HAEMATINIC AND ANTI-ANEMIC EFFECTS OF THE METHANOL EXTRACT OF SARACA INDICA STEM BARK AGAINST PHENYL HYDRAZONE-INDUCED HEMOLYTIC ANEMIA IN RATS


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ABSTRACT

Saraca indica (Caesalpiniaceae) is one of the most renowned and a religious tree of India. This versatile plant shows anti-cancer, anti-menorrhagic, anti-oxytocic, anti-microbial activity and has extended uses in Ayurveda, Unani and Homeopathy System of Medicine. Aims of the study were to evaluate the hematinic and anti-anemic effects of the methanol extract of Saraca indica stem bark (SI) against phenylhydrazine-induced hemolytic anemia in rats. Adult male rats were divided into 5 groups (Gr.I-V) containing 6 rats each. Rats in Gr. I served as control. Gr. II rats were treated with SI (250 mg/kg) extract for 14 days. Rats in Gr. III, IV and V were pre-treated with phenylhydrazine (PHZ, 10 mg/kg for 7 consecutive days) so as to induce anemia, followed by 0, 250 and 500 mg/kg doses of SI extract respectively up to 14 days. To investigate hematinic and anti-anemic profile of plant extract, blood samples were examined for various hematological parameters viz. Hgb, RBCs, WBC, MCV, MCHC, MCH, RDW, Platelet count, etc. Biochemical analysis of marker enzymes for general metabolic, liver and kidney function, and histological examination was done in control and treated-groups of rats. Antioxidant enzymes and level of lipid peroxidation was studied in blood samples from treated and control animals. Results showed that PHZ (10 mg/kg) treatment for 7 consecutive days caused a significant increase in MDA activity.
but, declined SOD and Catalase activity in blood serum. Further, PHZ- treatment caused significant decrease in Hgb (%), RBC, MCV, but increased TLC count in Gr. III-V rats. There was significant increase in Hgb (%), RBC, MCV and TLC count in Gr. IV and V rats treated with SI extract at 250 and 500 mg/kg doses from day 8-14, as compared to PHZ-treated (Gr.III) rats. Rats treated with SI extract alone did not alter any haematological/biochemical/histological aspects and organ weights as compared to controls. In conclusion, the results of the study clearly indicate antioxidant, hematinic and anti-anemic activity of the methanolic extract of SI stems bark. Findings provide scientific evidence for traditional use of SI extract as natural antioxidant and hematoprotective agent.

**KEYWORDS:** Antioxidant, hematinic, anti-anemic activity, *Saraca indica.*

**INTRODUCTION**

Herbal plants and phytomedicines are symbolized for safety of mankind, serving several purposes like health and protection from diseases and/or nutrition. In the present scenario the knowledge of traditional medicine has always guided the search for new cures and clues. Therefore, the demand for herbal products is growing exponentially throughout worldwide for the discovery of valuable drugs because of its non-toxicity and safety in spite of modern high throughput drug discovery and screening techniques. Nowadays, attention has been focused on the investigation, isolation and fractionation of drugs from plant origin for pharmacological basis of traditionally used plants.

Ashoka is the most ancient tree of India, generally known as “Ashok briks”, botanical name *Saraca asoca* (Roxb.), De.wild or *Saraca indica* (SI) belonging to family Caesalpinaceae. It is found throughout India, especially in Himalaya, Kerala, Bengal and entire southern region. As a medicinal tree the utility of Ashoka seems to have been recognized first in the Agnivesa Caraka Samhita which is supposed to have been compiled somewhere near 1000 B.C. Ayurvedic physicians of the present century are using Ashoka in different female diseases especially for uterine infections and pain traditionally as per Charka Samhita (100 A.D.). The leaves of *S. asoca* have been used in the treatment of diabetes. The plant contains many flavonoids, sterols and triterpenoids as its main constituents, which are known bioactive principles for antidiabetic potential, regenerate the damaged β-cells in diabetic mice. SI used in the Ayurvedic system of medicine as hypothermic and diuretic, as a blood purifier and in stomach ache. Asokarista, Asokaghrta, Asoka decoction and Asoka pills are the famous pharmaceutical preparations used in bleeding piles and bacillary
dysentery. The drug Asoka Aristha is traditionally used in India and Sri Lanka to treat menorrhagia.[15] The antibacterial activity of the extracts of leaves, stem and flowers of *S. asoca* has been reported elsewhere.[16-18] Petroleum ether extract of *SI* leaves exhibits potential antitumor and antioxidant activities.[19] Methanol extracts of *SI* leaves shows better antihelmintic and analgesic activity.[20,21] The stem bark of *SI* shows various pharmacological actions like antibacterial[22], antiulcer[23], anticancer[24], larvicidal[25], antidepressant[26] and chemo-protective activities.[27]

The use of antioxidants for inhibition of oxidative damage is one of the important approaches towards the prevention of health problems. Several studies have been indicated the antioxidant activities of herbal plants and correlated with their total phenolic contents.[28-30] The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals or their effects, quenching singlet and triplet oxygen or decomposing peroxides.[31,32] The flavonoids and terpenoids have multiple biological effects including an antioxidant activity, plays an important role in the defense against free radicals.[33] The oxidative environment of living organism possess a range of free radicals including superoxide radical, hydroxyl radical, hydrogen peroxide, nitric oxide and peroxynitrite, which are essential for the production of energy for various biological processes. However, excessive production of free radicals results in the development of carcinogenesis[1], neurodegenerative diseases[2], inflammatory diseases[34], atherosclerosis, aging, immunosuppression, ischemic heart disease, diabetes, hair loss, Alzheimer’s disease, cataract and many other problems.[34-39] The human body possesses innate defense mechanisms to counter free radicals in the form of enzymes such as superoxide dismutase, catalase, and glutathione peroxidase. Vitamin C, vitamin E, selenium, β-carotene, lycopene, lutein and other carotenoids have been used as supplementary antioxidants.[32, 40-42] The major phytoconstituents of *SI* stem bark extract are reported to be the flavonoids, terpenoid, lignin, cardiac glycosides, phenolic compounds, tannins and leucoanthocyanidins.[43-46] It also shows antioxidants by preventing the oxidation of low-density Lipoproteins (LDL), platelet aggregation and damage of red blood cells.[47] The ethyl acetate fraction of *Saraca ashoka* flowers exhibited free radical scavenging activity against the 1,1-diphenyl-2-picrylhydrazyl radical and superoxide radical, along with hydroxyl radical scavenging activity and Lipid peroxidation inhibitory potential and significant xanthine oxidase(XO) (key enzyme linked to inflammation) inhibitory activity.[48]
Phenyl hydrazine (PHZ)-induced hemolytic anemia hereditary or acquired haemolytic anemia in humans results from reduced life span or destruction of red blood cells (RBCs) and a failure of the bone marrow compensatory responses. Haemolysis of the RBCs reduces the efficiency of oxygen delivery which stimulates increased erythropoiesis and because of the deficit in the Fe supply and haemoglobin (Hgb) levels, the haemopoietic cells are numerically and morphologically abnormal. Other features include spherocytosis, polychromasia, red-cell reticulocytosis, an increase in urinary urobilinogen and porphyrins. PHZ induces a reactive oxygen species formation, peroxidation of lipids and oxidative degradation of spectrin in the membrane skeleton. PHZ-induced haemolytic injury seems to be derived from oxidative alternations to RBCs proteins. This compound can modulate immune reactions. PHZ-induced anemia has been used as a model to study of hematinic effects and for the evaluation of its influence on therapeutic effectiveness, like antitumor therapy or for reticulocyte research or erythrocyte senescence under abnormal physiological conditions. It causes ten folds increase in DNA-dependent RNA polymerase activity of developing erythropoietic spleen in haemolytic anemia. Our earlier studies have shown amelioration of such effects by flower extract of *Hibiscus rosa sinensis* and coconut oil in rats.

The present study was conducted to evaluate the antioxidant potential and anti-anemic effects of the methanol extract of *SI* stem bark against PHZ-induced hemolytic anemia in rats.

**MATERIALS AND METHODS**

**Chemicals**

The kits for biochemical analysis of SOD, Catalase and MDA and phenyl hydrazine were purchased from Sigma-Aldrich, USA. All other chemicals used in this study were available locally.

**Collection of plant material**

The fresh plant material - stem bark of *Saraca indica* (*SI*; family-Caesalpiniaceae) was collected locally nearby Lucknow region, Uttar Pradesh, India. Plant material was identified by Dr. K. R. Arya, Principal Scientist, Ethnobotany Division, CSIR-Central Drug Research Institute Lucknow and authenticated in Institutional Herbarium (Voucher Specimen No. KRA/23998) and kept at the medicinal plant repository of the institute.
Preparation of plant extract
The stem bark of *SI* was dried at room temperature (at 37°C) and milled into fine powder using electric Laboratory grinder. Dry powder (2kg) was macerated in 2000 ml Absolute Methanol for 48 h and subsequently the mixture was filtered, semi-dried under reduced pressure (Rotor vapor, Buchi, Germany) at 35°C (yield: 8.357% yield(w/w). The reconstituted extract was used for haemato-protective/ant anemic activity. SI extract was administered orally using metal oropharyngeal cannula to the animals at different dose schedule.

Experimental Protocol
Test animals
Sprague–Dawley rats (150-175 gm) of both sexes were obtained from National Laboratory Animal Center (NLAC), CSIR-Central Drug Research Institute, Lucknow, India. Animals were allowed to acclimatize to uniform husbandry conditions (22±3 °C, 12h light: 12h dark cycle) for 1 week prior to the experiment. The animals were fed with a standard pellet diet (supplied by Hindustan Lever Ltd., Bangalore) and access to water *ad libitum*. Animal studies were conducted according to the regulations of the Institutes Animal Ethics Committee and the protocol was approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals, New Delhi, India (IAEC No.- IAEC/2012/86).

Phenyl hydrazine (PHZ)-induced haematotoxicity/haematoprotective activity of *Saraca indica* (SI)
Rats (Males and females) were randomly divided into five groups (Gr.) containing five animals each of either sex. Rats in Gr. I (Control) was administered with 1% gum acacia orally (p.o.). The rats in Gr. II were orally administered with methanol extract of SI (500mg/kg) for 7 days. Rats in Gr. III, IV and V were treated with PHZ (10 mg/kg/day, p.o) for 7 days to induce haematotoxicity.[51,52,59] Group III rats severed as PHZ-induced haematotoxicity Control (STD Control). Animals in Gr. IV and V (pre-treated with PHZ for 7 days) were administered orally with SI extract at 250mg/kg and 500mg/kg doses respectively for another 7 consecutive days up to day 14. On day 15, body weights of control and treated animals were recorded. Blood was collected in pre-coated EDTA-vials for hematology and then autopsied by anesthetizing with solvent anesthetic ether. The vital organs (viz. heart, liver, lungs, spleen, kidney and brain) were dissected out, weighed and fixed in 10% Formalin (24h) for histological examination.
Hematological analysis
Blood samples from control and treated groups were collected on days 0, 7 and 14 of treatment by puncturing tail vein. The hematological parameters were analyzed using MS-9 Fully Automated Hematology Analyzer (Melet Schloesing, France). The parameters included were hemoglobin (Hgb), total erythrocyte-red blood cells (T-RBCs), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), platelet count (PC) and total leucocytes count (TLC).

Biochemical estimations
Blood samples were collected by cardiac puncture and retro-orbital plexus method from all animals into the EDTA sprinkled tubes and were centrifuged at 3000 rpm for 20 min. Serum was separated and stored at -20°C until analysis was performed. Serum samples were analyzed for general metabolic function viz. Glucose(GLU), cholesterol(CHO), triglycerides(TG), total proteins(TP) and albumin(ALB) levels. For liver function, serum biochemistry for alanine trasparase (ALT), Aspartate transferase (AST), alkaline phosphatase (ALP) and total billirubin (T-BIL) was done. For kidney function, estimations of blood urea and nitrogen (BUN), creatinin (CRTN), calcium (Ca) and phosphate (P) were carried out using the diagnostic kit (ERBA Diagnostics Mannheim, Germany) in Auto analyzer.

Enzymatic antioxidant assays
On day 15, blood was collected in pre-coated EDTA-vials, centrifuged at 10000xg for 10 min and serum was collected and stored at -20°C in refrigerator till estimation of antioxidant enzyme activity.

Superoxide dismutase (SOD) activity: SOD activity was performed by 19160 SOD determinations Kit (Sigma-Aldrich, USA). Kit contained WST Solution (5ml), Enzyme solution (100μl), Buffer Solution (100ml) and Dilution buffer (50ml). Preparation of working solution was done by diluting 1 ml of WST solution with 19 ml Buffer solution. For enzyme working solution, enzyme solution was centrifuged for 5 sec, mixed thoroughly and diluted 15 μl of enzyme solution with Dilution buffer. SOD was diluted with dilution buffer to prepare SOD STD solution as 200U/ml, 50U/ml, 10 U/ml, 5 U/ml, 1U/ml, 0.05 U/ml, 0.01U/ml,0.001U/ml.

Methods: 20μl of sample solution was added to each sample and blank well 2 and 20 μl of double distilled water (DDH₂O) was added in each blank well 1 and 3. Then 200μl of WST
working solution was added in each well and mixed. Then added 20 μl of Enzyme working solution to each sample and blank 1 well, and mixed thoroughly. Incubated the Eliza plate at 37°C for 20 minutes and read the absorbance at 450nm using a micro plate reader. The SOD activity (inhibition rate %) was calculated using the equation: SOD activity (inhibition rate %) = \[\frac{[(A_{blank1}-A_{blank3})-A_{sample}-A_{blank2})]}{(A_{blank1} – A_{blank3})} \times 100.\]

**Catalase enzyme activity:** Catalase enzyme activity was performed using Catalase Assay Kit (CAT 100) from Sigma-Aldrich, USA. Tissue samples were prepared in 1X Assay buffer solution (500mM potassium phosphate buffer, pH 7.0), then diluted with enzyme dilution buffer(50mM potassium phosphate buffer, pH7.0 containing 0.1% TritonX-100) in a microcentrifuge tube and added calorimetric assay substrate solution(200mM H2O2) and mixed and incubated for 5 min in incubator at 37°C. Then added 900 μl of stop solution and inverted the tube. 10μl aliquot of catalase enzyme reaction was taken in another centrifuge tube and added 1 ml of Color reagent and mixed by inversion and kept for 15 minutes at room temperature for color development and read the absorbance at 520 nm.

**Lipid peroxidation (MDA) activity:** MDA activity was assayed by using lipid peroxidation assay kit (Catalog no. MAK085, Sigma-Aldrich, USA). Briefly, blood serum (10μl) was gently mixed with 500 μl of 42 mM H2SO4 in a microcentrifuge tube. Then added 125 μl phosphotungstic acid solution, mixed by vortexing and incubated for 5 minutes and then centrifuged at 13000xg for 3 minutes. In a separate tube 2μl of BHT (100x) to 100μl H2O. Resuspended the pellet on ice with water/BHT solution and adjusted the volume for 200 μl with water.

Assay reaction: To form MDA-TBA adduct, added 600 μl of the TBA solution into each vial containing standard and sample. Incubated at 95°C for 60 min. Cooled to room temperature in a ice bath for 10 minutes, pipetted 200 μl from each reaction mixture into 96 well plates for analysis and read the absorbance at 532 nm.

**Histology of vital organs**

Tissues from liver, kidney and spleen were fixed in Bouins’ fluid (24 hr) for histology purpose. Further, they were dehydrated in graded series of ethanol, cleared in xylene and infiltrated and embedded in paraffin wax (at 58°C). Transverse tissue sections (5 μm) were stained with routine Haematoxylin-eosin, observed the histological changes under Olympus Trinocular Microscope (Tokyo, Japan) and photo micro graphed.
Statistical analysis
Analysis for significance of differences between control and treated group of animals was done by Students ‘t’ test and one-way ANOVA (one factor analysis of variance). Values for antioxidant assays represented in a triplicate manner and were expressed as Mean ± SD. Values with p < 0.05 were considered as significant.

RESULTS
Body and organ weights
There were no significant differences observed in the body weights of treated male rats as compared to controls. The absolute organ weights viz. adrenal, brain, testis, heart and kidney of treated rats were comparable to controls. However, PHZ (10mg/kg) treatment for 7 days caused significant increase in weights of liver (p<0.01), lung (p<0.01) and spleen (p<0.001) as compared to control rats. Treatment with SI extract alone did not show any significant difference in body and organ weights. But, oral administration SI stem bark extract at 250 or 500mg/kg doses to PHZ-induced anemic rats, caused significant decrease (recovery) in weights of liver, lung and spleen comparable to that of control rats (Tables 1 & 2).

Table 1. Body and Absolute organ weights (g) in control, PHZ-treated and PHZ+SI-treated rats after 14 days.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Bodyweight</th>
<th>Adrenal gland</th>
<th>Brain</th>
<th>Gonads</th>
<th>Heart</th>
<th>Kidney</th>
<th>Liver</th>
<th>Lungs</th>
<th>Spleen</th>
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<td>(g)</td>
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<tr>
<td>I. Control</td>
<td>301.0±19.13</td>
<td>0.02±0.004</td>
<td>1.50±0.32</td>
<td>1.04±0.43</td>
<td>0.97±0.13</td>
<td>0.86±0.18</td>
<td>6.54±1.71</td>
<td>1.65±0.11</td>
<td>0.65±0.17</td>
</tr>
<tr>
<td>II. SI (250)</td>
<td>302.0±18.64</td>
<td>0.02±0.004</td>
<td>1.80±0.26</td>
<td>1.48±1.03</td>
<td>0.96±0.10</td>
<td>0.87±0.10</td>
<td>8.92±1.62</td>
<td>1.80±0.24</td>
<td>0.76±0.11</td>
</tr>
<tr>
<td>III. PHZ (10)</td>
<td>288.6±43.40</td>
<td>0.04±0.025</td>
<td>1.99±0.12</td>
<td>1.46±0.33</td>
<td>1.21±0.40**</td>
<td>1.06±0.07**</td>
<td>11.70±0.52**</td>
<td>2.31±0.31</td>
<td>4.44±0.44***</td>
</tr>
<tr>
<td>IV. PHZ+SI (250)</td>
<td>321.2±16.25</td>
<td>0.04±0.08</td>
<td>1.71±0.22</td>
<td>1.50±0.17</td>
<td>0.96±0.08</td>
<td>0.87±0.08</td>
<td>8.06±1.71</td>
<td>1.77±0.28</td>
<td>1.18±0.31</td>
</tr>
<tr>
<td>V. PHZ+SI (500)</td>
<td>308.6±43.40</td>
<td>0.04±0.01</td>
<td>1.76±0.10</td>
<td>1.54±0.13</td>
<td>0.95±0.09</td>
<td>0.84±0.02</td>
<td>8.04±0.54</td>
<td>1.96±0.29</td>
<td>1.16±0.31</td>
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</table>

Significance, Control vs. PHZ-treated, *p<0.05; **p<0.02; ***p<0.001, NS- Not significant. Gr. I- Control; Gr. II- SI(250mg/kg); Gr. III – PHZ(10mg/kg); Gr. IV- PHZ+ SI(250mg/kg); Gr. V- PHZ+SI(500mg/kg).

Table 2. Body and Relative organ weights (%) in control, PHZ-treated and PHZ+SI-treated rats after 14 days.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body weights</th>
<th>Adrenal gland</th>
<th>Brain</th>
<th>Gonads</th>
<th>Heart</th>
<th>Kidney</th>
<th>Liver</th>
<th>Lung</th>
<th>Spleen</th>
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<td>(g)</td>
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<td>(g)</td>
<td>(g)</td>
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<td>(g)</td>
</tr>
<tr>
<td>I.</td>
<td>301.0±19.13</td>
<td>0.017±0.0013</td>
<td>0.59±0.12</td>
<td>0.67±0.15</td>
<td>0.43±0.05</td>
<td>0.28±0.06</td>
<td>2.19±0.61</td>
<td>0.42±0.05</td>
<td>0.19±0.06</td>
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<td>II.</td>
<td>302.0±18.64</td>
<td>0.0174±0.002</td>
<td>0.58±0.08</td>
<td>0.49±0.05</td>
<td>0.32±0.03</td>
<td>0.29±0.03</td>
<td>2.96±0.60</td>
<td>0.60±0.08</td>
<td>0.25±0.05</td>
</tr>
<tr>
<td>III.</td>
<td>288.6±43.40</td>
<td>0.024±0.02</td>
<td>1.10±0.27**</td>
<td>0.81±0.27**</td>
<td>0.70±0.36NS</td>
<td>0.58±0.33*</td>
<td>6.43±1.28**</td>
<td>1.29±0.38**</td>
<td>2.42±0.41***</td>
</tr>
<tr>
<td>IV.</td>
<td>321.2±16.25</td>
<td>0.0173±0.004</td>
<td>0.77±0.09</td>
<td>0.68±0.10</td>
<td>0.41±0.05</td>
<td>0.39±0.03</td>
<td>3.87±0.56</td>
<td>0.80±0.12</td>
<td>0.53±0.13</td>
</tr>
<tr>
<td>V.</td>
<td>308.6±43.40</td>
<td>0.024±0.01</td>
<td>0.93±0.194</td>
<td>0.65±0.20</td>
<td>0.42±0.11</td>
<td>0.46±0.14</td>
<td>4.05±1.11</td>
<td>1.07±0.24</td>
<td>0.65±0.26</td>
</tr>
</tbody>
</table>

Significance, Control vs. PHZ-treated , *p<0.05; ** p<0.005 , ***p<0.001, NS- Not significant. Gr. I- Control; Gr. II- SI(250mg/kg); Gr. III – PHZ(10mg/kg); Gr. IV- PHZ+ SI(250mg/kg); Gr. V- PHZ+SI(500mg/kg).
Histology of vital organs

Transverse sections of kidney in control rat showed normal distinct glomeruli, Bowman’s capsule. PHZ-treated rats showed shrinkage and glomerular and renal damage in kidney of some animals. SI treatment to PHZ-induced anemic rats showed distinct glomerular and tubular structure with improvement as compared to PHZ-treated rats but similar to controls.

In control rats, histology of liver was normal showing hepatocytes, portal tract and leucocytic infiltration. PHZ-treatment caused distortion of hepatocytes, portal tract dilation and inflammatory infiltration. SI treatment to PHZ-induced anemic rats showed normal structure similar to in controls. In control rats, spleen showed normal structure. PHZ treatment caused higher erythroblastic islands in spleen with a higher excessive erythrocytic congestion, inflammatory infiltration than in liver and kidney in anemic rats. SI treatment showed repair of all changes which were comparable to normal control rat spleen (Figs. 1-3).

Figure 1. A, B. Transverse sections of kidney in control rat showing normal distinct glomeruli and Bowman’s capsule (Arrow). PHZ-treated rats showing shrinkage and glomerular/renal damage in kidney (Thick Arrow) of some animals (C, D). SI extract
treatment at the dose of 250 mg/kg (E) or 500mg/kg (F) to PHZ-induced anemic rats showed normal glomerular and tubular structure with improvement as compared to PHZ-treated rats which is similar to controls. Magnification: x400 (A,C,E, F) & x1000 under oil immulsion(B,D) ; H-E stained.

![Figure 2](image1.png)

**Figure 2.** In control rats, histology of liver showing normal hepatocytes, portal tract and leucocytes infiltration (A). PHZ-treatment caused slight infiltration of leucocytes in liver (B). SI treatment at 250mg/kg(C) and 500mg/kg(D) to PHZ-induced anemic rats showed normal liver structure similar to in control rats. Hepatic portal duct-(*), leucocytes (Arrow). Magnification; x 400 for all figures. H-E stained.

![Figure 3](image2.png)

**Figure 3.** In control rats, spleen showed normal structure(A). PHZ treatment caused higher erythroblastic islands in spleen with a higher excessive erythrocytic congestion,
inflammatory infiltration(*) in anemic rat (B). SI treatment at 250mg/kg(C) and 500mg/kg (D) showed repair of all changes which were comparable to control rat spleen. Magnification: x400; H-E stained.

Antioxidants enzymes activity
PHZ (10mg/kg) treatment caused a significant decrease in SOD and CAT enzyme activity, whereas MDA activity was significantly increased by PHZ treatment as compared to control rats. SI extract alone did not show any significant change in these enzymes activity compared to controls. However, there was a significant increase in SOD and CAT enzyme activity in PHZ-induced anemic rats treated with SI extract at 250 or 500 mg/kg (Figs. 4-6).

Figure 4. Superoxide dismutase enzyme activity in blood serum of control, PHZ-treated and SI+PHZ treated rats after 14 days treatment (Mean±SD, n=5 number of animals).

Figure 5: Catalase enzyme activity in blood serum of control, PHZ-treated and SI+PHZ treated rats after 14 days treatment (Mean±SD, n=5 number of animals).
Figure 6: MDA activity in blood serum of control, PHZ-treated and SI+PHZ treated rats after 14 days treatment (Mean±SD, n=5 number of animals).

**Haematology**

Adult male rats treated with *SI* (250mg/kg) for 14 consecutive days (Gr. II) did not show any significant change in Hgb, RBC and MCV, Platelets(x10\(^3\)/mm\(^3\)) and TLC(x10\(^3\)/mm\(^3\)) concentration in blood as compared to Control rats (Gr.I). Rats in Gr. III treated with PHZ (10mg/kg/day) for 7 consecutive days, caused significant decrease in Hgb (%) (p<0.01), RBC (x10\(^6\)/mm\(^3\)) (p<0.001) and MCV (micron\(^3\)) (p<0.001) concentration as compared to control rats. The MCHC (g%) and Platelets(x10\(^3\)/mm\(^3\)) number did not differ to that of controls, but, increase in number of TLC(x10\(^3\)/mm\(^3\)) was observed in PHZ-treated rats. When PHZ-treated groups of rats administered *SI* at 250 or 500mg/kg, showed a significant decline in Hgb, RBC and MCV concentration comparable to in controls (Table 3). The TLC values increased by PHZ treatment were normalized by *SI* extract treatment comparable to controls. The Hct, MCHC and platelets did not show any significant change in PHZ or *SI* treatment alone or in combination with PHZ when compared with control rats (Table 3).
Table 3. Haematological parameters in control, PHZ-treated and PHZ+SI-treated male rats on day 0, 7 and 14 (n=5 number of animals, Mean ± S.D.).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Days</th>
<th>Group I (Control)</th>
<th>Group II (SI (250mg/kg))</th>
<th>Group III (PHZ (10mg/kg))</th>
<th>Group IV (PHZ+SI (250mg/kg))</th>
<th>Group V (PHZ+SI (500mg/kg))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hgb(g%)</td>
<td>Day 0</td>
<td>12.42±0.22</td>
<td>12.42±0.36</td>
<td>12.52±0.70</td>
<td>12.64±1.13</td>
<td>12.78±0.19</td>
</tr>
<tr>
<td></td>
<td>Day 7</td>
<td>12.17±0.12</td>
<td>12.80±0.15</td>
<td>8.12±0.49**</td>
<td>9.21±0.91**</td>
<td>9.22±0.43**</td>
</tr>
<tr>
<td></td>
<td>Day 14</td>
<td>12.31±0.50</td>
<td>12.58±0.41</td>
<td>9.22±0.48***</td>
<td>12.56±0.78</td>
<td>12.52±0.41</td>
</tr>
<tr>
<td>T-RBC (x10^12/mm³)</td>
<td>Day 0</td>
<td>7.04±0.43</td>
<td>6.85±0.60</td>
<td>7.15±0.52</td>
<td>7.21±0.75</td>
<td>7.42±0.35</td>
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<tr>
<td></td>
<td>Day 7</td>
<td>7.27±0.59</td>
<td>6.94±0.68</td>
<td>3.02±0.42***</td>
<td>3.21±0.42***</td>
<td>3.20±0.61***</td>
</tr>
<tr>
<td></td>
<td>Day 14</td>
<td>7.16±0.16</td>
<td>7.13±0.89</td>
<td>3.15±0.25***</td>
<td>5.95±0.50</td>
<td>6.55±0.20</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>Day 0</td>
<td>41.34±1.73</td>
<td>42.92±2.63</td>
<td>42.46±5.57</td>
<td>41.64±4.97</td>
<td>43.14±1.16</td>
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<tr>
<td></td>
<td>Day 7</td>
<td>45.64±3.27</td>
<td>43.82±3.15</td>
<td>35.28±2.17</td>
<td>41.64±4.97</td>
<td>43.14±1.16</td>
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<td>Day 14</td>
<td>45.76±5.57</td>
<td>44.22±4.90</td>
<td>39.42±2.04</td>
<td>45.50±4.33</td>
<td>45.50±4.71</td>
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<tr>
<td>MCV (micron³)</td>
<td>Day 0</td>
<td>60.64±3.53</td>
<td>62.66±2.43</td>
<td>58.90±3.83</td>
<td>57.74±2.12</td>
<td>58.14±1.67</td>
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<td></td>
<td>Day 7</td>
<td>59.60±1.75</td>
<td>62.24±2.71</td>
<td>108.80±4.82***</td>
<td>108.52±5.40***</td>
<td>107.99±3.77***</td>
</tr>
<tr>
<td></td>
<td>Day 14</td>
<td>60.46±2.30</td>
<td>62.14±2.66</td>
<td>104.40±4.62***</td>
<td>75.50±3.80</td>
<td>80.00±3.71*</td>
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<tr>
<td>MCHC (g%)</td>
<td>Day 0</td>
<td>28.58±1.81</td>
<td>29.04±1.91</td>
<td>29.82±2.42</td>
<td>30.56±2.86</td>
<td>28.54±0.75</td>
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<tr>
<td></td>
<td>Day 7</td>
<td>25.85±1.40</td>
<td>27.60±2.05</td>
<td>25.33±2.29</td>
<td>25.85±3.05</td>
<td>24.26±4.02</td>
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<td>Day 14</td>
<td>27.21±2.53</td>
<td>28.00±1.10</td>
<td>31.28±2.35</td>
<td>26.86±0.83</td>
<td>27.52±3.54</td>
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<tr>
<td>PC (x10^9/mm³)</td>
<td>Day 0</td>
<td>433.20±22.26</td>
<td>426.20±28.53</td>
<td>406.40±22.59</td>
<td>421.20±23.19</td>
<td>430.80±18.09</td>
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<td></td>
<td>Day 7</td>
<td>480.80±31.20</td>
<td>475.20±34.52</td>
<td>399.40±40.97</td>
<td>442.40±27.79</td>
<td>465.80±21.16</td>
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<tr>
<td></td>
<td>Day 14</td>
<td>434.00±28.45</td>
<td>408.00±10.04</td>
<td>353.80±35.62</td>
<td>439.00±30.04</td>
<td>489.20±19.28</td>
</tr>
<tr>
<td>TLC(x10^9/mm³)</td>
<td>Day 0</td>
<td>15.41±1.94</td>
<td>14.55±1.33</td>
<td>13.45±2.63</td>
<td>14.28±3.63</td>
<td>12.02±1.51</td>
</tr>
<tr>
<td></td>
<td>Day 7</td>
<td>13.45±2.20</td>
<td>13.06±2.32</td>
<td>22.15±2.63***</td>
<td>24.53±2.31***</td>
<td>24.83±2.50***</td>
</tr>
<tr>
<td></td>
<td>Day 14</td>
<td>14.35±1.30</td>
<td>14.59±3.38</td>
<td>25.51±2.63**</td>
<td>16.77±1.21</td>
<td>13.51±3.80</td>
</tr>
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</table>

Significance level, *P < 0.05, **P < 0.01, ***P < 0.001. Hgb- hemoglobin, T-RBC- total red blood cells, Hct- hematocrit, MCV- mean corpuscular volume, MCHC- mean corpuscular hemoglobin concentration, TLC- total leucocytes count, PC- platelet count.

Biochemical analysis

Oral administration of methanol extract of SI stem bark at 200mg/kg body weight did not show any significant changes in the general metabolic function, liver function or kidney function as evident by analysis of marker enzymes viz. GLU, CHOL, TG, TP, ALB, ALT, AST, ALP, T-BIL, BUN, CRTN, Ca and P as compared to control rats. Treatment of PHZ (10mg/kg body weight) alone caused significant increase in blood GLU concentration and in marker enzymes of Liver and kidney function e.g. ALT, AST, ALP, T-BIL, and in CRTN and P as compared to control or SI (500mg/kg) alone treatment. Administration of SI stem bark extract at 250 or 500 mg/kg to PHZ-induced anemic rats caused decline in GLU, AST, ALP, T-BIL, CRTN and P levels as compared to PHZ alone treatment, which were comparable to controls (Table 4).
DISCUSSION

In present study, PHZ-induced anemic rats showed significant increase in MDA activity and decrease in SOD and CAT enzyme activity in blood serum. Oral administration of the methanol extract of SI stem bark to PHZ-induced anemic rats caused significant decrease in MDA activity, but increased SOD and CAT enzyme activity equivalent to in SI- treated or normal rats. Previous studies have been shown the antioxidant properties of SI stem bark extract using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and SOD assay system.\[58, 60-61\] Further, the antioxidant, antiglycation and inhibitory potential of flavonoid fraction of the flower extract of this plant (SAF) against alpha-glucosidase and alpha-amylase (the enzymes linked to type 2 diabetes) and LDL oxidation have been reported.\[62\] Pre-treatment of C2C12 cells with SAF prevented the increased formation of MDA, ROS and depletion of GSH induced by H2O2.\[62\] The ethyl acetate fraction of SAF have also shown the free radical scavenging activity against the DPPH and superoxide radical, along with hydroxyl radical scavenging activity and lipid peroxidation inhibitory potential indicating significant antioxidant and xanthin oxidase (XO; key enzyme linked to inflammation) inhibitory potential.\[48\] SI leaves extracts (e.g. petroleum ether, chloroform and methanol) administration for 21 days have also
been reported to show significant (P <0.05) antioxidant activity at the dose of 500 μg/ml.[12]
In concurrence, previously reported studies with herbal extracts e.g. A. pterocarpoides, M. arboreus and H. madagascariensis[63,64] have been demonstrated to show an increase in the rate of inhibition of DPPH, as a function of time and the extract’s concentration which reflect on the kind of scavenging radical kinetic: the fast, intermediate and slow kinetics. The ameliorative effects of SI leaves ethanolic extract on hyperglycemia and lipid profile have been demonstrated to be due its contents including phytosterols, phenols, tannin and flavonoids.[65-67] In plant tissues many phenolic compounds (in addition to tocopherols) are potential antioxidants, flavonoids, tannins and lignin precursors may work as ROS-scavenging compounds. Antioxidants act as a cooperative network, employing a series of redox reactions. Interactions between ascorbic acid and glutathione, and ascorbic acid and phenolic compounds are well known.[68] Therefore, the radical scavenging activity of SI extract may to be due to its polyphenolic constituents that play an important role in antioxidative effects, act as reducing agents/antioxidants via several mechanisms including the scavenging of free radicals, chelation of transition metals, as well as the mediation and inhibition of enzymes.[46, 69]

The body and organ weights (viz. adrenal, brain, testes, heart and kidney) did not show any significant changes in SI, PHZ and PHZ+SI extract treated rats as compared to controls. But, there was a significant increase in weights of liver, lung and spleen in PHZ-treated anemic rats as compared to control. Oral administration of SI extract (250 or 500mg/kg) caused a significant decrease in these organ weights gain to normalcy. Histopathological studies showed higher erythroblastic islands in spleen with a higher excessive erythrocytic congestion, abundant macrophages than in liver and kidney in PHZ-treated anemic rats. Previous study has been demonstrated that PHZ treatment induces an increase in spleen, kidney and liver weights at 60mg/kg dose and showed severe splenomegaly and marked splenic erythroid hyperplasia on day 6 of post injection.[58, 70] Erythroblastic islands reported in the spleen, liver and kidney, this being indicative of compensatory erythropoietic activity. In ultrastructural study, sequential transformation of PHZ-induced hemolytic anemia as well as transformation of typical erythrocytes into erythrocytes full of protuberances and transformation of Heinz bodies into damaged erythrocytes by splenic lytic activity have been reported.[70] The treatments with SI stem bark extract show recovery of these changes similar to controls. Previous study on SI leaves extract also shown favorable histopathological changes in pancreas, liver and kidney in STZ-induced diabetic mice.[12]
The terminal serum biochemistry on day 15 of autopsy, showed significant increase in GLU, ALT, AST, ALP, T-BIL, CRTN and P levels by PHZ-treatment as compared to control or SI(250mg/kg) alone treated rats. This increase in by SI treatment was decreased to normal level by oral administration of SI extract (500 mg/kg) in PHZ-treated male rats. Previously, Kumar et al.\textsuperscript{[12]} has been shown a significant (P<0.01) reduction in blood GLU levels, improved body weight and altered biochemical parameters associated with diabetes in diabetic mice treated with SI leaves extracts (e.g. petroleum ether, chloroform and methanol) for 21 days. Estimation of blood GLU level has been used as marker of hyperglycemia. Hyperglycemia induced-oxidative stress caused by free radical generation and decrease in antioxidant defense system.\textsuperscript{[71-72]} Dyslipidaemia is associated with elevated total cholesterol, triglycerides and low level of high density lipoprotein (HDL).\textsuperscript{[73]} Treatment with ethanolic extract of SI leaves has been reported to normalize the altered lipid profile, reduce the elevated glucose level and attenuates the diabetes-induced renal oxidative stress in dose dependent manner.\textsuperscript{[12]}

Administration of SI extract (250mg/kg) alone for 14 days to normal rats did not alter Hgb, RBC, MCV, Platelets(x10\(^3\)/mm\(^3\)) and TLC(x10\(^3\)/mm\(^3\)) concentration as compared to controls. However, PHZ-treatment (10mg/kg/day) for 7 consecutive days, caused significant decrease in Hgb(\%(p<0.01 ), RBC(x10\(^6\)/mm\(^3\)) (p<0.001 ) and MCV(micron\(^3\))(p<0.001 ) concentration and increased TLC(x10\(^3\)/mm\(^3\)) infiltration. Treatment of SI extract at 250 or 500mg/kg body weight to PHZ-induced anaemic rats, showed significant increase in Hgb, RBC and MCV concentration which was comparable to in control rats. The Hct, MCHC and platelets did not show any significant change in PHZ or SI treated either alone or in combination with PHZ when compared with controls. Similarly, previously reported studies have been demonstrated that PHZ decreases the Hgb, RBC and MCV and the rate of haematocrit (HCT) below controls and impairs erythrocyte deformability. It also induces reticulocytosis, increases osmotic resistance, free plasma Hgb, MCH, MCHC and erythropoietin levels, and extramedular haematopoiesis in the spleen and liver.\textsuperscript{[59, 74-79]} The numbers of erythrocyte-committed progenitors and colony-forming units increases during PHZ-induced acute anaemia.\textsuperscript{[80]} PHZ induce vascular dysfunction and haemodynamic disturbance, but, decreases in mean arterial pressure and hindlimb vascular resistance.\textsuperscript{[81]} Uncompensated respiratory alkalosis, increased arterial CO2 tensions and acidosis were also reported following PHZ administration.\textsuperscript{[49, 82-83]} The plant extracts of Hibiscus cannabinus\textsuperscript{[52]}, hydroethanolic extract of H. madagascariensis\textsuperscript{[64]} and ethanolic extract of Swertia chirata leaves\textsuperscript{[84]} have been
shown anti-anaemic activity evidenced by an increase in the contents of Hb, RBC, HCT and reticulocytes rate in PHZ-induced anaemia. This action could be the result from the highest antioxidant potential and the presence of iron as reported earlier.[63] The aqueous extract of *Sorghum bicolor* (L.) Moench stem bark at the doses of 200, 400 and 800 mg/kg body weight on iron sufficient and iron deficient weaning rats had also produced a significant increase in Hgb, packed cell volume and RBCs in iron sufficient and iron deficient groups. There was also a significant increase in the catalase activity of rat liver and kidney indicating restoration of anaemic condition in the iron deficient group.[85] Results of the study with SI extract administration to PHZ-treated rats also explain the recovery of the decreased haematological parameters (RBCs, Hgb, and MCV) and increased TLC level to normalcy, indicating the action of the plant extract vis-a-vis haemolysis. Further, the toxic effects of PHZ have been shown to increase in ROS, Lipid peroxidation, and decrease in GSH which to be reversed by N-acetyl cysteine, a known ROS scavenger.[86,87] The sub chronic intoxication of rats with PHZ, results in marked anemia, reticulocytosis, methemoglobinemia and increases hemocatheresis, total iron content, hepatic ferritin and DNA fragmentation, increases levels of 8-oxo-7,8-dihydro-2’-deoxyguanosine (8-oxodGuo), a specific marker of oxidative DNA damage, and hepatocyte y-glutamyl transpeptidase (y-GT, EC 2.3.2.2) activity.[88] Acute haemolysis induced by PHZ, exhibited the extramedullary haemopoiesis and increased erythrophagocytosis in the spleen. In consequence, morphological changes include splenomegaly and congestion of haemosiderin deposits.[89] Catabolism of haemolysed RBCs is associated with increased expression and activity of haem oxygenase 1 which suggests increased capacity for degradation of Hb products, iron level and DMT1 and TFR1 mRNA expressions in the spleen by PHZ treatment.[79,88,90] Increased splenic erythrophagocytosis results in a net flux of iron into the circulation from haemolysed RBCs. While the mechanism of iron exchange between macrophages and transferrin is still undefined, ferroportin mRNA increased in the spleen of PHZ-treated mice[79], consistent with efflux of iron being mediated by this protein. Hepatic non-haem iron levels increases significantly after PHZ treatment and sustained for 7 days after haemolysis[91] which is due to increased absorption driven by haematopoeitic activity in PHZ-treated animals. Liver iron accumulates initially due to haemolysis, later on absorbed iron may be diverted to the liver hepatocytes via serum transferrin.[92] Iron deposition in the liver could in addition to the TFR/DMT1 routes, be due to influx of non-transferrin bound iron (NTBI). Furthermore, as PHZ-induced haemolysis is stressful and produces haemderived toxic reactants, the expressions of acute phase proteins hemopexin, haptoglobin and CD163 in the hepatocytes and the kidneys are increased.[93,94]
These organs mop up the haem-derived products from circulation.\[^{49}\]\ A flavonoid, silybin dihemisuccinate, an anti-hepatotoxic agent caused protective effects on the hepatic glutathione depletion and lipid peroxidation induced by PHZ.\[^{95}\]\ The protective effects of quercetin in a model of PHZ-induced oxidant stress, vascular dysfunction and hemodynamic disturbance, shown to protect the blood glutathione, suppressed plasma malondialdehyde levels and nitric oxide metabolites and superoxide anion production in rats.\[^{81}\]\ A significant increase in thiobarbituric acid (TBA)-reactivity in the circulating RBC of PHZ-treated rats increases 3-fold in RBC obtained from the spleen. Since lipid peroxidation accompanies formation of TBA-reactive malonyldialdehyde, phenylhydrazine induces anemia as a consequence of peroxidation of RBC membrane lipids and this effect may be a result of the autoxidation of the drug and the interaction of oxygen radicals with membrane lipids.\[^{96}\]

**CONCLUSION**

In summary, results of the present study provide the evidence that the methanolic extract of *S. integrifolia* stem bark could reduce oxidative stress evidenced by its antioxidant activity. Further, *S. integrifolia* extract also showed protective effects on decreased hematological parameters viz. Hgb, RBC and MCV concentration and increased TLC by PHZ- treated anemic rats. It also normalizes the increased biochemical parameters such as blood GLU level and marker enzymes for Liver function (viz. ALT, AST, ALP, T-BIL) and kidney function (CR TN and P) by PHZ treatment. The increase in organ weights of spleen, liver and kidney and histopathological alterations induced by PHZ-treatment were also restored to normalcy by oral administration of *S. integrifolia* extract. The prevention of PHZ-induced anemia and related changes in vascular dysfunction and dynamics by *S. integrifolia* extract, explore the traditional use of this plant as an antioxidant, antianaemic and haematoprotectant.

**Authors’ declaration:** Authors have no declaration of interest.

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