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Therapeutic efficacy of *Punarnavadi mandura* against phenylhydrazine-induced hemolytic anemia in rats

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ABSTRACT

Background & aim: Hemolytic anemia is a blood disorder whose incidence is increasing in the world in recent years especially after the pandemic. Conventional treatments include use of steroids and immunosuppresants that are accompanied by numerous adverse effects. With growing interest in using complex multi-component formulations for multi-targeted therapy, the present study aims to investigate the therapeutic efficacy of a traditional *Ayurvedic* herbomineral preparation, *Punarnavadi Mandura*, which has been traditionally used as a supplement in iron-deficiency anemia, against phenylhydrazine-induced hemolytic anemia in rodent models.
Experimental approaches: We employ a combination of *in vivo* and *in silico* methods in this work to study the therapeutic potential and to understand the possible molecular targets of this traditional formulation. Conventional drugs prednisolone and ferrous sulphate were used for comparison.
Results and conclusion: The *in vivo* studies confirm the ability of *Punarnavadi Mandura* to reverse pathological changes associated with hemolytic anemia at 100 mg/kg and 200 mg/kg concentration. It restored hemoglobin, bilirubin and white blood cell levels to normal and reduced reticulocytes, hemosiderin and Gamna Gandy bodies in the liver, spleen and kidney. *In silico* studies suggested that the key constituents in *Punarnavadi Mandura* interact with high affinity to erythropoietic receptor which could contribute to erythropoiesis. The *in silico* study also predicted that the phytoconstituents of *Punarnavadi Mandura* could inhibit TNF-α activity which was validated using gene expression studies.

1. Introduction

Punarnavadi Mandura (PM) is an *Ayurvedic* formulation that is prepared using the different parts of the plants namely, *Boerhavia diffusa, Operculina turpethum, Zingiber officinale, Phyllanthus emblica, Piper longum, Piper nigrum, Embilia ribes, Cedrus deodara, Plumbago zeylanica, Curcuma longa, Saussurea laupa, Berberis aristate, Terminalia chebula, Terminalia bellerica, Baliospermum montanum, Piper chaba, Holarrhena antidysenterica, Picorrhiza kurroa, Cyperus rotundus*, and iron containing *Mandura Bhasma*. Cow's urine is employed to bind these organic and inorganic constituents in to a paste that is later pelletized to form tablets. According to classical texts, PM is used for treatment of anemia, splenomegaly, and hemorrhoids due to its iron content. It is also used to treat chronic fever, inflammatory conditions, skin infections, dermatitis, malabsorption and intestinal worms (helminthiasis).[1] A clinical study had shown the effectiveness of PM on geriatric patients of anemia after a 90-day treatment period.[2] Most common use of PM is as a therapeutic agent for iron-deficiency anemia.[3] However, the therapeutic potential of this herbomineral formulation remains underexplored for other forms of anemia.

Anemia, a prevalent condition that affects nearly one third of the world's population, is caused due to insufficient red blood cells (erythrocytes) in the blood that adversely impacts the oxygen transport process.[4] Children, elderly and women are more prone to anemia arising due to nutritional deficits, genetic impairment, infections or

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List of abbreviations

EPO receptor	Erythropoeitin receptor
TNF-alpha	Tumour necrosis factor alpha
IMPPAT	Indian Medicinal Plants, Phytochemistry And Therapeutics
MCHC	mean corpuscular haemoglobin concentration
MCV	mean corpuscular volume
RDW	red cell distribution width
PDW	Platelet distribution width
MPV	mean platelet volume
GGB	Gamna – Gandy bodies
JAK	Janus kinase
STAT	Signal transducer and activators of transcription
TNFR1	Tumour necrosis factor receptor 1
GATA2	GATA Binding Protein 2
HIF1α	Hypoxia-inducible factor 1-alpha
Glu	Glutamic acid
Asp	Aspartic acid
Thr	Threonine
Ala	Alanine
Ser	Serine
Phe	Phenylalanine
His	Histamine
Pro	Proline

autoimmune disorders. As a result, the affected individual suffers from breathlessness, fatigue, dizziness, cardiac arrhythmia, etc.[5] There are four main classes of anemia namely, iron-deficiency anemia, vitamin B12-deficiency anemia, aplastic anemia and hemolytic anemia. The most prevalent form of anemia is the iron-deficiency form that predominantly arises due to blood loss and an iron-deficient diet. It is treated by administering iron supplements. Vitamin B12 or cobalamin is essential for erythropoiesis (erythrocyte production) by participating in the purine and thymidylate metabolic pathways. A diet deficient in vitamin B12 results in anemia. A special case of anemia known as pernicious anemia arises due to body's inability to absorb vitamin B12. Both conditions are usually addressed by administering vitamin supplements.[6] Similarly, vitamin B9 or folic acid deficient diet also results in anemia and together with vitamin B12 deficiency anemia, this condition is classified as megaloblastic anemia where nuclear division is inhibited. Aplastic anemia is a relatively less common autoimmune condition that impairs production of red blood cells (RBCs) in the bone marrow. This condition can be treated by bone marrow transplants or blood transfusions.[7] Another type of anemia is hemolytic anemia, which occurs when there is systemic inflammation and a cytokine storm triggered by the immune cells resulting in an imbalance in the rates of RBC destruction and production. Among these four major types, hemolytic anemia has a high mortality risk owing to organ failure as a result of hypoxia. Further, treatment of hemolytic anemia with immunosuppressants makes the individual prone to other infections. Another new facet that has emerged in the post-pandemic era is that the infection by SARS-COV-2 resulted in development of hemolytic anemia through induction of auto-antibodies.[8] This further exacerbates the mortality risk of the affected individual. Similarly, infections by human immunodeficiency virus (HIV) and mycoplasma also have been associated with the onset of hemolytic anemia.[9,10] Treatment with immunosuppressants in these conditions is not practical. Hence, alternate and more effective therapeutic options are the need of the hour. In the present study, we have investigated the potential of PM to treat hemolytic anemia using phenylhydrazine-induced anemia rodent model. Phenylhydrazine (PHZ) caused anemia by inducing membrane peroxidation and hemolysis. This leads to lowered hemoglobin, hematocrit and packed cell volume due to hydrogen peroxide and ferrihemochrome-mediated oxidative stress. It also results in Heinz body formation and oxidative damage of spectrin which inhibits the erythrocyte deformability due to continuous exposure of the blood cells to phenyl radicals and protoporphyrin. Furthermore, methemoglobin is converted to hemichrome as a result of oxidation of oxyhemoglobin during RBC disintegration. Additionally, PHZ plays a part in the deregulation of the erythropoietin receptor, which impacts specific intracellular signalling mechanism such as JAK/STAT and Ras/MAPK, and inhibits the transcription of gene and the production of erythrocytes.[11] The schematic representation of the mechanism involved in PHZ-induced hemolytic anemia is shown in Supplementary Fig. S1. This study uses the PHZ-induced rodent model of hemolytic anemia to compare the therapeutic efficacy of PM with standard anti-anemic drugs.

2. Materials & methods

2.1. Materials

Punarnavadi Mandura (PM) was procured from IMPCOPS, India. Phenylhydrazine hydrochloride was purchased from Merck, India. Male Wistar rats aged 5 months and weighing about 250 g were obtained from the Central Animal Facility, SASTRA Deemed University and used in the study. The animals were acclimatized for seven days before commencing the study. Standard rodent diet was procured from ATNT Laboratories, Thane. All animals were provided water and food *ad libidum*. All animal experiments were performed after obtaining approval from the Institutional Animal Ethics Committee (IAEC Approval Number: 688/SASTRA/IAEC/RPP).

2.2. Dose selection for PM

The therapeutic dose of PM in humans is 1000–1500 mg for an adult according to the [13]Ayurvedic Pharmacopeia of India. Assuming the average adult weight as 60 kg, the dose of PM in humans is calculated to be 16.66 mg/kg. The rat equivalent dose conversion factor was calculated using the following equation:

$$Rat\ dose\ (mg/kg) = Human\ equivalent\ dose\ (mg/kg) \times \frac{Human\ K_m}{Rat\ K_m}$$

where rat K_m and human K_m are 6 and 37 respectively.

Using the above equation, the rat dose for PM was calculated to be 100 mg/kg. This was considered as the therapeutic dose for rats. Half this dose (50 mg/kg) and twice the therapeutic dose (200 mg/kg) were chosen as the low dose and high dose respectively for the therapeutic study. The solvent used for dissolving the *Punarnavadi mandura* was a 2:3 ratio of water:honey.[12]

2.3. Creation of the hemolytic anemia model

Hemolytic anemia was induced through intraperitoneal administration of 40 mg/kg PHZ. The PHZ dose was calculated based on previous reports. The PHZ injections were administered to the animals for the first two days followed by a booster dose on day 7[13,14] (Supplementary Fig. S1). The animals were euthanized on day 16 following approved protocols and the hematological parameters were measured to confirm the onset of hemolytic anemia. Supplementary Table S1 summarizes the values obtained for animals in the normal and PHZ-administered groups. It is observed from Supplementary Table S1 that when compared to the normal group, the RBC and hemoglobin content were significantly reduced in the diseased animals with a considerable increase in reticulocytes. This is caused by continuous RBC disintegration due to membrane peroxidation, resulting in an increase in reticulocytes in the

peripheral circulation to compensate for the RBC loss. The granulocytes also show an increase in the diseased animals suggesting inflammation. Similar observations have also been reported in literature.[15] These changes suggest the onset of hemolytic anemia in the animals.

Following the confirmation of the animal model, the experimental animals were randomly divided in to seven groups comprising eight animals each as described in Table 1.

All the anemic rats were treated with test substance/standard drug according to the treatment schedule for 15 days. After 15 days of test substance treatment, blood was collected and all the rats were euthanized following approved protocol. Whole blood collected by retro-orbital plexus under mild anesthesia was subjected to hematological analysis. The liver, spleen and kidneys were collected and weighed for histopathological studies.

2.4. Prussian blue staining

The spleen and kidney fixed in 10% buffered formalin solution were processed using an automatic tissue processor (Leica TP1020, Germany). The processed samples were embedded in paraffin wax using a tissue embedding station (Leica EG1150C, Germany). Tissue sections of 3 μm thickness were obtained using a microtome (Leica RM2125RTS, Germany) and fixed to a gelatin-coated slide. The sections were incubated with Prussian blue stain (1:1 potassium ferricyanide: HCl) for 20 min to visualize the hemosiderin deposits in the tissues.[16] Similarly sections were stained by hematoxylin and eosin.

2.5. Hemoglobin assay

Hemoglobin concentration was determined by adding 250 μL of Drabkin's reagent to 20 μL of whole blood and standard hemoglobin. The mixture was incubated for 10 min at room temperature. Drabkin's reagent converts hemoglobin to cyanomethemoglobin which was measured at 540 nm (Epoch 2, Biotek, USA).[17]

Table 1
Animal grouping for the therapeutic study using *Punarnavadi Mandura*.

Group	Description	Number of Animals	Treatment
I	Normal	7	Only Water
II	Diseased control	7	Phenyl hydrazine hydrochloride (PHZ, 40 mg/kg, i.p.) for day 1,2 and 7 + distilled water on day 3 up to day 16
III	Standard drug I (Std-Fe)	7	Phenyl hydrazine hydrochloride (PHZ, 40 mg/kg, i.p.) for day 1, 2 and 7 + ferrous sulphate (0.0214 mg/kg b. wt.) on day 3 up to day 16 by oral gavage
IV	*PunarnavadI Mandura* (Low dose)	7	Phenyl hydrazine hydrochloride (PHZ, 40 mg/kg, i.p.), for day 1,2 and 7 + PM (50 mg/kg b. wt.) on day 3 up to day 16 by oral gavage
V	*PunarnavadIi Mandura* (Intermediate dose)	7	Phenyl hydrazine hydrochloride (PHZ, 40 mg/kg, i.p.), for day 1, 2 and 7 + PM (100 mg/kg b. wt.) on day 3 up to day 16 by oral gavage
VI	*PunarnavadIi Mandura* (High dose)	7	Phenyl hydrazine hydrochloride (PHZ, 40 mg/kg, i.p.), for day 1,2 and 7 + PM (200 mg/kg b. wt.) on day 3 up to day 16 by oral gavage
VII	Standard drug – II (Std-PD)	7	Phenyl hydrazine hydrochloride (PHZ, 40 mg/kg, i.p.) for day 1, 2 and 7 + prednisolone (10 mg/kg b. wt.) on day 3 up to day 16 by oral gavage

2.6. Reticulocyte count

150 μL of reticulocyte stain was added to 150 μL of whole blood and incubated at 37 °C for 30 min. The cells were then gently re-suspended, and blood smears were formed on glass slides, air-dried, and studied under a microscope at 100× magnification in various fields of view. Reticulocytes were counted using NIS Elements imaging software (Nikon Eclipse, Japan).[18]

2.7. Lactate dehydrogenase assay

Serum levels of lactate dehydrogenase (LDH) was measured by adding the serum from different groups to a substrate mixture containing imidazole buffer and pyruvate solution and the co-enzyme reduced nicotinamide adenine dinucleotide (NADH) followed by incubation for 5 min at 37 °C. Then, the absorbance was read at 340 nm (Epoch 2, Biotek, USA). The assay was performed using LDH Kit -DGKC method (Coral Clinical Systems, Tulip Diagnostics, India).

2.8. Gene expression studies

RNA was extracted from spleen tissues from the animals in different groups using the standard procedure.[12] Then, cDNA was extracted from RNA using a kit and labelled with fluorescent probe (SyBR Green, Biorad, USA) and the forward and reverse primers for TNF-α were added and the samples were subjected to the steps in the RT-PCR[19] (Qiagen, USA). The TNF-α primers used in the study were procured from Genurem Bioscience LLP, Trichy, and have the following sequence (Forward primer: GAG TAA GGG GAT GCA GCT AAG A; Reverse primer CAG TTT CAG GGC AAG AAG TAC C). GAPDH was used as the housekeeping gene and the primer sequences used were: Forward primer GGAGTCCACTGGCGTCTT; Reverse primer AGGCTGTTGTCATACTTCTCAT. $2^{(-\Delta\Delta CT)}$ method was used to calculate the fold change in the expression of the gene of interest.

2.9. Statistical analysis

All values were expressed as mean ± SD. The changes in body weight, feed consumption, hematology, biochemistry and organ weights of experimental animals belonging to the treatment groups were compared with the control group using one way ANOVA followed by Tukey Test using Graph pad Prism 6 software.[20]

2.10. Molecular docking analysis

Molecular docking analysis reveals molecular interactions between proteins and ligands and helps in identifying ligands that may bind to the proteins.

2.10.1. Protein preparation

The three-dimensional coordinates of the protein targets identified in this study were retrieved from the PDB database (PDB id of EPO Receptor: 1EBP, PDB id of TNF-α: 2AZ5). The protein preparation wizard in Maestro of the Schrödinger suite was used to add hydrogen atoms, ensure proper protonation state for histidine residues and optimize hydrogen bonds. The protein was then subject to energy minimization using the OPLSe forcefield. Water molecules beyond 5 Å of the protein surface were removed.

2.10.2. Ligand preparation

The chemical structure of the phytoconstituents (about 300 molecules) were retrieved from the PubChem or IMPPAT databases. The list of the phytoconstituents used in the study are given in the Supplementary Table SXY. The three-dimensional coordinates and conformers for all the compounds were generated using the LigPrep module of Schrödinger.

2.10.3. Docking

Receptor grid was generated using the Schrödinger suite based on the ligand present in the crystal structure of the receptor. The prepared ligands were then docked to the generated grid protein using the Glide module in Extra Precision (XP) mode. The resulting docked poses were viewed and the interacting residues were identified from the docked poses in the ligand interaction diagrams.

3. Results

3.1. Organ weight

The weights of the liver, spleen and kidney isolated from animals of different groups are shown in Fig. 1A. No significant changes were observed in the weights of liver and kidney from the different groups. However, the spleen weight in the disease control group (0.974 ± 0.183 g) exhibited a significant increase by nearly two-fold when compared to normal animals ($p < 0.05$) While all treatment groups exhibited a significant reduction in the spleen weight that is comparable to the normal animals (0.499 ± 0.115 g).

3.2. Hematological parameters

Fig. 1B depicts the changes in the various hematological parameters in animals from different treatment groups In hematological assays, increased white blood cells (WBC) count have been implicated in inflammation, a typical feature of PHZ-induced anemia. WBC count in disease control group (12,867 ± 2230 cells/mm^3) was increased significantly compared to normal control (8240 ± 2690 cells/mm^3). Among the treatment groups, PM200 had an average WBC count of 6350

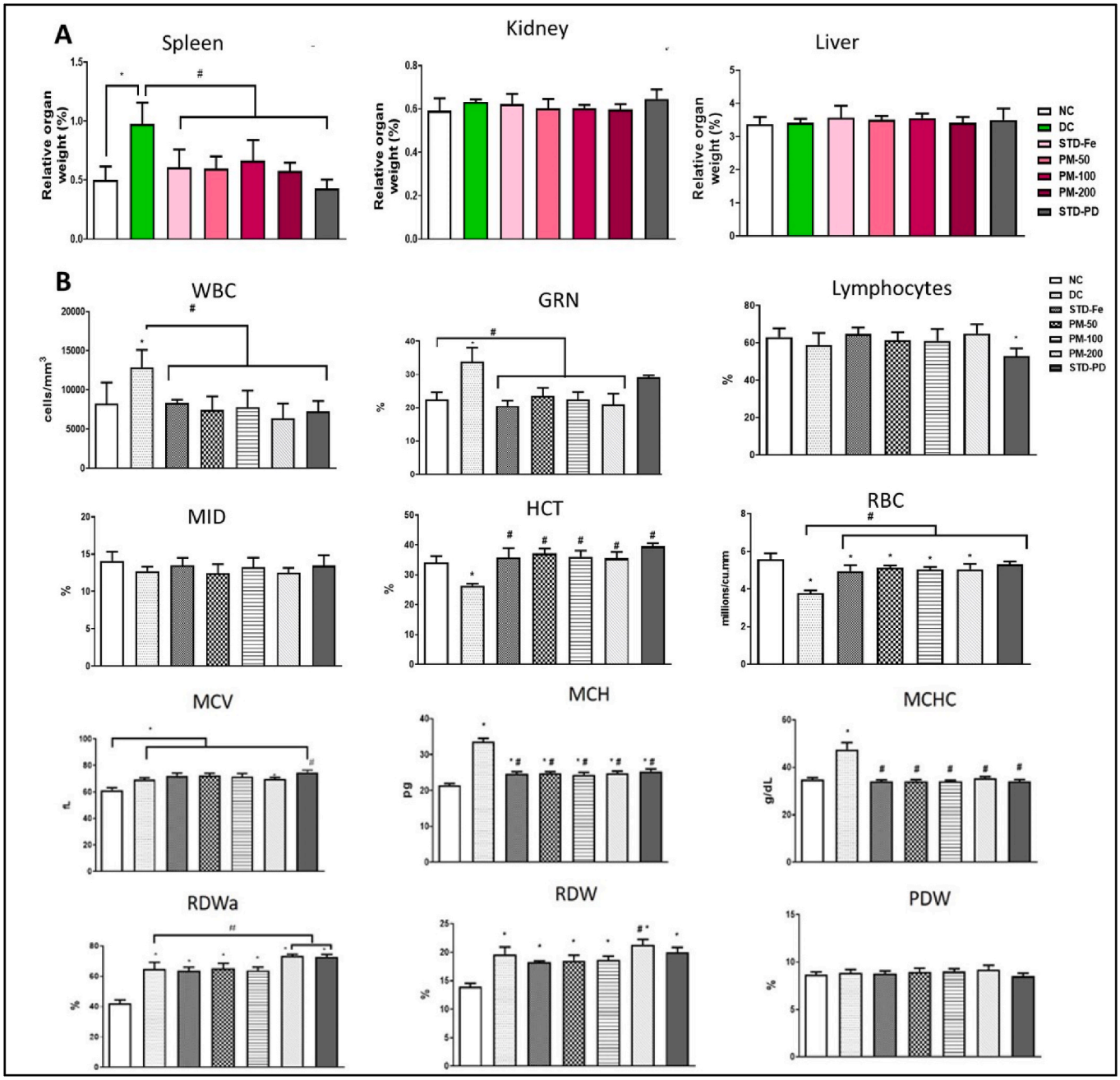

Fig. 1. Relative weights of spleen, liver and kidney isolated from animals of different treatment groups. N = 7, *p < 0.05. B. Effect of various treatments on hematological parameters in animals induced with hemolytic anemia; n = 6; # denotes p < 0.05.

± 1890 cells/mm^3 while PM50 and PM100 groups showed average WBC counts of 7410 ± 1740 cells/mm^3 and 7800 ± 2090 cells/mm^3 respectively that were comparable to the normal animals. The two standard groups also exhibited an average WBC count of 8300 ± 400 mm^3 and 7240 ± 1324 cells/mm^3 respectively that were comparable to the normal group.

Because PHZ has the ability to cause RBC lysis, the RBC count in blood was determined. The PHZ administered group had 3.79 ± 0.14 million RBC/mm^3, which was much lower than the normal group (5.57 ± 0.33 million cells/mm^3). The treatment groups PM50 mg/kg, PM100, and PM200 restored the RBC counts to normal values (Fig. 1B). A similar trend was also observed in the hematocrit values. Interestingly, the MCHC (mean corpuscular hemoglobin concentration) showed a significant elevation in the disease control group (47.54 ± 2.85 g/dL) in our study when compared to normal animals (34.9 ± 0.723 g/dL). All the treatment groups exhibited MCHC values that were comparable to the normal animals. The MCH value in the diseased group (33.64 ± 0.912 pg) showed a significant increase when compared to the normal group (21.4 ± 0.52 pg). Treatment with different doses of PM and standard drugs resulted in restoration of the MCH to normal values. Taken together, the likely cause for increase in MCH and MCHC values in diseased animals could be due to increased release of reticulocytes and chronic hemolysis which are successfully reversed by the treatment with PM (Fig. 1B).

Granular cells in WBC were quantified in the different groups to determine the effect of hemolytic anemia. The disease control group exhibited a higher percentage of total granulocytes (33.82 ± 4.2%) than the normal group (22.48 ± 2.1%). When compared to the diseased group, the standard drug-treated groups and the PM-treated groups exhibited normal values (Fig. 1B).

3.3. Total bilirubin and glucose levels

Table 2 shows the values of serum glucose, direct and total bilirubin in different groups. Our results show that the diseased animals exhibit increased levels of direct bilirubin (0.36 ± 0.12 mg/dL) with a concomitant increase in the indirect bilirubin levels that is nearly twice the values obtained for the normal animals. The group treated with prednisolone also exhibited elevated levels of total bilirubin comparable to the disease control. This is in agreement with earlier reports that have demonstrated that corticosteroids tend to increase bilirubin levels.[21] Similarly, the ferrous sulphate group also exhibited increased total bilirubin levels which correlates with previous findings in literature that reported increased risk of jaundice in neonates due to high bilirubin levels in ferrous sulphate-supplemented pregnant women.[22] The PM treated groups exhibited lower total bilirubin levels when compared to the diseased animals. The direct and total bilirubin values of PM100 group were comparable to the normal animals.

The changes in serum glucose in animals from various treatment groups reveal that the diseased group exhibited an elevated glucose level of 112.38 ± 17.18 mg/dL that is significantly higher than their normal counterparts which showed a glucose level of 95.58 ± 3.50 mg/dL. PM 50 group that is half the therapeutic dose did not reduce the glucose levels but both PM100 (therapeutic dose, 90.91 ± 8.22 mg/dL) and PM 200 (twice the therapeutic dose, 105.81 ± 13.547 mg/dL) were found to restore the glucose levels back to normal. Both standard groups also exhibited glucose levels comparable to the normal animals.

Table 2
Effect of PM treatment on the total, direct bilirubin levels, and serum glucose in animals with hemolytic anemia. N = 4.

Groups	Serum Glucose (mg/dL)	Direct bilirubin (mg/dL)	Total bilirubin (mg/dL)
Normal	95.58 ± 3.502	0.29 ± 0.11	0.46 ± 0.18
Disease control	112.38 ± 17.185	0.36 ± 0.12	0.60 ± 0.18
STD PD	114.37 ± 4.0415	0.24 ± 0.11	0.75 ± 0.26
PM50	130.88 ± 19.425	0.18 ± 0.05	0.13 ± 0.05
PM 100	90.914 ± 8.2267	0.17 ± 0.02	0.33 ± 0.10
PM 200	105.81 ± 13.547	0.25 ± 0.02	0.53 ± 0.14
STD FE	88.05 ± 8.753	0.32 ± 0.16	0.58 ± 0.15

3.4. Histopathological studies

3.4.1. Haematoxylin and eosin stain

Tissue sections from different groups were stained with hematoxylin and eosin to assess the safety profile of PM and disease indices in the spleen, which is the principal organ injured in hemolytic anemia (Fig. 2A). The high magnification image of the spleen section from the normal animal clearly showed the dense follicular zone in the white pulp with a distinct artery and marginal zone. The spleen section from the diseased animal showed marked reduction in the area of the white pulp. However, there was significant congestion in the red pulp and decreased cellularity in the white pulp in the disease control group. PM200 showed considerable decrease in the inflammatory infiltrates when compared to other groups. In addition, distinct yellow granular deposits were visible in the red and white pulp in the diseased group (Fig. 2B).These yellow deposits are the Gamna-Gandy bodies (GGB) that are a typical feature of hemolytic anemia.[23] The density, distribution foci and intensity of GGBs were found to increase in the prednisolone-treated group. A marked dose-dependent reduction in GGBs was observed in the sections obtained from the animals treated with PM100 and PM200, and a comparable reduction was also observed in the ferrous sulphate treated group.

The histopathological studies on the liver tissue sections obtained from animals of different treatment groups are shown in (Fig. 3A). The liver section from the normal animals exhibited well defined polygonal hepatocytes with prominent nucleus and no discernible abnormal lesions. The liver sinusoids were distinct and the high magnification image reveals the presence of normal biconcave RBCs. In contrast, the liver section of the diseased animal showed marked infiltration of inflammatory cells and the liver sinusoids exhibited lesser number of RBCs that possess abnormal morphology like echinocytes, acanthocytes, stomatocytes, etc. (Fig. 3B). The PM treated groups display a restoration of the normal morphology of the RBCs confirming its ability to overcome the pro-inflammatory and inhibitory environment for erythropoiesis.

3.4.2. Prussian blue staining for hemosiderin deposits

Prussian blue staining was used to confirm the iron deposits in the spleen and kidney caused by hemolysis (Fig. 4). The spleen and kidney tissue sections from the diseased animal showed a marked deposition of hemosiderin as evident from the blue deposits distributed throughout the tissues. Prednisolone-treatment resulted in numerous iron deposits distributed throughout the spleen but with lesser load in the kidney. The ferrous sulphate treated group also showed reduced iron deposits in the spleen when compared to the diseased and prednisolone groups. The kidney deposits in the ferrous sulphate treated group however, were denser than the prednisolone group but lower than the disease control. Iron compounds are not the primary preference for treatment of hemolytic anemia because of their propensity to exacerbate renal damage. Our results corroborate this fact. The low dose administration of PM (PM50) showed only a slight reduction in the iron deposits in both spleen and kidney when compared to the diseased group. Both therapeutic (PM100) and high dose (PM200) administration resulted in a significant reduction in the iron deposits in both spleen as well as kidney clearly indicating the ability of PM to suppress RBC breakdown unlike corticosteroids or ferrous sulphate.

3.5. Hemoglobin, reticulocytes and lactate dehydrogenase

Since, our experiments showed best results with PM200, its effect on reticulocytes, hemoglobin, and lactate dehydrogenase in rats with

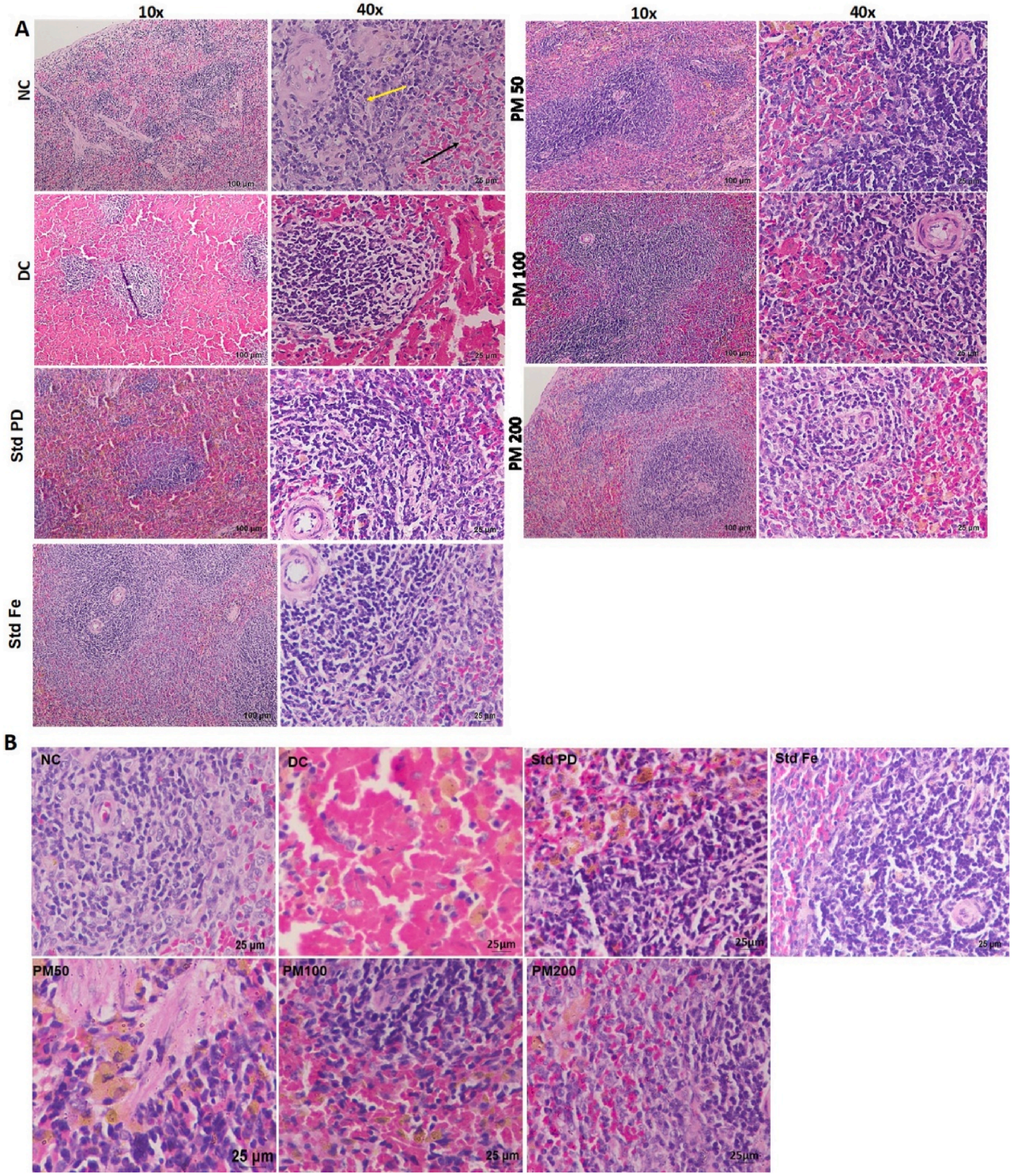

Fig. 2. A. Low and high magnification images of representative spleen sections of animals from various groups. NC: Normal animals with yellow arrow indicating white pulp and black arrow pointing to red pulp; DC: Animals with hemolytic anemia; Std PD: Anemic animals treated with Prednisolone; Std Fe: Anemic animals treated with ferrous sulphate; PM50: Anemic animals treated with 50 mg/kg b.wt. Of PM; PM100: Anemic animals treated with 100 mg/kg b.wt. Of PM; PM 200: Anemic animals treated with 200 mg/kg b.wt. Of PM. B. H&E stained sections of spleen showing the Gamna-Gandy bodies in the diseased and treatment groups.

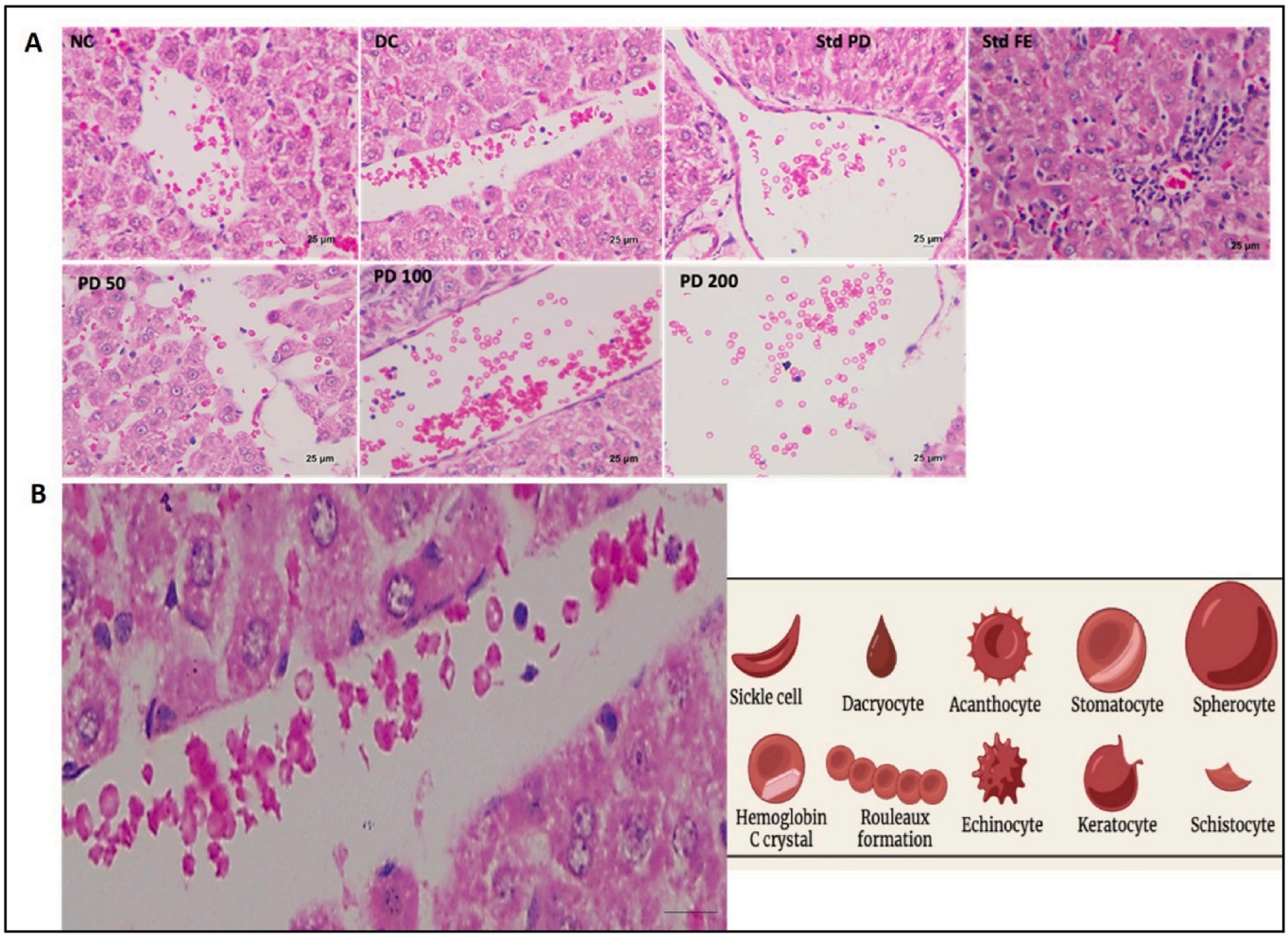

Fig. 3. A. H & E stained liver tissue sections obtained from animals of different treatment groups. B. High magnification image showing abnormal morphology of RBCs in the liver sinusoids of diseased animals.

hemolytic anemia was investigated and the results are shown in Fig. 5. The hemoglobin level in the disease control group was measured to be 9.51 g/dL which was significantly lower than the normal group (13.82 g/dL) (Fig. 5C). This is on expected lines as reduced hemoglobin levels is a typical characteristic of anemia. When compared to the disease control, the treatment groups namely, PM200 (14.36 g/dL) and prednisolone (13.8 g/dL) demonstrated a significant increase in hemoglobin level which were comparable to the normal group. Another characteristic of hemolytic anemia is elevated levels of reticulocytes. To assess the treatment efficacy of PM against hemolytic anemia, reticulocytes from different groups were imaged and quantified (Fig. 5A and B). The disease control exhibited increased number of reticulocytes compared to normal. Both the treatment groups restored the reticulocyte levels to the normal level.

The lactate dehydrogenase (LDH) levels indicative of RBC damage were found to be elevated in the disease control group when compared to the normal group (Fig. 5D). Treatment with PM200 restored the values to normal suggesting its ability to reverse RBC damage. Prednisolone also reduced the LDH levels comparable to the normal group.

3.6. Gene expression studies

In the present study, we explored the effect of PM treatment on the expression levels of the gene encoding for TNF-α and the results are presented in Fig. 6A. It is observed that the diseased group exhibited elevated levels of the TNF-α gene indicating a highly pro-inflammatory and anti-erythropoietic environment in the anemic animals. The PM-treated groups down-regulated TNF-α with respect to the disease control and standard treatment groups. Our results reveal the dose-dependent down-regulation of TNF-α by PM indicating its anti-inflammatory property which can help to overcome the PHZ-induced inhibition of erythropoiesis.

3.7. Molecular docking studies

The GC-MS of the hexane and methanolic extracts of PM revealed the presence of over 100 compounds. The major constituents identified (with peak area >1%) using GC-MS are listed in Supplementary Table S2. Our group had earlier identified chebulagic and chebulinic acids using LC-MS from *Terminalia* species.[24] The multiple herbs in PM may exert a synergistic effect on its anti-anemic properties. In order to understand this facet better, we carried out an *in silico* study with reported phytoconstituents from the herbs in PM and their ability to activate erythropoietin receptor (EPOR) (Fig. 7A). The active constituents of the plants in the PM formulations were retrieved from the Pubchem and IMPPAT databases and molecular docking was performed for the identification of the top 22 hits.

Erythropoietin receptor plays a central role in erythropoiesis pathway leading to formation of RBCs. The two polypeptide chains of EPOR dimerize to become the functional protein. The JAK2 is located in the cytoplasmic side of this protein. Research has revealed that the two binding sites 1 and 2 are important for activation of the JAK/STAT

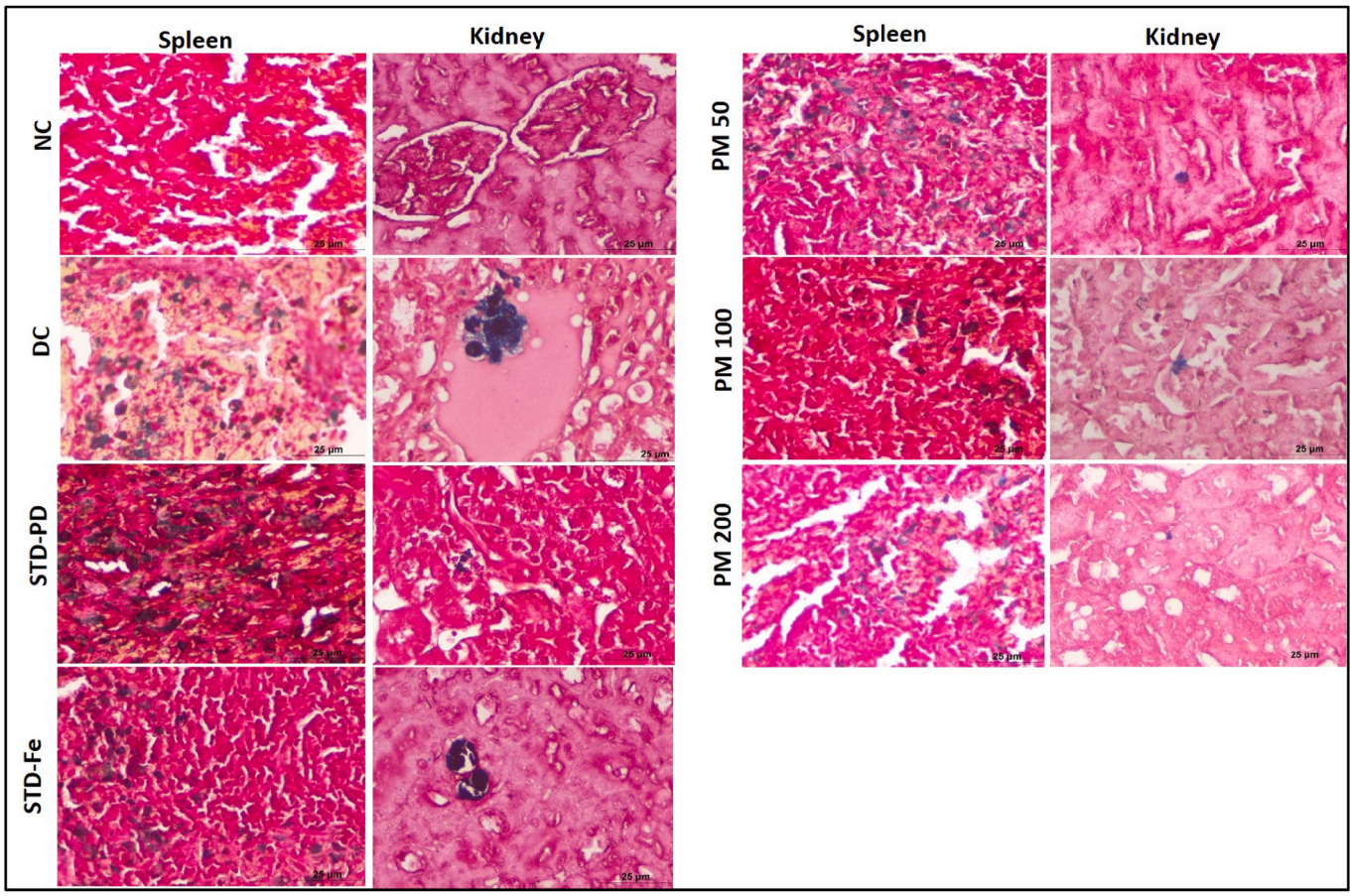

Fig. 4. Prussian blue staining of spleen & kidney isolated from different treatment groups.

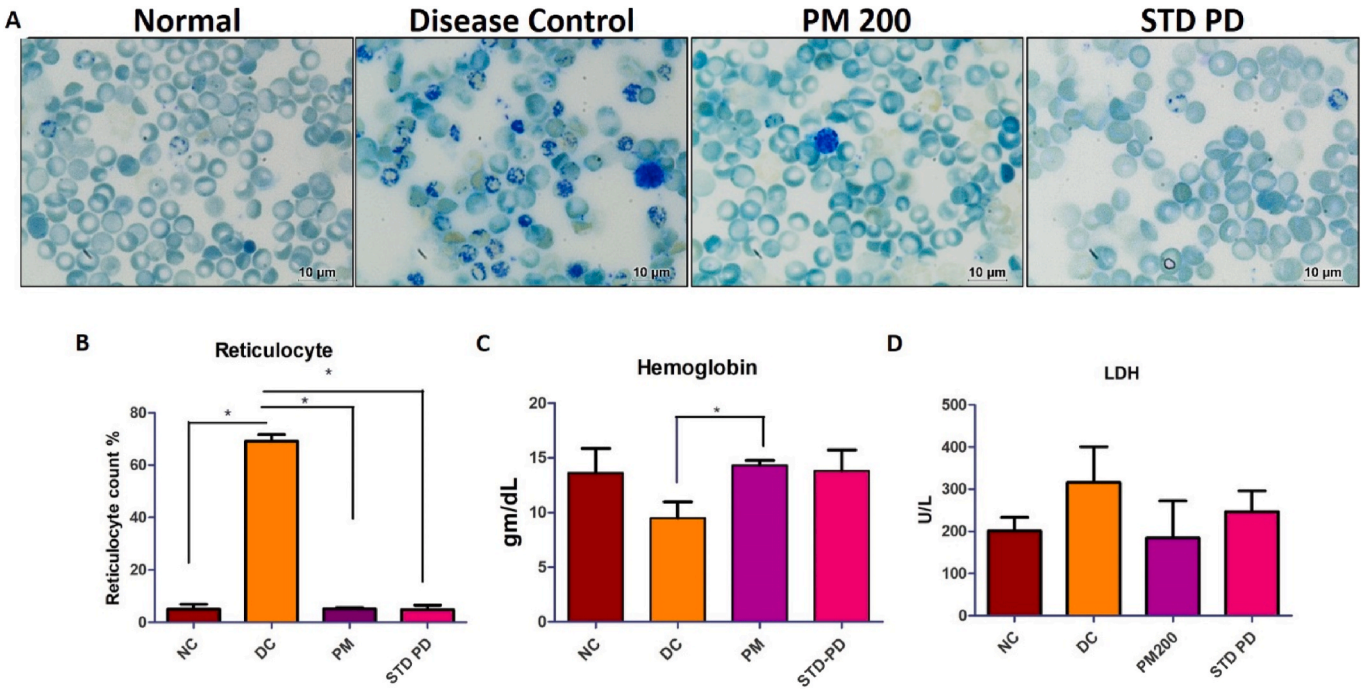

Fig. 5. A. Images of reticulocytes stained using methylene blue and viewed under 100× magnification. B. Quantification of reticulocytes in different visual field n = 4, mean ± SD, *p < 0.05, C. Hemoglobin levels of animals from different groups n = 4, mean ± SD, *p < 0.05. D. Lactate dehydrogenase leves of animals from different groups n = 3.

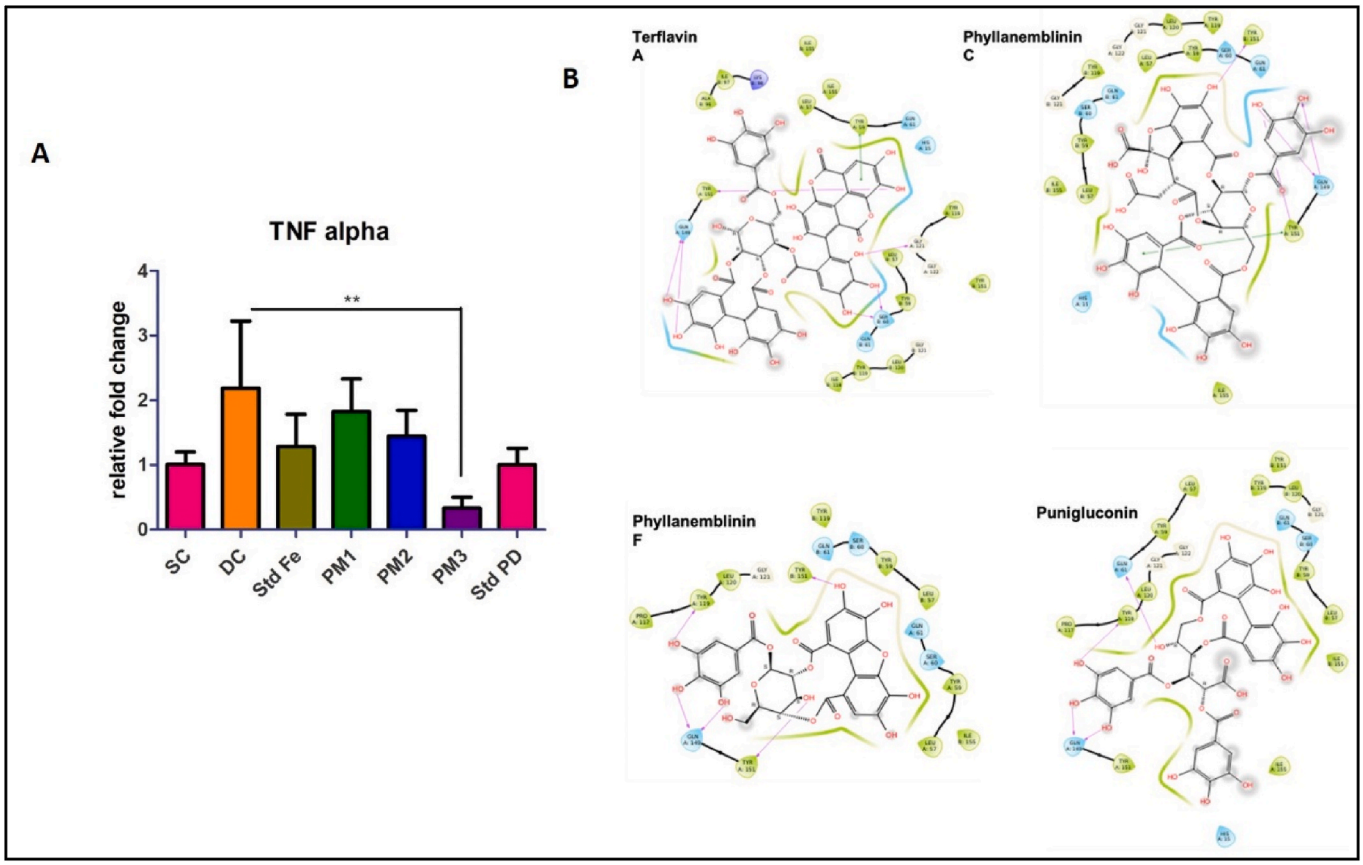

Fig. 6. (A) Expression levels of TNF-α gene in the spleen of animals from various treatment groups, PM1- PM 50, PM2 - PM 100, PM3 - PM 200; n = 3; ** denotes $p < 0.001$. (B) Ligand interaction diagram of selected phytoconstituents with TNF-α.

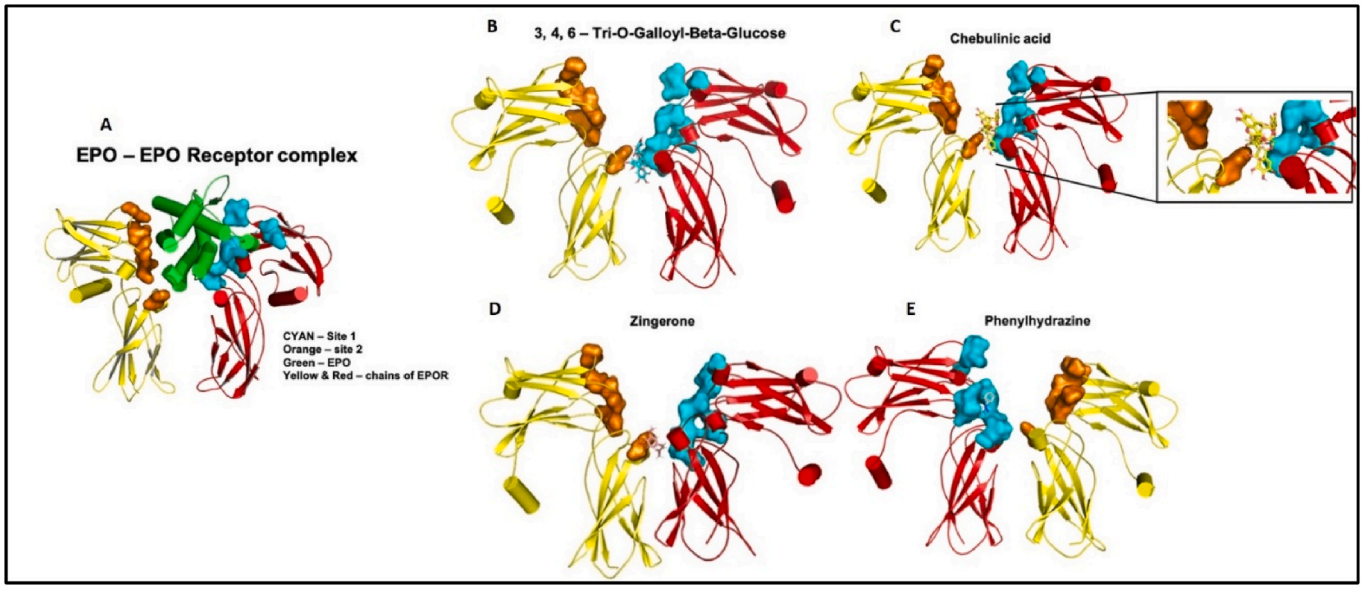

Fig. 7. (A) Visualization of EPO – EPO Receptor complex. (B) Docked poses of selected phytoconstituents of *Punarnavadi Mandura* 3,4,6 –Tri-O-galloyl-Beta-Glucose, Chebulinic acid, Zingerone and the toxin Phenylhydrazine used to induce hemolytic anemia.

pathway.[25] The binding of the ligand happens with high affinity to the binding site 1. The major residues involved in this binding are Glu60, Asp61, Glu62, Thr87, Ala88, Ser92, Phe93, His114, Glu117, Glu202, Pro203 and Ser204.[26,27] The ligand then interacts with site 2 through the residues Leu33, Glu34, Glu62, Ala88, Asp89, Ser91, Ser92 and His 153. Interactions with both sites will bring about a conformation change in the EPOR that is transmitted through its transmembrane domain to activate JAK2 for autophosphorylation of its tyrosine residue. The phosphorylated JAK2 then phosphorylates STAT5 to trigger the signalling pathway leading to erythropoiesis. Binding to both sites has been

implicated for JAK2 activation and interactions with a single site are found to inhibit the signalling pathway.[25] Among the 300+ phytoconstituents of the PM ingredients drawn from IMPPAT and Pubchem databases, the top 22 ingredients based on their docking score are listed in Supplementary Table S3.

A few representative docking poses of the top hit compounds are presented in Fig. 7B. It is observed that PHZ interacts only with site 1 while zingerone, another phytoconstituent of PM identified in the GC-MS studies was found to interact only with site 2 indicating that they may not activate the JAK2 signalling pathway. However, chebulinic acid, a major constituent present in *Terminalia chebula, Terminalia bellerica* and *Phyllanthus emblica*, was found to interact with 4 and 2 key residues in sites 1 and 2 respectively. Similarly, the top hit trigallylglucose, a key ingredient in *Terminalia* species interacted with 2 and 1 major residues in sites 1 and 2 while phyllanembilin, a marker compound from *Phyllanthus emblica* interacted with 2 key residues each in sites 1 and 2. Two other phytoconstituents from *Phyllanthus emblica,* punigluconin and emblicanin, interacted with one key residue each in site 1 and 2 implicated in JAK2 activation. Thus, it is evident that the polyherbal formulation PM has many phytoconstituents capable of activating the JAK2/STAT/MAPK pathway responsible for erythropoiesis.

A similar study was also carried out for the pro-inflammatory cytokine TNF-α and the top hits are summarized in Supplementary Table S4 and the docked poses of some of the major ingredients are depicted in Fig. 6B. The docking results showed that the phytoconstituents that activate the EPO receptor may also bind strongly with TNF-α and thus inhibiting it. Taken together, our studies reveal that PM has multiple phytoconstituents that possess strong anti-inflammatory properties and also can modulate the erythropoiesis signalling pathway to offset anemia. Our studies have shown for the first time the promise of PM to treat the autoimmune hemolytic anemia through a combination of anti-inflammatory effect, promotion of erythropoiesis and ameliorating Gamna Gandy bodies.

4. Discussion

Several studies have shown the therapeutic efficacy of PM against iron deficiency anemia. A clinical study involving PM had shown a significant improvement in serum iron, ferritin, hemoglobin, MCV, MCHC in anemic patients from the baseline.[28] The present study was conducted to evaluate the therapeutic efficacy of PM against hemolytic anemia. The efficacy of PM has not been scientifically validated against this type of anemia till date. Hemolytic anemia can be an inherited auto-immune disorder or could be chemically-induced.[29,30] In this condition, the rate of RBC destruction exceeds its rate of formation resulting in oxygen deprivation and multi-organ failure. Chronic conditions require blood transfusions to replenish the RBC levels.

Deregulation of WBC counts is associated with different types of hemolytic anemia. It is seen that the WBC count showed a significant increase in the disease control when compared to the normal animals. Interestingly, the PM treated as well as the standard groups restored the WBC levels to normal. This result suggests that the PM treatment could decrease inflammation in hemolytic anemia. A significant increase is observed in the granulocyte levels in the diseased animals that is reduced in all treatment groups. This also supports the earlier inference that the PM treatment has the potential to reduce inflammation. Lymphocyte levels did not show any change in the disease and treated animals. A small but insignificant reduction in lymphocytes is however, observed in the prednisolone treated animals. This may be due to its immunosuppressive nature.

The RBCs show a significant reduction in the disease control group when compared to the normal animals. This is a typical feature of hemolytic anemia which is characterized by lysis of RBCs. All treatment groups are found to restore the RBC counts to normal values post-treatment. The same trend is also observed in the hematocrit values that represents the percentage of RBCs in blood. Treatment with all doses of PM as well as the standard drugs restores the hematocrit to normal values (Fig. 1B). Earlier reports have shown that glucocorticoids including prednisolone increase RBC production and hematocrit values.[31] This may be attributed to accelerated maturation of reticulocytes. The increase in hematocrit by PM may also be due to a similar mechanism. MCHC elevation in disease control group may be attributed to the formation of abnormal shaped erythrocytes like spherocytes which has been reported in cases of hemolytic anemia. However, all treatment groups restored the MCHC values to normal indicating their therapeutic efficacy in treatment of hemolytic anemia and restoring normal RBC phenotype. The MCV of all the groups did not show much variation since hemolytic anemia is a normocytic anemia. The MCH values of the diseased group shows a significant increase when compared to the normal group. Such characteristics have been associated with folate deficiency.[32] Hemolysis can bring about folate deficiency suggesting a link between different types of anemia. This facet remains relatively unexplored and further research to understand the molecular pathways and inter-relationships between the different forms of anemia could be useful in developing better therapeutic strategies to treat this condition. Interestingly, treatment with PM results in restoration of the MCH values to normalcy suggesting that the likely cause for increase in MCV and RDW could be increased release and maturation of reticulocytes.

The loss of RBC by lipid peroxidation and oxidative stress causes hemolysis, resulting in lower hemoglobin levels in PHZ-injected animals compared to normal. When compared to the disease control, the therapy groups demonstrate a significant increase in hemoglobin levels which correlates well with our findings on the hematological parameters namely, RBC, hematocrit, MCH and MCHC. These results suggest that PM has the ability to reduce lipid peroxidation thereby restoring hemoglobin levels in the blood. To compensate RBC loss, more reticulocytes will be released from the bone marrow. This is reflected in the increased average reticulocyte count in the disease group when compared to the normal group, indicating that the PHZ administration caused a greater loss of RBC in circulation and retarded reticulocyte maturation. It is likely that the restoration of reticulocyte levels to normal by PM200 and standard treatment groups was through reduction in oxidative stress and RBC membrane damage brought about by PHZ and by augmenting reticulocyte maturation.

Hemolytic anemia results in the release of hemoglobin from the destructed RBCs, which are further transformed to bilirubin by hemeoxygenases.[33,34] When there is excessive release of hemoglobin, the rate of formation of bilirubin exceeds the capability of the liver enzymes to form conjugated (direct) bilirubin thereby leading to an accumulation of unconjugated (indirect) bilirubin in blood. This is a typical phenomenon associated with hemolytic anemia. Spleen is a key site for the destruction of abnormal RBCs in diseased animals. The histopathological examination of the spleen isolated from animals in the normal control showed characteristic white and red pulp regions of the spleen with no pathological changes. The red pulp region is the site for destruction of RBCs while the white pulp is responsible for production of leucocytes. In contrast, both red pulp and white pulp show pathological changes due to the oxidative stress mediated by PHZ in the diseased group. The appearance of distinct and unique yellow Gamna-Gandy bodies (GGB) in the spleen of the diseased animals are a typical feature of hemolytic anemia.[23] The GGBs are hemosiderin deposits containing iron, calcium and fibrotic tissue and are formed from the hemoglobin released due to the inflammation and hemorrhagic destruction of RBCs.[23] The density, distribution foci and intensity of GGBs are found to increase in the prednisolone-treated group. This is in agreement with literature reports of corticosteroids increasing the incidence of hemosiderin deposits.[35] In contrast, the ferrous sulphate treated group shows a marked reduction in GGBs. Interestingly, the PM treated groups showed negligible GGBs in the spleen suggesting that it has the propensity to restore the morphology of deformed RBCs which

also is evident from the histopathological sections of the liver tissue. Prussian blue staining reveals hemosiderin accumulation in the spleen and kidney. The breakdown of hemoglobin into heme during RBC lysis also promotes production of hemeoxygenase which transforms heme to iron, which is stained blue by Prussian blue dye. Similarly, iron in the form of hemoglobin liberated from damaged RBCs is retained in ferritin, which is transformed into hemosiderin that accumulates in the spleen. The appearance of distinct regions stained with Prussian blue in the spleen and kidney sections from the diseased animals concur with the hemosiderin accumulation. The reduction in the hemosiderin deposits in the PM treated groups suggest their propensity to curb hemolysis.

One of the two pathways by which PHZ induces destruction of RBCs is through induction of oxidative stress or through inhibition of the JAK/STAT signalling pathway.[36] Tumour necrosis factor – alpha (TNF-α) is a common player in both these pathways. TNF-α is a major cytokine molecule involved in multiple functions including mediating pro-inflammatory responses in the biological system. The role of TNF-α in anemia has been described by few groups. The binding of TNF-α to its receptor (TNFRI) on the membrane results in activation of GATA2 that inhibits erythropoiesis.[37] In an indirect mode, TNF-α inhibits the binding to erythropoietin to its receptor through GATA2-mediated suppression of HIF1α.[37] The pro-inflammatory role of TNF-α also contributes to the oxidative stress cascade in RBCs which subsequently triggers apoptosis.[38] The significant reduction in TNF-α by PM clearly suggests that the therapeutic benefits of PM arises due to its ability to curb oxidative stress and inflammation. This was also predicted by the *in silico* docking studies of the major reported phytoconstituents of this formulation. Additionally, the ability of these constituents to positively modulate erythropoietin receptor involved in RBC production, as predicted from *in silico* studies imply that the restoration of hemoglobin and RBC levels by PM may involve this target.

5. Concluding remarks

This work has demonstrated that the traditional *Ayurvedic* formulation *Punarnavadi Mandura* containing numerous phytoconstituents and iron oxide can be effective in treatment of hemolytic anemia. Our results show that apart from restoring the hematological parameters to normal, *PunarnavadiMandura* down-regulates the pro-inflammatory cytokine TNF-alpha. It also restores the morphology of the RBCs to normal and reduced the Gamna Gandy hemosiderin deposits in the spleen. The treatment with *Punarnavadi Mandura* also offset the splenomegaly induced by hemolytic anemia. Our *in silico* studies also reveal that the multiple constituents in the formulation could positively modulate erythropoiesis through interactions with both polypeptide chains of erythropoietin receptor in addition to inhibiting TNF-alpha mediated inflammation. Taken together, our study has revealed additional therapeutic applications for this polyherbomineral formulation relevant in the post-pandemic world.

Taxonomy

In vivo studies.

Disease

Hemolytic anemia.

Experimental approach

In vivo studies supported by *in silico* and gene expression studies.

Methodology

Hemolytic anemia model was created in male Wistar rats followed by treatment with PM for 15 days. At the end of treatment period, blood was collected for assessment of haematological and biochemical parameters. After euthanisation, the organs from different groups were collected and fixed in formalin for histopathological analysis. Spleen was also snap-freezed for performing the gene expression studies. In silico studies were carried out to understand plausible mechanism of action of PM.

Declarations of interest

None.

Declaration of competing interest

The authors discloses that there is no potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtcme.2024.03.017.

References

1. Samal J, Kumar Dehury R. A review of literature on Punarnavadi mandura: an ayurvedic herbo-mineral preparation. *Phcog J*. 2016;8(3):180–184. https://doi.org/10.5530/pj.2016.3.2.
2. Pandya MG, Dave AR. A clinical study of Punarnava Mandura in the management of Pandu Roga in old age (geriatric anemia). *Ayu*. 2014;35(3):252–260. https://doi.org/10.4103/0974-8520.153735.
3. Gupta C, Tiwari S. Role of Punarnava mandura in iron deficiency anaemia (Pandu Roga) with special reference to Pradhana Lakshana. *J Stress Physiol Biochem*. 2021;17(4):111–118.
4. Chaparro CM, Suchdev PS. Anemia epidemiology, pathophysiology, and etiology in low- and middle-income countries. *Ann N Y Acad Sci*. 2019;1450(1):15–31. https://doi.org/10.1111/nyas.14092.
5. Chen HF, Ho TF, Kuo YH, Chien JH. Association between anemia severity and ischemic stroke incidence: a retrospective cohort study. *Int J Environ Res Publ Health*. 2023;20(5):3849. https://doi.org/10.3390/ijerph20053849.
6. Esposito G, Dottori L, Pivetta G, Ligato I, Dilaghi E, Lahner E. Pernicious anemia: the hematological presentation of a multifaceted disorder caused by cobalamin deficiency. *Nutrients*. 2022;14(8):1672. https://doi.org/10.3390/nu14081672.
7. Brodsky RA, Jones RJ. Aplastic anaemia. *Lancet*. 2005;365(9471):1647–1656. https://doi.org/10.1016/S0140-6736(05)66515-4.
8. Jacobs J, Eichbaum Q. COVID-19 associated with severe autoimmune hemolytic anemia. *Transfusion*. 2021;61(2):635–640. https://doi.org/10.1111/trf.16226.
9. Virot E, Duclos A, Adelaide L, et al. Autoimmune diseases and HIV infection. *Medicine*. 2017;96(4), e5769. https://doi.org/10.1097/MD.0000000000005769.
10. Iwamoto Y, Anno T, Koyama K, Shirai R, Kaneto H, Tomoda K. Rapidly exacerbating autoimmune hemolytic anemia together with marked cytokine storm triggered by Pneumonia infection: a case report. *Front Immunol*. 2020;11. Accessed January 4, 2024 https://www.frontiersin.org/articles/10.3389/fimmu.2020.574540.
11. Pandey K, Meena A, Jain A, Singh R. *Molecular mechanism of phenylhydrazine induced haematotoxicity: a review*, 2. 2018:390–394.
12. Rajagopal P, Chellappan DR, Sridharan S, et al. Microarray analysis of genes from animals treated with a traditional formulation ChandraprabhaVati reveals its therapeutic targets. *J Tradit Complement Med*. 2020;10(1):36–44. https://doi.org/10.1016/j.jtcme.2019.08.001.
13. Okafor AI, Atsu CU. Ficus glumosa Del. reduces phenylhydrazine-induced hemolytic anaemia and hepatic damage in Wistar rats. *J Compl Integr Med*. 2022;19(3):661–668. https://doi.org/10.1515/jcim-2021-0306.
14. de Souza DW, Ceglarek VM, Siqueira BS, et al. Phenylhydrazine-induced anaemia reduces subcutaneous white and brown adipose tissues in hypothalamic obese rats. *Exp Physiol*. 2022;107(6):575–588. https://doi.org/10.1113/EP089883.
15. Gheith I, El-Mahmoudy A. Laboratory evidence for the hematopoietic potential of *Beta vulgaris* leaf and stalk extract in a phenylhydrazine model of anemia. *Braz J Med Biol Res*. 2018;51, e7722. https://doi.org/10.1590/1414-431X20187722.

16. Dhandapani R, Sethuraman S, Krishnan UM, Subramanian A. Self-assembled multifunctional nanotheranostics against circulating tumor clusters in metastatic breast cancer. *Acta Pharm Sin B*. 2023;13(4):1711–1725. https://doi.org/10.1016/j.apsb.2022.12.003.
17. Mustafa I, Hadwan TAQ. Hemoglobin oxidation in stored blood accelerates hemolysis and oxidative injury to red blood cells. *J Lab Physicians*. 2020;12(4):244–249. https://doi.org/10.1055/s-0040-1721156.
18. Gaur M, Sehgal T. Reticulocyte count: a simple test but tricky interpretation. *Pan Afr Med J*. 2021;40:3. https://doi.org/10.11604/pamj.2021.40.3.31316.
19. Qu Y, Shen Y, Teng L, et al. Chicoric acid attenuates tumor necrosis factor-α-induced inflammation and apoptosis via the Nrf2/HO-1, PI3K/AKT and NF-κB signaling pathways in C28/I2 cells and ameliorates the progression of osteoarthritis in a rat model. *Int Immunopharm*. 2022;111, 109129. https://doi.org/10.1016/j.intimp.2022.109129.
20. El-Hadary ARE, Sulieman AM, El-Shorbagy GA. Comparative effects of Hibiscus leaves and potato peel extracts on characteristics of fermented orange juice. *JFQHC*. 2023;21. https://doi.org/10.18502/jfqhc.10.1.11988. Published online February.
21. Kobayashi T, Takeba Y, Ohta Y, et al. Prenatal glucocorticoid administration enhances bilirubin metabolic capacity and increases Ugt1a and Abcc2 gene expression via glucocorticoid receptor and PXR in rat fetal liver. *J Obstet Gynaecol Res*. 2022;48(7):1591–1606. https://doi.org/10.1111/jog.15235.
22. Salehi L. Effect of iron supplementation in pregnant women with high hemoglobin on neonatal jaundice: a randomized double-blind clinical trial. *Iran J Obstetr, Gynecol Infertil*. 2019;22:18–22. https://doi.org/10.22038/IJOGI.2019.13441.
23. Piubelli MLM, Clemente LC, Duarte-Neto AN. Gamna-Gandy bodies of the spleen in sickle cell disease. *Autops Case Rep*. 2019;9(2), e2018076. https://doi.org/10.4322/acr.2018.076.
24. Selvaraj S, Ramanathan R, Vasudevaraja V, et al. Transcriptional regulation of the pregnane-X receptor by the Ayurvedic formulation Chandraprabha Vati. *RSC Adv*. 2014;4(110):64967–64976. https://doi.org/10.1039/C4RA13553A.
25. Tóthová Z, Tomc J, Debeljak N, Solár P. STAT5 as a key protein of erythropoietin signalization. *Int J Mol Sci*. 2021;22(13):7109. https://doi.org/10.3390/ijms22137109.
26. Middleton SA, Barbone FP, Johnson DL, et al. Shared and unique determinants of the erythropoietin (EPO) receptor are important for binding EPO and EPO mimetic peptide. *J Biol Chem*. 1999;274(20):14163–14169. https://doi.org/10.1074/jbc.274.20.14163.
27. Singh V, Kumar N, Kalsan M, Saini A. In silico designing and optimization of EPO mimetic using combinatorial library. Accessed July 18, 2023 https://www.semanticscholar.org/paper/In-silico-Designing-and-Optimization-of-EPO-Mimetic-Singh-Kumar/cb1fa4764ade364126bba38748502cbdf41d4d15; 2015.
28. Kori VK, Patel KS. Efficacy of Pandughni Vati & Punarnavadi Mandura Vati in the managment of Pandu w.s.r. iron deficiency anemia in children- an open labelled comparative clinical trial. *Ayu*. 2020;41(4):218–224. https://doi.org/10.4103/ayu.AYU_206_20.
29. Kim Y, Park J, Kim M. Diagnostic approaches for inherited hemolytic anemia in the genetic era. *Blood Res*. 2017;52(2):84–94. https://doi.org/10.5045/br.2017.52.2.84.
30. Parker CJ. Paroxysmal nocturnal hemoglobinuria. *Curr Opin Hematol*. 2012;19(3):141. https://doi.org/10.1097/MOH.0b013e328351c348.
31. Li H, Natarajan A, Ezike J, et al. Rate of progression through a continuum of transit-amplifying progenitor cell states regulates blood cell production. *Dev Cell*. 2019;49(1):118–129.e7. https://doi.org/10.1016/j.devcel.2019.01.026.
32. Hadi KA, Al-douri SDS, Hadree DH. Effect of folic acid on some physiological parameters in female rabbits treated with methotrexate. *Iraqi J Agric Sci*. 2023;54(3):730–734. https://doi.org/10.36103/ijas.v54i3.1754.
33. Olayemi E, Awodu OA, Bazuaye GN. Autoimmune hemolytic anemia in HIV-infected patients: a hospital based study. *Ann Afr Med*. 2008;7(2):72–76. https://doi.org/10.4103/1596-3519.55677.
34. Singh S, Nirala SK, Bhadauria M. Comparative role of acetaminophen, carbon tetrachloride and thioacetamide in development of fibrosis in rats. *Toxicol Res*. 2024;13(1), tfad114. https://doi.org/10.1093/toxres/tfad114.
35. Yang CT, Chiang BL, Wang LC. Aggressive corticosteroid treatment in childhood idiopathic pulmonary hemosiderosis with better outcome. *J Formos Med Assoc*. 2021;120(2):838–846. https://doi.org/10.1016/j.jfma.2020.05.022.
36. Shwetha BR, Siddalingaprasad HS, Swamy S, Nagalakshmi NC, Hariprasad MG. Mechanism of haematotoxicity induced by phenylhydrazine: a review. *J Appl Pharm Res*. 2019;7(4):1–6. https://doi.org/10.18231/j.joapr.2019.v.7.i.4.001.
37. Weiss G, Ganz T, Goodnough LT. Anemia of inflammation. *Blood*. 2019;133(1):40–50. https://doi.org/10.1182/blood-2018-06-856500.
38. Page MJ, Bester J, Pretorius E. The inflammatory effects of TNF-α and complement component 3 on coagulation. *Sci Rep*. 2018;8(1):1812. https://doi.org/10.1038/s41598-018-20220-8.