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RESEARCH ARTICLE

Evaluation of Haemopocitic Activity of *Crotalaria juncea* Seed Powder Suspension in Phenylhydrazine Induced Haemolytic Anaemia in Rats

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Abstract

Objectives: To investigate the anti-anaemic activity of *Crotalaria juncea* seed powder suspension against phenylhydrazine induced haemolytic anaemia in rats. **Methods**: The seeds of *Crotalaria juncea* was subjected to preliminary phytochemical analysis. Anemia was induced by administering phenylhydrazine (40 mg/kg, i.p.) for 2 consecutive days. Antianemic activity of *Crotalaria juncea* was investigated at the dose of 200 mg/kg and 400 mg/kg, once daily for 21 days by estimating blood parameters and pathological changes in kidney, liver, heart, spleen, and heart. **Findings**: *Crotalaria juncea* was showed the presence of alkaloids, carbohydrates, tannins, flavonoids, and phenolic compounds. Iron content was found to be 5 mg/100 ml in *Crotalaria juncea*. Anemia induction by phenylhydrazine injections to rats caused significant decrease in red blood cells, hemoglobin, and packed cell volume. These decreased levels of RBCs, hemoglobin and PCV in blood was significantly improved by the treatment with *Crotalaria juncea* ($p<0.05$). A significant normalized clotting time equivalent to normal control groups was observed in group treated with *C. juncea*. Furthermore, *Crotalaria juncea* restored pathological changes in kidney, liver, and heart tissues near to normal. **Novelty**: The *C. juncea* can be used as anti-anemic treatment with fewer side effects.

Keywords: Anaemia; Crotalaria juncea; Phenylhydrazine; Haemoglobin; Anti-anaemic activity

1 Introduction

Anemia, a common public health issue, characterized by erythrocyte mass or haemoglobin concentration reduction in the blood leading to decrease in its oxygen carrying capacity which impacting one-third of the global population [(1)].Hemolytic anemia is a blood condition that occurs when your red blood cells are destroyed faster than they can be replaced. Haemopoiesis can develop quickly or slowly and symptoms may include tiredness, dizziness, weakness, and a spleenor liver that is larger than normal. The clinical severity of the anemia depends on whether the onset of hemolysis is gradual or abrupt as well as the extent of erythrocyte destruction. Mild hemolysis can

be asymptomatic while the anemia in severe hemolysis can be life threatening and lead to angina and cardiopulmonary decompensation. Haemopoiesis has multiple causes, and the clinical presentation can differ depending on the etiology.

Anaemia is a more prevalent medical condition in underdeveloped nations[2].Iron supplementation and dietary modifications are typically recommended for the treatment of anaemia. There are numerous drawbacks to oral iron therapy, including poor absorption as well as noncompliance[3]. Additionally, using large amounts of these iron supplements can result in major health issues including cancer and neurogenic disorders[4].These facts collectively highlight the necessity of safe and efficient alternatives for the treatment of anaemia.

From many ages, medicinal plants have been a source to manage various diseases and anaemia is not an exception. Many plants are said to be effective for anaemia in traditional systems including Ayurveda[5].Earlier research revealed that a number of Indian medicinal plants have anti-anaemic properties[6]. Few polyherbal formulations are reported to be effective for the treatment of anemia[7].These herbal-based formulations are preferred by the community as they are cost-effective and have less side effects. *Crotalaria juncea* (Fabaceae), distributed throughout tropical Asia and Africa, is an annually renewable, multi-purpose fiber crop whose extract is used as food as well as medicine by many tribal communities. Generally, in the folk and Ayurvedic medicines, it is used as a blood purifier, abortificient, astringent, demulcent, emetic, purgative, and also in the treatment of anemia, impetigo, menorrhagia, and psoriasis. Considering the traditional uses of the plant the present study has not so far been scientifically evaluated for its haemopoietic activity. Hence, an attempt has been made to evaluate the haemopocitic activity of *C. juncea* in phenyl hydrazine induced Anemia model.

2 Methodology

2.1 Animals

Wistar albino rats, weighing 150–200 grams, were used for this study. The animals were obtained from Krupanidhi College of Pharmacy's Central Animal House. The animals were kept in propylene cages with six rats per cage and husk changed every 24 hours at ambient temperature (25±2°C), relative humidity (55±5%) and 12 hrs/12 hrs light-dark cycles. Animals were acclimatized and had free access to commercial brand rat pellet diet and water given *ad libitum*. Animal The protocol of the experiment was approved by the Institutional Animal Ethical Committee (IAEC) Ethical Committee approved Number: KCP/IAEC/PCOL/107/2022 as per the guidance of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. The studies were conducted according to the guidelines of CPCSEA.

2.2 Collection of *C. juncea* seeds and authentication

The fresh seeds of *C. juncea* seeds were collected from local market of Aizawl, Mizoram. The collected seeds were devoid of pollutants and pesticides. The collected plant material has been housed in a secure container maintaining the temperature and humidity of the storage environment. The seeds were identified and authenticated by Dr. Sulochana Bhat, Assistant Director In-charge, Central Ayurveda Research Institute, Uttarahalli Manavarthe Kaval, Thalaghattapura post, Bengaluru, and authentication no: SMPU/CARI/BNG/2022-23 (RRCBI-10207). The biochemical parameters were estimated using Erba diagnostic kits in Semi-autoanalyzer provided in the college laboratory (Krupanidhi College of Pharmacy).

2.3 Preparation of herbal suspension

Seeds of *C. juncea* was washed thoroughly with water, cut in small pieces, and permitted room temperature for drying. After drying, the seed of *C. juncea* was grinded and passed through sieve mesh size of 100 (100 holes per square inch) to obtain very fine uniform powder. Before being used, the dried powder samples were kept in polyethylene bags in a refrigerator at 4°C. Dose selection of *C. juncea* was done according to the acute toxicity studies (OECD 425)[8].

Suspension was prepared freshly prior to use. The finely powdered herbal powder (4 gm) was mixed properly by triturating in mortar and pestle and consecutively 2% carboxy methyl cellulose was added to it slowly and steadily. The mixture was triturated with further addition of 20 ml distilled water to obtain the desired suspension consistency and the concentration of suspension was made to 200 mg/ml.

2.4 Preliminary phytochemical analysis[4,9,10]

Finely powdered *C. juncea* seed powder was extracted (solvent extraction) in 70% ethanol (allowed to stand for 1hr) the content was filtered through filter paper. The ethanol used here is similar with the herbal preparation. The prepared extract was subjected

for phytochemical investigation for various constituents.

2.4.1 Test of carbohydrates

2.4.1.1 Benedicts test. In a test tube, the test solution and Benedict's reagent was mixed in an equal volume, stirred thoroughly, and heated for five minutes over a boiling water bath. Yellow colour was obtained. Yellow, green, or red colour solution shows the presence of reducing sugar.

2.4.1.2 Molich's test. 2 ml of Molisch's solution with the crude plant extract and 2 ml of concentrated solution sulphuric acid was mixed and poured along the side of test tube. Violet ring was obtained. A violet ring appearance at the test tube's inner surface shows that presence of carbohydrates.

2.4.2 Test for proteins

2.4.2.1 Biuret test. 4% NaOH and a few drops of 1% $CuSO_4$ solution was poured into a 3 ml test tube with the plant extract. Pink or violet color shows the presence of proteins.

2.4.2.2 Millon's test. 5ml of Millon's reagent was added to 3ml of test solution. White precipitate shows the presence of proteins.

2.4.3 Test for Steroids

2.4.3.1 Salkowski reaction. 2 ml each of the extract, chloroform and concentrated H_2SO_4 were mixed. The acid layer was yellow, whereas the chloroform layer was observed red in colour. Greenish yellow shows the presence of steroids.

2.4.3.2 Liebermann Burchard test. A small amount of acidic anhydride and crude extract were heated and cooled. Concentrated sulfuric acid was added from the side of the test tube. Yellow colour was obtained in the upper layer and deep red colour was obtained in the lower layer. Green colour shows the presence of steroids.

2.4.4 Test for Glycosides

2.4.4.1 Liebermann's test. 2 ml each of acetic acid and chloroform were combined with plant extract. When the mixture was cooled, concentrated H_2SO_4 was added. The aglycone steroidal portion of the glycoside were indicated by the colour green. Green colour shows the presence of glycosides.

2.4.4.2 Salkowski test. Concentrated H_2SO_4 was added to the plant extract. A reddish-brown colour was obtained which shows the entity of aglycone steroidal part of glycoside.

2.4.5 Test for Flavonoids

2.4.5.1 Alkaline reagent test. The test solution becomes more intensely yellow after being treated with sodium hydroxide solution; The presence of flavonoids was indicated when a few drops of diluted HCl were added.

2.4.6 Test for Alkaloids

2.4.6.1 Dragendroff's test. Few drops of Dragendroff's reagent were added to 2-3 ml of filtrate. Precipitates of orange-brown color was formed which shows the presence of alkaloids.

2.4.6.2 Mayer's test. To 2-3 ml of filtrate, few drops of Mayer's reagent was added. Precipitates was formed which shows the presence of alkaloids.

2.4.7 Test for Tannins

2.4.7.1 Ferric chloride test. 5% $FeCl_3$ solution was added to 2-3 ml of alcoholic or water extract. Deep blue colour was formed. Deep blue or black colour indicates the presence of tannins.

2.4.8 Test for Saponins

2.4.8.1 Foam test. The stock solution was diluted vigorously in a test tube filled with 20ml of distilled water. No layer was formed. The presence of a foam layer on top of the test tube shows the presence of saponins.

2.5 Induction of anaemia[4,10]

Analytical grade phenylhydrazine and ferrous ascorbate were procured from Sigma-Aldrich Chemical Private Limited, Bangalore. Prior to use, the phenyl hydrazine hydrochloride solution was produced in situ, neutralized in 0.1M potassium phosphate buffer (pH7.4) and filtered to ensure sterilization. Anemia was induced by intraperitoneal injections of Phenylhydrazine (PHZ) (40 mg/kg) for 2 consecutive days. Haematological parameters like PCV and Haemoglobin content of rats were tested before and after induction of Phenylhydrazine. When packed cell volume (PVC) level as well as hemoglobin content of the rats got reduced by 35% and 14g/dl, they were selected for the study.

2.6 Acute toxicity study

With a limit dose of 2000mg/kg, an acute toxicity test was conducted using Wistar albino rats (either sex) with *C. juncea* seed powder suspension prepared.

2.6.1 Experimental design[4,10]

The rats were divided into five groups, each with six rats (n = 6). Group I was normal control and did not receive any treatment [Table 1]. Group II was anaemic control and received 40 mg/kg of Phenylhydrazine (PHZ). Group III received low dose (200 mg/kg) of *C. juncea* twice daily, Group IV received high dose (400 mg/kg) of *C. juncea* twice daily. Group V was a standard drug treatment group and received ferrous ascorbate (9mg/kg twice daily). Ferrous ascorbate and *C. juncea* were given from day 3-21 by oral gavage after first injection of PHZ. At the end of the experimental period i.e. on day 21, the animals were sacrificed after giving anesthesia. The blood sample and serum were collected for further biochemical assessments.

Table 1. Treatment groups

Groups	Treatment	*n*
I	Normal (vehicle)	6
II	Anemic Control (PHZ 40 mg/kg)	6
III	Low dose (200mg/kg p.o) of C. juncea extract	6
IV	Low dose (400mg/kg p.o) of C. juncea extract	6
V	Ferrous ascorbate (9mg/kg)	6

2.7 Physical parameter

2.7.1 Body weights[4,10]

Body weights were measured on day 0, 3, 7, 14 and 21. The average weight gained or lost for the total experimental period was calculated with statistical significance.

2.8 Blood parameter estimation[4,10,11]

Under diethyl ether anaesthesia, blood was drawn from the retro-orbital sinus prior to the first injection of PHZ and on day 3, 7, 14, and 21. Blood was analyzed for red blood cells, haemoglobin, mean corpuscular volume, clotting time and haematocrit (HCT) levels. Blood samples were estimated at Pharmacology laboratory, Krupanidhi college of Pharmacy, Bangalore and serum was tested by using Erba diagnostic kits in semi-autoanalyzer provided in Pharmacology laboratory, Krupanidhi college of Pharmacy.

2.8.1 Estimation of RBC using Neubauer's chamber

Neubauer's chamber is a thick glass plate that is about the size of a glass slide, measuring 30 by 70 by 4 mm. The two square-shaped regulated sections comprise the counting region. The moats or depressions between the designated squares and on both sides provide a "H" shape. A watch glass received an adequate amount of RBC diluting solution. Capillary blood was carefully drawn up to 0.5 markings using an RBC pipette, and the pipette tip was subsequently cleaned. The exact absorption of the RBC dilution solution reached 101 points. The blood and liquids were well mixed to create an erythrocyte suspension. After five minutes, the liquid was charged into the counting chamber, and the pipette was held vertically to remove the first few drops. Two or three minutes were provided for it to calm down. The light was adjusted for the middle large square and the twenty-five small squares using a low power (10X) objective. The objective is now set to 40X high power. In each of the four corner squares are red blood cells.

2.8.2 Estimation of haemoglobin by Sahli's haemoglobinometer (Acid Haematin method)

N/10 HCl was added to the diluting tube until it reached the 20 mark. After that, blood was pipetted to the 20 cubic millimetre mark, bent into the tube, and washed for ten minutes. Drop by drop, distilled water was added until the tint perfectly matched the need. Next, the haemoglobin percentage reading is noted.

2.8.3 Packed cell volume (PVC) determination

To calculate the rats' PCV, blood from a tail that had been slightly chopped was utilized. After that, the blood was let to pass through a capillary tube until it was more than two thirds full. The bloodied end was then sealed off with plasticine. The capillary tubes were centrifuged for 15 minutes at 3,000 rpm after being placed inside. The capillary tubes were placed on top of the micro haematocrit to measure the packed cell volume.

2.8.4 Clotting time

A capillary tube had blood in it. The capillary glass tube is then sandwiched between the palms of both hands for 30 seconds in order to maintain body temperature. After 30 seconds of the tube being withdrawn and a small portion of the tube being broken at regular intervals of 30 seconds, a thread of clotted blood developed between the two pieces of capillary glass tube. The number of minutes that elapsed between the appearance of the blood drop and the blood clot thread was used to determine the rat clotting time.

2.8.5 Collection of serum

Using 1 mm glass capillaries, blood was drawn from the rat's retroorbital plexus while it was under a mild anaesthesia. Heparin-containing 2 ml Eppendorf tubes were used to collect blood. It was centrifuged at 5000 rpm for 20 minutes in order to separate the serum after being let to coagulate in the open for 15 minutes. The acquired serum was kept at -20°C until additional biochemical parameters, including AST, ALT, and ALP, were estimated.

2.9 Preparation for estimation of biomarkers

Using Erba diagnostic kits, the serum obtained from the animals at the conclusion of their therapy was used to estimate a number of liver and kidney enzymes, including Alkaline Phosphatase (ALP), Alanine Transaminase (ALT), and Aspartate Transaminase (AST).

2.10 Histopathology

After blood collection on last day, animals were sacrificed to isolate heart, liver, kidney, and lung[11,12]. The collected tissues were fixed immediately in 10% formalin and sent for histopathological study to Koushik laboratory and clinic, Bengaluru.

2.11 Statistical analysis

Statistical comparison was analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's test using Graph pad prism 10. Values were reported as mean ± SEM. The significance levels were expressed as $p<0.05$.

3 Results and Discussion

3.1 Preliminary phytochemical analysis

Phytochemical screening of *C. juncea* showed that alkaloids, carbohydrates, tannins, phenolic compounds, and flavonoids were present in the ethanolic extracts [Table 2].

Table 2. Phytochemical screening for *C. juncea* seed

Sr. No	Test	Interference	Results
		Alkaloids	
1.	Mayer's Test	Cream Precipitate	+
2.	Dragendroff's Test	Orange Precipitate	+
		Carbohydrates	
1.	Fehling's Test	Brick Red Colour	+

Continued on next page

Table 2 continued

2.	Molisch's Test	Violet Colour	+
3.	Barfoed's Test	Red Colour	+
	Steroids		
1.	Salkowski Test	Red Colour	-
2.	Keller Killani Test	Red Colour	-
	Tannins		
1.	Ferric Chloride Test	Blue Colour	+
	Saponins		
1.	Foam Test	1cm Foam	-
	Flavonoids		
1.	Alkaline Reagent Test	Yellow Colour	+
	Proteins and amino acids		
1.	Biuret Test	Violet Colour	-
2.	Millon's Test	Red Colour	-

+ indicates positive result for the test; - indicates negative result for the test

3.2 Acute toxicity study

The acute toxicity studies using guideline OECD 425 revealed that the herbal suspension of *C. juncea* was safe at a dose of 2000mg/kg. Hence, 2000mg/kg was LD50 cut-off value considered for the herbal suspension prepared. In order to screen for anti-anaemic properties, 1/10th (200 mg/kg) and 1/5th (400 mg/kg) of the cut-off dose were chosen for the investigation.

3.3 Body weights

Effect of powder suspension of *C. juncea* treatment at 200mg/kg (low dose) and 400mg/kg (high dose) and standard drug ferrous ascorbate (9mg/kg body weight) for a period of 21days demonstrated a substantially ($P<0.05$) increased body weight which is shown in Table 3. High dose (400mg/kg) of *Crotalaria juncea* had much significant effect than low dose (200mg/kg) of *Crotalaria juncea*) but not much significant when compared to standard drug ferrous ascorbate (9mg/kg). Following the induction of anaemia and the initiation of medication treatment, weight loss or gain was assessed for each group. The PHZ-injected anemia-induced group (control group) had a significantly lower body weight than the normal group ($P<0.05$). The groups treated with herbal suspension experienced a statistically significant increase in body weight as compared to the anaemic control group [Table 3].

Table 3. Effect of powder suspension of *C. juncea* seed on body weight

Group	No. of days				
	0	**3rd**	**7th**	**14th**	**21st**
Normal	183.23±1.02	184.38±1.15	182.16±0.96	183.21±1.13	183.23±0.87
Positive Control (PHZ) (40mg/kg)	183.23±1.02*	180.85±1.70***	178.31±2.12***	165.04±1.62****	163.14±1.03****
Low dose (*C. juncea*) (200mg/kg)	184.11±0.78*	182.42±1.29**	186.10±1.65***	189.24±1.64****	192.35±1.75****
High dose (*C. juncea*) (400mg/kg)	182.25±1.15**	181.11±1.59*	185.50±1.13***	191.26±1.35****	195.04±1.22****
Standard (Ferrous ascorbate) (9mg/kg)	183.13±0.87*	182.24±1.05***	185.51±1.02***	190.12±0.12****	193.23±1.25****

(Note: Values are expressed as mean ± SEM, n=6, analysed by one way ANOVA, followed by Dunnett's test, here ****p<0.0001 normal group vs. positive control group (PHZ=Phenyl hydrazine) and *p<0.05, **p<0.01, ***p<0.001 respectively were used to denote significance levels when compared all treated groups vs positive control group on corresponding days.)

3.4 Blood parameter

When comparing the PHZ control group to the normal group, there is a notable decline in the quantity of RBCs ($P<0.05$). Reduced haemoglobin levels are caused by a decrease in red blood cells. When compared to the anaemic control group, the groups treated with a herbal suspension of *C. juncea* for 21 days exhibited a statistically significant increase in the quantity of red blood cells [Table **??**]. Rats in the PHZ control group had a lower mean haemoglobin (Hb) content (g%) than those in the normal group ($P<0.05$). When compared to the anaemic control group, the groups treated with a herbal suspension of *C. juncea* for 21 days exhibited a significant increase in the number of haemoglobin levels similar to normal control groups [Table 4].

Table 4. Effect of powder suspension of *C. juncea* seed on RBC and Haemoglobin

Group	RBC content (106/μl)				
	No. of days				
	0	3rd	7th	14th	21st
Normal	8.19±0.26	8.34±0.49	7.28±0.30	7.28±0.30	7.63±0.37
Positive control (PHZ) (40mg/kg)	8.84±0.14*	3.78±0.15****	3.45±0.23****	5.15±0.34**	5.89±0.15**
Low dose (C. juncea) (200mg/kg)	8.48±0.27*	3.15±0.27*	5.16±0.18**	6.45±0.27**	7.02±0.17**
High dose (C. juncea) (400mg/kg)	8.19±0.34*	3.90±0.23*	5.25±0.13**	6.83±0.21**	7.04±0.24**
Standard (Ferrous ascorbate) (9mg/kg)	8.90±0.38*	3.17±0.23*	5.58±0.33**	6.92±0.32**	7.23±0.17***
	Haemoglobin content(g/dl)				
	0	3rd	7th	14th	21st
Normal	13.15±0.32	13.48±0.96	12.62±0.23	12.90±0.28	13.00±0.30
Positive control (PHZ) (40mg/kg)	14.52±0.25*	9.05±0.43****	9.23±0.31****	11.39±0.47**	12.90±0.34*
Low dose (C. juncea) (200mg/kg)	14.95±0.15*	9.13±0.34*	11.03±0.23***	12.38±0.20**	13.24±0.25**
High dose (C. juncea) (400mg/kg)	14.68±0.26*	10.33±0.43**	11.18±0.15***	12.64±0.22**	13.65±0.38**
Standard (Ferrous ascorbate) (9mg/kg)	15.01±0.30**	9.90±0.19*	11.62±0.37***	13.26±0.38***	14.27±0.21***

Rats in the PHZ control group have a lower packed cell volume (%) than those in the normal group (P<0.05). When compared to the anaemic control group, the groups treated with a herbal suspension of *C. juncea* for 21 days shown a significant rise in PCV comparable to normal control groups [Table 5].

Table 5. Effect of powder suspension of *C. juncea* seed on PCV, MCV and clotting time

Group	Packed cell volume (%)				
	No. of days				
	0	3rd	7th	14th	21st
Normal	42.25±2.40	42.30±2.37	41.18±1.10	39.44±2.25	40.95±1.14
Positive control (PHZ) (40mg/kg)	45.29±0.50*	25.13±1.10****	29.60±1.22****	30.70±1.78****	35.30±1.00****
Low dose (*C. juncea*) (200mg/kg)	45.95±1.17*	22.57±1.25***	33.52±1.23***	33.25±1.52***	36.92±1.02**
High dose (*C. juncea*) (400mg/kg)	47.94±1.13***	22.45±1.76***	33.19±1.50***	34.44±1.12***	38.25±1.14***
Standard (Ferrous ascorbate) (9mg/kg)	46.66±1.15**	21.20±0.98***	38.71±1.30****	39.72±0.18****	41.09±0.79****
	Mean corpuscular volume (fL)				
	0	3rd	7th	14th	21st
Normal	59.99±2.87	58.01±2.33	55.64±2.73	54.30±2.90	56.43±2.22
Positive control (PHZ) (40mg/kg)	57.30±2.65**	73.33±3.22****	72.74±3.23****	69.90±3.45****	69.57±2.41****
Low dose (*C. juncea*) (200mg/kg)	62.37±2.97***	64.34±5.61****	63.70±2.2***	63.54±2.89***	62.20±2.89***
High dose (*C. juncea*) (400mg/kg)	60.32±2.33***	65.08±5.76****	64.09±2.17****	64.45±3.38***	63.45±2.87***
Standard (Ferrous ascorbate) (9mg/kg)	62.00±3.19****	63.36±3.08****	62.07±2.10****	60.28±3.18****	60.15±2.78****
	Clotting time(secs)				
	0	3rd	7th	14th	21st
Normal	125.10±1.12	124.15±1.26	119.27±1.19	125.10±1.21	122.30±1.21
Positive control (PHZ) (40mg/kg)	124.18±0.9*	152.47±1.09****	146.18±1.22****	143.01±1.21****	148.67±1.14****
Low dose (*C. juncea*) (200mg/kg)	124.15±1.08*	154.27±1.09**	143.87±1.41***	132.82±1.32****	127.33±1.23****
High dose (*C. juncea*) (400mg/kg)	124.03±1.23*	156.15±1.32***	140.79±1.24***	132.92±1.41****	126.40±1.23****
Standard (Ferrous ascorbate) (9mg/kg)	123.18±1.16*	151.18±1.61*	131.76±1.31****	124.08±1.26****	123.18±1.47****

Rats in the PHZ control group have a higher mean corpuscular volume (fL) than those in the normal group (P<0.05). When compared to the anaemic control group, the groups treated with a herbal suspension of *C. juncea* for 21 days had a significant drop in MCV comparable to normal control groups [Table 5].

Treatment with *C. juncea* improved this drop in blood Hb. According to reports, PHZ causes anaemia by oxidatively denaturing Hb, which is started by free radicals[13–15]. The ability of blood to carry oxygen is reduced when haemoglobin levels are low, so haemoglobin levels are a key criterion for antianemia medication screening. Previous research has indicated that the experimental period of this examination saw a fall in the blood's Hb level following the injection of PHZ[15]. Consequently, the observed impact of *C. juncea* to improve blood Hb content may be due to its antioxidant capacity. In the present study, phenylhydrazine administration caused decline in RBCs count of blood during experimental period which is consistent with earlier report[4]. This decrease in RBCs count was improved by treatment with *C. juncea*. Phenylhydrazine causes selective destruction of matured RBCs through oxidative stress. Therefore, the beneficial effect of *C. juncea* to improve RBCs count may be due to its ability to prevent phenylhydrazine-induced hemolysis. Hb is a major parameter for screening of antianemic drugs as low level of Hb causes decline in oxygen-carrying capacity of blood. In the present study, phenylhydrazine administration caused decrease in Hb content of blood during experimental period which is consistent with earlier report[4]. This decline in blood Hb content was improved by treatment with *C. juncea*. Phenylhydrazine is reported to induce anemia by oxidative denaturation of Hb initiated by free radicals. Therefore, antioxidant potential of *C. juncea* may be responsible for the observed effect of *C. juncea* to improve blood Hb content. This is consistent with the study of Sheth et al. the beneficial effect of herbal formulations improved RBCs count[4].

HCT is the ratio of the packed RBC volume to the total blood volume. An anaemic state is indicated by a low HCT[10–12]. Because of phenyl hydrazine-induced haemolysis, there was a considerable drop in HCT in the current study's anaemic control rats, which is consistent with other results. Treatment with *C. juncea* corrected this decrease in HCT. This result could be the result of *C. juncea*'s ability to defend against haemolysis brought on by PHZ. HCT, also known as the packed cell volume, is the ratio of volume of packed RBCs to the total blood volume. A low HCT is an indicator of anemic condition. In the present study, there was significant decrease of HCT in anemic control rats due to phenylhydrazine-induced hemolysis which is consistent with earlier report[4]. This decrease in HCT was reversed by treatment with *C. juncea* which is consistent with the study of Sheth et al. The mean HCT significantly increased in females treated with 200 and 400 mg/kg of *A. vera* whole leaf extract[16]. The recovery of the hematocrit level is favored by the administration of the plant extracts tested, the highest recovery rate being that induced by *J. secunda* (97.87%) followed by nearly *S. bicolor* (93.95%) then *G. barbadense* (87.41%) and *H. sabdariffa* (80.35%), on the 18th day of treatment[17]. This, effect may be due to protective effect of *C. juncea* against phenylhydrazine-induced hemolysis.

It is well-known that PHZ raises MCV (Mean Corpuscular Volume), MCH (Mean Corpuscular Haemoglobin) and MCHC (Mean Corpuscular Haemoglobin Concentration) levels while lowering Hb and RBC levels[4,10–12]. *C. juncea* significantly lowered this rise in MCV. When comparing the PHZ control group to the normal group, the clotting time is longer ($P<0.05$). When compared to the anaemic control group, the groups treated with a herbal suspension of *C. juncea* for 21 days had a significant normalized clotting time equivalent to normal control groups [Table 5].

When comparing the PHZ control group to the normal group, the PHZ control group's increases ($P<0.05$). When compared to the anaemic control group, the groups treated with herbal suspension of *C. juncea* for 21 days displayed a substantial decreased in serum ALT and AST while ALP levels increase equivalent to normal groups [Table 6]. Anaemic control animals' serum had significantly higher levels of AST, ALT, and ALP ($P<0.05$) than normal control animals. This could be because hepatocytes leaking occurs when their membranes are damaged by peroxidation, increasing the permeability of the membrane. An increase in AST levels, which are involved in the conversion of amino acids to keto acids, always coincides with an increase in ALT levels. The biochemical proof of substantial liver damage produced by PHZ is provided by the high increase in ALT[18,19]. Treatment with *C. juncea* improved the tissue damage to some extent.

Table 6. Effect of *C. juncea* herbal suspension on various liver parameters in phenylhydrazine induced anaemia rats

Group	Liver Parameters		
	AST(IU/L)	ALT (IU/L)	ALP (IU/L)
Normal	137.28±2.19	36.29±0.92	52.05±1.47
Positive control (PHZ) (40mg/kg)	170.23±1.43****	51.30±0.58****	52.37±1.20*
Low dose (*C. juncea*) (200mg/kg)	150.56±1.25****	24.15±1.27****	59.45±1.02***
High dose (*C. juncea*) (400mg/kg)	144.65±0.87***	29.38±0.68****	53.65±1.78**
Standard (Ferrous ascorbate) (9mg/kg)	145.63±1.72***	24.30±0.60****	53.65±1.72**

According to reports, PHZ causes reactive oxygen species to develop more frequently, which in turn damages red blood cells through oxidative stress. Flavonoids, on the other hand, have strong antioxidant properties and the ability to stop or reverse this red cell damage[14,15]. Tannins, phenolic compounds, flavonoids, and alkaloids were detected by phytochemical study of

C. juncea. Therefore, it seems that the anti-anaemic action of *C. juncea* that has been reported may be due to the presence of flavonoids or other active components.

After treating with herbal suspension of *C. juncea* seed for 21 days there was increase in body weight, red blood cell and packed cell volume. This also indicate that *C. juncea* seed may contain some bioactive agents that may have brought about erythrocytes formation by stimulating the kidney to release renal erythropoietin factor, thereby causing the conversion of blood protein to erythropoietin which stimulates the red bone to produce more red blood cell. After treating with herbal suspension of *C. juncea* seed there is a decrease in clotting time when compared with the control group. This decrease maybe due to anti-thrombocytopenic activity.

3.5 Histopathology

In this present study, histopathological evaluation was carried out in the Liver, Heart, Kidneys & Spleen to evaluate the treated standard drug – PHZ, test drug – *C. juncea* seed and normal group response [Figures 1, 2, 3 and 4]. According to a histopathological analysis, the liver, heart, and kidneys experienced moderately graded pathological alterations as a result of the PHZ-induced anaemia. Treatment with *C. juncea* corrected these pathological alterations and returned the histomorphology features of these tissues almost to normal. The strong anti-anaemic properties of *C. juncea* may be the cause of the observed effect. This is similar to the study of Sheth et al. Histopathological evaluation indicated that anemia-induction by phenylhydrazine administration caused pathological changes of moderate grade in kidney, liver, heart, and spleen tissues. These pathological changes were reversed by *C. juncea* treatment and restored micromorphological features of these tissues near to normal. This maybe due to the presence of flavonoids, alkaloids and other essential components which fixed the morphological features. The observed effect may be due to potent antianaemic potential of *C. juncea* countered[4].

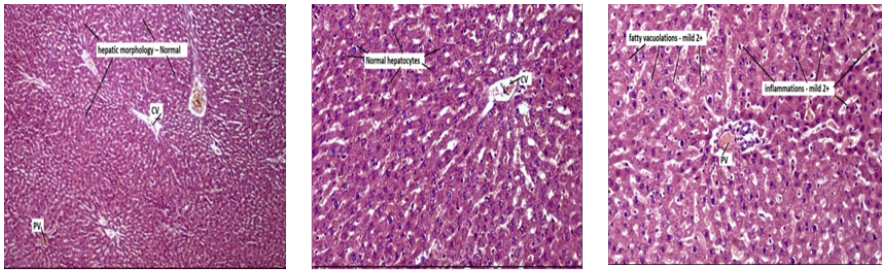


Fig 1. Representative image of liver section from rats a) Normal group showing hepatic morphology-Normal Central vein (CV) &portal vein (PV)-NAD+ (X50) b) Test drug (Crotalaria juncea): showing normal hepatocytes, central vein (CV)-NAD+ (X100) c) Treated drug Phenylhydrazine: showing inflammations with fatty vacuolations-portal vein (PV) (X100)

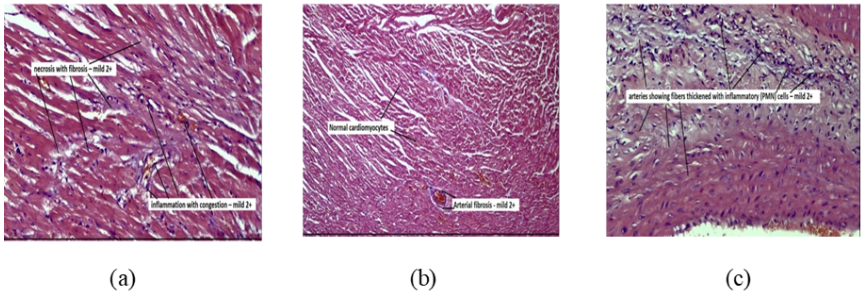
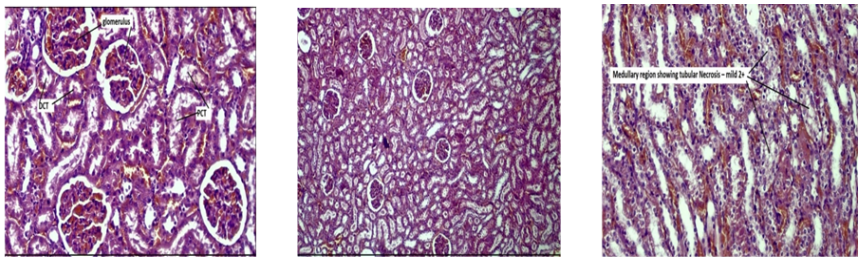
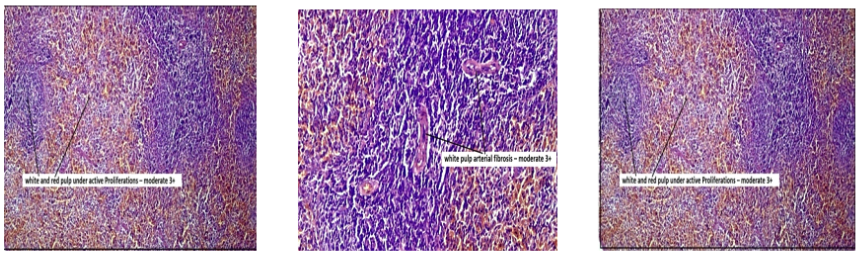


Fig 2. Representative image of heart section from rats a) Treated standard drug Phenylhydrazine: Necrosis with fibrous and inflammation with congestion-mild 2+ (X100) b) Test drug: Showing Normal cardiomyocytes-NAD+ with Arterial fibrosis-mild 2+ observed (X50) c) Treated drug: arteries showing inflammatory (PMN) cells with fibers thickened-mild 2+ (x100)



(a) (b) (c)

Fig 3. Representative image of kidney section from rats. a) Normal: showing normal glomeruli, tubules were normal- NAD+ (X100) DCT-Distal Convoluted Tubule PCT: Proximal Convoluted Tubule b) Test drug: showing normal glomeruli, tubules and vessels were evident-NAD+ (X50) c) Treated drug Phenylhydrazine: Medullary region showing tubular Necrosis-mild 2+ (X100)



(a) (b) (c)

Fig 4. Representative image of kidney section from rats a) Test drug showing white and red pulp under active proliferations-moderate 3+ (X50) b) Treated drug Phenylhydrazine: showing white pulp arterial fibrosis-moderate 3+ (X100) c) Normal: showing white and red pulp under active proliferations-moderate 3+ (X50)

4 Conclusion

C. juncea improved phenylhydrazine-induced decrease in RBCs, Hb and PCV levels. Furthermore, it also reversed pathological changes in tissues of kidney, liver, heart, and spleen. Hence, *C. juncea* has significant antianemic activity against Phenylhydrazine induced anemia in rats. Herbal formulations can replace chemical drugs with cost-effective, availability and fewer side effects. Further studies on quantitative estimation of phytoconstituents along with *in vivo* evaluations are necessary for elucidating the exact mechanism of action of the observed antianemic effect.

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