (dead/total)	Observations
I	10	0/3	Active and physically stable; no signs of toxicity
I	100	0/3	Active and physically stable; no signs of toxicity
I	1000	0/3	Active and physically stable; no signs of toxicity
II	1600	0/3	Active and physically stable; no signs of toxicity
II	2900	0/3	Calm and hypoactive for ~25 min, then fully active
II	5000	0/3	Calm and hypoactive for ~3 h, then fully active

Note: signs of toxicity assessed — agitation, piloerection, depression, writhing and death. LD₅₀ > 5000 mg/kg.

3.3. Body weight

Cyclophosphamide almost completely abolished body weight gain (Table 4, Figure 1). Over 28 days, rats in the normal control group gained 32.57±1.97 g (26.88±1.69%), whereas those in the cyclophosphamide group gained only 0.97±1.15 g (0.87±0.98%) ($p \leq 0.05$). Weight gain in the extract-treated groups was significantly higher than in the cyclophosphamide control: 7.13±0.50 g (6.28%), 7.53±1.30 g (6.20%) and 8.47±0.89 g (7.18%) at 250, 500 and 1000 mg/kg, respectively. The differences among the extract-treated groups were not significant, and weight gain in all of them remained markedly lower than in the normal control.

Table 4

Effect of *J. carnea* leaf extract on body weight in cyclophosphamide-treated rats

No.	Group	Initial weight, g	Final weight, g	Weight gain, g	Weight gain, %
1	Normal control (NC)	121.33±0.94 ^c	153.90±1.89 ^c	32.57±1.97 ^c	26.88±1.69 ^c
2	Cyclophosphamide 30 mg/kg (CP)	116.57±1.03 ^{ab}	117.53±0.74 ^a	0.97±1.15 ^a	0.87±0.98 ^a
3	JC 250 mg/kg + CP	113.50±1.93 ^a	120.63±2.20 ^a	7.13±0.50 ^b	6.28±0.42 ^b
4	JC 500 mg/kg + CP	122.43±0.87 ^c	129.97±0.43 ^b	7.53±1.30 ^b	6.20±1.10 ^b
5	JC 1000 mg/kg + CP	118.37±1.84 ^{bc}	126.83±1.83 ^b	8.47±0.89 ^b	7.18±0.78 ^b

Note: values are mean ± SEM; within a column, values with different superscript letters differ significantly ($p \leq 0.05$). JC — *J. carnea* leaf extract; CP — cyclophosphamide.

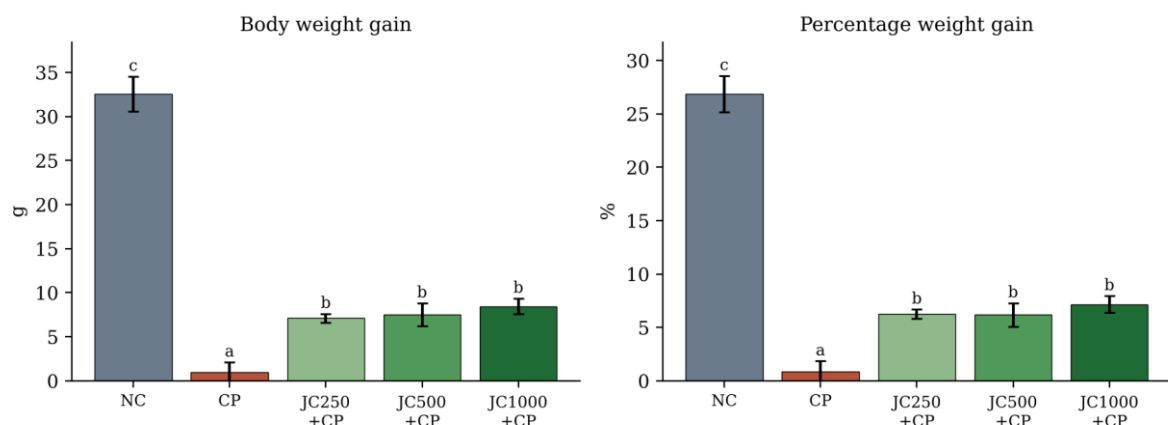


Figure 1. Effect of *J. carnea* leaf extract on body weight gain in cyclophosphamide-treated rats (mean ± SEM). NC — normal control; CP — cyclophosphamide 30 mg/kg; JC250/500/1000+CP — extract at the indicated dose plus cyclophosphamide. Different letters indicate significant differences ($p \leq 0.05$).

3.4. Serum vitamin B₉ and B₁₂

Cyclophosphamide caused a sharp decline in serum folate and cobalamin (Table 5, Figure 2). Compared with the normal control, vitamin B₉ in the cyclophosphamide group fell from 4.12±0.05 to 1.63±0.03 (by 60.4%) and vitamin B₁₂ from 6.53±0.09 to 2.83±0.03 (by 56.7%) ($p \leq 0.05$). This confirms that the vitamin deficiency characteristic of megaloblastic anaemia was established in the model.

Treatment with the extract increased both vitamins in a dose-dependent manner. Vitamin B₉ rose to 2.25±0.04 at 250 mg/kg (+38.0% vs. the cyclophosphamide group), 3.18±0.03 at 500 mg/kg (+95.1%) and 3.21±0.04 at 1000 mg/kg (+96.9%); the difference between the 500 and 1000 mg/kg doses was not significant. Vitamin B₁₂ differed significantly at every dose, rising to 5.18±0.05, 5.50±0.02 and 5.98±0.07, respectively (+83.0%, +94.3% and +111.3%). Nevertheless, even at the highest dose neither vitamin reached normal control values.

Table 5

Effect of *J. carnea* leaf extract on serum vitamin B₉ and B₁₂ concentrations

No.	Group	Vitamin B ₉ , mg/L	Vitamin B ₁₂ , mg/ml
1	Normal control (NC)	4.12±0.05 ^d	6.53±0.09 ^e
2	Cyclophosphamide 30 mg/kg (CP)	1.63±0.03 ^a	2.83±0.03 ^a
3	JC 250 mg/kg + CP	2.25±0.04 ^b	5.18±0.05 ^b
4	JC 500 mg/kg + CP	3.18±0.03 ^c	5.50±0.02 ^c
5	JC 1000 mg/kg + CP	3.21±0.04 ^c	5.98±0.07 ^d

Note: values are mean ± SEM; within a column, values with different superscript letters differ significantly ($p \leq 0.05$). JC — *J. carnea* leaf extract; CP — cyclophosphamide.

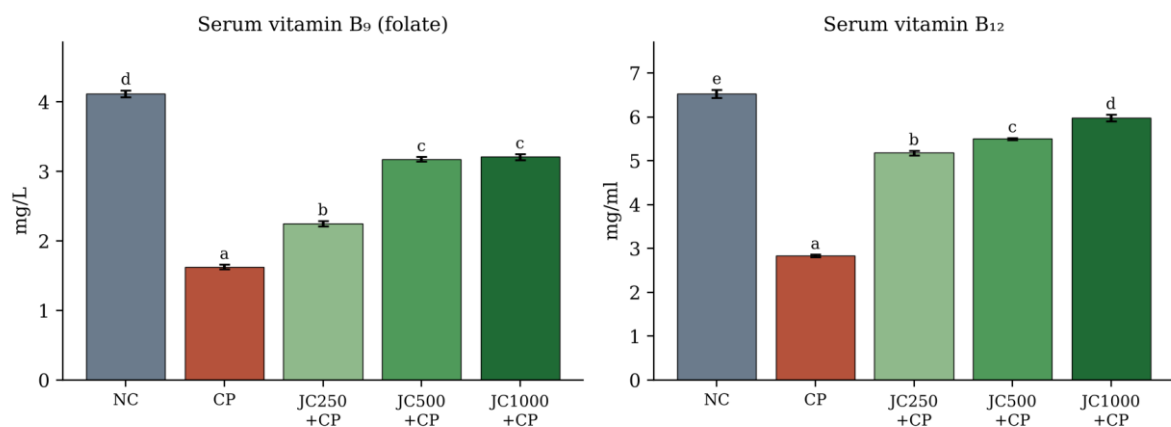


Figure 2. Effect of *J. carnea* leaf extract on serum vitamin B₉ and B₁₂ concentrations (mean ± SEM). Abbreviations as in Figure 1.

3.5. Haematological parameters

Haematological parameters are presented in Table 6 and Figure 3. Compared with the normal control, the cyclophosphamide group showed a fall in RBC count from 7.07±0.05 to 3.27±0.02 × 10⁶/mm³ (by 53.7%), in haemoglobin from 15.50±0.16 to 8.24±0.04 g/dl (by 46.8%) and in PCV from 44.00±0.32% to 27.00±0.32% (by 38.6%) ($p \leq 0.05$). At the same time, MCV increased from 62.21±0.09 to 82.64±0.48 fl (by 32.8%) and MCH from 21.91±0.08 to 25.23±0.11 pg, whereas MCHC decreased from 35.22±0.11 to 30.53±0.22 g/dl. The combination of reduced RBC count and haemoglobin with macrocytosis corresponds to the typical haematological picture of megaloblastic anaemia.

Treatment with the extract improved all anaemia parameters dose-dependently. RBC count increased to 4.44±0.06, 5.31±0.09 and 5.52±0.08 × 10⁶/mm³ at 250, 500 and 1000 mg/kg, respectively (+35.8%, +62.4% and +68.8% vs. cyclophosphamide); haemoglobin to 11.90±0.21, 12.87±0.07 and 13.27±0.13 g/dl (+44.4%, +56.2% and +61.0%); and PCV to 32.00±0.63%, 36.00±0.32% and

36.67±0.48%. Macrocytosis also diminished dose-dependently: MCV was 71.98±0.46, 67.82±0.58 and 66.43±0.08 fl, respectively (−12.9%, −17.9% and −19.6% vs. cyclophosphamide). MCHC was significantly higher than in the cyclophosphamide group in all extract-treated groups (35.75–37.20 g/dl). MCH decreased relative to the cyclophosphamide group at 500 and 1000 mg/kg (24.25±0.31 and 24.04±0.10 pg) but remained elevated at 250 mg/kg (26.77±0.13 pg).

Table 6

Effect of *J. carnea* leaf extract on haematological parameters in cyclophosphamide-induced megaloblastic anaemia

No.	Group	RBC, $\times 10^6/\text{mm}^3$	PCV, %	Hb, g/dl	MCV, fl	MCH, pg	MCHC, g/dl
1	Normal control (NC)	7.07±0.05 ^e	44.00±0.32 ^d	15.50±0.16 ^d	62.21±0.09 ^a	21.91±0.08 ^a	35.22±0.11 ^c
2	Cyclophosphamide 30 mg/kg (CP)	3.27±0.02 ^a	27.00±0.32 ^a	8.24±0.04 ^a	82.64±0.48 ^e	25.23±0.11 ^c	30.53±0.22 ^a
3	JC 250 mg/kg + CP	4.44±0.06 ^b	32.00±0.63 ^b	11.90±0.21 ^b	71.98±0.46 ^d	26.77±0.13 ^d	37.20±0.12 ^d
4	JC 500 mg/kg + CP	5.31±0.09 ^c	36.00±0.32 ^c	12.87±0.07 ^c	67.82±0.58 ^c	24.25±0.31 ^b	35.75±0.17 ^b
5	JC 1000 mg/kg + CP	5.52±0.08 ^d	36.67±0.48 ^c	13.27±0.13 ^c	66.43±0.08 ^b	24.04±0.10 ^b	36.19±0.12 ^c

Note: values are mean ± SEM; within a column, values with different superscript letters differ significantly ($p \leq 0.05$). JC — *J. carnea* leaf extract; CP — cyclophosphamide.

RBC — red blood cell count; PCV — packed cell volume; Hb — haemoglobin; MCV — mean corpuscular volume; MCH — mean corpuscular haemoglobin; MCHC — mean corpuscular haemoglobin concentration.

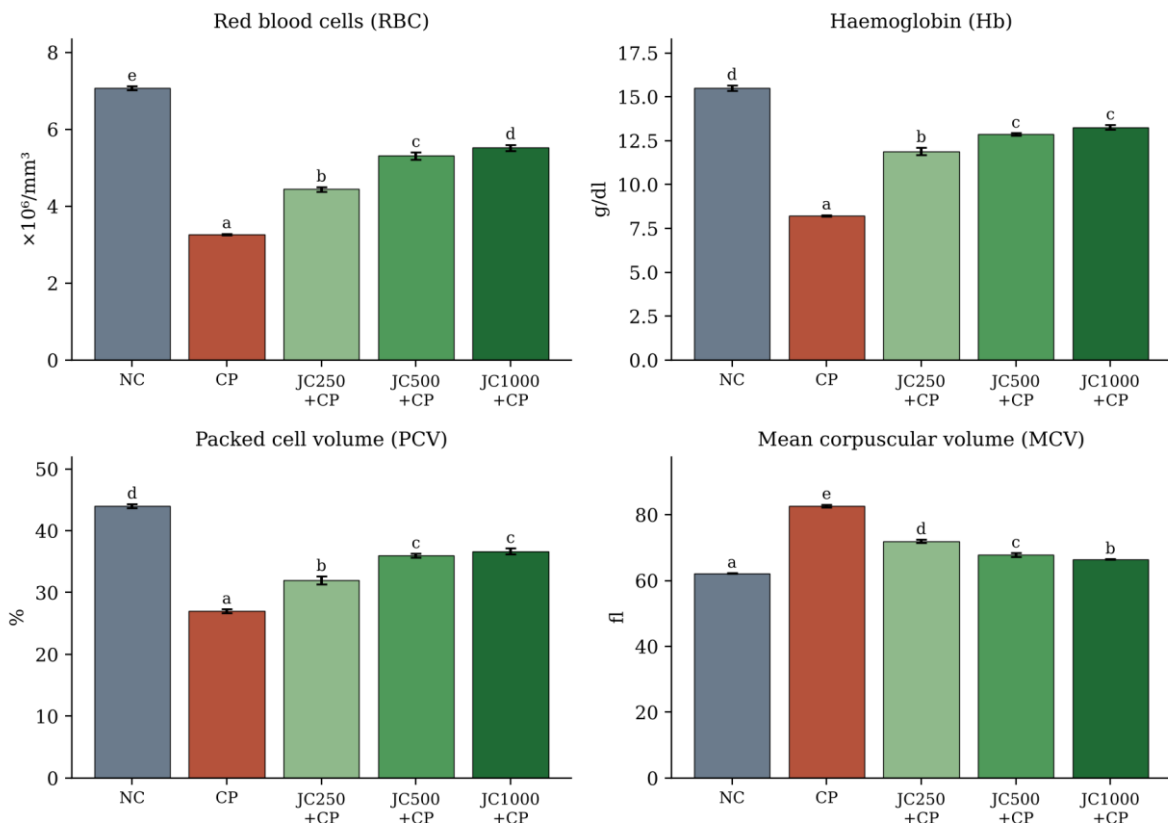


Figure 3. Effect of *J. carnea* leaf extract on the main haematological parameters in cyclophosphamide-induced megaloblastic anaemia (mean ± SEM). Abbreviations as in Figure 1.

3.6. Erythrocyte osmotic fragility

Osmotic fragility data are shown in Table 7 and Figure 4. In the cyclophosphamide group, the haemolysis curve shifted to the right, towards higher NaCl concentrations, indicating increased osmotic fragility. In 0.5% NaCl, haemolysis was $52.77 \pm 0.08\%$ in the normal control but $70.33 \pm 0.07\%$ in the cyclophosphamide group; in near-isotonic 0.85% NaCl, haemolysis rose from $1.67 \pm 0.05\%$ to $19.60 \pm 0.08\%$, i.e. almost twelve-fold. The interpolated MCF was 0.518% NaCl in the normal control and 0.638% NaCl in the cyclophosphamide group.

The extract restored erythrocyte osmotic resistance dose-dependently. Haemolysis in 0.85% NaCl decreased to $8.63 \pm 0.12\%$, $7.23 \pm 0.26\%$ and $5.83 \pm 0.07\%$ at 250, 500 and 1000 mg/kg, respectively. The corresponding MCF values were 0.555%, 0.533% and 0.490% NaCl; at the highest dose, MCF reached, and was even slightly below, the normal control value. At 0.3% NaCl, haemolysis in the 1000 mg/kg group ($73.70 \pm 0.31\%$) did not differ from the normal control ($73.73 \pm 0.07\%$).

Table 7

Effect of *J. carnea* leaf extract on erythrocyte osmotic fragility (haemolysis, %)

NaCl, %	NC	CP	JC250 + CP	JC500 + CP	JC1000 + CP
0	98.07 ± 0.04^a	100.00 ± 0.00^b	97.90 ± 0.32^a	98.27 ± 0.24^a	98.27 ± 0.13^a
0.1	97.27 ± 0.07^b	100.00 ± 0.00^c	97.53 ± 0.07^b	97.27 ± 0.24^b	96.07 ± 0.04^a
0.2	93.53 ± 0.13^c	99.73 ± 0.07^e	94.37 ± 0.07^d	92.90 ± 0.21^b	90.50 ± 0.14^a
0.3	73.73 ± 0.07^a	89.40 ± 0.18^d	76.97 ± 0.22^c	75.87 ± 0.27^b	73.70 ± 0.31^a
0.4	60.23 ± 0.07^a	79.67 ± 0.10^d	66.07 ± 0.09^c	63.83 ± 0.42^b	64.03 ± 0.22^b
0.5	52.77 ± 0.08^b	70.33 ± 0.07^e	59.57 ± 0.12^d	54.97 ± 0.21^c	48.43 ± 0.41^a
0.6	37.23 ± 0.07^a	58.00 ± 0.18^d	42.27 ± 0.10^c	39.83 ± 0.27^b	37.10 ± 0.24^a
0.7	21.83 ± 0.16^a	36.90 ± 0.14^e	28.17 ± 0.07^d	26.90 ± 0.17^c	24.90 ± 0.03^b
0.8	8.70 ± 0.06^a	30.17 ± 0.05^e	14.43 ± 0.04^d	13.93 ± 0.14^c	11.43 ± 0.07^b
0.85	1.67 ± 0.05^a	19.60 ± 0.08^e	8.63 ± 0.12^d	7.23 ± 0.26^c	5.83 ± 0.07^b
MCF, % NaCl	0.518	0.638	0.555	0.533	0.490

Note: values are mean $\pm$ SEM; within a column, values with different superscript letters differ significantly ($p \leq 0.05$). JC — *J. carnea* leaf extract; CP — cyclophosphamide.

MCF — median corpuscular fragility (NaCl concentration producing 50% haemolysis), calculated by linear interpolation between adjacent points.

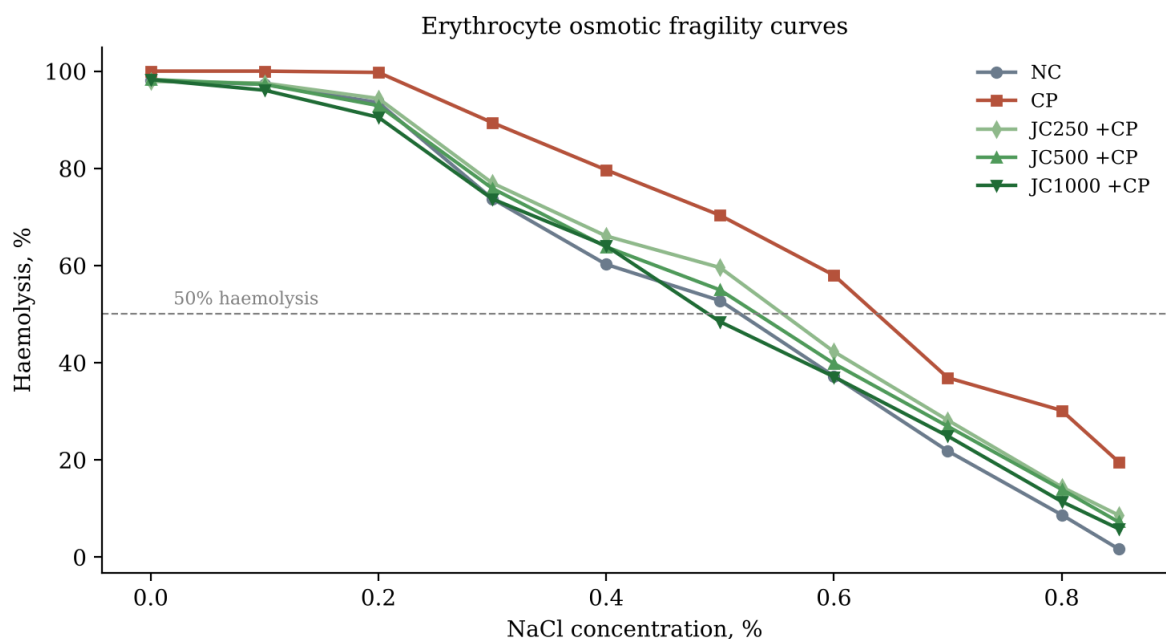


Figure 4. Erythrocyte osmotic fragility curves in the experimental groups (means). The dashed line marks 50% haemolysis; its intersection with each curve corresponds to the median corpuscular fragility (MCF).

3.7. Relative liver weight and liver function

Cyclophosphamide increased relative liver weight from $4.63 \pm 0.03\%$ to $5.84 \pm 0.09\%$ (by 26.1%), suggesting hepatic swelling or inflammatory enlargement (Table 8). The extract reduced this parameter dose-dependently to $5.41 \pm 0.06\%$ (250 mg/kg), $5.29 \pm 0.01\%$ (500 mg/kg) and $5.01 \pm 0.03\%$ (1000 mg/kg).

In the cyclophosphamide group, serum ALT activity increased from 24.33 ± 0.37 to 113.33 ± 2.54 U/l (4.7-fold), AST from 31.67 ± 0.48 to 141.67 ± 0.97 U/l (4.5-fold), ALP from 77.67 ± 0.48 to 175.00 ± 3.11 U/l (2.3-fold) and total bilirubin from 0.57 ± 0.01 to 1.37 ± 0.02 mg/dl. Total protein (from 7.00 ± 0.04 to 4.68 ± 0.11 g/dl) and albumin (from 4.20 ± 0.06 to 2.38 ± 0.08 g/dl) decreased, indicating impaired hepatic synthetic function.

In the extract-treated groups, ALT decreased dose-dependently to 78.33 ± 1.11 , 70.67 ± 0.66 and 68.33 ± 0.91 U/l, ALP to 106.00 ± 0.95 , 102.33 ± 0.80 and 99.00 ± 2.21 U/l, and total bilirubin to 1.08 ± 0.02 , 1.05 ± 0.01 and 1.01 ± 0.01 mg/dl. AST was lowest at 250 and 500 mg/kg (76.00 ± 1.14 and 76.67 ± 0.48 U/l) and was 98.00 ± 1.14 U/l at 1000 mg/kg. Albumin recovered dose-dependently (2.90 ± 0.07 , 3.08 ± 0.01 and 3.43 ± 0.09 g/dl). Total protein increased significantly at 500 and 1000 mg/kg (5.41 ± 0.02 and 6.21 ± 0.11 g/dl) but did not differ from the cyclophosphamide group at 250 mg/kg. Globulin returned to the normal control level at 1000 mg/kg (2.79 ± 0.03 g/dl).

Table 8

Effect of *J. carnea* leaf extract on relative liver weight and liver function

Parameter	NC	CP	JC250 + CP	JC500 + CP	JC1000 + CP
Relative liver weight, %	4.63 ± 0.03^a	5.84 ± 0.09^d	5.41 ± 0.06^c	5.29 ± 0.01^c	5.01 ± 0.03^b
Total protein, g/dl	7.00 ± 0.04^d	4.68 ± 0.11^a	4.48 ± 0.10^a	5.41 ± 0.02^b	6.21 ± 0.11^c
Albumin, g/dl	4.20 ± 0.06^d	2.38 ± 0.08^a	2.90 ± 0.07^b	3.08 ± 0.01^b	3.43 ± 0.09^c
Globulin, g/dl	2.80 ± 0.03^c	2.29 ± 0.04^b	1.58 ± 0.05^a	2.33 ± 0.01^b	2.79 ± 0.03^c
ALT, U/l	24.33 ± 0.37^a	113.33 ± 2.54^d	78.33 ± 1.11^c	70.67 ± 0.66^b	68.33 ± 0.91^b
AST, U/l	31.67 ± 0.48^a	141.67 ± 0.97^d	76.00 ± 1.14^b	76.67 ± 0.48^b	98.00 ± 1.14^c
ALP, U/l	77.67 ± 0.48^a	175.00 ± 3.11^d	106.00 ± 0.95^c	102.33 ± 0.80^{bc}	99.00 ± 2.21^b
Total bilirubin, mg/dl	0.57 ± 0.01^a	1.37 ± 0.02^d	1.08 ± 0.02^c	1.05 ± 0.01^{bc}	1.01 ± 0.01^b

Note: values are mean $\pm$ SEM; within a column, values with different superscript letters differ significantly ($p \leq 0.05$). JC — *J. carnea* leaf extract; CP — cyclophosphamide.

ALT — alanine aminotransferase; AST — aspartate aminotransferase; ALP — alkaline phosphatase.

3.8. Antioxidant status and lipid peroxidation

Cyclophosphamide induced marked oxidative stress (Table 9, Figure 5). Compared with the normal control, MDA increased from 0.32 ± 0.01 to 0.60 ± 0.02 mMol/L (by 87.5%), while endogenous antioxidant defences declined: GSH by 26.9% (from 11.49 ± 0.11 to 8.40 ± 0.13 mg/dl), GPx by 35.1%, SOD by 20.9% and CAT by 21.3%.

The extract restored antioxidant status dose-dependently. MDA decreased to 0.45 ± 0.01 , 0.36 ± 0.00 and 0.33 ± 0.01 mMol/L at 250, 500 and 1000 mg/kg, respectively; at the highest dose it did not differ significantly from the normal control. GSH increased to 9.32 ± 0.09 , 9.88 ± 0.03 and 10.13 ± 0.11 mg/dl, and GPx activity to 38.33 ± 0.91 , 41.67 ± 0.80 and 42.00 ± 0.63 U/l. SOD activity at 1000 mg/kg

(30.33±0.66 U/l) returned fully to the normal control level, and CAT activity at 500 and 1000 mg/kg (20.00±0.32 and 20.67±0.37 U/l) did not differ from the normal control.

Table 9

Effect of *J. carnea* leaf extract on serum antioxidant parameters and lipid peroxidation

No.	Group	GSH, mg/dl	GPx, U/l	SOD, U/l	CAT, U/l	MDA, mMol/L
1	Normal control (NC)	11.49±0.11 ^d	49.33±1.32 ^d	30.33±0.48 ^d	20.33±0.48 ^b	0.32±0.01 ^a
2	Cyclophosphamide 30 mg/kg (CP)	8.40±0.13 ^a	32.00±0.63 ^a	24.00±0.55 ^a	16.00±0.32 ^a	0.60±0.02 ^d
3	JC 250 mg/kg + CP	9.32±0.09 ^b	38.33±0.91 ^b	26.00±0.63 ^b	17.00±0.84 ^a	0.45±0.01 ^c
4	JC 500 mg/kg + CP	9.88±0.03 ^c	41.67±0.80 ^c	27.67±0.37 ^c	20.00±0.32 ^b	0.36±0.00 ^b
5	JC 1000 mg/kg + CP	10.13±0.11 ^c	42.00±0.63 ^c	30.33±0.66 ^d	20.67±0.37 ^b	0.33±0.01 ^{ab}

Note: values are mean ± SEM; within a column, values with different superscript letters differ significantly (p≤0.05). JC — *J. carnea* leaf extract; CP — cyclophosphamide.

GSH — reduced glutathione; GPx — glutathione peroxidase; SOD — superoxide dismutase; CAT — catalase; MDA — malondialdehyde.

3.9. Pro-inflammatory cytokines

Cyclophosphamide markedly raised serum pro-inflammatory cytokines (Table 10, Figure 5): IL-1β increased from 0.12±0.00 to 0.37±0.01 pg/ml (3.1-fold) and TNF-α from 34.43±1.31 to 77.20±0.91 pg/ml (2.2-fold). The extract reduced both cytokines dose-dependently: IL-1β to 0.18±0.00, 0.17±0.00 and 0.15±0.00 pg/ml (−51.4%, −54.1% and −59.5%) and TNF-α to 53.60±0.58, 52.57±1.36 and 40.80±1.24 pg/ml (−30.6%, −31.9% and −47.2%). The effect on cytokines was most pronounced at 1000 mg/kg.

Table 10

Effect of *J. carnea* leaf extract on serum pro-inflammatory cytokines

No.	Group	IL-1β, pg/ml	TNF-α, pg/ml
1	Normal control (NC)	0.12±0.00 ^a	34.43±1.31 ^a
2	Cyclophosphamide 30 mg/kg (CP)	0.37±0.01 ^d	77.20±0.91 ^d
3	JC 250 mg/kg + CP	0.18±0.00 ^c	53.60±0.58 ^c
4	JC 500 mg/kg + CP	0.17±0.00 ^c	52.57±1.36 ^c
5	JC 1000 mg/kg + CP	0.15±0.00 ^b	40.80±1.24 ^b

Note: values are mean ± SEM; within a column, values with different superscript letters differ significantly (p≤0.05). JC — *J. carnea* leaf extract; CP — cyclophosphamide.

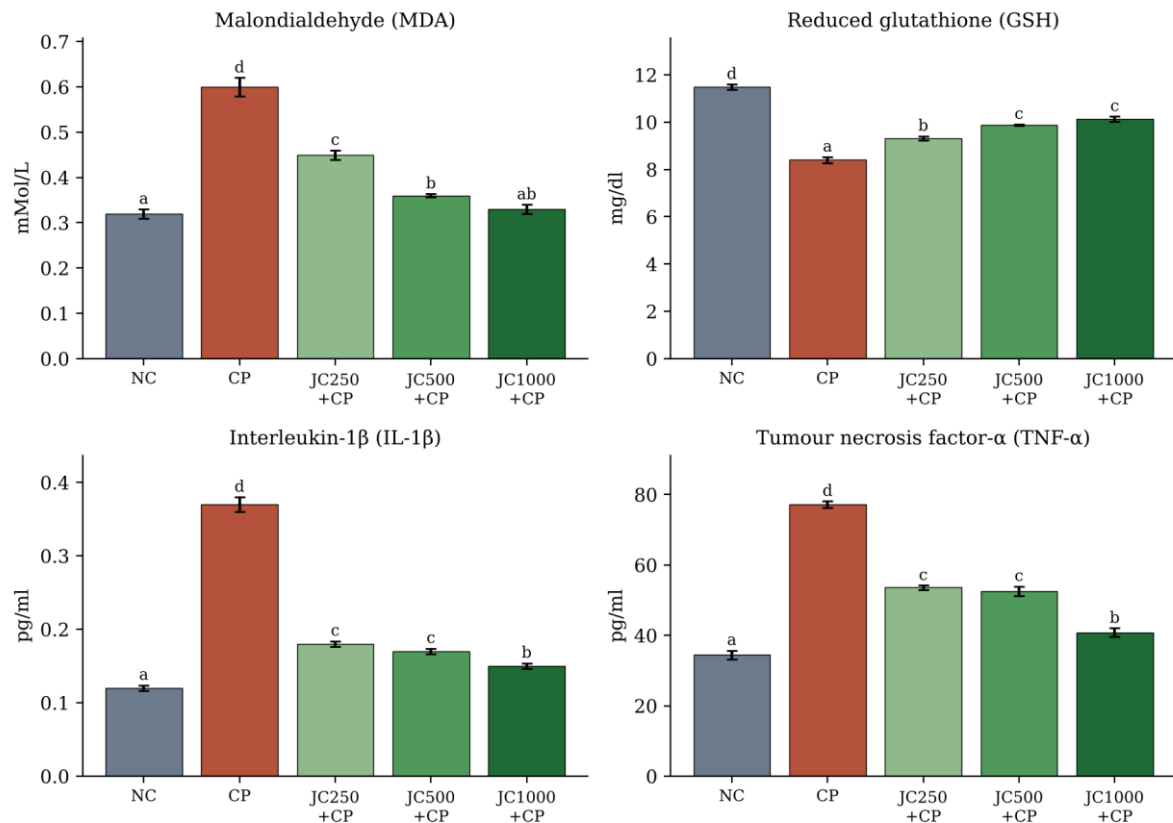


Figure 5. Effect of *J. carnea* leaf extract on lipid peroxidation (MDA), reduced glutathione (GSH) and pro-inflammatory cytokines (IL-1 β , TNF- α) (mean $\pm$ SEM). Abbreviations as in Figure 1.

3.10. Overall assessment of the protective effect

Protection indices calculated to compare the degree of recovery across parameters are given in Table 11. For almost all parameters, the protection index increased with dose. At 1000 mg/kg, recovery reached 88–108% for the antioxidant markers (SOD, CAT, MDA) and IL-1 β , 85% for vitamin B₁₂ and TNF- α , and 79% for MCV. Recovery of erythropoietic parameters (RBC, PCV, Hb) was moderate (57–69%), while that of aminotransferases (ALT, AST) and total bilirubin remained at 40–51%. These results indicate that the antioxidant and anti-inflammatory effects of the extract were more pronounced than its effects on erythropoiesis and liver function.

Table 11

Cyclophosphamide-induced changes and protection indices (PI, %) of *J. carnea* extract

Parameter	Change in CP vs. NC, %	PI, % (JC250)	PI, % (JC500)	PI, % (JC1000)
RBC	-53.7	30.8	53.7	59.2
Hb	-46.8	50.4	63.8	69.3
PCV	-38.6	29.4	52.9	56.9
MCV	+32.8	52.2	72.5	79.3
Vitamin B ₉	-60.4	24.9	62.2	63.5
Vitamin B ₁₂	-56.7	63.5	72.2	85.1
MCF	+23.2	69.2	87.5	123.3
ALT	+365.8	39.3	47.9	50.6
AST	+347.3	59.7	59.1	39.7
ALP	+125.3	70.9	74.7	78.1
Total bilirubin	+140.4	36.3	40.0	45.0
Albumin	-43.3	28.6	38.5	57.7
Relative liver weight	+26.1	35.5	45.5	68.6
MDA	+87.5	53.6	85.7	96.4
GSH	-26.9	29.8	47.9	56.0
GPx	-35.1	36.5	55.8	57.7
SOD	-20.9	31.6	58.0	100.0
CAT	-21.3	23.1	92.4	107.9
IL-1 β	+208.3	76.0	80.0	88.0
TNF- α	+124.2	55.2	57.6	85.1

Note: $PI = (X_{JC} - X_{CP}) / (X_{NC} - X_{CP}) \times 100$, calculated from group means. $PI > 100\%$ indicates that the value overshot the normal control level.

4. Discussion

In the present study, administration of cyclophosphamide at 30 mg/kg for 28 days produced anaemia in rats characterised by marked reductions in RBC count, haemoglobin and PCV, an increase in MCV (macrocytosis), and depletion of serum folate and cobalamin. This constellation of findings matches the classical laboratory picture of megaloblastic anaemia [2, 3] and confirms that the model was successfully established. Co-administration of *J. carnea* leaf extract with cyclophosphamide attenuated most of these changes in a dose-dependent manner. Taken together, the results suggest that the protective effect of the extract is mediated by several interrelated mechanisms: improvement of vitamin status, reduction of oxidative stress, stabilisation of the erythrocyte membrane, suppression of inflammation and preservation of liver function.

4.1. Pathogenesis of cyclophosphamide-induced megaloblastic anaemia

Phosphoramidate mustard, the active metabolite of cyclophosphamide, alkylates guanine bases in DNA and forms interstrand cross-links that block replication during the S phase of the cell cycle [5]. Erythroid precursors in the bone marrow are among the most rapidly proliferating cell populations and are therefore particularly sensitive to impaired DNA synthesis. When DNA replication slows while RNA and protein synthesis continue, the cytoplasm matures ahead of the nucleus, giving rise to large erythroid precursors and macrocytes in the peripheral blood [3]. The 32.8% increase in MCV in the cyclophosphamide group reflects precisely this process.

The 57–60% reduction in serum folate and cobalamin in the cyclophosphamide group is also an important finding. Although cyclophosphamide is not a direct vitamin antagonist, it can produce vitamin

deficiency through several routes. First, it damages the intestinal epithelium and thereby impairs folate and cobalamin absorption. Second, the liver — the main storage organ for cobalamin and folate — is injured by acrolein and reactive oxygen species generated during cyclophosphamide metabolism [5, 6]; in our study this was confirmed by the 4.5–4.7-fold rise in aminotransferases and the fall in albumin. Third, enhanced DNA repair in response to alkylating damage, together with ineffective erythropoiesis, increases the consumption of folate and cobalamin for nucleotide synthesis [4]. The cyclophosphamide model therefore combines direct cytotoxicity with secondary vitamin deficiency, which makes it a valuable model of key aspects of chemotherapy-associated anaemia in clinical practice.

4.2. Effect of the extract on vitamin status

The extract increased serum folate up to two-fold and serum cobalamin up to 2.1-fold. The effect on folate plateaued at 500 mg/kg, whereas the dose–response for cobalamin persisted up to 1000 mg/kg. The rise in folate may be partly explained by the extract itself acting as a source of folate: green leafy plants are natural sources of folate, and Asogwa et al. reported substantial amounts of vitamins and trace elements in *J. carnea* leaves [19]. The dissertation on which this article is based also attributes a high vitamin B₉ content to the leaves [27].

The cobalamin findings require cautious interpretation. Vitamin B₁₂ is synthesised exclusively by microorganisms and is virtually absent from plant foods [28]. The recovery of cobalamin in the extract-treated groups is therefore unlikely to reflect direct supply of vitamin B₁₂ by the plant; more plausible are indirect mechanisms, namely preservation of hepatic storage capacity, protection of the intestinal mucosa with improved absorption, and reduced vitamin consumption as a result of lower oxidative stress. This hypothesis is supported by the concurrent recovery of liver function markers and albumin. Further studies measuring the vitamin B₁₂ content of the extract directly and assessing hepatic vitamin stores are needed to clarify the mechanism.

4.3. Effect on erythropoiesis and red cell indices

The extract increased RBC count, haemoglobin and PCV dose-dependently, and at 1000 mg/kg the protection index for haemoglobin reached 69%. These results are consistent with earlier reports that *J. carnea* restores blood parameters in phenylhydrazine-induced haemolytic anaemia [21, 22]. Onyeabo et al. showed that *J. carnea* leaf extract increases RBC count, haemoglobin, PCV and platelet count in a dose-dependent manner [21], and Anthonia et al. observed similar effects with an aqueous extract [22]. Comparable anti-anaemic activity has been described for other medicinal plants, including *Tectona grandis* [29], *Xylopia aethiopica* [30] and several African plant species [31]. Our study shows that this effect is not limited to haemolytic anaemia but also extends to megaloblastic anaemia associated with impaired DNA synthesis.

Several factors may account for the positive effect of the extract on erythropoiesis. Phytochemical analysis revealed saponins, alkaloids, flavonoids and tannins, classes of compounds that have been associated in the literature with stimulation of erythropoietin production and haematopoietic cell activity [22, 31]. In addition, *J. carnea* leaves are rich in iron [19], an essential substrate for haemoglobin synthesis [32]. Finally, protection of erythroid cells against oxidative injury and attenuation of pro-inflammatory cytokine effects may limit ineffective erythropoiesis (see Section 4.5).

The dose-dependent decline in MCV (protection index 79% at 1000 mg/kg) indicates that the extract attenuated megaloblastic changes, in agreement with the improvement in folate status. Certain features of the red cell indices, however, warrant comment. In the extract-treated groups, MCHC values (35.75–37.20 g/dl) were equal to or slightly above the normal control, and at 250 mg/kg MCH remained even higher than in the cyclophosphamide group. MCHC values close to the physiological maximum may reflect the sensitivity of calculated indices to small measurement errors in RBC count and PCV [26]; direct assessment of erythrocyte morphology (peripheral blood film, bone marrow aspirate) is

therefore required in future studies. It should also be noted that even at the highest dose RBC count, haemoglobin and PCV did not reach normal control values; the extract thus markedly attenuates, but does not abolish, the myelotoxic effect of cyclophosphamide.

4.4. Erythrocyte membrane stability

The osmotic fragility test is an integral measure of erythrocyte membrane integrity and of the cell's surface-area-to-volume ratio [26]. In the cyclophosphamide group, the shift of MCF from 0.518% to 0.638% NaCl and haemolysis of nearly 20% even in near-isotonic medium (0.85% NaCl) indicate substantial weakening of the erythrocyte membrane. Two factors may explain this. First, macrocytes have a reduced surface-area-to-volume ratio and rupture more readily in hypotonic media. Second, peroxidation of unsaturated fatty acids in the membrane — confirmed by the 87.5% rise in MDA — impairs membrane permeability and deformability [6].

The extract returned MCF to normal in a dose-dependent manner, and at 1000 mg/kg erythrocytes were even slightly more resistant than in the normal control. This finding points to a membrane-stabilising effect of the extract. Flavonoids and other polyphenols are known to interact with the polar head-group region of the lipid bilayer and to protect the membrane against free-radical attack [33]. Anthocyanin extracts from the closely related species *J. secunda* have been shown to stabilise the membrane of sickle erythrocytes [20]. The purplish-red colour that develops when *J. carnea* leaves are boiled also suggests the presence of anthocyanins, which strengthens the plausibility of this mechanism.

4.5. Antioxidant and anti-inflammatory effects

Acrolein formed during cyclophosphamide metabolism conjugates with glutathione, depleting intracellular stores and allowing reactive oxygen species to accumulate [5, 6]. The simultaneous decline in GSH, GPx, SOD and CAT and the rise in MDA observed in the cyclophosphamide group reflect this mechanism. The extract restored these parameters dose-dependently, and at 1000 mg/kg SOD, CAT and MDA were practically normalised (protection index 96–108%). These results agree well with the high phenol and flavonoid content of the extract: polyphenols scavenge free radicals directly, chelate transition-metal ions and enhance the expression of antioxidant enzymes [33]. Akintimehin et al. likewise reported that the ethanol extract of *J. carnea* increases antioxidant enzyme activity in healthy rats [23].

Pro-inflammatory cytokines are important negative regulators of erythropoiesis. IL-1 β and TNF- α directly suppress the proliferation of erythroid progenitors, reduce renal erythropoietin production, shorten erythrocyte survival and, by stimulating hepcidin synthesis, limit the availability of iron for erythropoiesis [8]. The 3.1-fold rise in IL-1 β and 2.2-fold rise in TNF- α in the cyclophosphamide group indicate that an inflammatory component also contributed to the anaemia [7]. The extract lowered IL-1 β by up to 59.5% and TNF- α by up to 47.2%. Reduced cytokine levels probably lessened the suppression of erythropoiesis and thereby contributed to the recovery of haematological parameters. Because oxidative stress and inflammation reinforce each other, the antioxidant and anti-inflammatory effects of the extract are likely to be complementary.

4.6. Hepatoprotective effect

Cyclophosphamide is metabolised mainly in the liver, which is therefore the primary target of its toxic metabolites [5]. In our study, the multiple-fold increases in ALT, AST and ALP, the rise in bilirubin, the falls in albumin and total protein and the increase in relative liver weight indicate hepatocellular membrane damage and impaired hepatic synthetic function. The extract reduced ALT, ALP, bilirubin and relative liver weight dose-dependently and increased albumin. Preservation of liver function is also relevant to haematological protection, because the liver is the main store of folate and cobalamin, the central organ of iron metabolism and the site of hepcidin synthesis [8, 32].

The higher AST activity at 1000 mg/kg compared with 250 and 500 mg/kg is noteworthy. Because AST is not liver-specific and is also present in skeletal muscle, myocardium and erythrocytes, this finding may reflect an effect of the high extract dose on other tissues or individual variability. ALT, a more liver-specific marker, was lowest at 1000 mg/kg. Histological studies are needed to assess the long-term safety of high doses.

4.7. Safety and body weight

An oral LD₅₀ above 5000 mg/kg indicates that the extract is practically non-toxic by the oral route [25]. This is consistent with reports on the safety of the ethanol extract of *J. carnea* in healthy Wistar rats [23]. The transient sedation observed at high doses (2900 and 5000 mg/kg) may be related to alkaloids or other neuroactive constituents of the extract and merits further investigation.

Cyclophosphamide almost completely prevented body weight gain, which is attributable to reduced appetite, damage to the gastrointestinal mucosa and the general catabolic effect of the cytotoxic drug [5]. Although the extract increased weight gain approximately seven- to nine-fold, the effect was not dose-dependent and weight gain remained well below normal control levels. This suggests that the effect of the extract on nutritional status is limited and that the general toxicity of cyclophosphamide largely persists.

4.8. Limitations

Several limitations should be considered when interpreting these results. First, the number of animals per group was small ($n = 4$), which limits statistical power, and because males and females were analysed together, sex-related differences could not be assessed. Second, the diagnosis of megaloblastic anaemia was based on peripheral blood parameters and vitamin levels; bone marrow morphology, reticulocyte counts and peripheral blood film findings were not reported. Third, the design did not include a positive control group receiving a reference treatment (e.g. folic acid or cyanocobalamin), so the efficacy of the extract could not be compared with existing therapy. Fourth, the extract was not standardised and active constituents were not isolated, so the observed effects cannot be attributed to specific compounds. Fifth, the folate, cobalamin and iron content of the extract itself was not measured directly. Finally, the units of measurement for vitamin concentrations and phytochemical parameters need to be harmonised with international standard units.

4.9. Practical implications and future directions

The present findings provide scientific support for the traditional use of *J. carnea* leaves as a “blood booster” and open the prospect of studying the plant as an adjunctive phytotherapeutic agent for preventing or attenuating anaemia associated with cytotoxic therapy. In this context, it will be essential to demonstrate that the extract does not reduce the antitumour efficacy of cyclophosphamide, since antioxidants may in some cases compromise the effectiveness of chemotherapy. Future research should focus on isolating and identifying the active constituents, standardising the extract, elucidating the mechanisms of action at the molecular level (erythropoietin, hepcidin, and the Nrf2 and NF- κ B signalling pathways), histological evaluation of the bone marrow and liver, chronic toxicity testing, and assessment of interactions with chemotherapy in tumour-bearing models.

5. Conclusions

1. The ethanol leaf extract of *Justicia carnea* is rich in phenols, flavonoids, saponins and alkaloids and has low oral toxicity (LD₅₀ > 5000 mg/kg).
2. Cyclophosphamide (30 mg/kg for 28 days) induces megaloblastic anaemia in rats, characterised by reduced RBC count, haemoglobin and PCV, macrocytosis, folate and cobalamin deficiency, increased erythrocyte osmotic fragility, oxidative stress, systemic inflammation and liver injury.

3. *J. carnea* leaf extract (250–1000 mg/kg) attenuates cyclophosphamide-induced haematological changes in a dose-dependent manner: it increases RBC count, haemoglobin and PCV, reduces MCV, and approximately doubles serum vitamin B₉ and B₁₂ levels.
4. The extract stabilises the erythrocyte membrane (MCF reduced from 0.638% to 0.490% NaCl), reduces lipid peroxidation, restores the endogenous antioxidant system (GSH, GPx, SOD, CAT) and lowers pro-inflammatory cytokines (IL-1 β , TNF- α).
5. The extract partially protects the liver against cyclophosphamide toxicity, reducing aminotransferases, alkaline phosphatase and bilirubin and restoring albumin synthesis.
6. The most pronounced protection was observed at 1000 mg/kg. The protective effect of the extract appears to be related mainly to its antioxidant and anti-inflammatory properties and to its role as a source of micronutrients. These findings justify further investigation of *J. carnea* as a promising phytotherapeutic agent for the prevention and adjunctive treatment of megaloblastic anaemia, particularly forms associated with cytotoxic therapy.

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Conflict of Interest

The authors declare no conflict of interest.

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